Glucose-6-phosphatase is required for sperm energy metabolism, motility and fertility in *Drosophila*

Sheida Hedjazi, Tetsuya Miyamoto, Ji-Eun Ahn, Raquel Sitcheran and Hubert Amrein

Department of Cell Biology and Genetics

College of Medicine

Texas A&M University

Correspondence: amrein@tamu.edu

Summary

Glucose-6-Phosphatase (G6Pase), a key enzyme in gluconeogenesis and glycogenolysis in the mammalian liver and kidney, converts glucose-6-phosphate to glucose to maintain systemic blood glucose homeostasis during nutrient deprivation. However, its function has remained elusive in insects which have no need for G6Pase in sugar homeostasis, since they use trehalose as the main circulating sugar and convert glucose-6-phosphate to trehalose via trehalose phosphate synthase (TPS1). This study reveals an unexpected and essential role for G6Pase in *Drosophila* fertility. We report that *Drosophila G6P* mutant males are sterile, and show that G6Pase plays an essential role to produce motile sperm. While spermatogenesis and spermiogenesis in *G6P* mutant males appears to proceed normally, leading to the production and transfer of mature sperm, *G6P*$^{-}$ sperm tail beat frequency is severely diminished in the female reproductive tract, and sperm only rarely enter an egg. Moreover, when compared to wild type sperm, *G6P*$^{-}$ sperm are depleted earlier from spermatheca and seminal receptacle. Immunohistochemical analyses show that *G6P* mutant spermatocytes present with an enlarged and stressed endoplasmic reticulum (ER) and a diminished Golgi. Additionally, the acrosome, a Golgi derived organelle critical for sperm-egg interaction, is partially depleted of the transmembrane protein SNEAKY, which is essential to breakdown the sperm plasma membrane after fertilization. Metabolic analyses show that both basal and compensatory glycolysis and ATP production are compromised in testes of *G6P* mutant males. Based on these observations, we propose a model in which *Drosophila* G6Pase functions as a metabolite repair enzyme in spermatocytes. By removing a non-canonical metabolite, G6Pase facilitates glycolysis to meet the high energy demands of spermatogenesis and sperm cell differentiation. Thus, our investigations unveil a novel function for G6Pase in male fertility, highlighting its importance in regulating energy homeostasis in reproductive tissues.

Introduction

Meiosis is a fundamental process of virtually all metazoans, playing a critical role in the survival of sexually reproducing species, generating genetic diversity and facilitating adaptation to environmental changes over time through natural selection. Genetic screens in *Drosophila* have identified hundreds of genes that cause male sterility when mutated, and based on saturation screens it is estimated that the second chromosome covering roughly 40% of the genome harbors more than 250 different genes essential for male fertility (Wakimoto et al., 2004). Many more genes are likely to be necessary for male fertility, but their function cannot easily be assessed due to their essential roles during development.

Drosophila melanogaster has served as the main invertebrate model system for identifying genetic components essential for male gametogenesis (spermatogenesis). Spermatogenesis is a complex process during which germ cells undergo extensive physical changes, while also transitioning from diploid germline stem cells (GSCs) to mature haploid s (Figure 1A). In the male fruit fly, approximately 60 to 100 GSCs reside in the apical tip of each testis. Each GSC divides asymmetrically, producing a daughter stem cell and a gonioblast (Dansereau and Lasko, 2008; Fabian and Brill, 2012; Fuller, 1998). The gonioblast, surrounded by two cyst cells that provide support throughout meiosis, first undergoes four mitoses, producing 16 interconnected spermatogonia cells. These 16 cells grow extensively to become primary spermatocytes, before they synchronously enter meiosis to first form 32 secondary spermatocytes and eventually 64 spermatids, which differentiate into mature sperm. During mating, sperm are transferred to the female reproductive organs, where they are stored in the spermatheca and seminal receptacle until fertilization. Oocytes are fertilized as they pass from the oviduct to the bursa, and the eggs are then laid by the female on suitable substrates that provide nutrients to the larvae that hatch after 24 hours of embryonic development. Sperm stored in the female reproductive organs are viable and capable of fertilization for up to 20 days after mating, and their long-term viability is thought to dependent on nutritional sources provided by the female reproductive organs and the male seminal fluid that is transferred during the mating (Loppin et al., 2015).

While Glucose-6-Phosphatase (G6Pase) is crucial for glucose metabolism and homeostasis in mammals, its function in insects has remained enigmatic. G6Pase is a key enzyme in the liver during times of nutrient deprivation by hydrolyzing glucose-6-phospate in gluconeogenesis and glycogenolysis to generate glucose and maintain blood glucose homeostasis. Insects, including *Drosophila*, utilize trehalose as their primary circulating sugar, and they convert g-6-p to trehalose using the enzyme trehalose phosphate synthase (TPS) (Matsuda et al., 2015). Surprisingly however, many dipteran insects have maintained a *G6P* gene in their genome (Miyamoto and Amrein, 2019, 2017), and we recently reported that *G6P* has a novel, non-canonical function in *Drosophila* peptidergic neurons to facilitate neuropeptide release (Miyamoto et al., 2024). During these investigations, we uncovered a novel, non-canonical function of G6Pase in Drosophila male fertility. G6P homozygous mutant males are sterile, despite exhibiting normal courtship behavior and mating patterns.

Here, we show that G6Pase is a metabolic regulator during spermatogenesis. We find that sterile *G6P* mutant males exhibit no deficit in courtship and mate with the same vigor as wild type control males, implying that peptidergic neurons expressing G6Pase are not the cause of male sterility due to aberrant male mating behavior. Instead, and consistent with single cell RNAseq analysis (Lu et al., 2023), we find that G6Pase is expressed in spermatocytes during spermatogenesis. While sperm differentiation appears to proceed normally and *G6P* mutant males transfer sperm to females during mating just like wild type males, tail beat frequency of *G6P*$^{-}$ sperm stored in females is severely diminished, and *G6P*$^{-}$ sperm are depleted sooner from the female reproductive tract than wild type sperm. Cell biological investigations reveal that the endoplasmic reticulum (ER) in spermatocytes of *G6P* mutant males is more expanded and under increased ER stress, and their Golgi is diminished compared to wild type spermatocytes. Moreover, we find reduced amounts of the cell membrane protein Sneaky on the acrosome of *G6P*$^{-}$ sperm, a component necessary for sperm egg interaction and essential for the breakdown of the sperm plasma membrane after fertilization. Lastly, we show that testes of *G6P* mutant males have severely impaired metabolic activity and ATP production when compared to wild type testes. Based on these observations and the phenotypic similarities to *G6PC3* mutations in neutrophils of mammals, we propose that *Drosophila* G6Pase is a metabolite repair enzyme

necessary to remove a non-canonical metabolite, and that the accumulation of such metabolite in *G6P* mutant spermatocytes interferes with glycolysis and ATP production critical for the high energy demands during spermatogenesis and sperm cell differentiation.

RESULTS

G6Pase is expressed in male germ cells and required for male fertility

While constructing strains to characterize *G6P*'s function in peptidergic neurons, we noticed that wild type females failed to produce progeny when mated to homozygous *G6P* mutant males, while homozygous *G6P* mutant females were fully fertile when mated to wild type males. Because *G6P-GAL4* is expressed in several different subsets of neurosecretory cells in the brain and thoracic ganglion, including cells producing NPF, PDF, Orcokinin, FMRFamide and Nplp 1, and having established that G6Pase facilitates NP release from at least some of these neurons (Miyamoto et al., 2024), we wondered whether male sterility was associated with impaired male courtship and mating (Figure 2). Thus, we mated single *Y/yw; G6P^{-} /G6P^{-}* males, *Y/yw* control males and *G6P$^{-/-}$* males carrying a genomic rescue construct (*Y/yw; G6P^{-}/G6P^{-}; G6P_rescue/+*) with *G6P* wild type females and measured mating latency and mating time (Figure 2A). By these criteria, *G6P* mutant males and wild type control males were equally efficient in courtship and mating. We next quantified male fertility by measuring oviposition rate and hatching rate of eggs laid by females mated to the three types of males (Figure 2B, Supplementary Table 1). Regardless of the male genotype, females laid about the same number of eggs, but less than 2% developed when mated to *G6P* mutant males, compared to more than 80 % when mated to control males (Supplementary Table 1). Providing a genomic *G6P* transgene to *G6P$^{-/-}$* fully rescued fertility (Figure 2B). Together, these findings show that *G6P* plays no role in male courtship and mating behavior and is not required to increase mating-induced egg production in females.

Single cell transcriptome analysis (Lu et al., 2023) indicates that *G6P* is expressed in spermatogonia and spermatocytes (Figure 1B). To confirm that G6Pase is expressed in the male germline, we generated an antibody against a G6Pase peptide and carried out immunostaining experiments of testes from wild type, *G6P* mutant and *G6P* rescue males (Figure 1C-1E). G6Pase

is present in spermatogonia and spermatocytes, while no immunoreactivity is observed in later stages of spermatogenesis or in somatic cells of testes (Figure 1C). Importantly, no staining is observed in *G6P* mutant males, while staining was restored in spermatogonia and spermatocytes of fertile *G6P* mutant males carrying a genomic *G6P* rescue construct (Figure 1D and 1E). Together, these data indicate that *G6P* plays an essential role during early spermatogenesis. Of note, inspection of testes from *G6P* mutant males revealed no obvious defects, with clearly visible accumulation of spermatid and mature sperm (Figure 1C-1E and Supplementary Figure 1).

$G6P^-$ Sperms are transferred efficiently during mating, but move and survive poorly in the female reproductive tract

We next examined whether *G6P* mutant males exhibit phenotypes at later stages in spermatogenesis, including sperm production, sperm transfer and motility, and survival in the female reproductive organs. We introduced *don juan-GFP* (*dj-GFP*), a marker encoding a green fluorescent tagged flagellar protein that localizes to the sperm tail (Santel et al., 1997), into the *G6P* mutant strain, which permits quantitative visualization of spermatids and mature sperm in males and females after mating (Figure 3). *dj-GFP* labelled sperm is abundant in seminal vesicle of wild type and $G6P^{-/-}$ males (Figure 3B and 3C), with the latter showing a very modest reduction in sperm counts compared to wild type (Figure 3D-F). Both wild type and *G6P* mutant males transfer to *dj-GFP* sperm efficiently during mating (Figure 3H-3I). When we examined the eggs for the presence of *dj-GFP* sperm, we found that 85% of eggs laid by females mated to wild type males showed GFP signals, while only 8 % of eggs laid by females mated to *G6P* mutant males showed GFP signals (Figure 3J). However, these eggs, in contrast to eggs fertilized by wild type sperm, fail to develop, and DAPI staining identifies an elongated nucleus typical of that of sperm cells, indicating a failure in the breakdown of the sperm cell membrane (Supplementary Figure 2). Together, these observations indicate that mating and sperm transfer occurs normally in *G6P* mutant males, but most of their sperm is unable to enter an egg.

***G6P* mutant sperm have severely reduced tailbeat frequency**

Key factors for successful fertilization are the sperms' motility and long-term survival once transferred to the female reproductive tract. Fertilization requires the sperm to sense and move towards the micropyle located between the dorsolateral appendages at the anterior tip of the egg. Given that sperm transfer is not impeded during mating with *G6P* mutant males, but *G6P*$^{-}$ sperm are severely compromised in entering the egg (Figure 3D and 3J, respectively), we wondered whether sperm motility and survival were affected. We first measured tail beat frequency of wild type and *G6P*$^{-}$ sperm in the male (seminal vesicle), which revealed a vigorous tail beat activity for both types of sperm, albeit *G6P*$^{-}$ sperm had a small reduction (Figure 4A). Tail beat frequency in the spermathecae of females measured immediately after mating remained similar for wild type sperm, but was dramatically reduced in *G6P*$^{-}$ sperm, from 20 Hz to about 3 Hz (Figure 4B). Moreover, long term storage and/or survival of *G6P*$^{-}$ sperm in spermathecae and seminal receptacles was reduced. Specifically, abundance of *G6P*$^{-}$ sperm was already reduced after 5 days and almost completely depleted after 10 days in spermathecae, and 15 days after fertilization, neither spermathecae nor seminal receptacle contained any sperm (Figure 3C and 3D). In contrast, wild type sperm remained undiminished in spermathecae throughout 15 days after mating, and remained also abundantly present in the seminal receptacle for at least 10 days. Taken together, these observations indicate that *G6P* mutant males produce and transfer sperm in abundance during mating. However, once in the female reproductive tract, *G6P*$^{-}$ sperm rapidly lose motility, fail to enter the egg and are more rapidly depleted from the female storage organs when compared to wild type sperm.

Loss of *G6P* disrupts organelle reorganization during spermatogenesis

Drosophila spermatocytes undergo impressive growth during meiosis. They increase in size by about 25-fold in the G2 phase of the cell cycle (meiotic prophase), becoming larger than most somatic cells (Lindsey and Tokuyashu, 1980). Furthermore, extensive, energetically costly reorganization of organelles, such as the mitochondria, Endoplasmic Reticulum (ER) and Golgi, is necessary to facilitate the cellular transformation that occurs during spermiogenesis, from large spherical spermatocytes to the elongated soma with and exceedingly long tail (up to 1.8 mm) of mature sperm. During this physical transformation, the ER is broken down, mitochondria are

relocated to the mid piece where they fuse into the mitochondrial sheet powering the sperm tail during fertilization, and the Golgi compartment is mostly eliminated, with the remainder moved to the apical tip to form the acrosome (Fabian and Brill, 2012; Fitch and Wakimoto, 1998; Wilson et al., 2006). Thus, we assessed whether loss of *G6P* had any effect on this reorganization, with a focus on ER and Golgi, since this transformation relies heavily on RNA and protein synthesis. Consistent with mammalian G6Pases, which have been shown to reside in the ER (Burchell et al., 1994), we find that in *Drosophila* spermatocytes, G6Pase is perinuclear and co-localizes with the ER resident protein calnexin (Figure 5A). However, in spermatids and sperm, G6Pase is no longer detectable. Intriguingly, when we compared calnexin distribution, reflecting of the size of the ER, we noted a significant expansion in mutant spermatocytes when compared to wild type (Figure 5B). Since calnexin is upregulated under ER stress, we examined whether this expansion is associated with increased ER stress. Indeed, immunostaining of spermatocytes showed a significant total increase of calnexin in *G6P* mutants when compared to wild type (Figure 5C). We also quantified splicing of *XBP1* mRNA, which is increased during stress to generate more XBP1 protein which plays a key role in the Unfolded Protein Response (URP) (Cairrão et al., 2022; Uemura et al., 2009). Comparison between mRNA isolated from testes of wild type and *G6P* mutant males revealed that spliced *XBP1* mRNA is significantly increased in *G6P* mutants at the expense of non-spliced *XBP1* RNA (Figure 5D). Additionally, Binding immunoglobulin Protein (BiP), a UPR chaperone (Bertolotti et al., 2000), is also upregulated in *G6P* mutant spermatocytes (Supplementary Figure 3).

We next visualized the Golgi in wild type and *G6P* mutant spermatocytes using an antibody against the Golgi Matrix protein 130 (GM130) and found *G6P* mutant spermatocytes had a severely reduced number of Golgi compartments (Figure 6A and 6B), similar to peptidergic neurons of *G6P* mutant flies (Miyamoto et al., 2024). The Golgi gives rise to the acrosome, which is thought to be involved in sperm-egg (i.e. micropyle) recognition (Intra et al., 2006; Loppin et al., 2015; Perotti and Riva, 1988). To assess acrosome integrity, we crossed a *sneaky-GFP* (*snky-GFP*) transgene, which encodes a membrane protein localized to the acrosome (Wilson et al., 2006), into the *G6P* mutant strain. Indeed, the acrosome of *G6P$^-$; snky-GFP* sperm exhibited less GFP immunofluorescence compared to *GFP$^+$; snky-GFP* sperm (Figure 6C and 6D). In summary,

G6P mutant spermatocytes show an expanded ER exhibiting elevated stress, and a concomitant reduction of the Golgi and partial loss of SNEAKY, an integral transmembrane protein of the acrosome that is essential during and after fertilization (Fitch and Wakimoto, 1998; Wilson et al., 2006).

Testes of *G6P* mutant males exhibit reduced energy metabolism

Our studies have shown that *G6P*$^{-}$ sperm are incapable of fertilizing eggs due to severely compromised motility and reduced survival in spermathecae and seminal receptacle. The loss of motility suggests that *G6P*$^{-}$ sperm have an energy deficit, likely caused by increased ER stress and a diminished Golgi. To investigate potential metabolic dysfunction in *G6P* mutant male testes, we conducted ex vivo energy consumption measurements using a Seahorse XF analyzer (Agilent Technologies). We observed a >60% reduction in both basic and compensatory glycolysis when compared to wild type testes (Figure 7A), demonstrating an impaired ability to upregulate glycolysis when mitochondrial respiration is inhibited. Moreover, G6P mutants exhibit impaired mitochondrial function, with significantly reduced basal respiration, spare respiratory capacity and ATP production (Figure 7B). Reduced ATP levels in testes of G6P mutant males were independently confirmed by measuring total ATP levels using the ATPLite system (PerkinElmer), which revealed that *G6P* mutants produced only about 50% ATP when compared to testes of wild type males or G6P$^{-/-}$ males carrying the genomic rescue transgene (Supplementary Figure 5). Collectively, these results imply that sterility of *G6P* mutant males is caused by severe metabolic deficiencies in spermatocytes that impact both glycolytic and mitochondrial energy production pathways in the differentiation of mature sperm.

DISUCSSION

In this paper, we established a novel function for the metabolic enzyme G6Pase in *Drosophila* male fertility, in addition to its role we reported earlier as a facilitator in Neuropeptide release from several types of peptidergic neurons in the CNS (Miyamoto et al., 2024). We first considered the possibility that male sterility had a behavioral cause, whereby reduced

neuropeptide signaling would interfere with proper male courtship and mating behavior. However, *G6P* mutant males court and mate normally, and they produce and transfer sperm during copulation just like wild type males. Analysis of spermatogenesis established that *G6P* has a cell autonomous function in spermatogonia and spermatocytes that is necessary for proper organelle reorganization essential to produce capacitated sperm. Specifically, we showed that the *G6P* gene is expressed during early spermatogenesis and G6Pase protein is present in the ER of spermatogonia and spermatocytes. Second, we found that *G6P* mutant spermatocytes show ER stress and a compromised Golgi apparatus, and that the acrosome of *G6P*$^{-}$ sperm have diminished amounts of the essential membrane protein SNEAKY. Third, *G6P*$^{-}$ sperm are much less motile and vigorous after mating compared to wild type sperm, and their presence in two main female storage organs, the spermathecae and the seminal receptacle, is shortened. And fourth, testis of *G6P* mutant males have a sharply reduced energy metabolism.

G6Pases have mainly been studied in mammals, particularly in the context of gluconeogenesis and glycogenolysis in the liver and kidney mediated by *G6PC1*. There, G6Pase converts glucose-6-phosphate to glucose, which is released into the blood stream to ensures systemic glucose homeostasis during times of limited nutrient availability. Patients with mutations in *G6P1C* suffer from glycogen storage disease type I and present with hypoglycemia and hyperlipidemia, and eventually with hepatomegaly due to glycogen accumulation in the liver(Chou et al., 2015). In addition to *G6PC1*, mammals have two additional G6Pase encoding genes. *G6PC2* is mainly expressed in in the pancreas and has been associated with cell-mediated autoimmunity, and patients with a mutation in *G6PC2* and *G6PC2* knock out mice have lower fasting blood glucose levels, due to increased glycolysis in β-cells (Bosma et al., 2020). Lastly, *G6PC3* is broadly expressed in many tissues, but it has mainly been studied in neutrophils, where congenital *G6PC3* deficiency leads to neutropenia due to failure to clear cells from 1,5-anhydroglucitol phosphate (1,5AG6P; see below)(Veiga-da-Cunha et al., 2019). Interestingly, while all three mammalian G6Pase enzymes can hydrolyze g-6-p, their catalytic activities and substrate specificities differ, and consequently, so do their specific roles in metabolism (Morciano et al., 2021). We note that while *Drosophila* G6Pase can convert amino acids via g-6-p to glucose

in peptidergic neurons in the brain (Miyamoto and Amrein, 2019), it remains to be seen whether g-6-p is the main substrate for this enzyme in either peptidergic neurons or spermatocytes.

A non-canonical function for *Drosophila* G6Pase in the male germ line

Drosophila G6Pase in spermatogenesis and neurosecretory cells in the CNS is unlikely used for systemic glucose production, as is the case for hepatic G6Pase in mammals. Neurosecretory cells and spermatocyte perform energy consuming tasks – production of large amounts of neuropeptides and neural signaling in the CNS, proliferation, meiosis and cellular and organelle reorganization in the case of spermatogenesis, and hence are not equipped to engage in energy consuming processes of glucose production for the benefit of systemic glucose homeostasis. Moreover, other genes encoding enzymes required for either gluconeogenesis (1,6-bisphosphatase and phosphoenolpyruvate carboxykinase) or glycogenesis and glycogenolysis (glycogen synthase, debranching enzyme, glycogen phosphatase) are not expressed in the spermatocytes (Supplementary Figure 5) (Lu et al., 2023), which further supports a non-canonical function of *Drosophila* G6Pase in the male germ line.

We propose that *G6P* in spermatocytes has a role like *G6PC3* in neutrophils. Neutrophils lacking *G6PC3* are sensitive to the accumulation of 1,5-Anhdrogucitol (1,5AG), a glucose analog and common monosaccharide present in most foods. 1,5AG enters cells via glucose transporters, where it is phosphorylated by hexokinase to 1,5AG6P. However, 1,5AG is a non-metabolizable sugar that must be cleared from cells via G6Pase by converting it back to 1,5-Anhdrogucitol (1,5AG), and then exported via glucose transporters and excreted through the kidneys. In neutrophils of *G6PC3*$^{-/-}$ patients, 1,5AG6P accumulates and disrupts glycolysis, leading to the well-known cellular phenotypes, including ER stress, reduction of glycolytic metabolites such as lactate, and glycosylation deficits (Hayee et al., 2011; Jun et al., 2011, 2010), ultimately resulting in loss of neutrophils (Veiga-da-Cunha et al., 2019). Of note, *G6PC3* deficiency has also been associated with numerous additional pathologies, including urogenital malformations, heart defects, and learning disabilities (Banka et al., 2011; Boztug and Klein, 2009), suggesting that other tissues and organs are sensitive to 1,5-AG6P accumulation (Dienel, 2020). Thus, G6Pase encoded by *G6PC3* functions as a metabolite repair enzyme, like several other enzymes, in the

glycolysis pathway (Bommer et al., 2020). Thus, metabolite repair enzymes are necessary to eliminate non-canonical products that occur during glycolysis due to substrate promiscuity inherent to most glycolytic enzymes, including hexokinase (Veiga-da-Cunha et al., 2019). Based on the similarity of the cellular phenotypes between *G6P*$^{-/-}$ spermatocytes and *G6PC3*$^{-/-}$ neutrophils (ER stress, reduced Golgi compartments), and the severe disruption of glycolysis in testes of *G6P* mutant males, we suggest that failure of *G6P*$^{-}$ sperm to fertilize eggs is brought about by a non-metabolizable compound that interferes with glycolysis in male germ cells. Of note, the glucose transporter GLUT3 is robustly expressed in spermatocytes and spermatids (Lu et al., 2023), and hence, this transporter might participate as a metabolic repair protein, similar to the glucose transporter expressed in neutrophils *G6PC3*$^{-/-}$ neutrophils. It remains to be investigated what kind of sugar compound might be the cause for these defects in *Drosophila* spermatocytes.

Cascading effects *G6P*$^{-/-}$ spermatocytes affect pre-and post-fertilization properties of sperm

Regardless of the specific mechanism through which G6Pase functions to produce capacitated sperm, its loss has severe consequences on sperm metabolism, evidenced by the reduced metabolic activity in testes and the loss of sperm motility in the female reproductive organs. While impaired metabolism is likely a main reason for male sterility, failure to reorganize the cellular organelles during spermatogenesis may contribute to additional deficits that lead to a failure in sperm capacitation. For example, the severely diminished Golgi and deficits of important acrosome associated proteins might impede the post translational modification of proteins involved in secretory pathways and glycosylation.

We note that the acrosome in *Drosophila* sperm has essential roles, albeit different ones than the mammalian acrosome which is essential during sperm capacitation by facilitating the fusion of sperm and oocyte membranes (Okabe, 2018). In *Drosophila* and probably other insects, membrane fusion does not occur, as sperm enter an oocyte fully intact through the micropyle. It is not known, what specific role the acrosome plays during this process (see below), but it is essential in several processes after sperm entry for successful fertilization and initiation of development. Specifically, in eggs inseminated by *snky*$^{-}$ sperm, the male nucleus fails to

decondense and form a functional pronucleus, leading to failure in initiating embryonic development (Fitch and Wakimoto, 1998; Wilson et al., 2006). Interestingly, *snky*$^{-}$ inseminated eggs showed reduced sperm membrane permeability, indicating that the acrosome membrane protein SNEAKY is necessary to break down the sperm membrane after fertilization.

Additional roles for the acrosome during sperm entry is indicated by acrosome associated glycosylation enzymes (Perotti and Pasini, 1995). These modifications appear to be complementary with sugar residues mapped to the micropyle of the oocyte, suggesting interactions between the two entities (Intra et al., 2006; Perotti and Riva, 1988). Evidence that glycosylation is critical for sperm-egg interaction has been obtained from *Casanova* mutant males, which produce mature motile sperm that are, however, unable to fertilize the egg (Perotti et al., 2001). *Casanova* mutant sperm have only half of the β-hexosaminidase activity when compared to wild type sperm, with none of it on the membrane overlaying the acrosome itself. Indeed, examining single cell expression data in the male reproductive tract of key enzymes of the hexosamine biosynthetic pathway (HBP) shows that they are mainly expressed in spermatocytes (Supplementary Figure 6). Thus, it will be interesting to see whether the diminished Golgi of *G6P* mutant spermatocytes affects expression of HBP pathway enzymes in a manner similar to neutrophils of humans with mutations in *G6PC3*, which are highly defective in glycosylation (Hayee et al., 2011).

FIGURES

Figure 1: Expression of G6Pase in spermatogenesis

A) Diagram of Spermatogenesis and Spermiogenesis: in Males have approximately 60 to 100 undifferentiated germline stem cells (GSCs) located at the apex of each testis. GSCs divide and produce a primary spermatogonial cell (spermatognium) and another GSC. Spermatogonia undergo four synchronized mitoses, each producing 16 secondary spermatogonia, which grow into larger primary spermatocytes. These spermatocytes undergo synchronized meiosis, generating 64 spermatids, which enter spermiogenesis

and transform into mature sperm. Late spermatocytes and spermatids undergo extensive organelle reorganization that is necessary to differentiate into highly specialized sperm.

B) Single cell *G6P* RNA expression analysis derived from the "Aging Fly Cell" Atlas (Lu et al., 2023). *G6P* mRNA is abundantly present in spermatocytes, but absent in Late elongated spermatids, mature sperm or somatic testis tissue. Tissues/cells are positioned from high (left most) to low (right most) in the graph. Spermatocyte and spermatid cell groups with highest level of expression are shown in green and pink, respectively, while all other cell/tissue types are shown in blue.

C-E) G6Pase protein expression in wild type males (C), $G6P^{-/-}$ males (D) and $G6P^{-/-}$ males containing a genomic rescue construct (E) is visualized using immunostaining with a polyclonal antibody against G6Pase. Immunoreactivity (green) is observed in spermatogonia and spermatocytes of wild type and rescue males, but not in $G6P^{-/-}$ males. Note that G6Pase is not detected in germ stem cells and spermatids.

Figure 2: Sterile *G6P* mutant males exhibit normal mating behavior

A) Male courtship was quantified by assessing latency time (x; time from introducing the male and female into the mating arena to successful mounting) and duration of mating (filled circles). No difference was observed between $G6P^{-/-}$ and $G6P^{+/+}$ males, or mutant males carrying a genomic transgene (*G6P_rescue*), mated to mated to *yw* virgin females. n=20.

B) Females mated to either $G6P^{-/-}$ males and females mated to $G6P^{+/+}$ control males lay the same number of eggs/female per day. Less than 2% of larvae hatched from eggs sired by $G6P^{-/-}$ males, whereas approximately 85 % of larvae hatched from eggs sired by control males. Fertility is rescued fully in $G6P^{-/-}$ males carrying a genomic *G6P* rescue construct. n=20. See also Supplementary table 1.

Figure 3: *G6P* mutant males produces and transfer sperm to females during mating

A-C) Sperm storage in males: (A) shows a diagram of the male reproductive system: T=testis, SV=seminal vesicle, mAG: male accessory gland, EB: ejaculatory bulb. Visualization of

sperm carrying a dj-GFP transgene in wild type (B) and *G6P* mutant males (C) shows stored sperm in the seminal vesicle.

D-F) Spermatids present in testis of wild type (E) and *G6P* mutant (F) males. Elongated spermatids and mature sperm in the seminal vesicle were stained with Hoechst, which stains the elongated sperm nucleus. Sperm count (D) shows a slight reduction of in *G6P* mutants. n = 8 seminal vesicles, asterisks indicates ** $p<0.01$, using unpaired Mann-Whitney t-student, error bars represent SE.

G-H) Sperm transfer to females: (G) shows organization of the female reproductive system: OD = oviduct, U = uterus, ST = spermathecae, SR = seminal receptacle, fAG = female accessory gland); Wild type female reproductive organs (H) inseminated by sperm from control (*Y/yw; G6P$^+$/G6P$^+$; dj-GFP/dj-GFP*; left) or (I) *G6P* mutant males (*Y/yw; G6P$^-$/G6P$^-$; dj-GFP/dj-GFP*; right) is efficiently transferred to females and stored in spermatheca (*) and the seminal receptacle (red squares).

J) Sperm from homozygous *G6P* mutant males (*Y/yw; G6P$^-$/G6P$^-$; dj-GFP/ dj-GFP*) rarely enter the egg laid by mated females after successful mating, while sperm from control males (*Y/yw; G6P$^+$/G6P$^+$; dj-GFP/dj-GFP*) do so very efficiently. n = 40 seminal vesicles, asterisks indicates **** $p<0.0001$, using unpaired Mann-Whitney t-student, error bars represent SE.

Figure 4: *G6P* sperm exhibit severely reduced sperm tail beat and survival

A-B) Tail beat frequency of *G6P$^+$* and *G6P$^-$* sperm dissected from male seminal vesicle before mating (A) or dissected from seminal receptacle (B) n =30 , asterisks indicates **** $p<0.0001$, using one-way ANOVA with the Tukey post hoc test.

C) Survival of control sperm (top line, *Y or y w; G6P$^+$; dj-GFP*) and *G6P* mutant sperm (bottom line, *Y or y w; G6P$^-$; dj-GFP*) in the female reproductive organs after mating (0 days), or 5, 10 and 20 days after mating. *G6P* mutant sperm is depleted sooner in both sperm storage organs, compared to wild type sperm.

D) Total average *GFP* fluorescence derived from sperm of *Y or y w; G6P^{+}; dj-GFP* (blue) and *Y or y w; G6P^{-}; dj-GFP* (green) sperm in female reproductive tract immediately and 5, 10 and 15 days after mating. n=5

Figure 5: G6Pase protein expression and Endoplasmic Reticulum stress in spermatocytes lacking *G6P*

A) Anti-G6Pase antibody staining reveals perinuclear association with and co-localization with calnexin in the ER. DNA was visualized using Hoechst.

B and C) ER is enlarged in *G6P$^{-/-}$* spermatocytes, with concomitant elevation of calnexin levels. DNA was visualized using Hoechst in B. Quantification of calnexin is sown as Corrected total cell fluorescence (CTCF) using Image J.

D) *G6P$^{-/-}$* spermatocytes show ER stress evident from increased splicing of the transcription factor encoding *XBP1* mRNA and increased amount of Binding immunoglobulin Protein (BIP). Splicing was quantified by RT-PCR using RNA isolated from whole testes of wild type and *G6P$^{-/-}$* males non-spliced (XBP1-T) and spliced (XBP1-S) was normalized and compared to wild type (dotted line).

Figure 6: Loss of *G6P* leads to a reduction of Golgi in spermatocytes and the acrosome membrane associated protein SNEAKY

A) *G6P$^{-/-}$* spermatocytes (top, nuclei stained with Hoechst in blue) show a significant reduction of Golgi compartments, visualized using an antibody against the Golgi marker protein GM130 (red), in spermatocytes compared to wild type spermatocytes(bottom). Images to the right show higher magnification of a single spermatocyte.

B) Quantification of the average number of Golgi compartments/spermatocyte, determined by counting the cells and Golgi compartment of representative field image areas (n) as shown in A. n = 5, asterisks indicate ** $p<0.01$, using unpaired Mann-Whitney t-student, error bars represent SE.

C) *G6P*$^{-}$ sperm show reduced GFP-tagged SNEAKY protein in the acrosome, compared to wild type sperm. Top image shows sperm bundle in a cyst. Bottom images show free, mature sperm.

D) GFP intensity shown as RFU (relative fluorescence units)/sperm measured from representative images of dissected testes (n) using Image J. N = 5, asterisks indicate ** $p<0.01$, using unpaired Mann-Whitney t-student, error bars represent SE.

Figure 7: Testes of *G6P*$^{-/-}$ males have severely reduced energy metabolism

Metabolic capacity of testes from wild type (blue) and *G6P*$^{-/-}$ (green) males was determined using a Seahorse XF analyzer (Agilent Technologies). *G6P* mutant flies carrying a genomic rescue construct (*G6P*$^{-/-}$; *G6P_rescue*) are shown in red.

A) Basic glycolysis is impaired in testes of *G6P* mutant males, and inhibition of the ETC using rotenone/antimycin A shows that compensatory glycolysis is also reduced.

B) Overall oxygen consumption is reduced in testes of *G6P*$^{-/-}$, with significantly diminished basal respiration, spare respiratory capacity and ATP production. Note that *G6P* is not expressed in somatic tissues of testes, suggesting that germ line accounts for the energy deficit.

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METHODS

Drosophila Stocks and Husbandry: *Drosophila* stocks were maintained in 12 hour light/dark cycle on standard corn meal food in plastic vials at 25° C. The *y* w^{1118} strain was used as wild type control. Fly stocks used were: The *G6P* mutant strain ($G6P^{MIC}$) and the *dj-GFP* transgenic strain was generated from the Bloomington stock center (#57904) by back-crossing it *y* w^{1118} and removing the *protamineB-eGFP* transgene. The *dj-GFP*/*dj-GFP*; $G6P^{-}/G6P^{-}$ strain was generated by combing the $G6P^{MIC}$ allele (chromosome 2) and the *dj-GFP* transgene (chromosome 3), balanced with *GyO* and *TM3 Sb*.

Molecular Cloning: To generate the genomic *G6P* rescue transgene, a 4,925 base pair long fragment was amplified from genomic DNA including the entire coding sequence with introns, 3,109 base pairs upstream of the ATP start codon and 500 base pairs downstream of the stop codon using a forward primer (5'-GGACTAGCGGCCGCCTACTGCTCCACAAGAAGGAGATGACTT -3') and a reverse primer (5'- ACCTTAGGTACCTCCCATCATGGCTATCACGCGCTGCGAA -3'). NotI and KpnI (underlined) restriction enzyme sites were incorporated into primers for directional cloning. The PCR product were then subcloned into pCaSpeR transformation vector (Addgene). Transgenic fly lines were generated by a standard procedure with P-element mediated transformation of w^{1118} embryos (BestGene Inc., Chino Hills, CA).

Statistical analysis: Statistical significance was assessed using either the Student's t-test or a two-tailed Mann–Whitney U test for pairwise comparisons. For multiple comparisons, one-way ANOVA was applied with Tukey's post hoc test or the Kruskal–Wallis test with Dunn's post hoc test. Error bars represent the standard error of the mean (SEM), unless stated otherwise. Significance levels were denoted as $*P < 0.05$, $**P < 0.01$, with NS indicating no significance. All replicates were derived from distinct biological samples (different flies or cells). Image analysis was conducted using the NIS-Elements software suite, and statistical analyses were carried out with Real Statistics Add-Ins in MS Excel and GraphPad PRISM.

Fertility and mating test: All mating experiments were performed with males 4-7 days of age. Single pair matings were set up using a mating wheel. Naive males (collected and isolated within 24 hours after hatching) were kept in isolation for at least 3 days on standard food before used in mating assays. A single male was paired with a single virgin female (2 to 5 days old) in a mating chamber of the mating wheel. The latency time is defined as the time from the moment the male and female were introduced into the mating chamber until the male mounted the female. Mating or copulation time is defined as the time interval from mounting to dismounting the female.
To test for fertility, three females were crossed with three males for 48 h on a grape juice plate with yeast paste. After day 2, the males were removed from the chamber, and each female was transferred to a fresh grape juice plate with yeast paste. The number of eggs was counted after 24 h. The plates were then placed in a humidity chamber for the next 72 h, and the number of hatched larvae was recorded.

Immunostaining**:** Fly testes dissection, fixation, and staining were performed as described in Sitaram et.al. (*Cytological analysis of spermatogenesis: live and fixed preparations of Drosophila testes.*, 2014) with minor modifications. For the intact testes, a hydrophobic barrier pen (Super PAP Pen,71310, EMS) was used to border the testis and protect them from getting squished at the start of the process. 2-3 pairs of testes were dissected in 1x PBS and transferred to the glass microscope slide marked with a PAP pen. A coverslip glass was placed very gently on top of the preparation. For specific stages of cell staining, testes were dissected and transferred to a coverslip containing 20uL of PBS. Testes were cut open to retrieve spermatocytes. A glass slide was gently placed on top of the coverslip, and the excess PBS was removed using kim wipe, with ensuing pressure pushing spermatocytes out. The sample was immediately frozen in liquid nitrogen. Next, the coverslip was removed, and the glass slide was placed in cold 95% ethanol for 10 minutes in -20°C freezer. Samples were transferred to 4% Formaldehyde in PBS with 0.1% triton (PBST 0.1%) for 10 minutes, washed in PBS 3X each time for 5 minutes and then transferred to PBST for 45 minutes to permeabilize the cell membrane. Samples were washed again 3 times for 5 minutes each in PBS and blocked by immersing the slides in PBS with 1% BSA for 45 minutes and then incubated overnight in a 4°C fridge with primary antibody solution diluted in PBS

containing 1% BSA. The next day, preparations were washed once with PBST and twice with PBS for 5 minutes and then incubated with secondary antibodies diluted in PBS overnight at 4°C. The next day, samples were washed 3 times with PBS and incubated with Hoechst for 20 minutes to stain DNA/nuclei. Primary antibodies were chicken anti-GFP (Thermo Fisher Scientific), Rabbit anti-G6P (Boster Bio), Mouse Anti-CNX99A (DSHB Biology), Rabbit Anti-GM130 (abcam-ab30637). For DNA staining, either DAPI or Hoechst was used. The secondary antibodies that were used in this study were anti-chicken Alexa488 (Thermo Fisher Scientific), anti-rabbit Alexa Fluor 647 (abcam, ab150075), and anti-mouse Alexa 488 (abcam, ab150105). The polyclonal antibody against *Drosophila* G6Pase was generated using the peptide VREAFKWCPEPTTYLR (amino acid 248-263; Boster Bio).

Sperm tail-beat frequency analysis: For tailbeat frequency measurements, sperm was isolated from seminal vesicles 2 days after eclosion (males) or spermathecae (female) 1 day after mating, via dissection of reproductive organs in PBS. Reproductive organs were sliced open, and sperm was released into 20µl of PBS on a glass slide. Sperm motility video micrographs were taken on an Olympus FV3000 scanning confocal microscope using an Olympus 20x/0.75NA UPLSAPO air objective lens. Images were acquired by Olympus Fluoview Software with the transmitted detector in resonant scanning mode, imaging at 0.067 µSec/px, and pixel size of 0.211 µm x 0.211 µm. The total ROI was set to include the whole sperm tail and was aimed to be kept similar in dimension. 1000 frames were collected per image. 640 nm laser was used as the light source with a laser power of 0.55% and transmitted detector sensitivity of 272V. DIC prism and polarizer were included in the light path.

To measure sperm tail-beat frequency, .Oir time series were saved as .avi video clips. The frame per second (fps) was set, such as the number of frames divided by the length of the video. To measure the tail beat frequency, we followed the protocol by Hafezi and colleagues (Hafezi et al., 2024). Briefly, videos were analyzed using the ffmpeg plugin of FIJI (RRID:SCR_002285). The tail beat frequency of the fastest-beating sperm was measured by generating the kymograph. The number of beats and frames were counted for the sperm tail of interest. Beat frequency was measured using the below formula:

$$Beat\ Frequncy\ (Hz) = \frac{\#beats \times fbp}{\#frames}$$

Sperm Count in testes: Virgin males were separated after eclosion and kept isolated for 3 to 4 days, and the number of sperm in the seminal vesicle was counted using Imaris 10.1.1 software (RRID:SCR_007370) following the protocol of Hafezi and collaborators (Hafezi et al., 2024), with minor modifications. Briefly, testes were dissected, fixed, and stained for the nuclei using Hoechst. The entire seminal vesicle area were scanned, and accessory glands were excluded. Using the "Surfaces" function, sperm heads were detected using 1µm surface grain and sphericity set to less than 0.6).

Sperm Transfer: To assess sperm transfer after the mating was conducted as described by Köttgen et al. (Köttgen et al., 2011) with minor modifications. Briefly, virgin *dj-GFP*/*dj-GFP* (control) males and *dj-GFP*/*dj-GFP*; $G6P^{-}/G6P^{-}$ (experimental) males were kept separated for 24 to 36 hours before mating was set up to w^{1118} virgin females. Immediately after the mating, females were separated, and their lower reproductive tract was dissected in PBS. The ovaries and digestive tract were removed carefully. The reproductive tracts of females were transferred to a glass slide with 20µl of PBS. Real-time live imaging of fluorescently labeled sperm in females was performed using a Nikon T1 A1R inverted confocal microscope. Microscopic analysis was performed within two hour (0 days), 5, 10 and 15 days after mating termination for 10 females. Sperm entry into the eggs was assessed for virgin males (*dj-GFP* in a wild type and $G6P^{-/-}$ background). Males and females were left to mate for 24h, and after that, the females were left on grape juice agar plates to lay eggs for 1h. Every hour the flies were flipped and number of eggs were counted on the plate and they were transferred to the glass slide and checked for the presence of sperm in the embryo. The numbers were presented as percentages of GFP positive on each plate.

Seahorse Analysis: We used method described by Neville and colleagues to measure whole testes metabolism, with minor modifications (Neville et al., 2018). The testes were dissected as described for RNA extraction to measure the glycolytic rate. The software measures the

extracellular acidification rates of the media. 96 well Seahorse plates were twice coated with Cell-Tak (50µg/mL, Corning® Cell-Tak™ Cell and Tissue Adhesive, Cat. No. 354249). Testes were placed in the middle of the well and pushed down by forceps. To measure glycolytic rate proton efflux, we used the base medium without Phenol (Agilent, 103335-100), supplemented with HEPES (Agilent, 103337-100), 2mM Glutamine, 1mM sodium pyruvate, and 10mM glucose. Injection port A was used to supply 20µL of 50µM rotenone and antimycin-A. Injection port B was used to supply 22uL of 1M, 2-Deoxyglutarate. Analysis of glycolytic rate was done following Agilent guidelines (Agilent Technologies, Seahorse XF Glycolysis Stress Test Kit User Guide). After the assay, tissue was homogenized and lysed using default lysis buffer (50 mM Tris (pH 7.5), 125 mM NaCl, 5% glycerol, 0.2% IGEPAL, 1.5 mM $MgCl_2$, 1 mM DTT, 25 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA and 2 × Complete protease inhibitor (Roche, Indianapolis, IN) on ice for 10 min. The plate was then centrifuged, and the supernatant was collected.

Measurement of bulk ATP: Total ATP levels in testes were measured using the ATPlite luminescence assay system (PerkinElmer), a luciferase-based assay. Measurements were carried out as described by Nellas and colleagues (Nellas et al., 2022). *Drosophila* testes were dissected in PBS and transferred to the wells of flat bottom polystyrene assay plates (Costar® 3917) containing 100 ml of PBS (1 pair/well) . Samples were lysed by adding 50 ml of cell lysis solution and shaking at 700 rpm for 10 minutes. 50ml of luciferase substrate solution was added, shaking at 700 rpm for 5 minutes. After incubating in the dark for 10 minutes, luciferase activity was measured using a PerkinElmer EnVision plate reader. ATP concentration was calculated using a standard curve, generated through serial dilution of a 10 mM ATP stock solution.

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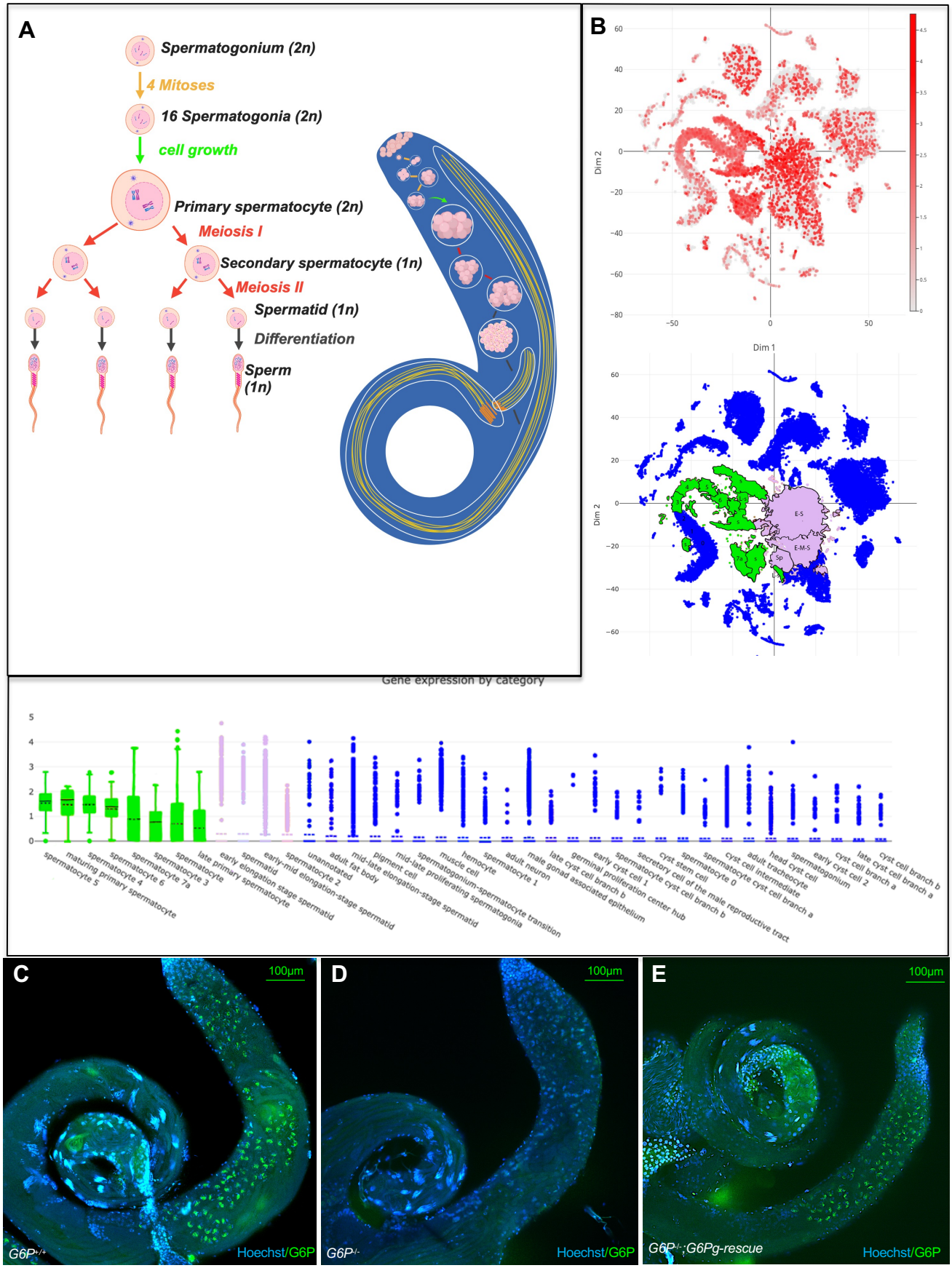
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Hoechst/G6P

Figure 1

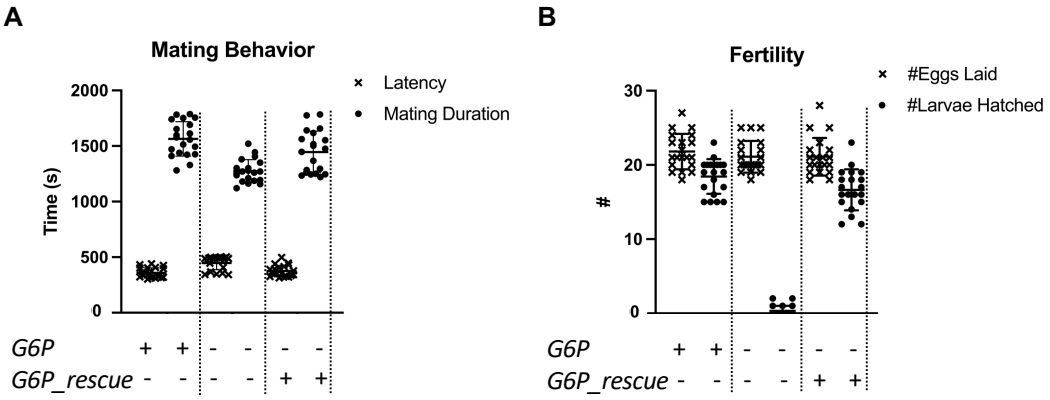
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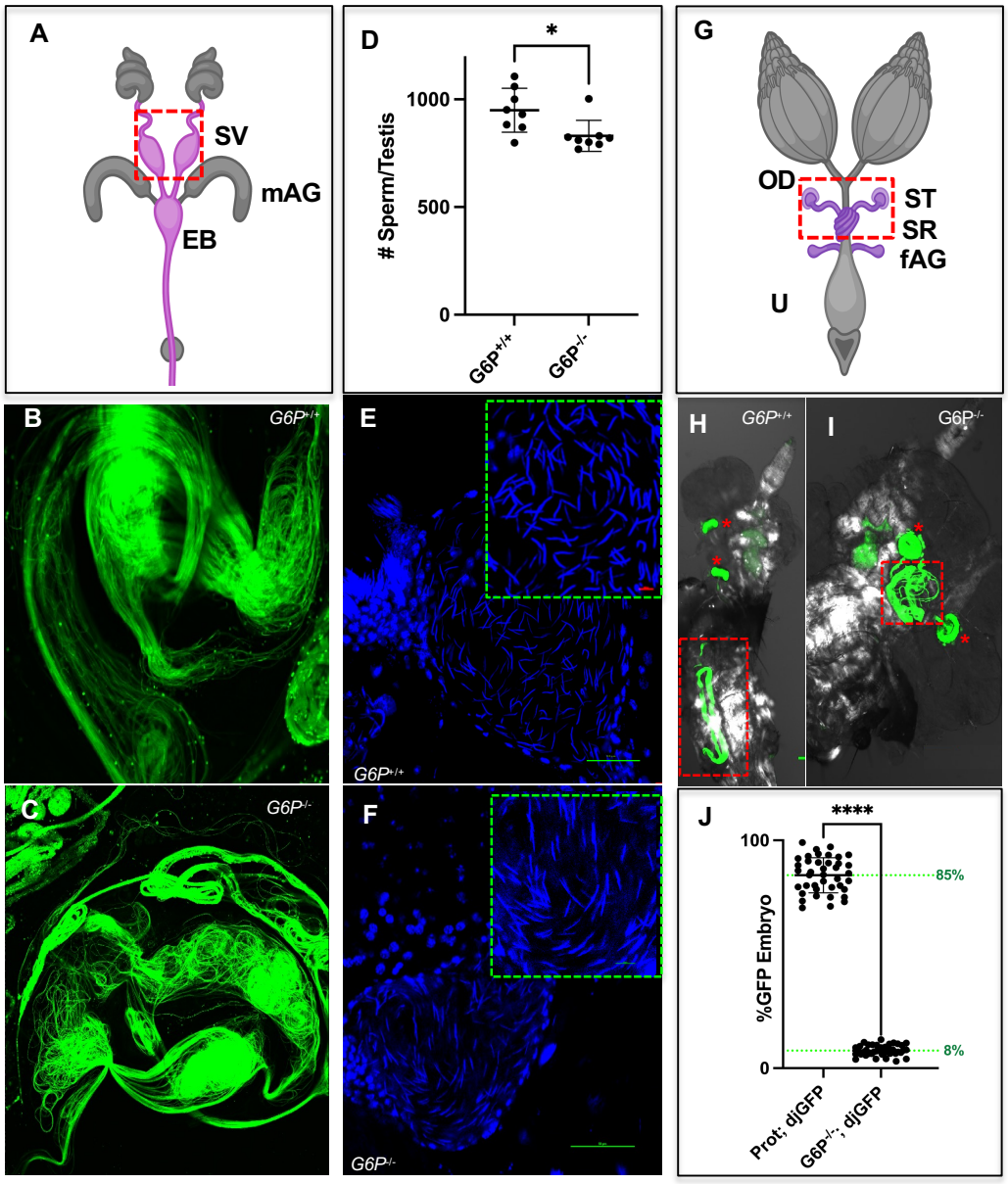
Figure 2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Prot; djGFP
G6P-/-; djGFP

Figure 3

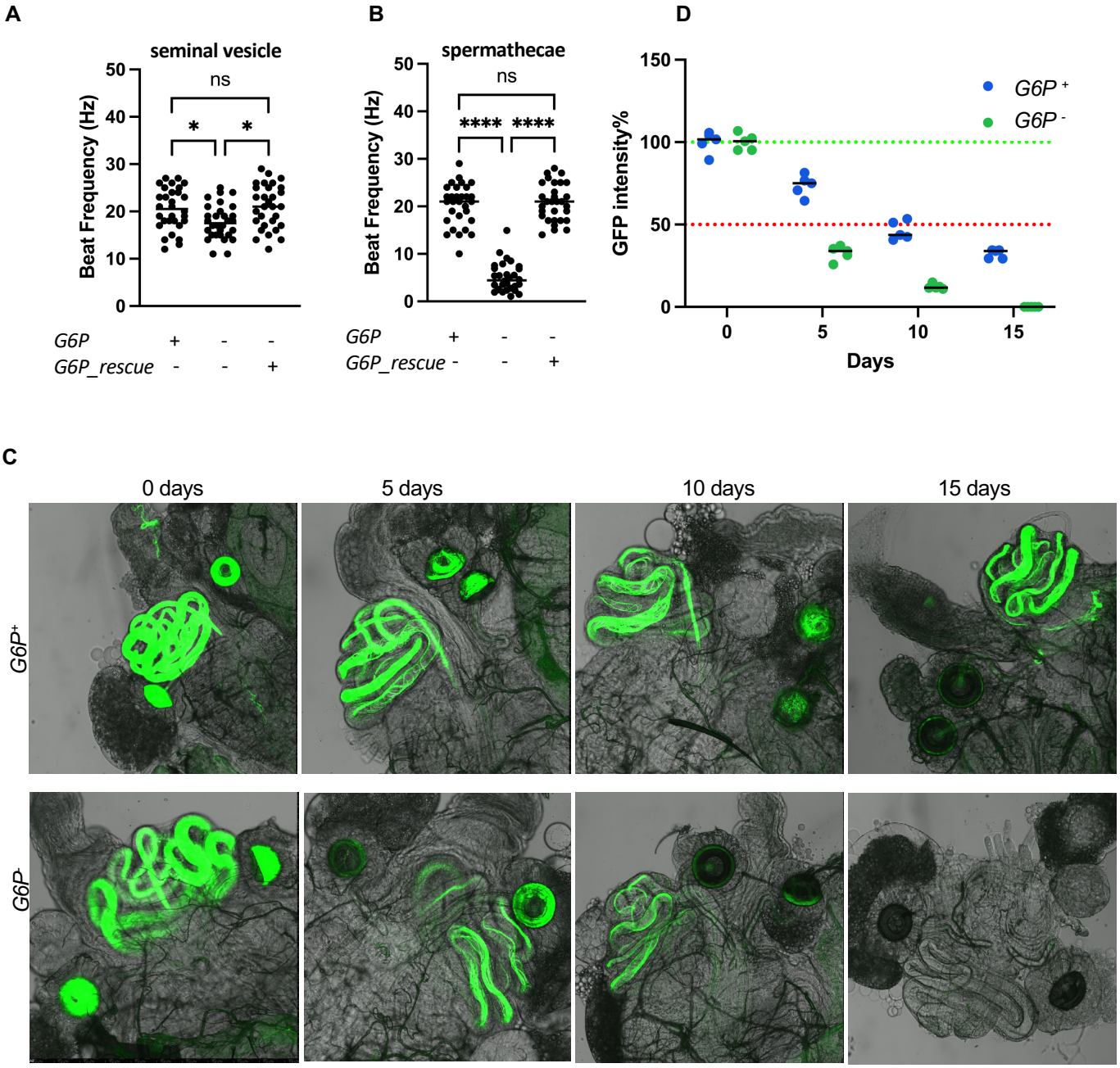
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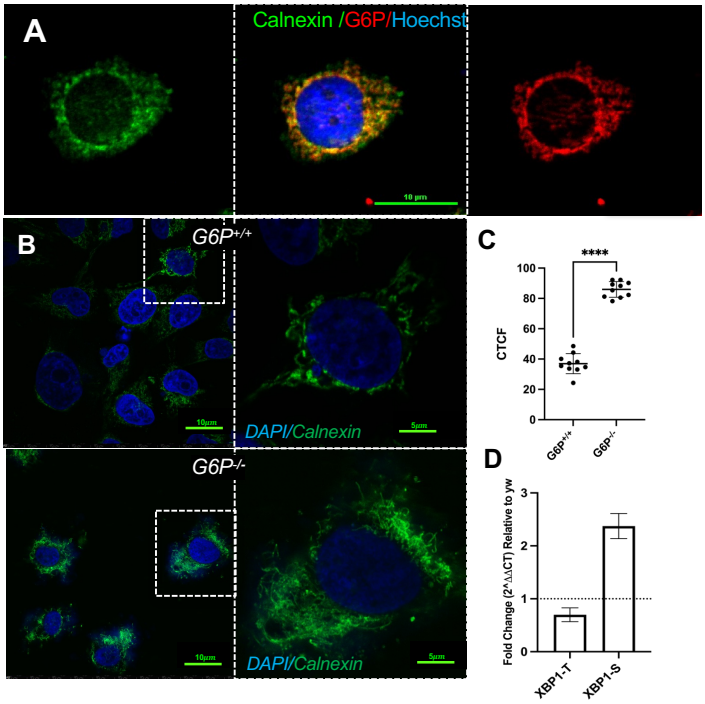
Figure 4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Calnexin /G6P/Hoechst
10 µm
B
G6P+/+
10µm
DAPI/Calnexin
5µm
G6P-/-
10µm
DAPI/Calnexin
5µm
C
****
CTCF
100
80
60
40
20
0
G6P+/+
G6P-/-
D
Fold Change (2^ΔΔCT) Relative to yw
3
2
1
0
XBP1-T
XBP1-S

Figure 5

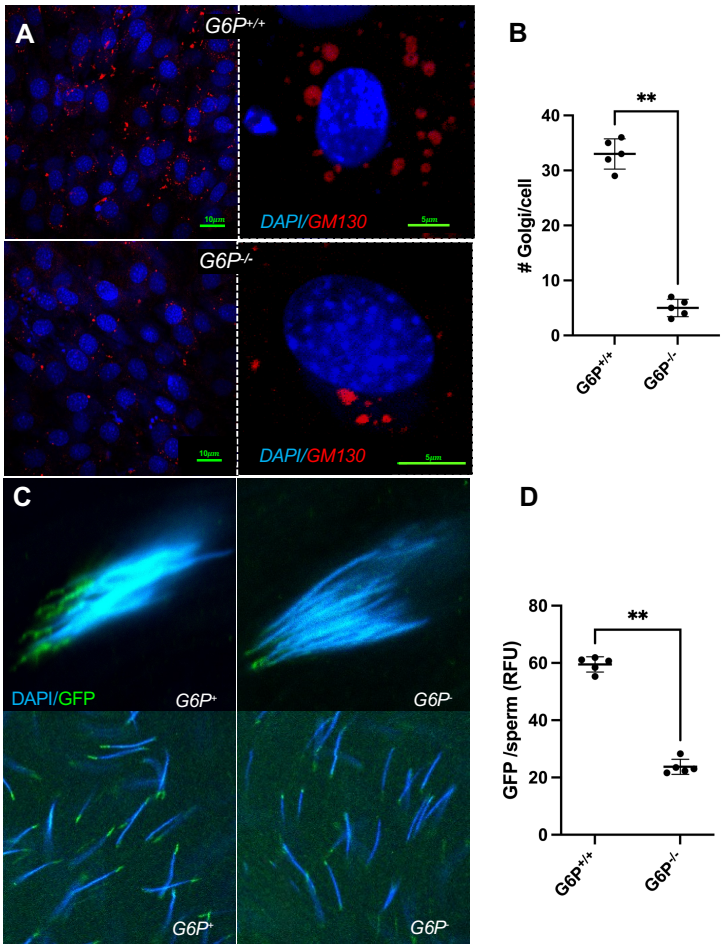
A
G6P+/+
DAPI/GM130
G6P-/-
DAPI/GM130
B
**
Golgi/cell
40
30
20
10
0
G6P+/+
G6P-/-
C
DAPI/GFP
G6P+
G6P-
G6P+
G6P-
D
**
GFP /sperm (RFU)
80
60
40
20
0
G6P+/+
G6P-/-

Figure 6

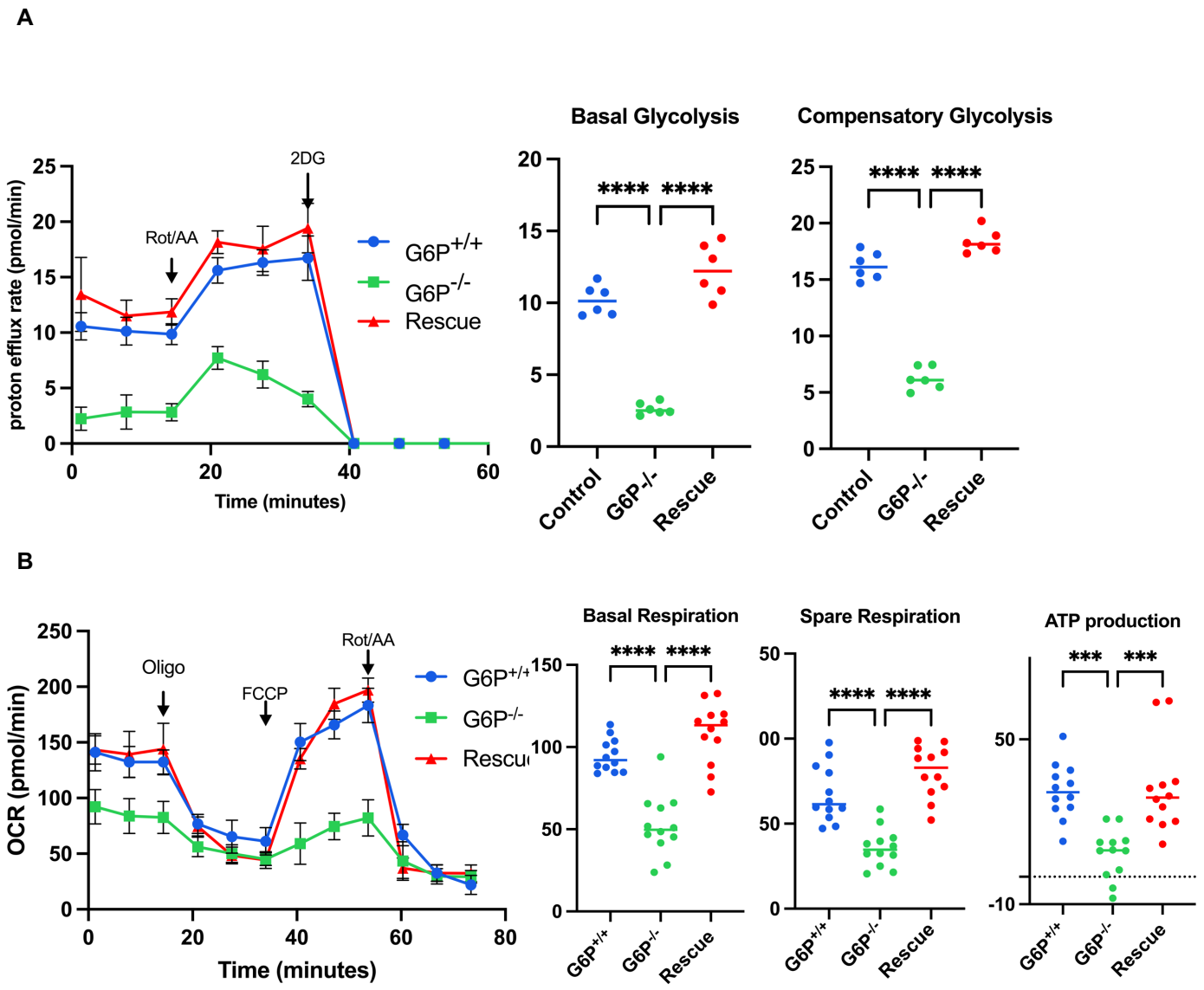
A
Basal Glycolysis
Compensatory Glycolysis
2DG
Rot/AA
proton efflux rate (pmol/min)
Time (minutes)
G6P+/+
G6P-/-
Rescue
****
Control
G6P-/-
Rescue
B
Basal Respiration
Spare Respiration
ATP production
Oligo
FCCP
Rot/AA
OCR (pmol/min)
Time (minutes)
G6P+/+
G6P-/-
Rescue
***

Figure 7