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Obesity-exposed oocytes accumulate and transmit damaged mitochondria due to an inability to activate mitophagy

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Abstract

Mitochondria are the most prominent organelle in the oocyte. Somatic cells maintain a healthy population of mitochondria by degrading damaged mitochondria via mitophagy, a specialized autophagy pathway. However, evidence from previous work investigating the more general macroautophagy pathway in oocytes suggests that mitophagy may not be active in the oocyte. This would leave the vast numbers of mitochondria – poised to be inherited by the offspring – vulnerable to damage. Here we test the hypothesis that inactive mitophagy in the oocyte underlies maternal transmission of dysfunctional mitochondria. To determine whether oocytes can complete mitophagy, we used either CCCP or AntimycinA to depolarize mitochondria and trigger mitophagy. After depolarization, we did not detect co-localization of mitochondria with autophagosomes and mitochondrial DNA copy number remained unchanged, indicating the non-functional mitochondrial population was not removed. To investigate the impact of an absence of mitophagy in oocytes with damaged mitochondria on offspring mitochondrial function, we utilized in vitro fertilization of high fat high sugar (HF/HS)-exposed oocytes, which have lower mitochondrial membrane potential and damaged mitochondria. Here, we demonstrate that blastocysts generated from HF/HS oocytes have decreased mitochondrial membrane potential, lower metabolites involved in ATP generation, and accumulation of PINK1, a mitophagy marker protein. This mitochondrial phenotype in the blastocyst mirrors the phenotype we show in HF/HS exposed oocytes. Taken together, these data suggest that the mechanisms governing oocyte mitophagy are fundamentally distinct from those governing somatic cell mitophagy and that the absence of mitophagy in the setting of HF/HS exposure contributes to the oocyte-to-blastocyst transmission of dysfunctional mitochondria.

Introduction

The oocyte has the unique role of generating all of the mitochondria an embryo will inherit and rely upon during pre-implantation development (Cummins, 2000) because sperm mitochondria are degraded immediately after fertilization (Al Rawi et al., 2011; Cummins, 2000; Song et al., 2016; Sutovsky et al., 1999). To achieve sufficient numbers of mitochondria for the embryo, oocytes massively increase their mitochondria during follicular development. This increase in mitochondrial biogenesis results in a mature oocyte containing 10^5-10^6 mitochondria, 100 times the average number of mitochondria in a somatic cell (Piko and Taylor, 1987). While mitochondria are the dominant organelle in the oocyte(Motta et al., 2000), only a small percentage of these mitochondria are metabolically active (Van Blerkom et al., 2002). Additionally, typical oocyte mitochondria are small and round, with few cristae that wrap the periphery of the organelle (Au et al., 2005). This is in contrast to active mitochondria found in somatic tissues that produce high levels of ATP such as the heart, liver, and skeletal muscle (Picard et al., 2016).

Accumulating evidence indicates that obesity can damage mitochondria in the oocyte (Boots et al., 2016; Boudoures et al., 2016; Igosheva et al., 2010; Luzzo et al., 2012; Wu et al., 2010; Wu et al., 2015). The mechanism for this damage and the influence it has on the embryo during development and adulthood are only beginning to be investigated. However, embryos fertilized from obese mothers show delayed preimplantation development. Additionally, embryo transfer experiments taking embryos fertilized from HF/HS fed mothers and allowing them to develop in a lean uterine environment still results in growth restriction in utero in addition to decreased glucose tolerance and increased body weight in the adult offspring(Huypens et al., 2016; Sasson et al., 2015). Because these phenotypes are determined by the metabolism, it suggests there may be mitochondrial defects that persist into adulthood.

Cells can utilize mitophagy, a subset of the macroautophagy pathway, which detects and recycles damaged mitochondria to maintain a functional cell (Lemasters, 2014). The most well studied pathway involves the stabilization of Pink1 on depolarized mitochondrial membranes (Jin et al., 2010; Narendra et al., 2010). This stabilization results in the ubiquitination of mitochondrial proteins, which recruits autophagy machinery (Lazarou et al., 2015). One of the main proteins involved in autophagosome formation in microtubule associated protein/light chain 3 (LC3), a well-established marker of autophagosomes (Mizushima et al., 2004). After the autophagosome closes, it fuses with the lysosome where the acidic pH and lysosomal hydrolayses facilitate degradation of its contents into macromolecules to be recycled back into the cell (Settembre et al., 2013).

Autophagy and specific subsets thereof have been described in almost every type of cell and has proven essential to maintain normal cell function (Roberts et al., 2016). Oocytes are an exception to this rule. Oocytes deficient in the autophagy protein Atg5 are viable and able to be fertilized but do not develop past the 4-8 cell stage (Tsukamoto et al., 2008a; Tsukamoto et al., 2008b). While autophagy is necessary for embryo survival post-fertilization, other groups have shown premature activation of autophagy in the oocyte via pharmacologic or genetic methods prior to fertilization impairs oocyte maturation, fertilization rate, and pre-implantation embryo development (Lee et al., 2016; Rojas-Rios et al., 2015).

Based on the evidence that oocytes do not activate autophagy, we hypothesized that oocytes fail to recognize and respond to mitochondrial damage by mitophagy activation. We further hypothesize that damage induced by obesity in the oocytes results in inheritance of damaged mitochondria in embryos prior to implantation and resumption of mitochondrial biogenesis.

Materials and Methods

Animals and Diet

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the IACUC-accredited Animal Studies Committee of Washington University School of Medicine (Study #20150034). Four-week-old female C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME) were fed either a high-fat/high-sugar (HF/HS; Test Diet 58R3, 35.8% hydrogenated coconut oil/17.5% sucrose by weight) diet or a diet of standard mouse chow (Lab Diet 5053, 5% Fat/3.25% sucrose by weight) *ad libitum* for six to eight weeks. Mice constitutively and ubiquitously expressing green fluorescent protein-tagged version myosin light chain 3 protein (GFP-LC3) were a gift from Dr. Conrad Wiehl and were initially described in (Mizushima et al., 2004).

Oocyte collection

To collect germinal vesicle (GV) stage oocytes and cumulus oocyte complexes (COCs), mice were intraperitoneally injected with 5 IU pregnant mare serum gonadotropin (PMSG; EMD Millipore, Billerica, MA). Mice were sacrificed by cervical dislocation 46-48 hours later, and ovaries were removed and placed in pre-warmed M2 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 5 µM milrinone (EMD Millipore) to maintain GV arrest and 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO). Large antral follicles were punctured with a 29 x ½ gauge needle, and cumulus cells were removed by multiple passes through a glass pipette to isolate GV oocytes. To collect metaphase II (MII) stage oocytes, mice were administered 5 IU PMSG. After 46–48 hours, they were administered 5 IU human chorionic gonadotropin (Sigma Aldrich), and 13 hours later, they were sacrificed as above, and ampullae were removed and placed in M2 medium + 1 µg/ml hyaluronidase (Sigma Aldrich) + 10% FBS (Sigma Aldrich). Ampullae were nicked with a 29 x ½ gauge needle and incubated for 10 min to remove cumulus cells. Oocytes were washed once in fresh M2 medium +10% FBS and used for assays.

Cell Culture
Murine embryonic fibroblasts (MEF) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma D579) with 2 mM L-glutamine, 100 U/ml Penicillin and Streptozocin, and 10% FBS. MEFs were a gift of Dr. Jason Weber and were isolated as described in (Kamijo et al., 1997).

Mitochondrial membrane potential
For JC-1 staining of oocytes, the MitoPT™ JC-1 Assay Kit (Immunochemistry Technologies, Bloomington, MN) was used according to the manufacturer's protocol with the following modification: oocytes were stained for 20 min in 200 µl of JC-1 diluted in M2 medium instead of assay buffer followed by a single manufacturer prescribed wash once for 5 min in pre-warmed EmbryoMax FHM with HEPES without phenol red (MR-025-D; EMD Millipore) with 5 µM milrinone and 10% FBS. Oocytes were immediately imaged live at 37°C, 5% CO_2 in a glass-bottomed culture dish on a Leica LASX scanning confocal microscope using a 63X oil immersion lens. To detect green and red J-aggregates, images were acquired with sequential scanning using a 488 nm laser with a 510nm-540nm bandpass filter followed by a 532nm laser with a 580-610 nm bandpass filter (Perelman et al., 2012). Fluorescent analysis was done by measuring the total fluorescence of the entire oocyte in each channel. Mean gray value for each channel was normalized to area of measurement using the corrected total cell fluorescence (CTCF) method (Parry and Hemstreet, 1988). Values are expressed as total red CTCF divided by total green CTCF for individual oocytes from three different mice in each diet group.

Oocytes and blastocysts were stained with 500 nM MitoTracker CMXRosamine (CMX Ros, Invitrogen, Carlsbad, CA) diluted in M2 medium for 30 min at 32°C, 5% CO_2. After staining, oocytes and blastocysts were fixed for 30 min in 4% paraformaldehyde, 1% bovine serum albumin and either mounted in VectaShield (Vector Labs, Burlingame, CA) or used for immunofluorescent staining. Oocytes and blastocysts were visualized on an Olympus Laser scanning confocal microscope with a 532 nm laser and a 60X oil immersion lens.

Metabolic Assays
Fifteen denuded germinal vesicle (GV) stage oocytes or blastocysts were collected from 6-8 mice per diet. Individual oocytes or blastocysts were frozen on glass slides in isopentane equilibrated with liquid nitrogen and freeze dried overnight under vacuum at -35°C. Individual oocytes or blastocysts were extracted under nanoliter volume and used for analysis of levels of citrate, ATP, or phosphocreatine (pCr) by enzyme-linked cycling assays as previously described (Chi et al., 2002; Chi et al., 1988).

Western Blots
For MEF protein, cells were lysed in RIPA buffer with three 15-second sonication pulses. BCA analysis was used to assess protein level. 10 µg of protein was diluted in 4x NuPage LDS sample buffer (Invitrogen, Carlsbad, CA) and loaded in each lane. Three hundred denuded GV oocytes from at least 15 mice or 35 blastocysts from 6-8 mice were washed twice with Complete Protease and Phosphatase Inhibitors (Roche, Indianapolis, IN) and added directly to Laemmli sample buffer, boiled for 5 min. All protein samples were subjected to SDS-PAGE on 10% acrylamide gels, and transferred to nitrocellulose by using the iBlot® 2 Dry Blotting System (Thermo Fischer Scientific, Waltham, MA) at 20 V for 7 min. Blots were processed according to standard Western blot procedures and probed with primary antibodies specific to Pink1 (1:1000, Abcam, Cambrige, United Kingdom, ab23707) Tom20 (1:1000, Santa Cruz Biotechnologies, Santa Cruz, CA, FL-145) and Gapdh (1:1000, Cell Signaling Technologies, Clone 14C10, catalog # 2118L). Anti-rabbit or anti-mouse secondary antibodies (1:10,000, Cell Signaling Technologies) were used as appropriate, and signal was detected with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fischer Scientific). All experiments were performed in triplicate. Relative protein levels were quantified in Image J and normalized to Tom20 levels.

Mitochondria Depolarization

GV oocytes were cultured for 2 hours in 10 µM Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma-Aldrich, C2759) reconstituted in dimethyl sulfoxide (DMSO, Sigma-Aldrich) or 10 µg/ml Antimycin A (AntA; Sigma-Aldrich, A8674) reconstituted in 100% ethanol. CCCP and Antimycin A (AntA) were diluted in M2 medium (Sigma-Aldrich) supplemented with 10% FBS and 10 µM milrinone in organ dishes overlain with mineral oil. MEFs were treated for up to 24 hours with 20 µM CCCP in previously defined culture medium. Vehicle-only controls omitted the CCCP or AntA.

Lysosome Quantification

To quantify lysosomal puncta, COCs from transgenic GFP-LC3 mice were collected as described above. COCs were cultured for two hours in 10 µM CCCP or 10 µg/ml AntA alone, 50 nM Bafilomycin A1 (BafA1; Cayman Chemical Company, Ann Arbor, MI) alone, or the one of the two mitochondrial depolarizing agents in combination with BafA1. BafA1 inhibits acidification of the lysosomes to prevent degradation of the contents within (Klionsky et al., 2016). 30 minutes prior to the end of chemical exposure, COCs were stained with 500 nM MitoTracker CMXRosamine (Invitrogen) diluted in M2 medium + 5 µM milrinone with or without the above agents for 30 min at 37°C, 5% CO_2 to confirm mitochondrial membrane depolarization. After staining, COCs were fixed in 4% PFA and stained for immunofluorescence as described below. COCs were visualized on a Leica LASX scanning confocal microscope with a 63X oil immersion lens, and 15 images of a 10 µm section of each oocyte were collected. Lysosome puncta in oocytes were quantified with LASX Analysis software, version 1.9, from at least 12 oocytes from 2-3 animals per treatment. Specifically, images were first processed with background subtraction and median noise removal before setting a threshold of accepted fluorescence to reject further background noise. Next, the edge removal algorithm was applied. Finally, to count objects, only puncta greater than 0.68 µm (the lower limit of autophagosome size; (Martin et al., 2013)) within the user-defined oocyte region were accepted by the pipeline. The averages of the sum of puncta from each 10 µm oocyte section in each treatment group were compared by using GraphPad Prism software to perform a two-way ANOVA with Tukey-Kramer correction for multiple comparisons. Lysosome puncta in cumulus cells were counted by a blinded observer and normalized to the total number of cumulus cells in that image. The biological assay was performed 3-4 times using 2-3 mice per assay.

Immunofluorescence

COCs were fixed for 15 min in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS + 1% bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO). Oocytes were transferred to a permeabilization solution (0.5% Triton X-100 in PBS) for 20 min, blocked with PBS + 0.1% Tween-20 + 0.01% Triton X-100 + 1% BSA for 15 min, and then incubated with an anti-Heat-shock protein 60 antibody (HSP-60; 1:250, Santa Cruz Biotechnologies, sc-1052 [N-20]) overnight at 4°C. Next, oocytes were washed three times in PBS + 0.1% Tween-20 + 0.01% Triton X-100 and then incubated in appropriate secondary antibodies conjugated to Alexa-647 (1:250, Invitrogen) and diamidino-2-phenylindole (DAPI; Thermo Fischer Scientific, 1 ng/ml) for one hour. Oocytes were washed twice in PBS + 0.1% Tween-20 + 0.01% Triton X-100 and mounted in VectaShield (Vector Labs, Burlingame, CA) on 12-well, 5 mm diameter Teflon Printed Slides (Electron Microscope Sciences, Hatfield, PA) .

Mitochondrial DNA copy number quantification

Mitochondrial DNA (mtDNA) was extracted from individual denunded GV oocytes after CCCP culture or Antimycin A culture by lysing individual oocytes for 2h at 55 °C in 50 mM Tris-HCl, pH 8.5, 0.1 mM EDTA, 0.5% Tween-20, and 200 µg/ml proteinase K. Enzyme was then inactivated by 10 min incubation at 95 °C. Alternately, mtDNA was extracted from MEFs after CCCP treatment using the Qiagen DNeasy kit (Cat. No. 69504). MtDNA copy number for each oocyte was calculated by using SYBR green to perform qPCR of each oocyte sample. Each reaction contained 5 µl SYBR, 0.3 µl ea primer (100 µM stock concentration), 3.4 µl H2O, and 1 µl DNA. Primer sequences were from the MT-ND5 gene on mitochondrial DNA; forward, 5'-AACCTGGCACTGAGTCACCA-3' and reverse, 5'-

GGGTCTGAGTGTATATATCATGAAGAGAAT-3'. Copy number was determined by measuring oocyte mtDNA against a standard curve. To standardize for cell numbers of MEFs, an additional standard curve containing a region of GAPDH was used. The primers for GAPDH were: Forward, 5'-TGAAATGTGCACGCACCAAG- 3' and Reverse, 5' – GGGAAGCAGCATTCAGGTCT – 3'. To calculate mtDNA abundance in somatic cells, 3 ng of total DNA was loaded in each well. As described in (Saben et al., 2016), a version of delta CT was used to quantify mitochondrial abundance:

$$2^{[Ct(A)\times Ef(A)\}-[Ct(B)\times Ef(B)]}$$

where Ct= cycle threshold, Ef= efficiency, A =MT-ND5 and B = GAPDH

The efficiency (out of 100%) was calculated using a standard curve which was generated as described below for both genes. Values were then expressed as fold change from the vehicle treated cell values.

To generate the mitochondrial and genomic DNA standard curves, the MT-ND5 or GAPDH regions were amplified from genomic DNA by using the primers above and the following PCR protocol: 98°C for 5min; 35 cycles of 98 °C for 10 sec, 52.5°C for 30 sec, 72°C for 30 sec; followed by 72°C for 10 min. The MT-ND5 or GAPDH PCR fragment was cloned into the TOPO-TA cloning vector per the manufacturer's protocol. Positive colonies were sequenced, and a plasmid containing one copy of the MT-ND5 or GAPDH region was diluted to 10^8 to 10^2 plasmids per µl by calculating the concentration on a nanodrop and converting to exact copy number by using the following equation (May-Panloup et al., 2005): $\frac{6.3\ X\ 10^9\ copies}{ng} = \ absolute\ number\ of\ plasmids\ in\ a\ reaction.$

Transmission electron microscopy of germinal vesicle stage oocytes

Oocytes were collected as described above and prepared for electron microscopy as described in (Boudoures et al., 2016).

In vitro fertilization and collection of blastocysts

MII oocytes were collected from the ampullae of C57B6/J females after superovulation as described above. Preparation of cauda epididymal sperm from male ICR mice, insemination, and embryo culture were performed as described previously (Kim and Moley, 2008). Blastocyst-stage embryos were collected for western blot, metabolic assays, and staining with MitoTracker CMX Rosamine as described above.

Results

To test our hypothesis that oocytes would not be able to activate mitophagy after mitochondrial damage, we isolated germinal vesicle (GV) stage oocytes from C57Bl6/J mice, chemically depolarized their mitochondrial membranes to induce damage, and assessed their mitophagy response. We began by assessing the first step in the mitophagy pathway, the stabilization of Pink1 on depolarized mitochondrial membranes, in GV oocytes. Ionophores such as CCCP or complex III inhibition with the enzyme Antimycin A induce a massive mitophagy response in somatic cells (Georgakopoulos et al., 2017), which results in a significant increase in Pink1 as soon as 30min after depolarization (Geisler et al., 2010). Therefore, we sought to determine if oocytes had a similar response. We first demonstrated that both CCCP and Antimycin A are effective in depolarizing oocyte mitochondria using MitoTracker CMX Rosamine, which accumulates only in polarized mitochondria (Figure 1A). In murine embryonic fibroblasts (MEFs), two hours of CCCP treatment induced a significant increase in Pink1 stabilization (Chen and Dorn, 2013; Geisler et al., 2010), Figure 1B). In oocytes, Pink1 was present even in the absence of bulk mitochondrial depolarization. Additionally, only a moderate increase in Pink1 occurred after treating oocytes with CCCP for two hours (Figure 1C).

In somatic cells, after Pink1 is stabilized and accumulates on damaged, depolarized mitochondria, these mitochondria are trafficked to the autophagosome (Yoshii and Mizushima, 2015). To determine whether this occurred in oocytes, we isolated GV stage cumulus cell –oocyte complexes (COCs) from mice ubiquitously expressing GFP-LC3, a well-established autophagosome marker. To monitor

autophagosome turnover bafilomyocin A1 (BafA1) can be used to stabilize LC3 by preventing acidification of autophagosomes after fusion with lysosomes. An increase in LC3 expression after BafA1 treatment indicates autophagic flux occurs in that cell type (Klionsky et al., 2016). 50 nM of BafA1 was shown to be sufficient to inhibit acidification of lysosomes using the pH sensitive dye LysoTracker (Supplementary Figure 1). To monitor if oocytes and cumulus cells activate mitophagy, COCs were treated with 50 nM of BafA1 and 10 μm CCCP or 10 μg/ml Antimycin A simultaneously.

In cumulus cells, treatment with BafA1 only results in an increase in the number of LC3 puncta, indicating these cells undergo a basal level of autophagy (Figure 2A, C). After mitochondrial membrane depolarization with CCCP or Antimycin A, there is a reduction in the number of puncta that is expected with flux. Depolarization in combination with BafA1, results in a subtle increase the number of LC3 positive puncta, albeit not to the level of vehicle treated cumulus cells (Figure 2B, D). However, these puncta also colocalize with mitochondria, evidenced by an overlap of red (Hsp60; a mitochondria marker) and green (LC3; autophagosomes) fluorescence (Figure 2B, D) suggesting the damaged mitochondria are trafficked to the autophagosomes measured and will be recycled after lysosomal acidification.

In oocytes, while LC3 puncta are present, there was no increase in LC3 expression with BafA1 (Figure 3A, C), indicating an absence of autophagic flux, as described previously (Tsukamoto et al., 2008a; Tsukamoto et al., 2008b). GFP-LC3 positive puncta were completely eliminated in GV oocytes but after both CCCP and AntA exposure (Figure 3). Additionally, co-localization of red and green fluorescence (Figure 3B, D) was not observed in GV oocytes following CCCP or AntA exposure, suggesting GV oocytes did not traffic damaged mitochondria to the autophagosome. This LC3 clearance was completely independent of the lysosome, as treatment BafA1 in combination with CCCP or AntA did not inhibit LC3-II clearance in the oocytes (Figure 3). This indicates that LC3 puncta are being removed independent of the lysosome and the canonical autophagy pathway.

To confirm that oocytes did not remove damaged mitochondria in response to membrane depolarization, we measured mtDNA copy number in individual oocytes after CCCP or AntA treatment. Because each oocyte mitochondrion harbors, on average, only one copy of mtDNA, mtDNA copy number quantification accurately assesses total number of mitochondria (Wai et al., 2008). Additionally, mtDNA copy number decreases in somatic cells and is detectable with our assay after exposure to CCCP ((Klionsky et al., 2016; Okatsu et al., 2010) Supplementary Figure 2). Two hours of treatment with CCCP did not affect mtDNA copy number (Figure 4A). After two hours of treatment with AntA, GV oocytes increased the mtDNA copy number (Figure 4B). To ensure that 2h treatment was not too brief to observe completely degraded mitochondria, we treated oocytes with CCCP or AntA for six hours, but this also had no effect on mtDNA copy number (Figure 4C), though it did greatly impair oocyte survival (Figure 4D).

We wanted to determine if an absence of mitophagy in oocytes had a functional consequence on oocyte mitochondria, fertilization, and blastocyst development in instances where animal physiology damages oocyte mitochondria. Feeding mice an obesogenic, HF/HS diet, has been shown to induce oocyte mitochondrial damage and is currently a relevant global health crisis (Boots et al., 2016; Boudoures et al., 2016; Igosheva et al., 2010; Luzzo et al., 2012; Margaret J. R. Heerwagen, 2010; Wu et al., 2010; Wu et al., 2015). Therefore, we fed mice a HF/HS diet for a minimum of 8 weeks to induce obesity and hyperglycemia (Supplementary Figure 3). After prolonged consumption of a HF/HS diet, the oocyte mitochondria from HF/HS mice have a smaller population of active mitochondria as demonstrated by a reduced red/green fluorescence ratio after staining with JC-1 dye (Figure 5A-B). JC-1 dye works by accumulating more readily in mitochondria with a more negative membrane potential (a feature of active mitochondria). These active mitochondria will fluoresce red. Alternately, less active mitochondria accumulate less JC-1 and will fluoresce green. Measuring the ratio of red/green fluorescence is indicative of the amount of mitochondrial activity in a given cell (Reers et al., 1995).

To demonstrate that mitochondrial damage causes the observed decrease in JC-1 red-green ratio, we analyzed EM images of oocytes collected from control and HF/HS mice. These images revealed that HF/HS diet consumption severely damages oocyte mitochondria. This was evidenced by lack of electron density in addition to ruptured membranes (Figure 5C, D). While this damage did not cause a reduction in

ATP production (Figure 5E), it did result in decreased total phosocreatine (pCr) content (Figure 5F), which is used to buffer ATP concentration in cells during times of stress and energy expenditure (Wallimann et al., 2011). Finally, we wondered whether the damaged mitochondria that accumulated in the oocytes of HF/HS-fed had an impact on the resulting embryos. To test this, we isolated oocytes from either control diet or HF/HS-fed mice and subjected them to in vitro fertilization with sperm from chow-fed males. The number of fertilized oocytes to reach the blastocyst stage was significantly reduced if the oocytes came from a HF/HS fed donor female (P=0.03, Figure 5G).

Until implantation, all mitochondria in an embryo are inherited directly from the oocyte; the embryo does not make any new mitochondria (Piko and Taylor, 1987). Therefore, we hypothesized the mitochondrial damage and dysfunction we observed in the oocytes may also be present in the blastocysts after fertilization. To test this hypothesis, we assessed the mitochondrial function of the blastocysts generated by IVF from control and HF/HS donor mice. This will eliminate any interference of uterine environment on embryo development and ensure all embryos are fertilized from the same male.

After IVF, the blastocysts from HF/HS donor oocytes had a significantly lower mitochondrial membrane potential, as measured by relative fluorescence of MitoTracker (Figure 6A). Consistent with this finding, the levels of both citrate and phospho-creatine were significantly lower in embryos from HF/HS females than in those from controls, though ATP levels were not affected (Figure 6B-D). As in GV oocytes, HF/HS blastocyst ability to maintain total ATP levels is likely due to utilization of pCr to replenish ATP (Wallimann et al., 2011). Finally, the level of Pink1 protein expression was significantly higher in embryos from HF/HS-exposed oocytes than in those from controls (Figure 6E), suggesting an increased rate of mitophagy in response to presumed inheritance of damaged mitochondria.

Discussion

In conclusion we show that oocytes do not activate mitophagy in response to mitochondrial damage. Therefore, when females consume a HF/HS diet for a sustained period, the dysfunctional mitochondria cannot be cleared from the oocytes. As a result, embryos inherit suboptimal mitochondria which persist at least until the blastocyst stage. There are several potential reasons oocytes do not activate mitophagy. First, only a small pool of mitochondria exhibits high membrane polarity (Fig 1 and (Van Blerkom and Davis, 2006; Van Blerkom et al., 2003; Van Blerkom et al., 2002). Instead, the majority appear to by metabolically quiescent. This can be visualized in the JC-1 stain, in which a sub-population of highly polarized mitochondria are present at the periphery of the GV oocyte (Figure 1A). Mitochondrial quiescence in the oocyte has been proposed to be in order to reduce oxidative damage to mitochondria and organelles prior to fertilization (Cummins, 2004). Because Pink1 is stabilized on depolarized mitochondrial membranes, normal but inactive mitochondria would be eliminated in oocytes, preventing fertilization.

Second, one of the primary changes that occurs to oocytes during cytoplasmic maturation is a massive increase in the number of mitochondria present (Piko and Taylor, 1987; Wang et al., 2009). Whereas somatic cells average 200-2,000 mitochondria per cell (depending on the cell type), a fertilization-competent, mature MII oocyte has approximately 100,000 mitochondria (Piko and Taylor, 1987; Reynier et al., 2001). After fertilization, mitochondria inherited from the mother are randomly partitioned to blastomeres as cell divisions occur. Only at the blastocyst stage is mitochondrial biogenesis re-initiated (Piko and Taylor, 1987). Therefore, there is an obvious conflict programmed in the oocyte to have high levels of mitochondria production and a majority of normal mitochondria with low membrane potential paired with a membrane-polarity dependent process for mitochondrial elimination.

Finally, research in multiple model organisms and human oocytes have shown that fertilization-competent oocytes must contain mtDNA copy numbers within a specific range. Falling outside of this range results in failed fertilization (Reynier et al., 2001; Wai et al., 2010). In oocytes, mtDNA copy number is representative of the number of mitochondria (Kasashima et al., 2014). Therefore, mitophagy initiation would disrupt this range and result in failed fertilization. The oocyte may be programmed to retain damaged mitochondria in order to successfully fertilize with sub-optimal mitochondria. This is

supported by the clearance of LC3B positive puncta but not mitochondria in the oocytes after CCCP treatment (Figure 3).

The autophagosome fuses with the lysosome to break down proteins and organelles, including mitochondria, for recycling or removal (Zhu et al., 2011). However, in oocytes, this does not occur. Although LC3 positive vesicles and/or pre-autophagic structures are present in the oocytes from GFP-LC3 mice (Figure 3), these puncta do not colocalize with mitochondria. Despite elimination of GFP-LC3 after CCCP or AntA treatment, mitochondria are still abundant in the oocytes, supporting a lack of mitophagy in oocytes. Further, when oocytes were treated with both CCCP and BafA1 to inhibit lysosomal degradation, oocytes still eliminated the LC3 puncta. If oocytes used autophagy to degrade the GFP-LC3, BafA1 would prevent this degradation. This indicates oocytes remove LC3 using a lysosome-independent mechanism, such as the ubiquitin proteasome system. While the mechanism for LC3 elimination is unclear, it is not surprising that the mechanism would be independent of the lysosome and autophagy. Although oocytes deficient for an essential autophagy protein, Autophagy- Related 5 protein (Atg5), are viable and fertilize, the Atg5 null embryos die at the 4-8 cells stage (Tsukamoto et al., 2008a; Tsukamoto et al., 2008b). Our data support these earlier findings and extends this research to now include an absence of mitophagy in oocytes.

It is reasonable to speculate that oocytes activate mitophagy independent of macroautophagy in response to mitochondrial damage. There are multiple ways cells can recognize and remove damaged mitochondria (Lemasters, 2014 (Lemasters, 2014)). However, oocytes do not reduce mtDNA copy number in response to CCCP, which makes activation of an autophagy-independent pathway for mitophagy unlikely. Additionally, multiple groups have shown HF/HS oocytes have increased numbers of mitochondria (Igosheva et al., 2010; Luzzo et al., 2012; Schrauwen et al., 2010), suggesting oocytes may recognize the damage and respond by increasing mitochondria numbers as opposed to activating mitophagy. Our data supports this notion, since treatment with AntA caused a significant increase in mtDNA copy number. Finally, because oocytes do have an increase in Pink1 protein in response to CCCP exposure after two hours, it is possible once embryos activate autophagy, the mitophagy pathway can also resume. An increase in Pink1 and a decrease in mitochondrial membrane potential at the blastocyst stage in HF/HS embryos supports the hypothesis that the embryos inherit damaged mitochondria because oocytes are incapable of activating mitophagy.

Recent publications show a variety of negative physiological effects in the offspring from obese mothers (Huypens et al., 2016; Sasson et al., 2015). Despite the potential upregulation of mitophagy in the embryo (as evidenced by moderately increased Pink1 expression, Fig 5), the damage may be long lasting. This could be due to the effects of developmental programming. Offspring from obese mothers have impaired peripheral insulin signaling paired with alterations to mitochondrial dynamics(Saben et al., 2016). Furthermore, these changes to the mitochondria persist for three generations, even if the only dietary exposure occurs maternally in the F0 generation, suggesting a transgenerational epigenetic mechanism for transmission.

After fertilization, DNA methylation is largely erased from the embryonic genome. Embryos rapidly re-establish DNA methylation at the blastocyst stage (Fulka et al., 2008). Methylation requires ATP, linking epigenetics and metabolism (Ulrey et al., 2005). Therefore, damaged and less active mitochondria in the blastocyst could alter DNA methylation patterns. While this was suggested in recent publications in which embryos from HF/HS mothers were transferred to lean recipients for development (Huypens et al., 2016; Sasson et al., 2015), data comparing early stage embryos from lean and obese mothers has yet to be published. This is most likely due to the technical limitations of sequencing technologies, which lack the sensitivity to accurately measure methylation changes in embryos. However, rapid development in the sequencing field should make these data accessible in the near future.

We are the first to address a cellular pathway that explains the retention of mitochondrial damage in oocytes due to prolonged consumption of a HF/HS diet. The data presented here suggests that the oocyte itself, independent of the uterine environment, can have effects on embryo quality and offspring health. The inheritance of damaged mitochondria and potential lasting consequences on future generations can explain the persistence of mitochondrial dysfunction and metabolic syndrome in our

population. This inheritance has a sustained impact on the offspring's embryonic development and adult life (Huypens et al., 2016; Saben et al., 2016; Sasson et al., 2015). Concluding definitively that the persistent mitochondrial dysfunction is due to lack of mitochondrial removal by autophagy will require restoration of mitochondrial function by microinjection of mitochondria, and persistence of decreased mitochondrial metabolic activity if mitochondria from HF/HS fed mouse oocytes are microinjected into healthy control oocytes. While we are currently unable to address this, we plan to in future experiments.

Two questions remain. First, do embryos from HF/HS mothers successfully remove all damaged mitochondria once autophagy and mitophagy are activated after fertilization? We plan to fully address the inability for oocyte to clear damaged mitochondria in future experiments where we will restore mitochondrial function in HF/HS oocytes by microinjection of healthy mitochondria and show persistence of mitochondrial damage by microinjection of damaged mitochondria into control oocytes. Second, what impacts on epigenetics result from inheritance of a large pool of damaged mitochondria? Is this independent of the obese uterine environment? Answers to these questions will be important in understanding how obesity can influence the health of multiple generations.

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Supplementary Figure 1: Dose response images of GV oocytes treated with 0 nM (DMSO vehicle) 50 nM BafA1 or 100 nM BafA1 to show 50 nM sufficiently prevents lysosome acidification. LysoTracker DND Red was used to assess lysosome pH, as the dye accumulates in lysosomes based on pH. Mitochondria were labeled with MitoTracker Green FM.

Supplementary Figure 2: (a) 20 μM CCCP or greater sufficiently depolarized mitochondria in murine embryonic fibroblasts. MitoTracker CMXRosamine was used to assess polarity. (b) mtDNA copy number is significantly reduced in MEFs after 24 hour treatment with CCCP.

Supplementary Figure 3: Phenotype of female HF/HS fed mice. (a) Body weights of mice fed a HF/HS diet for six weeks are significantly greater than mice fed standard mouse chow (Con). (b) Blood glucose is significantly elevated in HF/HS females after a 16 hour fast.

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Figure Legends

Figure 1: Pink1 protein is upregulated in MEFs and Oocytes in response to CCCP treatment. (A) Representative of GV oocytes stained with MitoTracker CMXRosamine (MTR) showing successful depolarization with 10 µM CCCP or 10 µg/ml AntA. (B) Representative Pink1 western blot of MEFs and GV oocytes incubated for two hours with vehicle (-) or CCCP (+). Gapdh is shown as a marker of total protein, and Tom20 as a marker of total mitochondrial protein. (C) Densitometry of MEF western blots, with Pink1 normalized to Tom20, a mitochondrial loading control (n=3).

Figure 2: Cumulus cells activate mitophagy in response to cumulus-oocyte complex mitochondria membrane depolarization. (A, C) Quantification of LC3B puncta in cumulus cells treated with vehicle (Vh), bafilomycin A1 (BafA1), CCCP, AntimycinA (AntA), or CCCP/AntA and BafA1 for two hours. Puncta counts were normalized to nuclei in the same image to control for cell number. **(**B, D) Representative images cumulus cells in the respective treatment groups; arrows indicate colocalization of mitochondria in autophagosomes. Scale bar = 10 µm. red: Hsp60 (mitochondria), green: GFP-LC3B, white: nuclei; 3 experimental replicates, 2-3 mice per experiment.

Figure 3: GV oocytes remove LC3 puncta but do not activate mitophagy in response to mitochondria membrane depolarization. (A, C) Quantification of LC3 puncta in a 10 µm Z-stack section of GV oocytes treated with (A) CCCP or (C) AntA with or without BafA1. (B, D) Representative images of GV oocytes. Scale bar = 25 µm. (d) Quantification of LC3B puncta; n=32-37 oocytes/group. In (B) and (D), red: Hsp60 (mitochondria), green: GFP-LC3B, white: nuclei; 3 experimental replicates, 2-3 mice per experiment.

Figure 4: CCCP treatment does not reduce mtDNA in oocytes. mtDNA copy number of GV oocytes after two hours of (A) 10 µM CCCP treatment (n=27), or (B) 10 µg/ml AntA treatment (n=45). (C) mtDNA copy number of GV oocytes treated 6 hours with CCCP (n= 6-11) or AntA (n=5-7). (D) Oocyte survival after treatment with CCCP (dark gray square) or AntA (light gray triangle) as compared to the respective vehicle (Vh; black circle) treatment.

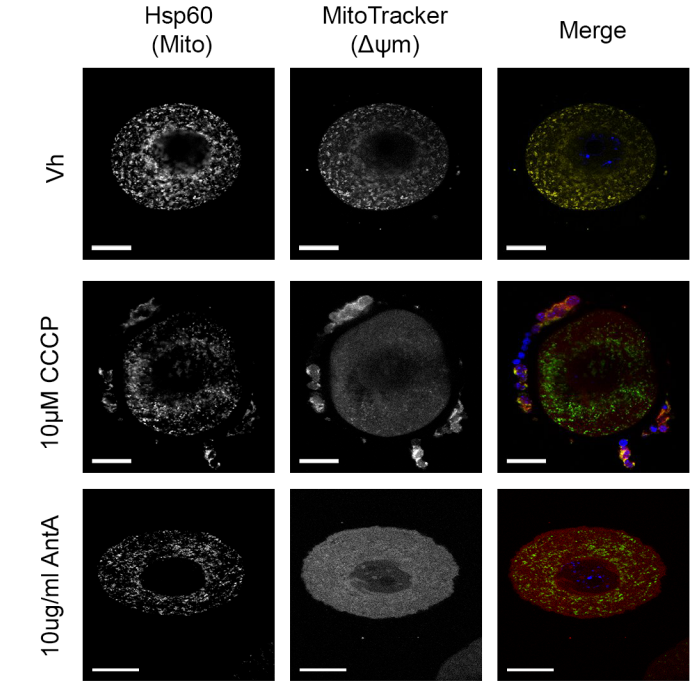
Figure 5: Germinal vesicle stage oocytes from HF/HS diet fed mice have impaired metabolism. (A) JC-1 staining of GV oocytes. (B) Quantification of JC-1 red/green ratio in GV oocytes from control (n =86) and HF/HS (90) GV oocytes; Experiment was done in triplicate, 3 mice per diet. (C) Representative EM images of oocyte mitochondria; 2,500x (scale bar = 2 µm) or 5,000 x (scale bar = 500 nm). Mitochondrion with ruptured membrane depicted with arrowhead. (D) Quantitation of oocyte mitochondria electron density (n=339 Con oocyte mitochondria and 393 HF/HS oocyte mitochondria from 11 oocytes per diet) (E) ATP content of GV oocytes (n=45/diet). (F) Phosphocreatine (pCr) content of GV oocytes (n=15/diet). (G) Blastocyst formation rate of embryos after *in vitro* fertilization of control or HF/HS oocytes. * $P<0.05$, ** $P<0.01$, **** $P<0.0001$

Figure 6: IVF-generated blastocysts from HF/HS fed donor mouse oocytes have impaired metabolism and increased mitophagy. (A) Representative images and (B) quantitation of MitoTracker in HF/HS and control blastocysts (n= 5 blastocysts). (C-E) Quantitation of levels of (C) citrate, (D) phosphocreatine (pCr) and (E) ATP of individual blastocysts (n=15 blastocysts). (F) Pink1 protein in HF/HS and control blastocysts (n=35 blastocyst/lane).

Highlights

- Oocytes are incapable of activating mitophagy to clear mitochondrial damage.
- Exposure to a high fat/high sugar diet damages oocyte mitochondria and metabolism.
- Lack of mitophagy in oocytes transmits damaged mitochondria to the embryo.
- Embryos may upregulate mitophagy in response to inherited mitochondrial damage.

Figure 1



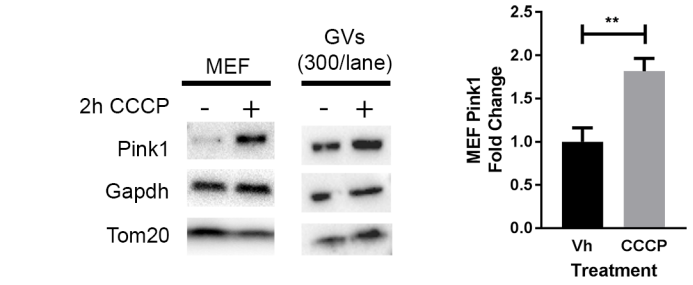


Figure 2

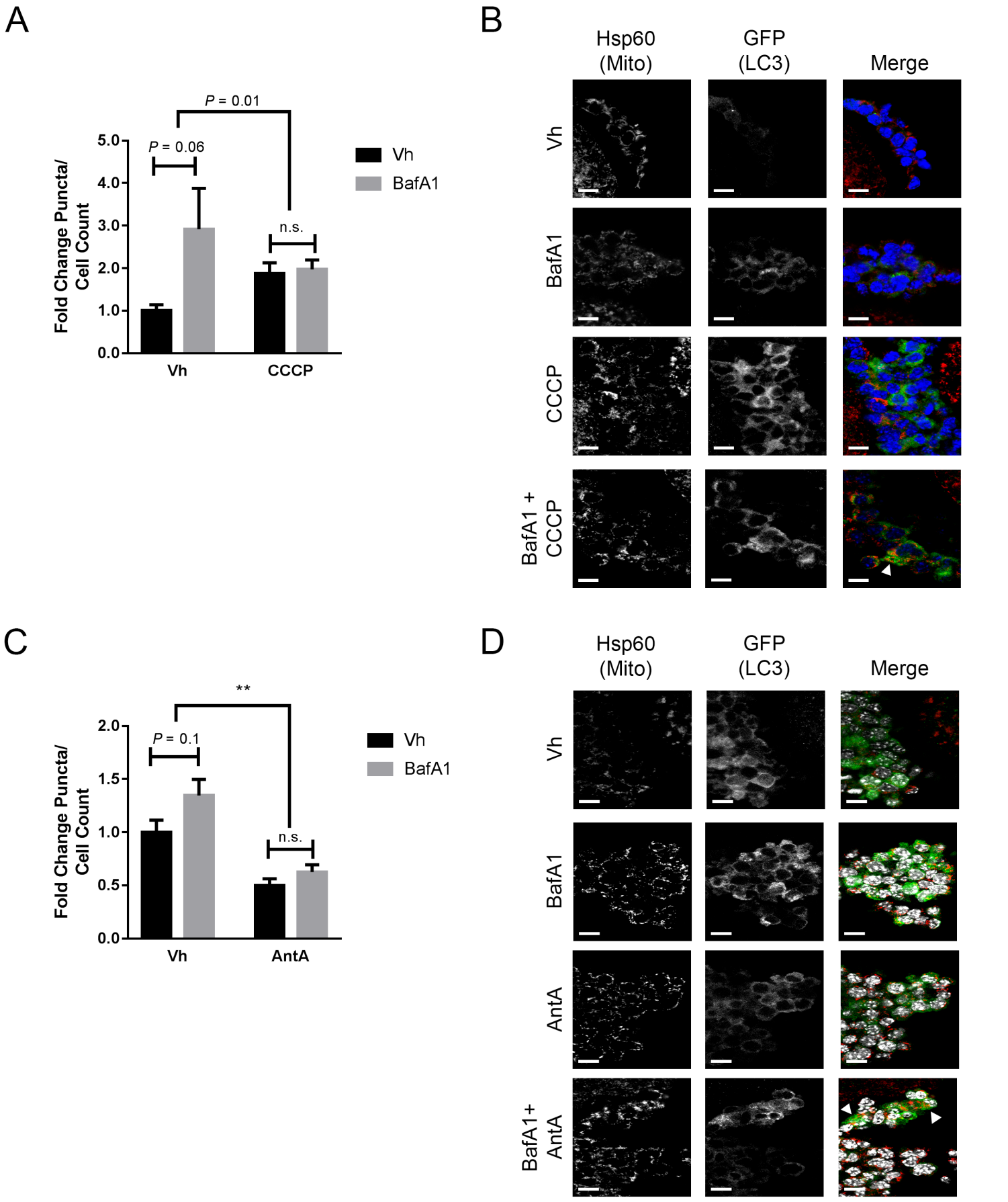


Figure 3

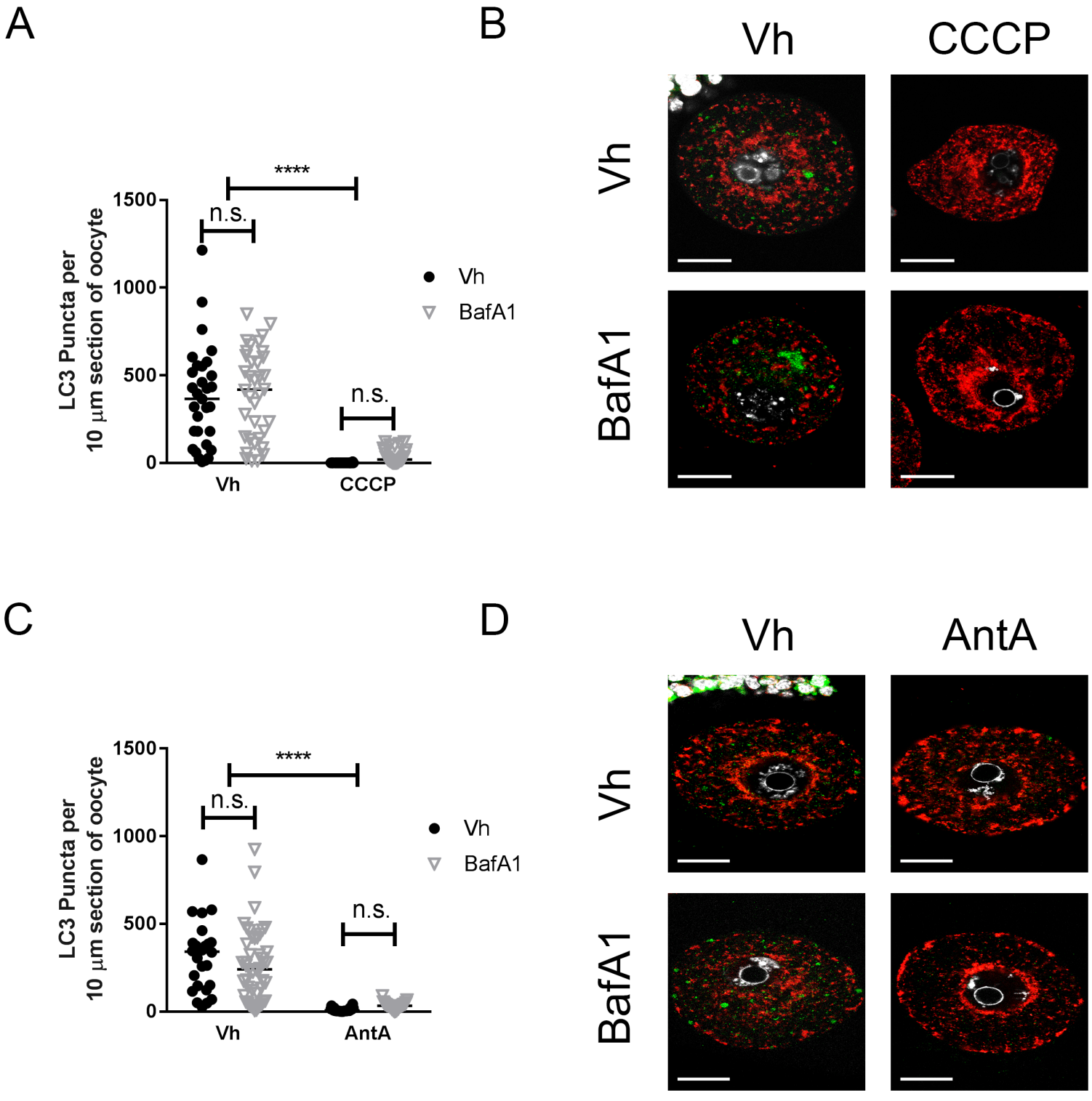

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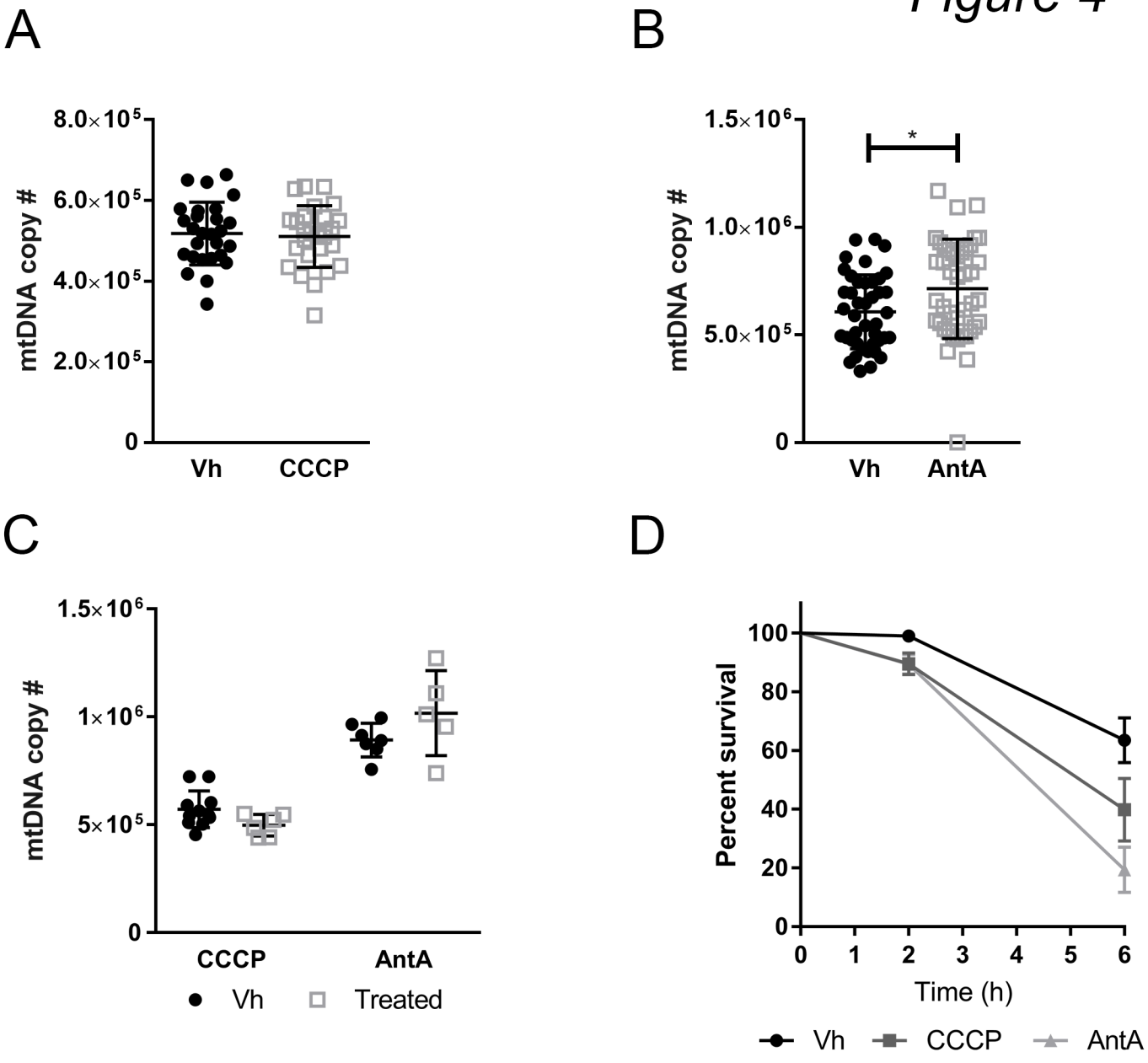
Figure 4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Time (h)
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CCCP
AntA

Figure 5

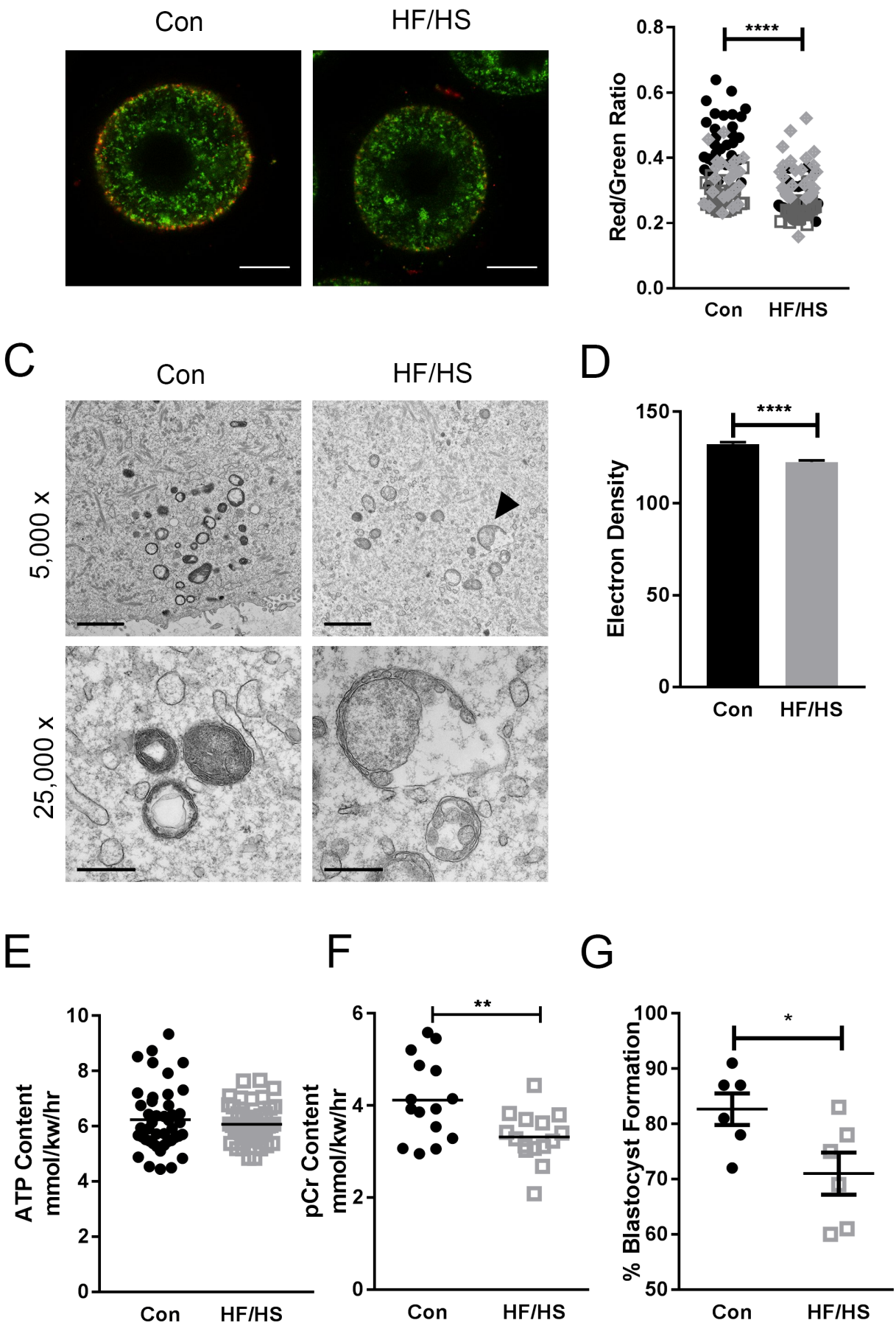
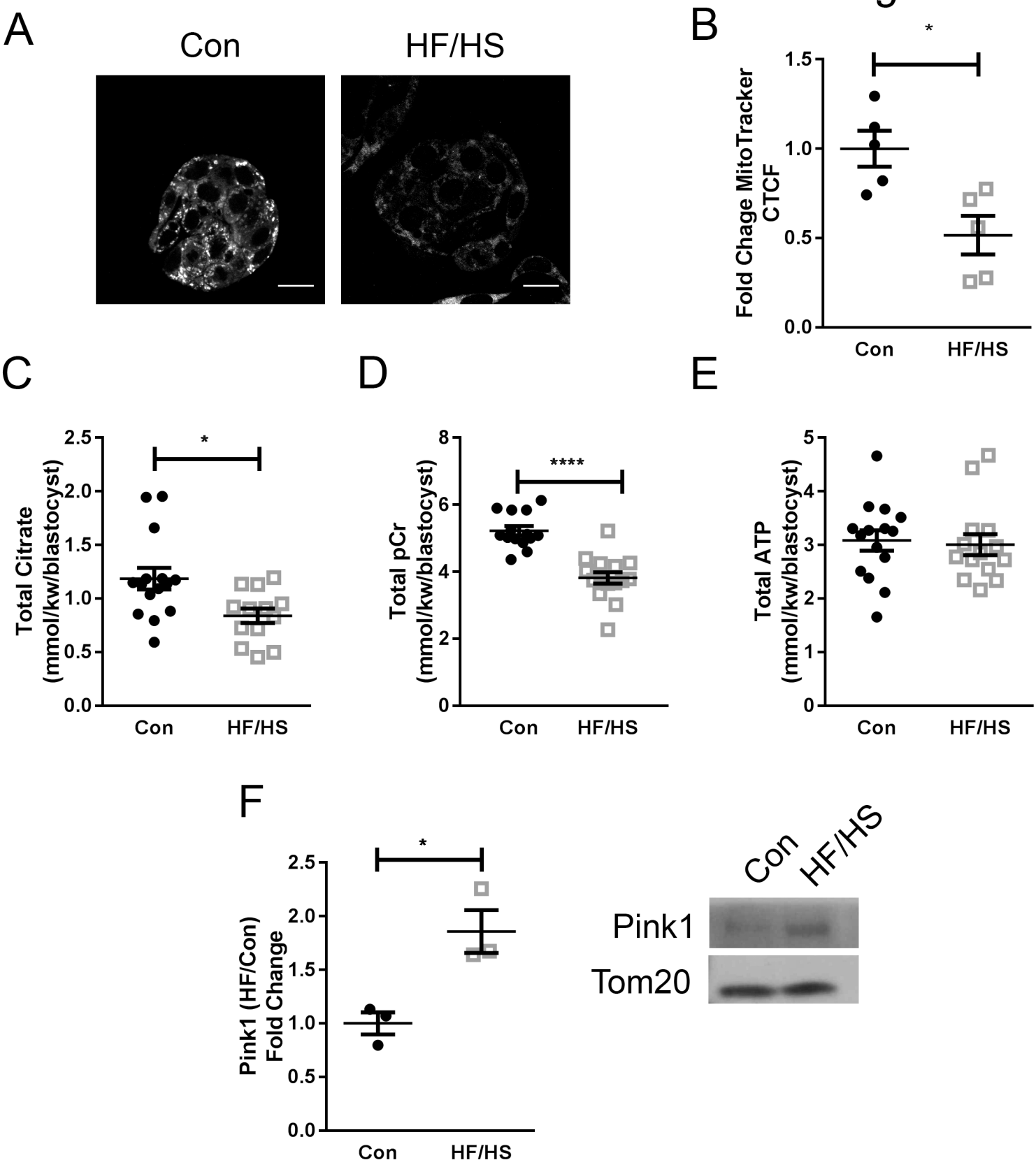


Figure 6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