

Elucidation of the role of inner mitochondrial membrane organization in *Drosophila* neural stem cell differentiation

A Thesis

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by

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From May 2023 to Mar 2024

Certificate

This is to certify that this dissertation entitled **Elucidation of the Role of inner mitochondrial membrane organization in *Drosophila* neural stem cell differentiation** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Atharva Sanjay Bhingare at Indian Institute of Science Education and Research under the supervision of Prof. Richa Rikhy, Professor, Department of Biology, during the academic year 2023-2024.



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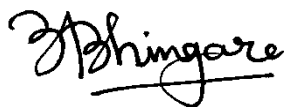
Prof. Richa Rikhy

Prof. Kalika Prasad

This thesis is dedicated to sea creatures and flowering plants and the sky

Declaration

I hereby declare that the matter embodied in the report entitled **“Elucidation of the role of inner mitochondrial membrane organization in *Drosophila* neural stem cell differentiation”** are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education & Research (IISER) Pune, under the supervision of Prof. Richa Rikhy, and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.



Atharva Sanjay Bhingare

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1. Abstract

Mitochondrial dynamics is essential for proper neural stem cell differentiation. Previous work in the lab suggests that the mitochondrial inner membrane plays a distinct role in type II neuroblast (NBII) differentiation in *Drosophila melanogaster*. Though a similar extent of mitochondrial fragmentation is observed when either OPA1, which is responsible for inner membrane fusion and cristae width regulation, or Marf, which is responsible for outer membrane fusion, is depleted in NBII, only OPA1 depletion leads to a striking differentiation defect. Forced fusion of mitochondria by depleting Drp1 in the background of OPA1 depletion rescues this defect by a hypothesized amelioration of cristae morphology in fused mitochondria. The cristae morphology context-dependently modulates the efficiency of the ETC complexes, informing mitochondrial activity. Considering the intimacy of this relationship, we investigated the role of cristae morphology regulatory proteins in NBII differentiation to discover the causal link between the two. Through my work, I was able to demonstrate cristae disruption by hampering ATP synthase dimerisation or L-OPA1 enrichment in NBII causes a significant decrease in the number of differentiated lineage cells. Furthermore, I was able to demonstrate that cristae amelioration is a prerequisite for hyperfusion-mediated rescue of differentiation defect. Furthermore, I was able to demonstrate ROS to be causally linking cristae morphology to NBII differentiation. Additionally, I was able to demonstrate the independent role of ROS in NBII differentiation. Through my work, I have provided strong evidence suggesting cristae morphology to be mediating the interaction between mitochondrial morphology and ETC which coregulate NBII differentiation.

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3. Contributions

Contributor name	Contributor role
Atharva Sanjay Bhingare, Richa Rikhy	Conceptualization Ideas
Rahul Kumar Verma, Richa Rikhy	Methodology
—	Software
Atharva Sanjay Bhingare	Validation
Atharva Sanjay Bhingare	Formal analysis
Atharva Sanjay Bhingare	Investigation
Richa Rikhy	Resources
Atharva Sanjay Bhingare	Data Curation
Atharva Sanjay Bhingare	Writing - original draft preparation
Atharva Sanjay Bhingare	Writing - review and editing
Atharva Sanjay Bhingare	Visualization
Richa Rikhy	Supervision
Atharva Sanjay Bhingare	Project administration
Richa Rikhy	Funding acquisition

Chapter 1 Introduction

1.1 Mitochondrial function

Colloquially called the “powerhouse of the cell,” the popularly recognised role of mitochondria is to manufacture ATP, the cell's energy currency, the end product of cellular metabolism (Osellame et al., 2012). Fats, proteins, and lipids, after being catabolised into Acetyl-CoA (or, as in the case of amino acids, into citric acid cycle intermediates), enter the mitochondrial matrix, getting oxidised through the citric acid cycle. As a result of each of their oxidation step, NADH or FADH₂ is produced as reduction products. These reduced molecules act as electron carriers to the electron transport chain complexes (Complex I, II, III, IV, and ATP synthase), organised on folds of the mitochondrial inner membrane called cristae. These complexes transfer the electrons, working as a chain of electron acceptors and donors, utilising the energy to drive protons via Complex I, III, and IV out of the mitochondrial matrix. The ATP synthase utilises the generated membrane potential to convert ADP to ATP as the final step of oxidative phosphorylation (Fig 1.1).

Besides ATP production, other roles of mitochondria are now gaining recognition. Mitochondria is a significant source of reactive oxygen species (Mailloux, 2020), a buffer for calcium ions (Matuz-Mares et al., 2022), and a warehouse for cytochrome C, a salient trigger for apoptosis (Garrido et al., 2006). ROS is predominantly produced due to the leakage of electrons from Complex I and Complex III. The leaked electrons are accepted by oxygen molecules, forming short-lived superoxide radicals that soon form a more stable form of ROS, the peroxide radical (Zhao et al., 2019).

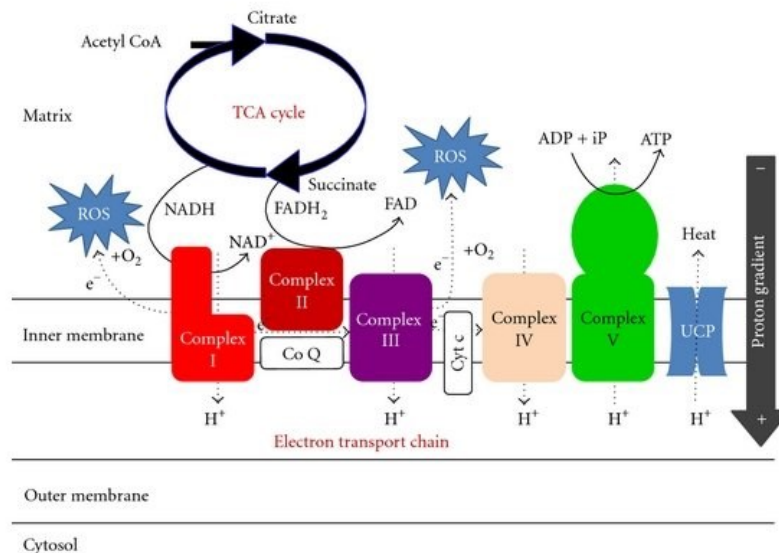


Fig 1.1: Schematic representation of electron transport chain function (Shinmura, 2013)

Recent advances have recontextualised the role of mitochondria from producing ATP and metabolic intermediates to a busy hub of cell signalling, sensing, integrating, and responding to environmental contexts at the level of both morphological and functional dynamics. Mitochondrial activity and morphology regulation are being investigated now not only as the consequence of cell signalling cascades but also as the initiator and transducer of cell signalling (Picard and Shrihail, 2022). Two key strategies by which mitochondria participate in cell signalling. Mitochondrial surface provides proteins a platform to interact upon. On the other hand, by modulating the levels of secondary cell signalling molecules such as ROS, calcium ions, etc, mitochondria are able to interact with a diversity of signaling pathways. (Tait and Green, 2012). For instance, by modulating its rate of ROS production, mitochondria modulate the levels of cytoplasmic ROS, determining the oxidation status and, therefore, the activity of various oxidisable proteins (Finkel, 2011). Therefore, mitochondria are gaining increasing recognition in cellular decision-making.

1.2 Cristae morphology as the structural unit of mitochondrial function

The productivity of electron transport amongst the ETC complexes determines the rate of mitochondrial respiration. For normal electron transport, the relative distance between the ETC complexes needs to be precisely regulated. Two strategies achieve such a regulation. Interactions between the ETC complexes lead to

supercomplex formation, ensuring efficient transport between the supercomplex members by reducing their distance (Berndtsson et al., 2020).

Mitochondrial inner membrane morphology and dynamics regulate oxidative phosphorylation by modulating the relative spatial organisation of ETC supercomplexes in the cristae (Cogliati et al., 2016). Indistinct, immature cristae, as observed in smaller, globular mitochondria, are associated with lower levels of membrane potential and ATP production; in contrast, mature, well-defined cristae, as observed in lamellar, interconnected mitochondria, are associated with higher levels of membrane potential and ATP production (Xu et al., 2013). Therefore, mitochondria exhibit immense intimacy between their morphological and functional dynamics, where mitochondria morphology influences its ultrastructure, which in turn informs its activity.

Additionally, mitochondrial morphology is dynamic, constantly undergoing cycles of fission and fusion. The overall mitochondrial morphology is regulated by modulating the frequency of these cycles by regulating distinct sets of dynamin-related GTPases which facilitate the fission and fusion events. Primarily, dynamin related-protein 1 (Drp1) polymerises into a constricting ring around mitochondria, leading to mitochondrial fission; on the other hand, fusion is caused by Optic Atrophy 1 (OPA1), and dynamin-superfamily GTPase proteins Mfn1, and Mfn2. Mfn 1 and 2 on the mitochondrial outer membrane facilitate membrane fusion by dimerising across corresponding membranes (Song et al., 2009).

Therefore, a nexus of cellular decision-making runs through mitochondrial morphology, ultrastructure, and activity, with the mitochondrial inner membrane dynamics at its crux.

1.3 Cristae dynamics regulation

Besides being globally regulated by mitochondrial morphology, cristae dynamics are precisely fine-tuned and locally regulated, as suggested by individual cristae having distinct membrane potentials (Wolf et al., 2019). Three main players, mitochondrial contact site and cristae organising system (MICOS) complex, OPA1 dimers, and ATP synthase dimers, localise in different parts of the cristae and regulate the cristae morphology and dynamics by stabilising the local inner membrane curvature.

The MICOS complex is a seven-subunit supercomplex localised at cristae junction sites (Fig 1.3), which coincides with endoplasmic reticulum-mitochondria contact sites. Studies in yeast have demonstrated that the MICOS complex comprises two independently organising subcomplexes. The MIC60 and the MIC10 subcomplex is connected via MIC13, with MIC60 determining the localisation of the complex (Friedman et al., 2015). The MICOS complex induces a negative curvature in the mitochondrial inner membrane, stabilising the cristae junction (as reviewed by Anand et al., 2021).

OPA1 exists as two isoforms: the inner membrane-bound long form and the soluble intermembrane short form. OPA1 is translated as a mitochondrial inner membrane protein and cleaved into two forms within the mitochondria by proteases such as YME1L, OMA1, etc. (Griparic et al., 2007). Long and short forms of OPA1 dimerise near the mouth of the cristae, with the short forms pointed towards each other in the intermembrane space across the cristae (Fig 1.3). These dimers interact with each other to regulate the cristae width, enabling the sequestering of cytochrome C within the cristae pockets (Patten et al., 2014; Cipolat et al., 2006). Altering the ratio of S-OPA1 and L-OPA1 leads to distinct cristae defects. L-OPA1 enrichment causes cristae stacking and more interconnected mitochondria, whereas S-OPA1 enrichment causes irregular cristae and mitochondrial fragmentation (Fry et al., 2023, preprint). On the other hand, L-OPA1 has been shown to be sufficient for inner membrane fusion but not for overall mitochondrial morphology (Del Dotto et al., 2018).

ATP synthase dimerises and self-organizes in rows, inducing positive membrane curvature, leading to the formation of the cristae tips (Blum et al., 2019; Fig 1.3). In *Drosophila*, subunits E and G have been determined to be exclusively responsible for ATP synthase dimerisation with ATP synthase α and β being responsible for both dimerisation and ATP production, with the rest of the subunits exclusively responsible for mitochondrial respiration. Therefore, ATP synthase E, G, α , and β are responsible for cristae dynamics in *Drosophila* (Teixeira et al., 2015). Studies in yeast have illustrated that the depletion of ATP synthase subunits E or G leads to the formation of onion-like cristae (Paumard et al., 2002).

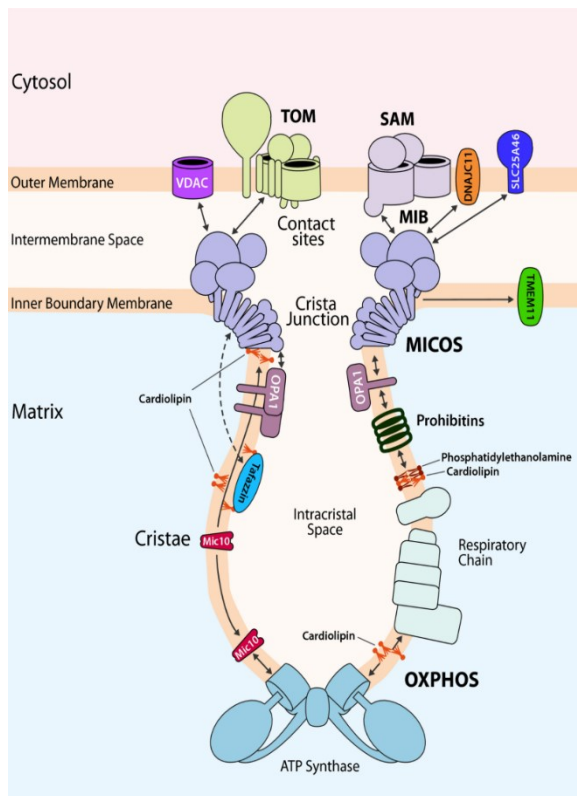


Fig 1.3: Organisation and interactions of mitochondrial inner membrane proteins in cristae (Colina and Tenorio et al., 2020)

In addition to the aforementioned players, cardiolipin (CL), a mitochondria-specific dimeric phospholipid, is essential for managing the cristae membrane curvature strain. CL is a cone-shaped phospholipid comprising four acyl chains connected to a single glycerol head. Such a conical constitution structurally stabilises its organisation in highly curved membranes (Ikou and Ryan, 2017). Furthermore, CL has been demonstrated to bind to essential mitochondrial inner membrane proteins such as ETC complexes (Jiang et al., 2022), MICOS complex (Weber et al., 2013), ATP synthase (Duncan et al., 2016), etc (Fig 1.3). Disrupting cardiolipin synthesis has been shown to cause fewer cristae, resembling those in undifferentiated mitochondria (Pineau et al., 2013). Therefore, the distribution of CL is crucial to localise these proteins properly and, consequently, facilitate proper mitochondrial function and cristae maturation.

1.4 Cristae morphology in stem cell differentiation

A proliferating cell (a stem cell) has anabolic and catabolic demands distinct from that of a non-proliferating cell (a differentiated cell). Stem cells rely on the less effective glycolysis over oxidative phosphorylation as the dominant mode of ATP production, shuttling some of the glycolysis intermediates into the anabolic pentose

sugar pathway for nucleotide production to enable DNA replication (Folmes et al., 2012).

Therefore, considering the intimacy of mitochondrial morphology and respiration, mitochondrial morphology differs significantly between stem cells and differentiated cells. While stem cells have globular, cristae-poor mitochondria, differentiated cells have more elongated, cristae-rich mitochondria. Such a morphological shift is coupled with a metabolic shift; stem cells have lower levels of mitochondrial ATP production and oxidative phosphorylation, whereas differentiated cells have higher levels of ATP production and oxidative phosphorylation (Xu et al., 2013; Fig 1.4).

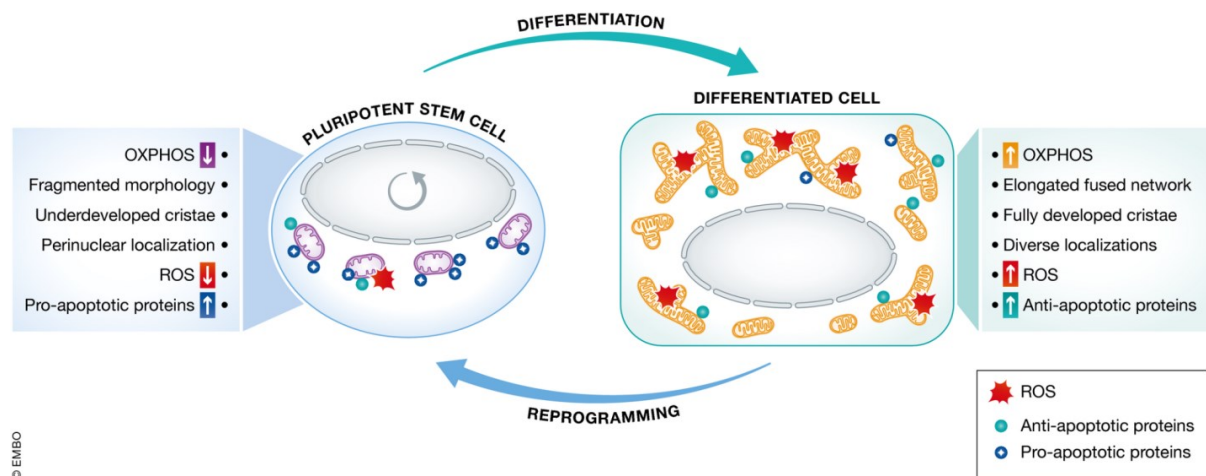


Fig 1.4: Mitochondrial dynamics and cellular respiration differences between a pluripotent stem cell and a differentiated cell (Lisowski et al., 2018)

Recent work has established the importance of such a morphological-metabolic shift in stem cell differentiation, as reviewed by Seo et al. (2018) and Ren et al. (2020). Disrupting mitochondrial dynamics by modulating fission or fusion-regulating proteins has been established to cause differentiation defects in murine models. Though the explicit role of cristae morphology in differentiation is relatively underexplored, the available literature strongly suggests it to be a promising line of questioning. For instance, Teixeira et al. (2015) demonstrated that ATP synthase dimerization-based cristae maturation regulates germline stem cell differentiation in *Drosophila* independent of oxidative phosphorylation. Merkwirth et al. (2008) established a relationship between prohibitin-based regulation of OPA1 processing and the consequent cristae maturation and cell proliferation in mouse embryonic fibroblasts.

Dubal et al. (2022) investigated the role of mitochondrial morphology in *Drosophila melanogaster* neuroblast differentiation. Despite causing similar mitochondrial

fragmentation, a strikingly more severe differentiation defect was observed with OPA1 depletion than with Marf depletion in type II neuroblasts, suggesting the mitochondrial inner membrane plays a distinct role in neuroblast differentiation. Furthermore, the defect was rescued by forced mitochondrial hyperfusion by the expression of Drp1^{SD}, a dominant negative form of Drp1, in type II neuroblasts. Considering the intimate association between mitochondrial and cristae morphology, such a hyperfusion-based rescue further adds to the plausibility of the mitochondrial inner membrane's importance in NB II differentiation. Furthermore, the mechanistic link between cristae morphology and differentiation is severely underexplored.

1.5 Introduction to model system

In *Drosophila melanogaster*, during the embryonic stages (9 to 11), discrete cells in the neuroepithelium are specified to become neural stem cells or neuroblasts. By Notch/Delta signaling-based lateral inhibition, proneural gene expression is refined to these cells, determining their cell fate as stem cells. Subsequently, these neuroblasts delaminate from the neuroepithelium and undergo cycles of asymmetric divisions, giving rise to neuroblast and glial mother cells, which divide into neurons and/or glial cells. These cycles of self-renewing divisions comprise the first of the two waves of neurogenesis and leads to the formation of the larval central nervous system (Homem and Knoblich, 2012).

After the first wave, the neuroblasts in the abdominal region undergo programmed cell death, whereas those in the cephalic and thoracic regions enter a quiescent phase. These quiescent cells reenter the cell cycle in the second wave of neurogenesis, beginning in the first instar larval stage, eight to ten hours post-hatching, and continue dividing into the pupal stage, giving rise to the adult central nervous system (Homem and Knoblich, 2012).

The neuroblasts involved in the second wave of neurogenesis can be divided into four categories: type I, type II, mushroom body, and optic lobe neuroblasts. Each brain lobe has ninety to hundred type I neuroblasts, which undergo asymmetric division to form a neuroblast and a glial mother cell, which, in turn, undergo division to form neurons and/or glial cells. On the other hand, type II neuroblasts are exactly eight in each brain lobe. They undergo asymmetrical division to create a neuroblast and immature intermediate progenitor cells, which, after maturation, undergo

asymmetrical division to form another mature INP and a GMC, which, in turn, divide to form neurons and/or glial cells (Homem and Knoblich, 2012; Fig 1.5).

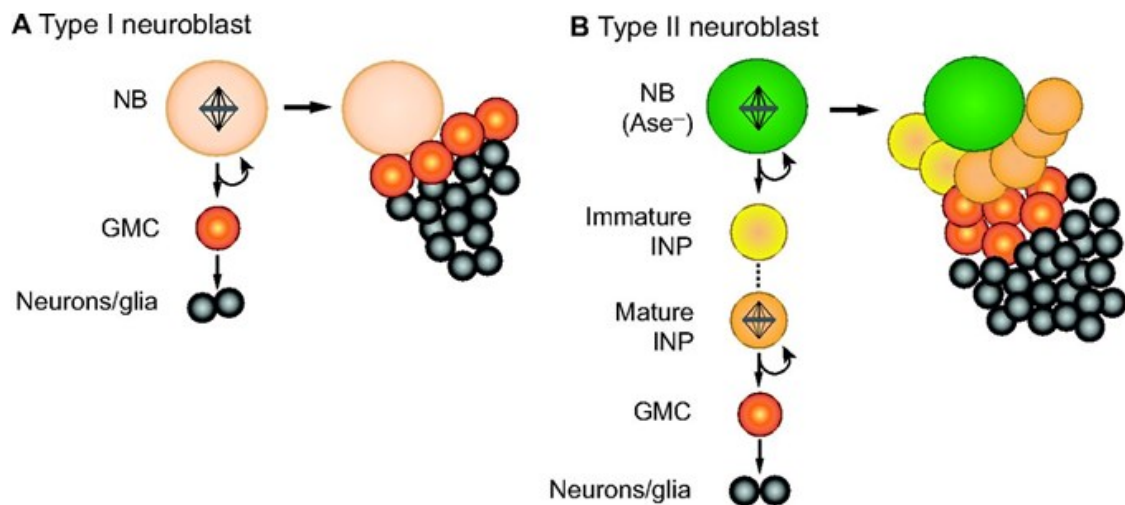


Fig 1.5: Lineage progression in third-instar larval central brain A. NBI. B. NBII (Homem and Knoblich, 2012)

Type II neuroblasts are an exceptionally amenable model system for investigating stem cell differentiation for two significant reasons. Firstly, the lineage cell types are easily tractable by staining for their characteristic transcription factors. Secondly, being dispensable to the fly's survival and viability, severe genetic disruptions could be performed in the type II neuroblasts without impinging the development of the fly to the desired stage.

1.6 Objectives

The objectives of my study are the following:

1. Investigate the role of cristae dynamics in type II neuroblast differentiation
2. Measure the cellular respiratory changes accompanying cristae dynamic disruption
3. Dissect the mechanistic link between cristae dynamics and neuroblast differentiation

In addition to the above-mentioned objectives, this study aims to expand upon the ongoing work in the lab concerning the role of ETC complex subunits in type II neuroblast differentiation. Upon complex I subunit depletion, for instance, ND51, in type II NB, under proliferation has been observed, accompanied by increased ROS and a loss of membrane potential. Mitochondrial hyperfusion using Drp1^{SD} rescues

the differentiation defect as well as the cellular respiratory defect. Furthermore, rescuing the ROS defect has been sufficient to rescue the differentiation defect, suggesting ROS to be the causal link between mitochondrial morphology and differentiation. The nature of the interaction between ETC activity and mitochondrial morphology is unclear. Considering the intimacy between mitochondrial morphology and cristae morphology and the role of cristae morphology in regulating ETC activity, we hypothesise cristae dynamics to mediate the interaction between mitochondrial morphology and respiration (Fig 1.6). Therefore, this study aims to verify the hypothesis, furthering our understanding of the mitochondrial morphology-activity interaction.

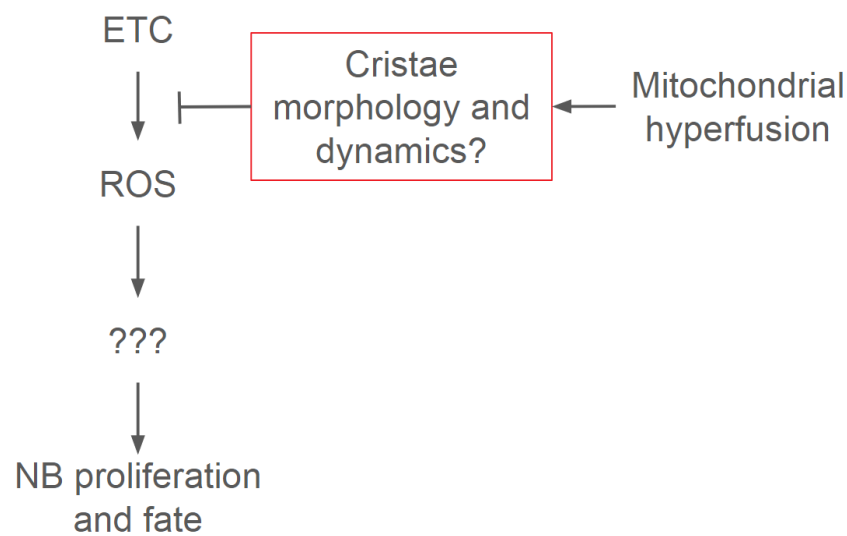


Fig 1.6: Schematic representation of the hypothesised mechanism of mitochondrial hyperfusion-mediated rescue of differentiation defect in NBII associated with ETC disruption

Chapter 2 Materials and Methods

2.1 Fly genetics

The following fly lines were used in this study: (1) *worniu*-Gal4,UAS-mCD8-GFP (*wor*-Gal4), (2) *pointed*-Gal4,UAS-mCD8-GFP (*pnt*-Gal4), (3) UAS-mCherry RNAi (Bloomington stock number BL35785), (4) UAS-MIC60 RNAi/CyO-GFP (BL63994), (5) UAS-MIC26-27 RNAi (BL67030), (6) UAS-MIC19 RNAi (BL51157), (7) UAS-MIC10 RNAi (BL63669), (8) UAS-hMIC25 RNAi (BL94796), (9) UAS-MIC13 RNAi (BL44634), (10) UAS-Yme1L (BL95272), (11) UAS-Yme1L RNAi (BL51752), (12) UAS-Spg7 RNAi (BL55324), (13) UAS-dATAD3a (BL65057), (14) UAS-ATP synthase $\square$ RNAi (BL28059), (15) UAS-ATP synthase O RNAi (BL65180), (16) UAS-ATP synthase B RNAi (BL28062), (17) UAS-ATP synthase β RNAi (BL28056), (18) UAS-ATP synthase E RNAi, (19) UAS-ATP synthase G RNAi (BL67347), (20) UAS-Letm1 RNAi (BL37052), (21) UAS-Phb1 RNAi (BL32404, BL32921), (22) UAS-Phb2 RNAi (BL40835), (23) phospholipase D RNAi (BL32839), (24) tafazzin RNAi (BL51484), (25) rhomboid-7 RNAi (BL35617). *wor*-Gal4 was used to express the RNAi in all the larval neuroblasts, and *pnt*-Gal4 to express in all the type II neuroblasts of the third-instar larval brains. Fly crosses were incubated at 29 °C. Each cross used ten virgin female flies from the Gal4 line and five males from the UAS line per vial. Two replicates of each cross were performed simultaneously, and the parents were transferred into fresh media vials every three days.

2.2 Immunostaining of brains

Dissection of wandering third instar larvae were performed in Schneider's medium, and the tissue of interest fixed for 25 minutes in 4% PFA solution. The tissue was washed with 1X PBS with 0.1% Triton X-100 (PBST) for 30 minutes, then blocked with 1% BSA for 1 hour. All these steps were carried out at room temperature. The tissue was stained with the primary antibody as described below for 14 to 15 hours at 4°C. They were then washed thrice with 0.1% PBST, each wash being 20, 10 and 10 minutes, respectively. The tissue was then incubated in fluorescently coupled secondary antibody for an hour at RT. Subsequently, the tissue was washed thrice (for 20, 10 and 10 minutes, respectively) with 0.1% PBST. The tissue was mounted in Anti-Fade, and the prepared slides were stored at 6 °C.

Primary antibodies	Dilution	Source
chicken-anti-GFP	1:1000	Invitrogen
rat-anti-Miranda	1:400	Abcam
rabbit-anti-phosphohistone 3	1:500	Invitrogen
rat-anti-Deadpan	1:150	Abcam
mouse-anti- Prospero	1:25	DSHB
rabbit-anti-Asense	1:10000	Yuh-Nung Jan, UCSF, USA
mouse-anti-cytochrome C	1:200	Cell Signalling
rat-anti-ATP synthase β	1:200	Abcam

Table 2.2: Primary antibodies used

The following fluorescently coupled secondary antibodies (Molecular Probes) were used: anti-Chicken 488, anti-Rat 568, anti-Rabbit 647, anti-Mouse 568, anti-Rat 647 were used in 1:1000 dilution.

2.3 DHE staining and live imaging of ROS

All dissections were performed in Schneider's medium before the third instar larval brains were incubated in Dihydroethidium (DHE) diluted in Schneider's medium (1:1000 of a 30mM stock, Molecular Probes) for 15 minutes at RT. Then, they were washed in Schneider's medium for 5 min before being mounted on poly-L-lysine coated LabTek chambers containing ice-cold Schneider's medium. They were immediately imaged with a 63x/1.4NA oil objective on Zeiss Anisotropy. In the Zeiss ZEN software, the DPSS (561 nm) laser was used excite the DHE and the dihydroethidium-1 filter settings to visualize. Laser power and gain were adjusted to prevent oversaturation or undersaturation of the image on an 8-BIT scale. Other parameters were retained across genotypes.

2.4 TMRM uptake for live imaging of mitochondrial membrane potential

All dissections were performed in Schneider's medium before the third instar larval brains were incubated in Limonene diluted in Schneider's medium (1:150) for 4 minutes at RT to permeabilise the cell membrane. Then, they were incubated in TMRM diluted in Schneider's medium (1:1000 dilution) for 30 min before being mounted in poly-L-lysine coated LabTek chambers containing ice-cold Schneider's medium. They were immediately imaged with a 63x/1.4NA oil objective on Zeiss Anisotropy. In the Zeiss ZEN software, the DPSS (561 nm) laser was used to excite and visualize TMRM. Laser power and gain were adjusted to prevent oversaturation or undersaturation of the image on an 8-BIT scale. Other parameters were retained across genotypes.

2.5 Microscopy and Image Analysis

2.5.1 Neuroblast proliferation analysis

Fixed samples were imaged at RT using Leica SP8 confocal microscope (Leica Microsystems, Inc.; IISER Pune microscopy facility) with a Plan apochromat 40x oil-immersion objective. In the LAS X software, the images were acquired at 1024x1024 pixels. Fluorescent intensity was adjusted to avoid under- and oversaturation. Line averaging was set to 2 and acquisition speed to 600. The excitation of various fluorophores was carried out using the following lasers: HeNe (633 nm) for Alexa Fluor 647, DPSS laser for Alexa Fluor 568, and 406 diode laser line at 488 nm for Alexa Fluor 488. The representative images are from the posterior side of the brain lobe, showing both type II and type I NBs.

2.5.2 Type II NB lineage analysis

Fixed samples were imaged at RT using Leica SP8 confocal microscope (Leica Microsystems, Inc.; IISER Pune microscopy facility) with a Plan apochromat 63x oil-immersion objective. In the LAS X software, the images were acquired at 1024x1024 pixels. Fluorescent intensity was adjusted to avoid under- and oversaturation. Line averaging was set to 2 and acquisition speed to 600. The excitation of various fluorophores was carried out using the following lasers: HeNe (633 nm) for Alexa Fluor 647, DPSS laser for Alexa Fluor 568, and 406 diode laser line at 488 nm for

Alexa Fluor 488. The representative images are from the posterior side of the brain lobe, showing one or more type II NB lineage.

2.5.3 Mitochondrial morphology analysis

Fixed samples were imaged at RT using Leica SP8 confocal microscope (Leica Microsystems, Inc.; IISER Pune microscopy facility) with a Plan apochromat 100x oil-immersion objective. In the LAS X software, the images were acquired at 1024x1024 pixels. Fluorescent intensity was adjusted to avoid under- and oversaturation. Line averaging was set to 2 and acquisition speed to 200. The 406 diode laser line was used to excite the Alexa Fluor 488. The representative images are from the posterior side of the brain lobe showing one type II NB.

2.6 Image Analysis

2.6.1 Neuroblast proliferation analysis

NBs in a brain lobe were counted using Miranda as a marker across genotypes. Mitotic NBs were counted by counting pH3-positive NBs and plotted using the GraphPad Prism software. Non-parametric Mann-Whitney test was used for statistical analysis.

2.6.2 Quantification of mature INPs, GMCs and neurons in type II NB lineages

Dpn-positive lineage cells are mature INPs; Dpn-negative, Ase-positive cells distal from the neuroblast are GMCs; Highly Pros positive cells distal from the neuroblast are the newly formed neurons. The lineage boundary was visualised using anti-GFP staining. The contrast and brightness adjustment tools in ImageJ were used to ensure the accurate quantification of images. Non-parametric Mann-Whitney test was used for statistical analysis.

2.6.3 DHE, TMRM and cytochrome C intensity quantification

The fluorescent intensity of cytoplasmic DHE, TMRM or cytochrome C was measured for each of the type II neuroblasts as well as that of a nearby type I neuroblast in the same plane by drawing regions of interest using the free-hand tool in ImageJ for all genotypes. The fluorescent intensity of type II neuroblast was divided by that of type I neuroblast in the same plane. The values were plotted using

GraphPad Prism software. Non-parametric Mann-Whitney test was used for statistical analysis.

Chapter 3 Results

3.1 Cristae dynamics plays a role in *Drosophila* type II neuroblast differentiation

3.1.1 Lethality screen

A wide variety of players cooperatively regulate the cristae dynamics, most of them simultaneously playing a role in processes besides cristae regulation (Table 1). To erect an argument from parsimony, I depleted proteins spanning four categories of cristae morphology regulation in all NBs, screening for lethality, the four categories being MICOS complex subunits (Table 3.1.1. A.), ATP synthase subunits (Table 3.1.1. B.), OPA1 modifiers (Table 3.1.1. C.), and proteins observed to be contributing to cristae regulation via unclear mechanisms (Table 3.1.1. D.). Each player regulates the cristae morphology differently and, therefore, is expected to produce unique cristae defects when knocked down. As I observe lethality across categories, such a screen suggests by parsimony that cristae morphology plays a role in NB differentiation. Non-lethal RNAi lines are expected to be non-functional, though supplementation with screening with ubiquitous Gal4 is necessary.

A

MICOS subunits	BL number	Function in cristae dynamics	Other function	Result: lethal or non-lethal	Remark
MIC60 (Mitofilin)	63994	Cooperatively inducing negative membrane curvature at the cristae junction determines the localisation of the cristae junction (Anand et al., 2021)	Participates in mitochondrial protein import (Callegari et al., 2019) and mitochondrial membrane lipid synthesis (Aaltonen et al., 2016)	Lethal	Pupal lethality
Mic 26-27	67030			Non-lethal	
MIC19	51157			Lethal	Flies die soon after emerging
MIC10 RNAi (Minos10)	63669			Non-lethal	
UAS-hMIC25 (CHCHD6)	94796			Non-lethal	
MIC13 (QIL1)	44634			Non-lethal	

B

OPA1 regulators	BL number	Function in cristae dynamics	Other function	Result: lethal or non-lethal	Remark
YME1L	51752	OPA1 processing (Gripovic et al., 2007)	Regulating mitochondrial protein homeostasis, maintaining ETC complex levels (Kan et al., 2022)	Lethal	Pupal lethality
Spg7 (paraplegin)	55324		Involved in the quality control of mitochondrial proteins, mitochondrial biogenesis, and assembly of ribosomes (Wali et al., 2020)	Non-lethal	
rho-7 (PARL)	35617			Non-lethal	

C

ATP synthase subunits	BL number	Function in cristae dynamics	Other function	Result: lethal or non-lethal	Remark
ATP synthase α	28059	Induces positive membrane potential at the cristae tips via ATP synthase dimerisation (Teixeira et al., 2015)	Essential for ATP synthase activity (Teixeira et al., 2015), ATP synthase dimers comprise the mitochondrial permeability transition pore (Giorgio et al., 2013)	Lethal	Pupal lethality
ATP synthase β	28056			Lethal	Pupal lethality
ATP synthase E			ATP synthase dimers comprise the mitochondrial permeability transition pore (Giorgio et al., 2013)	Lethal	Pupal lethality
ATP synthase G	67347			Lethal	Pupal lethality
ATP synthase O	65180		Essential for ATP synthase activity	Non-lethal	
ATP synthase B	28062			Lethal	Pupal lethality

D

Other cristae regulators	BL number	Function in cristae dynamics	Other function	Result: lethal or non-lethal	Remark
Letm1	37502	Regulates cristae invagination by an unknown mechanism (Nakamura et al., 2020)	Regulates mitochondrial ionic balance (Li et al., 2019)	Lethal	Pupal lethality
Phb1 (Prohibitin 1)	32404	Regulates OPA1 processing by an unknown mechanism (Merkwirth and Langer, 2009)	Participates in membrane signaling and transcriptional co-regulation (Ande et al., 2017)	Lethal	Pupal lethality
Phb1 (Prohibitin 1)	32912			Lethal	Pupal lethality
Phb2 (Prohibitin 2)	40835			Lethal	Pupal lethality
PLD (Phospholipase D)	32839	Provides substrates for cardiolipin synthesis (Lu et al., 2015)	Regulates the overall mitochondrial composition (Lu et al., 2015)	Non-lethal	
Tafazzin	51484	Modifies cardiolipin to facilitate inner membrane curvature (Schlame, 2013)		Non-lethal	
dATAD3A (belphegor)	65057	Regulates cristae morphology by physically scaffolding the two mitochondrial membranes (Chen et al., 2023)	Regulates mtDNA organisation, intracellular cholesterol transport, mitochondrial morphology and mitophagy (Chen et al., 2023)	Lethal	Flies die soon after emerging

Table 3.1.1: Lethality screen of RNAi lines. All the RNAi lines were expressed using *wor*-Gal4. A. MICOS subunit RNAi lines screened. B. OPA1 regulator RNAi lines screened. C. ATP synthase RNAi lines screened. D. Miscellaneous cristae regulatory RNAi lines screened (performed with Achyushman Samantha).

3.1.2 Depletion of MICOS complex subunits MIC60 and MIC19 in type II NBs does not cause a differentiation defect

I decided to quantify the differentiation defect as observed with the lethality RNAi lines of the first three categories. To investigate the role of MIC60 and MIC19 in NB proliferation, I used pH3 (phosphohistone 3) as a proliferation marker and quantified the number of dividing NBs. Phosphorylation of Serine 10 in Histone 3 is an epigenetic modification associated cell division (the late G2 and M phase of the cell cycle) (Ladstein et al., 2012). Furthermore, using combinations of lineage cell type-specific transcription factors as cell type markers, I could quantify the number of lineage cell types in the NBII lineages. My analysis demonstrates insignificant differentiation defects caused by MIC60 and MIC19 depletion in NBs (Fig 3.1.2a C-E, 3.1.2b). Though I am observing an increase in the number of proliferating NBs (Fig 3.1.2a A-B), considering the limited number of brains observed, it seems unlikely that it is a true trend. Therefore, the following results suggest that such MICOS-mediated regulation of cristae junction does not contribute to NB differentiation. The data also suggests the presence of partial MICOS complex to be sufficient for normal differentiation, warranting a double knockdown analysis. Alternatively, it is plausible that the MICOS complex comprise long-lived proteins, delaying the expected effects of the knockdown.

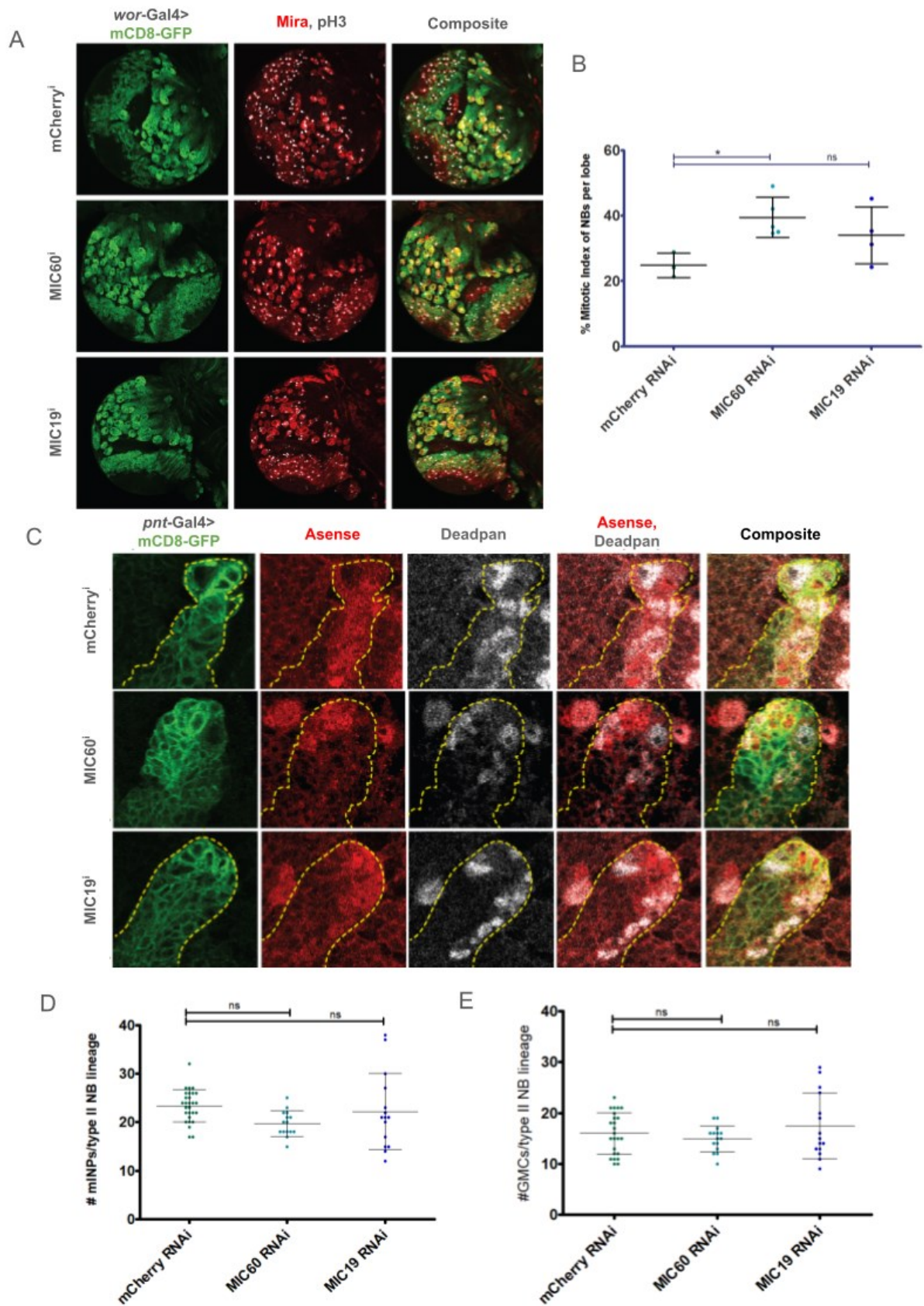


Fig 3.1.2a: Depletion of MICOS complex subunits MIC60 and MIC19 in type II NBs does not cause a differentiation defect. A. Confocal microscopy images representing brain lobe showing pH3 positive NBs in mCherry RNAi (1 lobe per brain, 3 brains), MIC60 RNAi (1,5), and MIC19 RNAi (1,4) each expressed with wor-Gal4, mCD8-GFP. B. Quantification of percentage dividing NBs per lobe in mCherry RNAi (1,3), mitofilin RNAi (1,5), and MIC19 RNAi (1,4). C. Confocal microscopy images representing type II neuroblast lineage showing Dpn and Ase positive cells in mCherry RNAi (n=5), UAS-MIC60 RNAi (4), and UAS-MIC19 RNAi (5) each expressed with pnt-Gal4, mCD8-GFP. D. Quantification of number of mature INPs per lineage in mCherry RNAi (n=5), UAS-MIC60 RNAi (4), and UAS-MIC19 RNAi (5). E. Quantification of number of GMCs per lineage in

mCherry RNAi (n=5), UAS-MIC60 RNAi (4), and UAS-MIC19 RNAi (5) Non-parametric Mann-Whitney test was used for statistical analysis. ns-non-significant ($p > 0.05$)

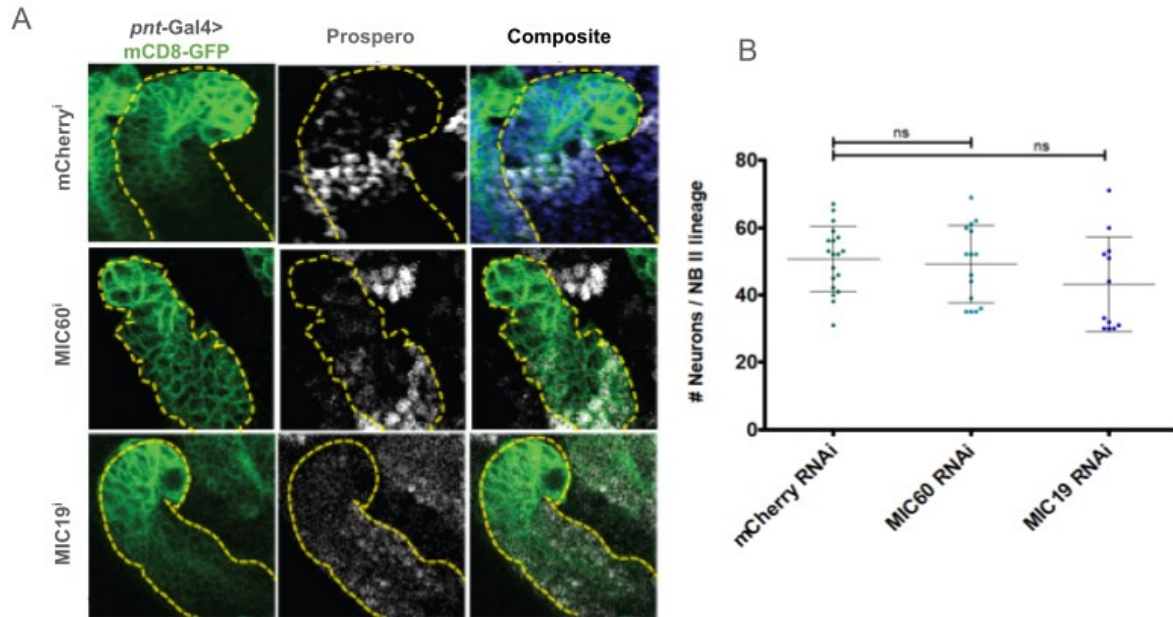


Fig 3.1.2b: Depletion of MICOS complex subunits MIC60 and MIC19 in type II NBs does not cause a differentiation defect. **A.** Confocal microscopy images representing type II neuroblast lineage showing Pros positive cells in mCherry RNAi (n=3), UAS-MIC60 RNAi (4), and UAS-MIC19 RNAi (3) each expressed with pnt-Gal4, mCD8-GFP. **B.** Quantification of number of neurons in mCherry RNAi (n=3), UAS-MIC60 RNAi (4), and UAS-MIC19 RNAi (3). Non-parametric Mann-Whitney test was used for statistical analysis. ns-non-significant ($p > 0.05$)

3.1.3 Yme1L depletion in type II leads to mitochondrial clumping and a decrease in the number of differentiated lineage cells

Along the lines of the previous analysis, I investigated the role of Yme1L in NB differentiation. Though I observed insignificant change in the NB proliferative index (Fig 3.1.3a A-B), I observed a significant decrease in the number of GMCs (Fig 3.1.3b C-E) and neurons (Fig 3.1.3b A-B), suggesting maintaining the ratio of long and short forms of OPA1 is crucial to normal NB differentiation. Furthermore, I observed extensive mitochondrial clumping with Yme1L depletion (Fig 3.1.3b C). Considering that L-OPA1 is sufficient for mitochondrial fusion (Del Dotto et al., 2018), Yme1L depletion-mediated L-OPA1 enrichment could explain the observed mitochondrial morphology.

Therefore, Yme1L depletion leads to a decreased number of differentiated lineage cells despite mitochondrial clumping. Since a previous study suggests that Drp1^{SD}-mediated mitochondrial hyperfusion does not affect neuroblast differentiation, the nature of mitochondrial morphology in Yme1L depletion is inferred to be different from that with Drp1^{SD}. It is highly plausible that despite mitochondrial clumping, the cristae morphology with Yme1L is defective, causing the observed defect.

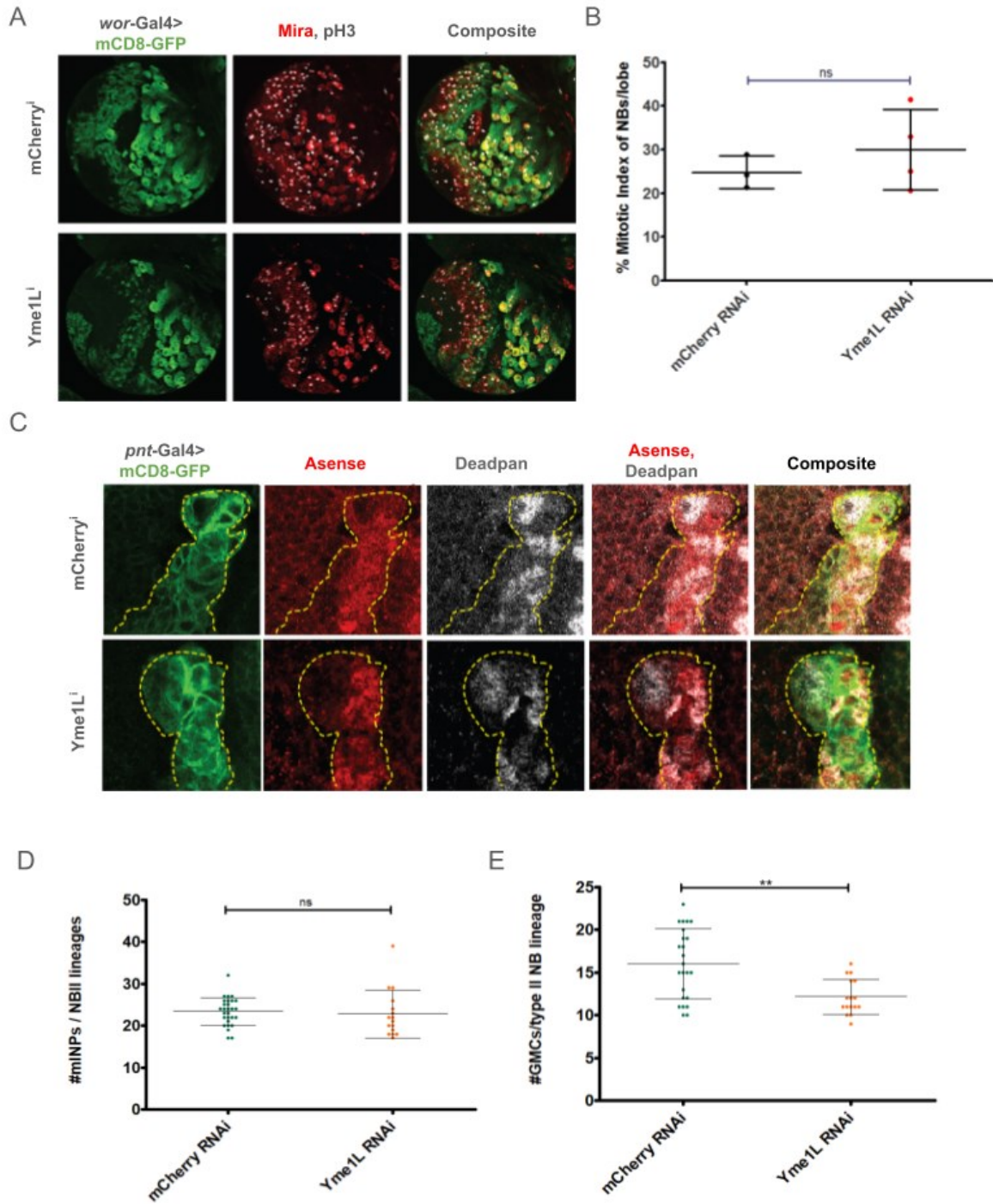


Fig 3.1.3a: Yme1L depletion in type II leads to mitochondrial clumping and a decrease in the number of differentiated lineage cells. A. Confocal microscopy images representing brain lobe showing pH3 positive NBs in mCherry RNAi (1 lobe per brain, 3 brains, reused images), and Yme1L RNAi (1,4) each expressed with wor-Gal4, mCD8-GFP. B. Quantification of percentage dividing NBs per lobe in mCherry RNAi (1,3), and Yme1L RNAi (1,4). C. Confocal microscopy images representing type II neuroblast lineage showing in Dpn and Ase positive cells in mCherry RNAi (n=5, reused images), and Yme1L RNAi (5) each expressed with pnt-Gal4, mCD8-GFP. D. Quantification of number of mature INPs per lineage in mCherry RNAi (n=5), Yme1L RNAi (5). E. Quantification of number of GMCs per lineage in mCherry RNAi (n=5), Yme1L RNAi (5) Non-parametric Mann-Whitney test was used for statistical analysis. ns-non-significant ($p > 0.05$)

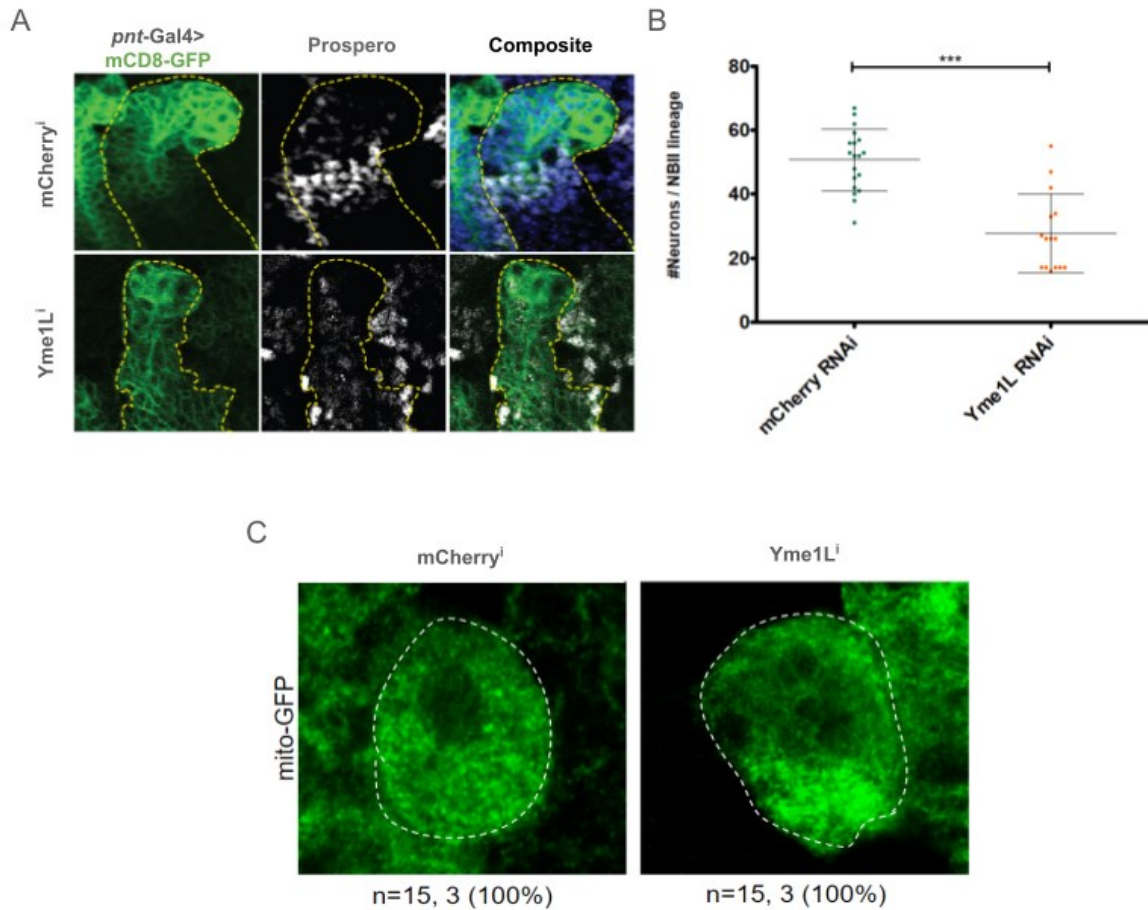


Fig 3.1.3b: Yme1L depletion in type II leads to mitochondrial clumping and a decrease in the number of differentiated lineage cells. A. Confocal microscopy images representing type II neuroblast lineage showing Pros positive cells in mCherry RNAi (n=3, reused images), and Yme1L RNAi (3) each expressed with pnt-Gal4, mCD8-GFP. B. Quantification of number of neurons in mCherry RNAi (n=3), and Yme1L RNAi (3). Non-parametric Mann-Whitney test was used for statistical analysis. ns-non-significant ($p > 0.05$) C. Confocal images of type II neuroblasts showing mito-GFP distribution in mCherry RNAi (n=3) and Yme1L RNAi (n=3) each expressed with pnt-Gal4, mCD8-GFP.

3.1.4 Depletion of ATP synthase subunits E, G, α , β , and B in type II NBs leads to a decrease in the number of lineage cells

In *Drosophila melanogaster*, ATP synthase subunits E, G, α , and β have been demonstrated to be responsible for dimerisation and consequently cristae maturation (Teixeira et al., 2015). I observed that depletion of these subunits, and presumably disruption of ATP synthase dimerisation, leads to a significant reduction in the number of mINPs, GMCs (Fig 3.1.4a C-E, 3.1.4c C-E) and neurons (Fig 3.1.4b, 3.1.4d). On the other hand, depleting ATP synthase B, a subunit uninvolved in dimerisation, also leads to a similar differentiation defect (Fig 3.1.4c C-E, 3.1.4d). Therefore, it is likely that both oxidative phosphorylation and cristae morphology dynamics are involved in NB differentiation, though in different ways.

Despite the severe differentiation defect, I did not observe any NB proliferation defect (Fig 3.1.4a A-B, 3.1.4c A-B). Such an inconsistency could be explained by cristae morphology playing a distinct role in NBII than in NBI. Since the proliferative

index represents NBI to a much greater extent than NBII, it might not reflect the differentiation defect observed in NBII.

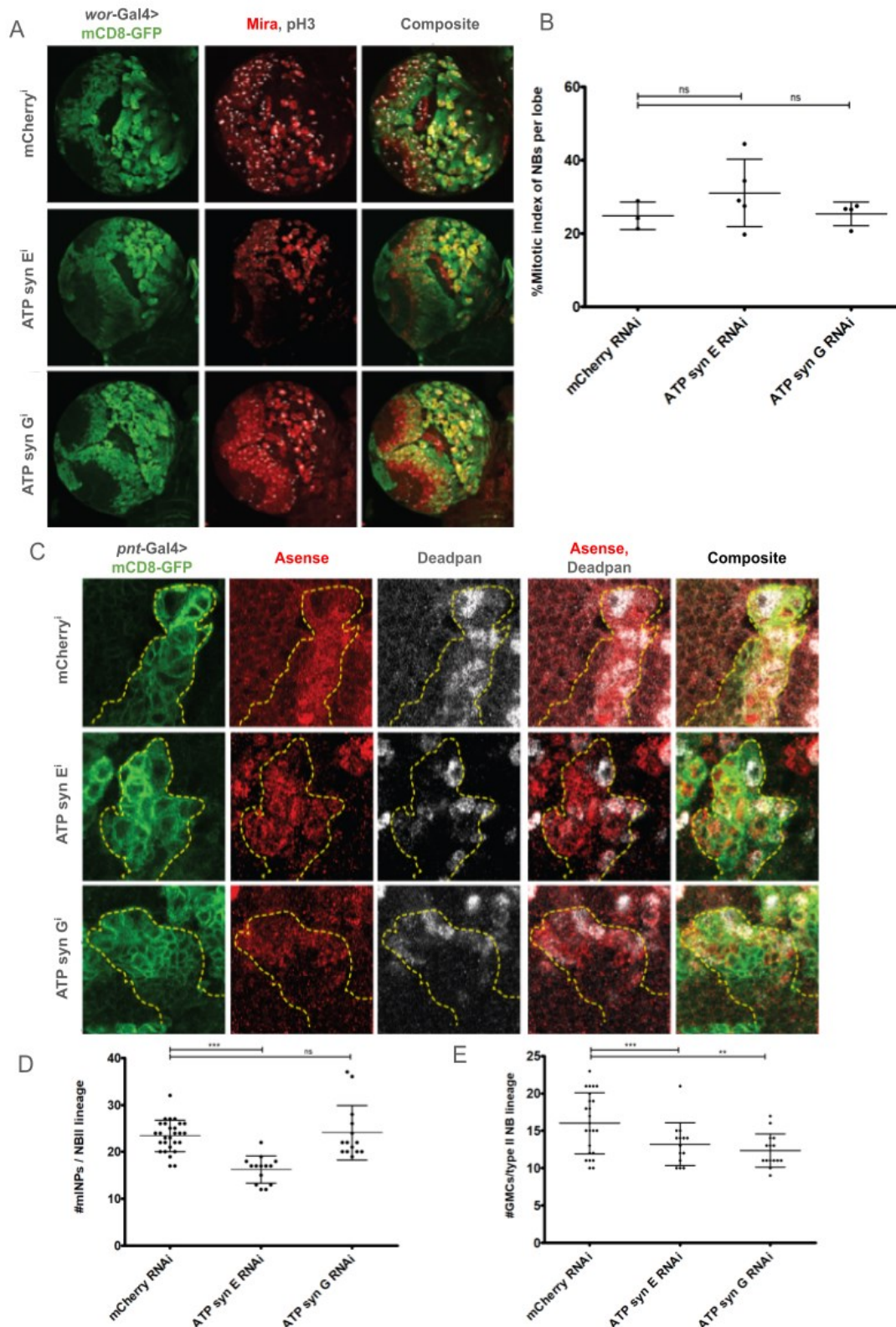


Fig 3.1.4a: Depletion of ATP synthase subunits E and G in type II NBs leads to a decrease in the number of lineage cells. **A.** Confocal microscopy images representing brain lobe showing pH3 positive NBs in mCherry RNAi (1 lobe per brain, 3 brains, reused images), ATP syn E RNAi (1,4), and ATP syn G RNAi (1, 4) each expressed with *wor-Gal4*, *mCD8-GFP*. **B.** Quantification of percentage dividing NBs per lobe in mCherry RNAi (1,3), ATP syn E RNAi (1,4), and ATP syn G RNAi (1, 4). **C.** Confocal microscopy images representing type II neuroblast lineage showing Dpn and Ase positive cells in mCherry RNAi (n=5, reused images), ATP syn E RNAi (1,4), and ATP syn G RNAi (1, 4) each expressed with *pnt-Gal4*, *mCD8-GFP*. **D.** Quantification of number of

mature INPs per lineage in mCherry RNAi (n=5), ATP syn E RNAi (1,4), and ATP syn G RNAi (1, 4). E. Quantification of number of GMCs per lineage in mCherry RNAi (n=5), ATP syn E RNAi (1,4), and ATP syn G RNAi (1, 4). Non-parametric Mann-Whitney test was used for statistical analysis. ns-non-significant ($p > 0.05$)

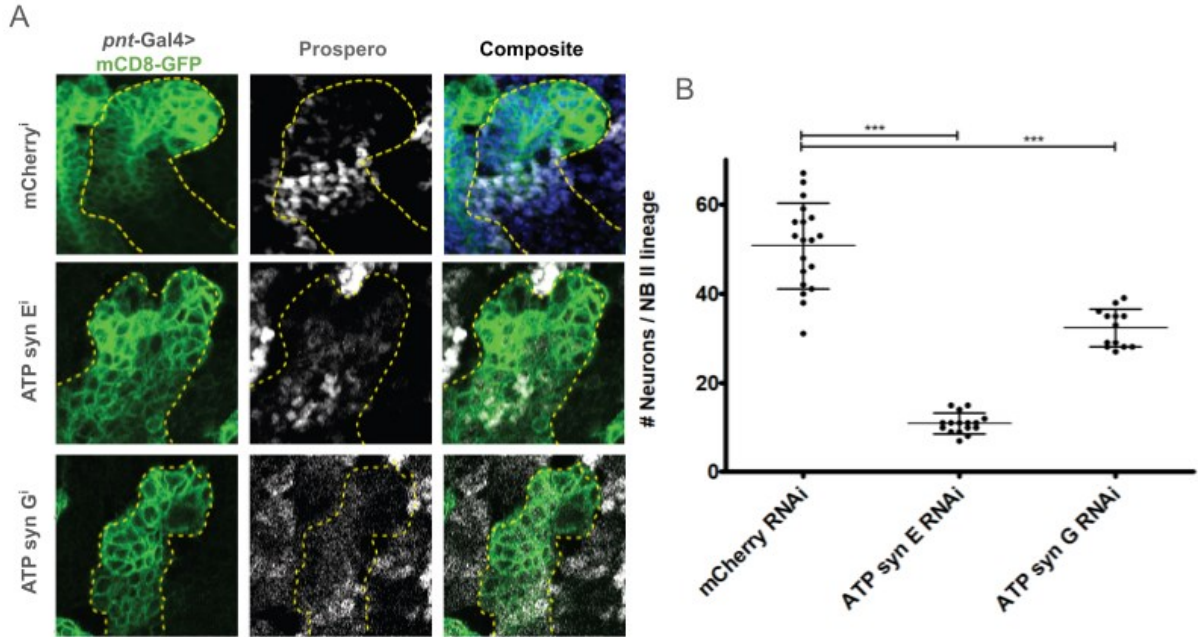


Fig 3.1.4b: Depletion of ATP synthase subunits E and G in type II NBs leads to a decrease in the number of lineage cells. .
A. confocal microscopy images representing type II neuroblast lineage showing in Pros positive cells in mCherry RNAi (n=3, reused images), ATP syn E RNAi (1,4), and ATP syn G RNAi (1, 4) each expressed with pnt-Gal4, mCD8-GFP. **B.** Quantification of number of neurons in mCherry RNAi (n=3 ATP syn E RNAi (1,4), and ATP syn G RNAi (1, 4). Non-parametric Mann-Whitney test was used for statistical analysis. ns-non-significant ($p > 0.05$)

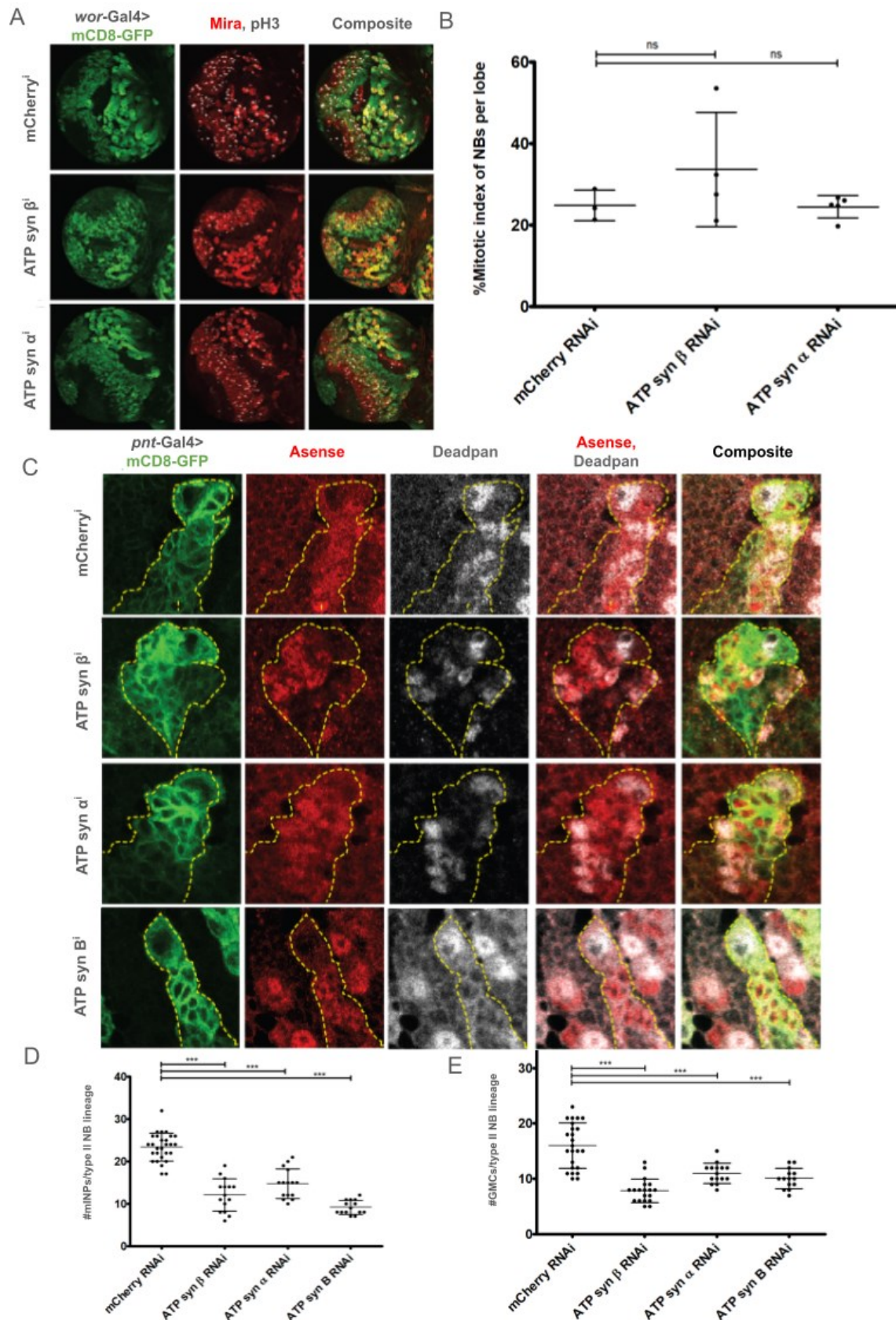


Fig 3.1.4c: Depletion of ATP synthase subunits α , β , and B in type II NBs leads to a decrease in the number of lineage cells. A. Confocal microscopy images representing brain lobe showing pH3 positive NBs in mCherry RNAi (1 lobe per brain, 3 brains, reused images), ATP syn β RNAi (1,4), and ATP syn α RNAi (1, 4) each expressed with *wor-Gal4*, *mCD8-GFP*. B. Quantification of percentage dividing NBs per lobe in mCherry RNAi (1,3), ATP syn β RNAi (1,4), and ATP syn α RNAi (1, 4). C. Confocal microscopy images representing type II neuroblast lineage showing Dpn and Ase positive cells in mCherry RNAi (n=5, reused images), ATP syn β RNAi (1,4), ATP syn α RNAi (1, 4), and ATP syn B RNAi each expressed with *pnt-Gal4*, *mCD8-GFP*. D. Quantification of number of mature INPs per lineage in mCherry RNAi (n=5), ATP syn β RNAi (1,4), ATP syn α RNAi (1, 4), and

ATP syn B RNAi. E. Quantification of number of GMCs per lineage in mCherry RNAi (n=5), ATP syn β RNAi (1,4), ATP syn α RNAi (1, 4), and ATP syn B RNAi. Non-parametric Mann-Whitney test was used for statistical analysis. ns-non-significant ($p > 0.05$)

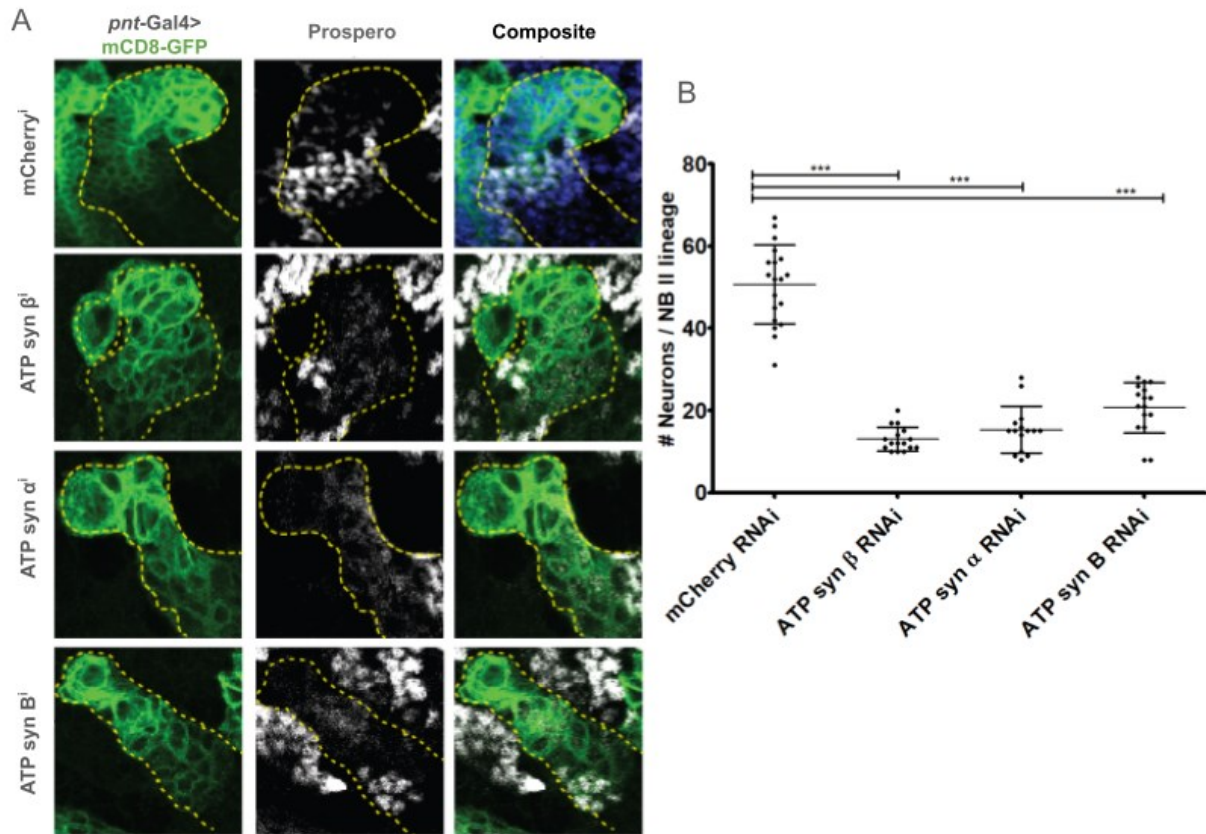


Fig 3.1.4d: Depletion of ATP synthase subunits α , β , and B in type II NBs leads to a decrease in the number of lineage cells. A. Confocal microscopy images showing type II neuroblast lineage showing Pros positive cells in mCherry RNAi (n=3, reused images), ATP syn β RNAi (1,4), ATP syn α RNAi (1, 4), and ATP syn B RNAi each expressed with pnt-Gal4, mCD8-GFP. B. Quantification of number of neurons in mCherry RNAi (n=3) ATP syn β RNAi (1,4), ATP syn α RNAi (1, 4), and ATP syn B RNAi. Non-parametric Mann-Whitney test was used for statistical analysis. ns-non-significant ($p > 0.05$)

3.2 Forced mitochondrial fusion using Drp1^{SD} does not rescue the differentiation defect

Mitochondrial hyperfusion mediated by Drp1^{SD}, a dominant negative form of Drp1, has been observed to rescue NBII differentiation defect associated with mitochondrial fragmentation (Dubal et al., 2022). Furthermore, ongoing work in the lab has observed such a hyperfusion to rescue NBII differentiation defect associated with ETC disruption. The mechanism underlying this hyperfusion-mediated rescue is currently unknown. To test the role of mitochondrial inner membrane dynamics in the implicated interaction between ETC and mitochondrial morphology, I attempted to rescue the observed NBII differentiation defect associated with cristae defects by hyperfusion. The analysed NBII lineages are expected to have hyperfused mitochondrial morphology and defective cristae.

I observed that hyperfusion cannot rescue the differentiation defect produced by ATP synthase α and β depletion (Fig 3.2a-b). In contrast, a partial rescue was observed in the case of ATP synthase B depletion (Fig 3.2a-b). Considering the involvement of

ATP synthase α and β subunits in ATP synthase dimerisation (and consequently cristae morphology), the analysis suggests ATP synthase dimerisation to be a necessary precondition for hyperfusion-mediated rescue.

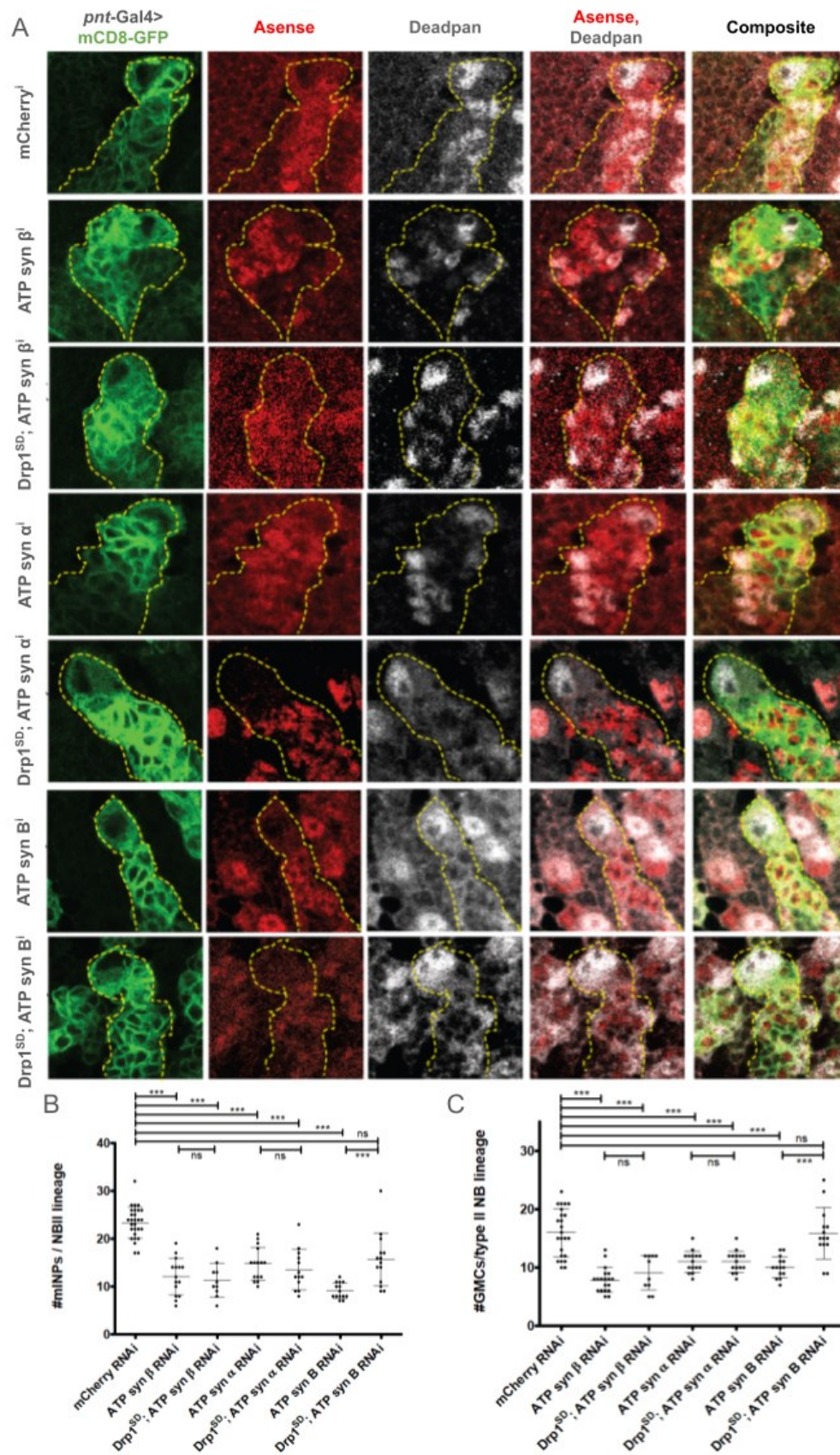


Fig 3.2a: Forced mitochondrial fusion using Drp1^{SD} does not rescue the differentiation defect. A. Confocal microscopy images representing type II neuroblast lineage showing Dpn and Ase positive cells in mCherry RNAi (n=5, reused images), ATP syn β RNAi (1,4), Drp1^{SD}; ATP syn β RNAi (1,4), ATP syn α RNAi (1, 4), Drp1^{SD}; ATP syn β RNAi (1,4), ATP syn α RNAi (1, 4), Drp1^{SD}; ATP syn α RNAi (1,4), ATP syn B RNAi, and Drp1^{SD}; ATP syn B RNAi each expressed with pnt-Gal4, mCD8-GFP. B. Quantification of number of mature INPs per lineage in mCherry RNAi (n=5), ATP syn β RNAi (1,4), Drp1^{SD}; ATP syn β RNAi (1,4), ATP syn α RNAi (1, 4), Drp1^{SD}; ATP syn α RNAi (1,4), ATP syn B RNAi, and Drp1^{SD}; ATP syn B RNAi. C. Quantification of number of GMCs per lineage in mCherry RNAi (n=5), ATP syn β RNAi (1,4), Drp1^{SD}; ATP syn β RNAi (1,4), ATP syn α RNAi (1, 4), Drp1^{SD}; ATP syn α RNAi (1,4), ATP syn B RNAi, and Drp1^{SD}; ATP syn B RNAi. Non-parametric Mann-Whitney test was used for statistical analysis. ns-non-significant ($p > 0.05$)

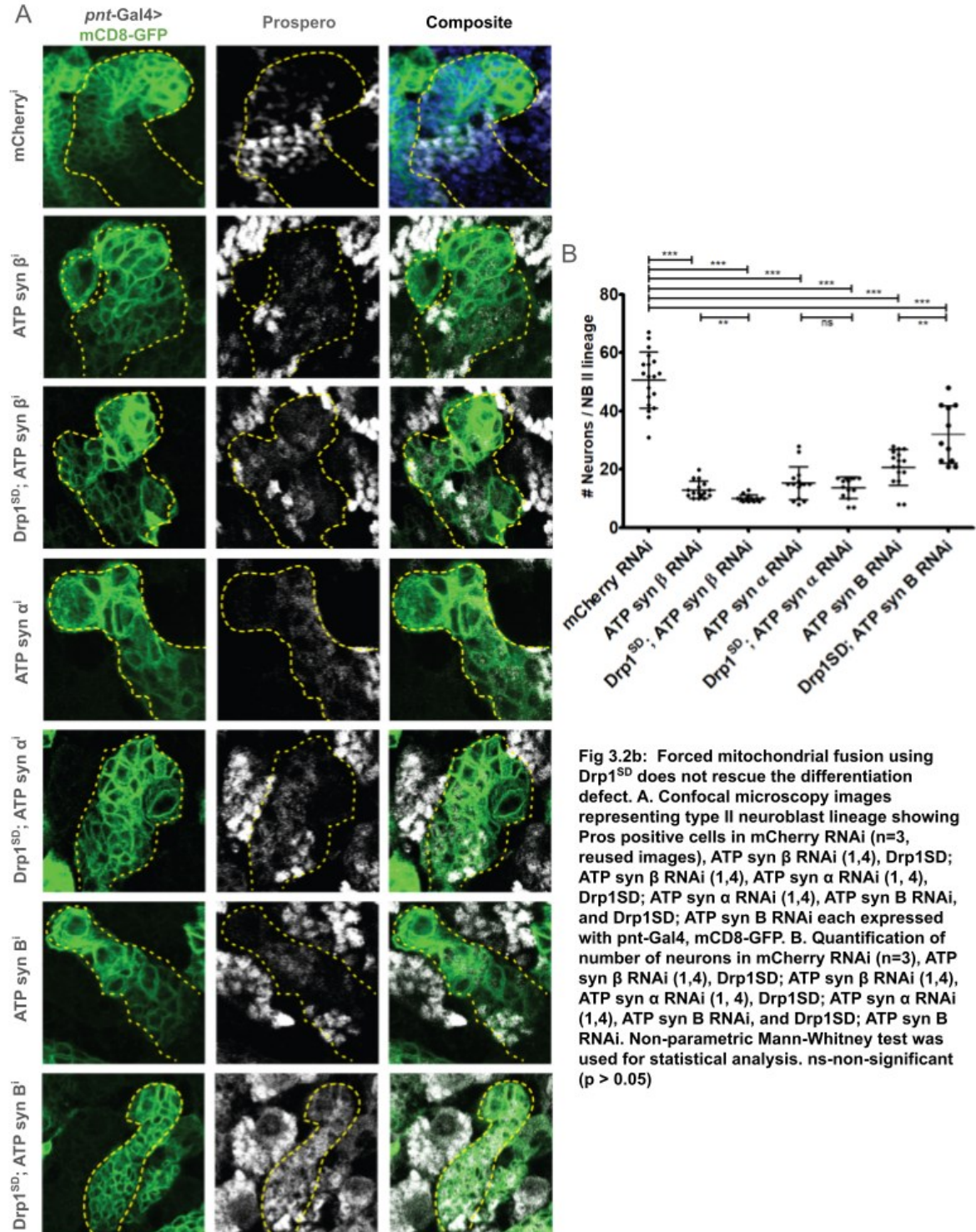


Fig 3.2b: Forced mitochondrial fusion using Drp1^{SD} does not rescue the differentiation defect. A. Confocal microscopy images representing type II neuroblast lineage showing Pros positive cells in mCherry RNAi (n=3, reused images), ATP syn β RNAi (1,4), Drp1SD; ATP syn β RNAi (1,4), ATP syn α RNAi (1, 4), Drp1SD; ATP syn α RNAi (1,4), ATP syn B RNAi, and Drp1SD; ATP syn B RNAi each expressed with pnt-Gal4, mCD8-GFP. B. Quantification of number of neurons in mCherry RNAi (n=3), ATP syn β RNAi (1,4), Drp1SD; ATP syn β RNAi (1,4), ATP syn α RNAi (1, 4), Drp1SD; ATP syn α RNAi (1,4), ATP syn B RNAi, and Drp1SD; ATP syn B RNAi. Non-parametric Mann-Whitney test was used for statistical analysis. ns-non-significant ($p > 0.05$)

3.3 Analysis of membrane potential, ROS levels, and cytochrome C levels with depletion of ATP synthase subunits and expression of Drp1^{SD} in NBII

Cristae defect in NBII is expected to tamper with the ETC activity. To decipher the causal mechanism behind observed cristae defect, I quantified cellular ROS and mitochondrial inner membrane potential. While membrane potential is a read-out of OXPHOS activity (Vander Heiden et al., 1997), mitochondrial ROS is the product of electron leakage from the ETC. Dihydroethidium (DHE) is a fluorescent probe that fluoresces when oxidised by ROS and is therefore utilised to measure cellular ROS levels quantifiably (Wang et al., 2018). Tetramethylrhodamine Methyl Ester (TMRM) is a potentiometric fluorescent dye that localises at regions of high electric potential, making it a suitable candidate probe for membrane potential quantification (Creed and McKenzie, 2019). On the other hand, cytochrome c has been visualised as an indirect read-out of cristae morphology. Cytochrome c is a peripheral inner membrane protein localised within the cristae pocket (Garrido et al., 2006). Since normal cristae remodelling is crucial for the containment of cytochrome c (Scorrano et al., 2002), we expect that disturbances in cristae morphology lead to an increase in the cytoplasmic cytochrome c levels.

I observed membrane depolarization in the case of ATP synthase E, B, and β depletion, which is rescued with Drp1^{SD} only with β . Furthermore, such a loss of membrane potential was not observed with ATP synthase α depletion, suggesting membrane potential is not playing a role in differentiation.

On the other hand, a much clearer trend is observed with ROS. A significant increase in ROS is observed with ATP synthase subunit depletion. Combining with Drp1^{SD}, a rescue was observed with ATP synthase B depletion but not with α and β depletion (Fig 3.3b). Considering the role of α and β subunits in ATP synthase dimerisation, the data suggests ATP synthase dimerisation to be a necessary condition for hyperfusion-mediated rescue with ROS as its downstream partner, the mechanistic link between cristae and NB differentiation.

The trend observed with cytochrome c intensity corresponds with the proposed hypothesis. An increase in cytoplasmic cytochrome c was observed with ATP synthase depletion in NBII, which was rescued with B but not α and β subunits (Fig 3.3c), suggesting an inability of hyperfusion to ameliorate cristae morphology in the absence of ATP synthase dimerisation causing the non-rescue.

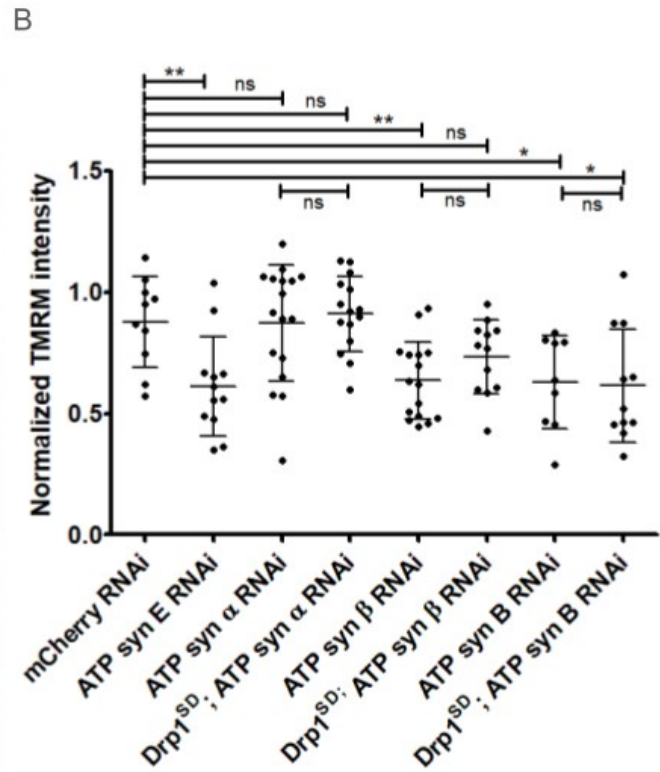
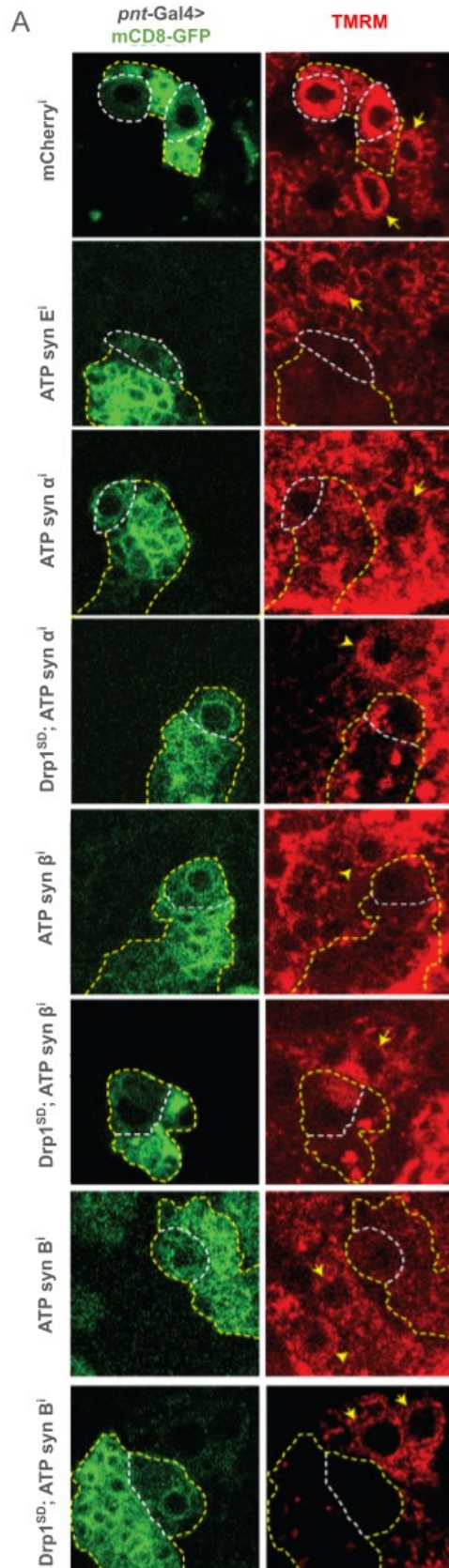
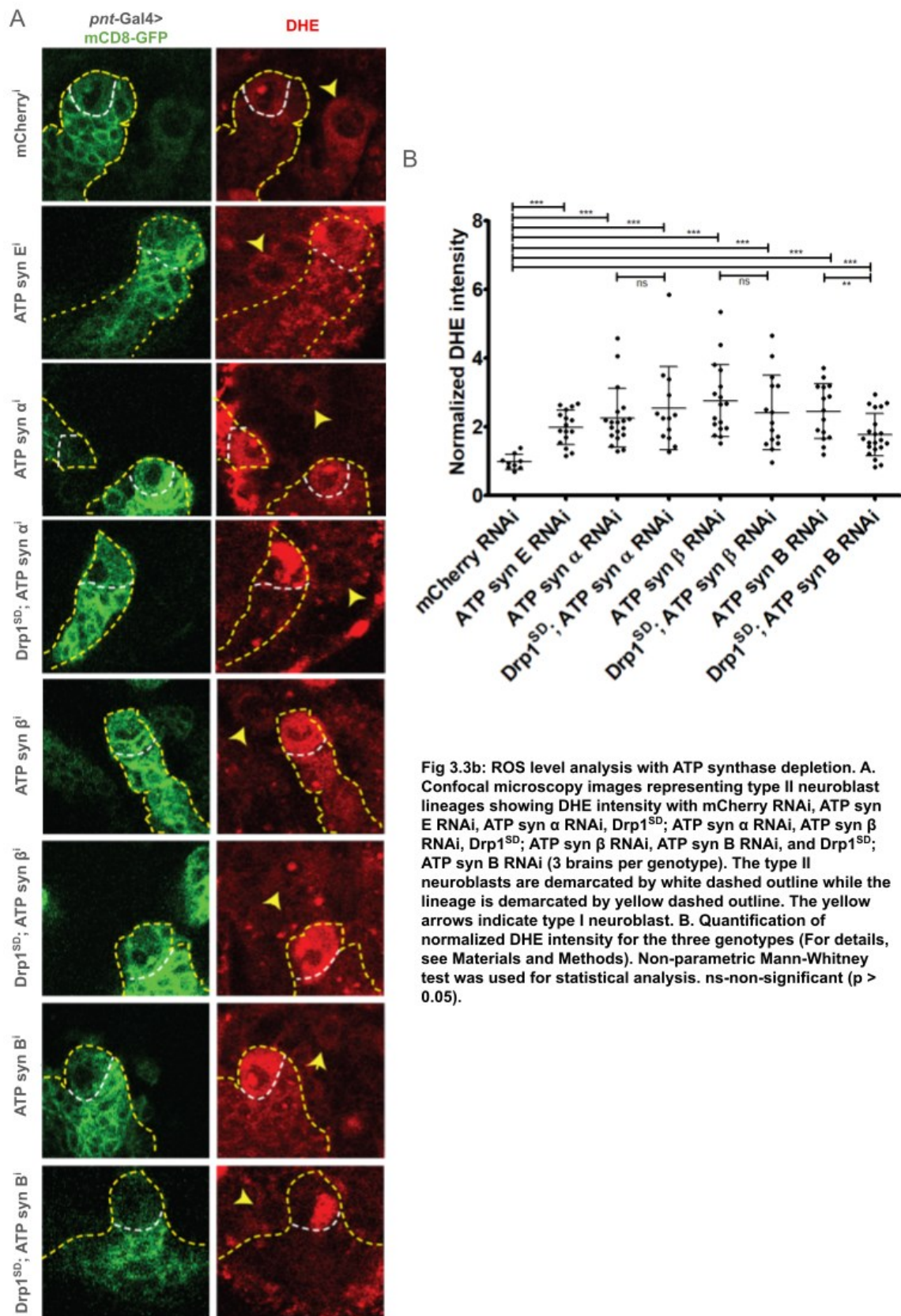
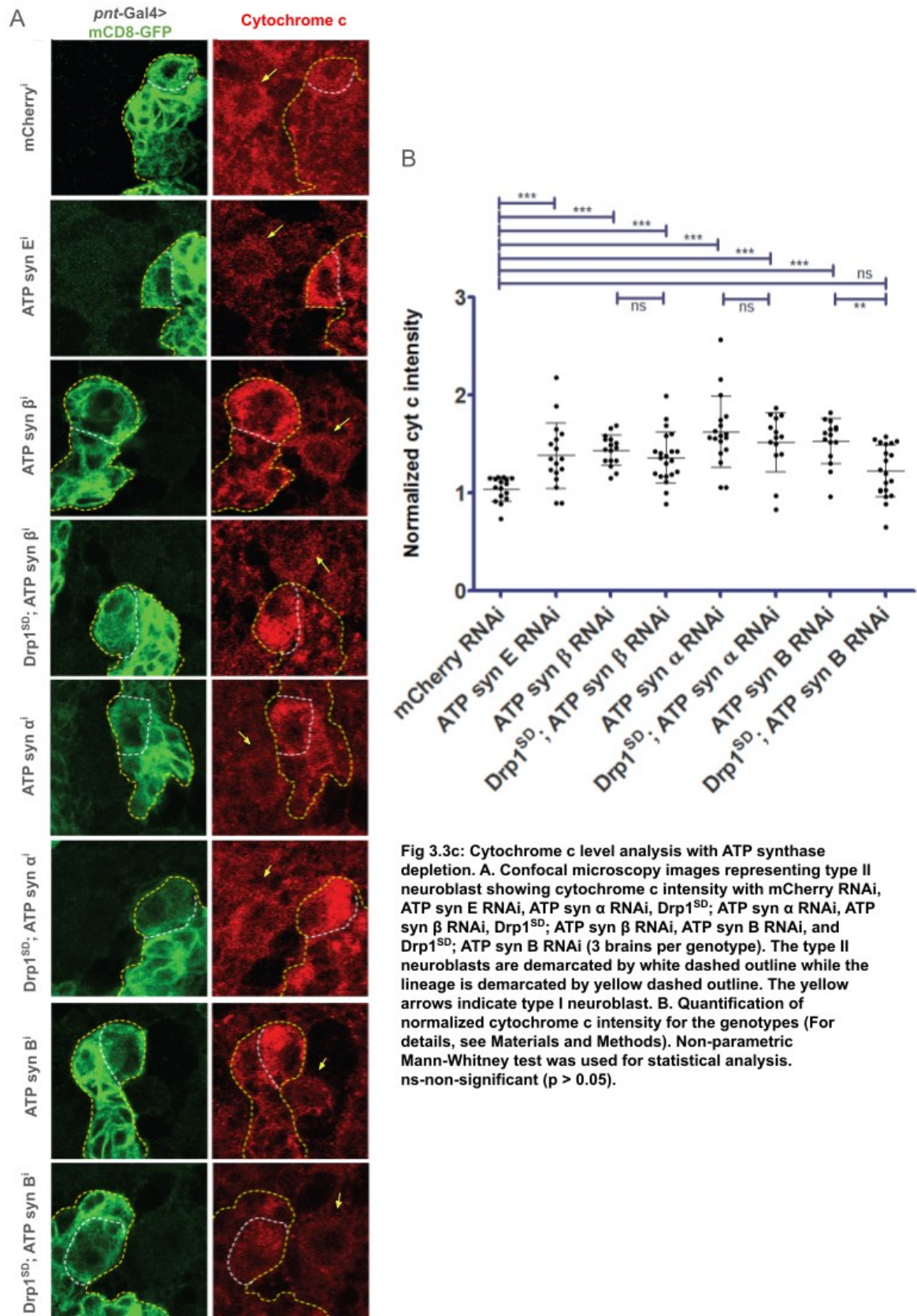


Fig 3.3a: Loss of membrane potential with ATP synthase subunit depletion. A. Confocal images representing type II neuroblast lineages showing TMRM intensity with mCherry RNAi (performed by Rahul Kumar Verma), ATP syn E RNAi, ATP syn α RNAi, Drp1^{SD}; ATP syn α RNAi, ATP syn β RNAi, Drp1^{SD}; ATP syn β RNAi, ATP syn B RNAi, and Drp1^{SD}; ATP syn B RNAi (3 brains per genotype). The type II neuroblasts are demarcated by white dashed outline while the lineage is demarcated by yellow dashed outline. The yellow arrows indicate type I neuroblast. B. Quantification of normalized TMRM intensity for the genotypes (For details, see Materials and Methods). Non-parametric Mann-Whitney test was used for statistical analysis. ns-non-significant ($p > 0.05$).





3.4 Yme1L regulates NBII differentiation differently than ATP synthase dimers

3.4.1 Drp1^{SD} does not rescue NBII differentiation defect associated with Yme1L depletion

Mitochondrial hyperfusion failed to rescue differentiation in Yme1L-depleted NBII lineages (Fig 3.4.1). I expect the hyperfused mitochondria to possess defective cristae due to L-OPA1 enrichment. The result further consolidates the involvement of cristae morphology in hyperfusion-mediated.

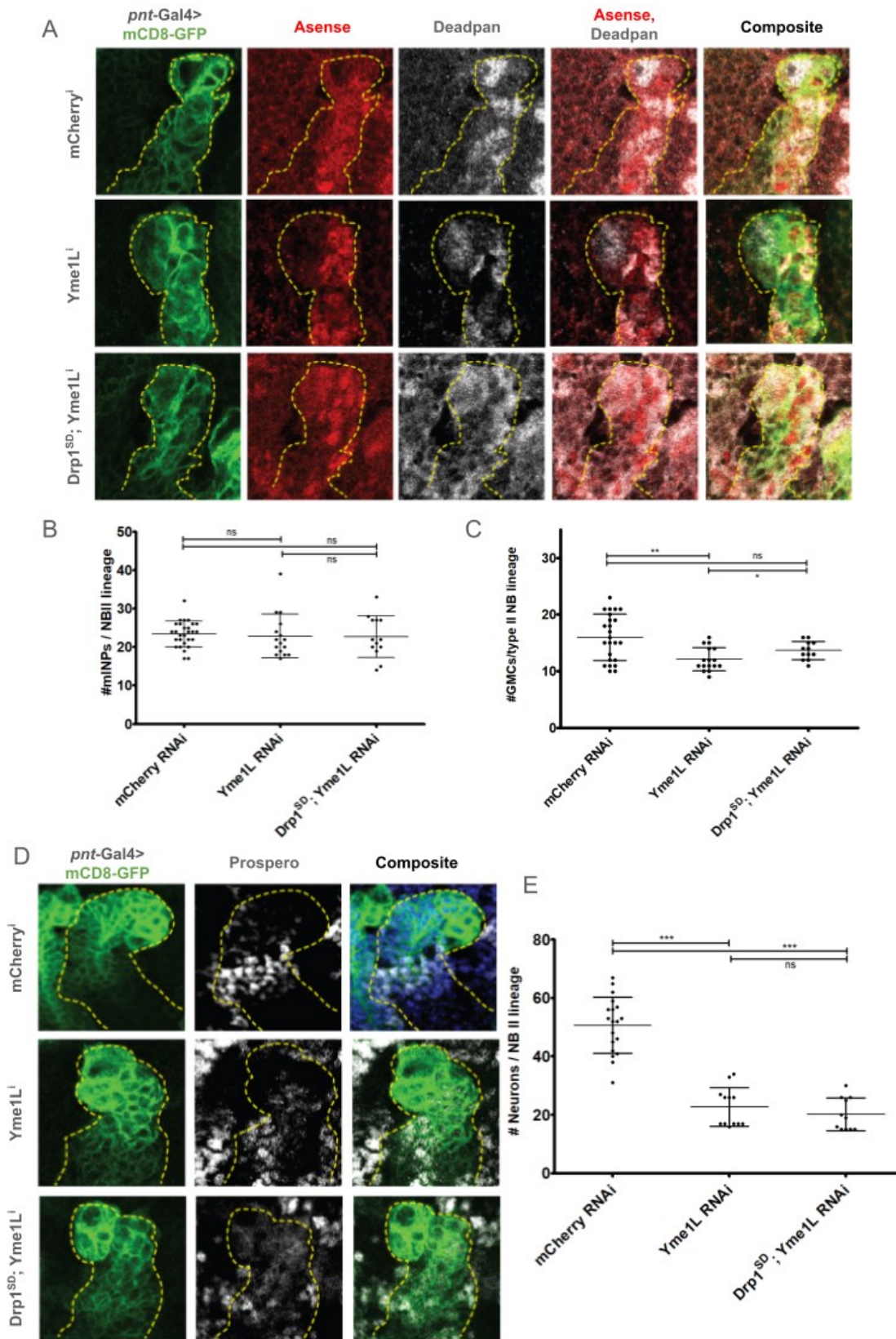


Fig 3.4.1: Drp1^{SD} does not rescue NBII differentiation defect associated with Yme1L depletion . A. Confocal microscopy images representing type II neuroblast lineage showing in Dpn and Ase positive cells in *mCherry* RNAi (n=5, reused images), *Yme1L* (1,4, reused images), and *Drp1*^{SD}; *Yme1L* RNAi (1,4) each expressed with *pnt-Gal4*, *mCD8-GFP*. **B.** Quantification of number of mature INPs per lineage in *mCherry* RNAi (n=5), *Yme1L* (1,4, reused images), and *Drp1*^{SD}; *Yme1L* RNAi (1,4). **C.** Quantification of number of GMCs per lineage in *mCherry* RNAi (n=5), *Yme1L* (1,4, reused images), and *Drp1*^{SD}; *Yme1L* RNAi (1,4). **D.** Confocal microscopy images representing type II neuroblast lineage showing in Pros positive cells in *mCherry* RNAi (n=3, reused images), *Yme1L* (1,4, reused images), and *Drp1*^{SD}; *Yme1L* RNAi (1,4) each expressed with *pnt-Gal4*, *mCD8-GFP*. **E.** Quantification of number of neurons in *mCherry* RNAi (n=3), *Yme1L* (1,4, reused images), and *Drp1*^{SD}; *Yme1L* RNAi (1,4). Non-parametric Mann-Whitney test was used for statistical analysis. ns-non-significant (p > 0.05).

3.4.2 Analysis of ROS levels and cytochrome c levels with Yme1L depletion and expression of Drp1^{SD} in NBII

When ROS and cytochrome c were measured Yme1L depletion and with mitochondrial hyperfusion in NBII, an unexpected result was observed. Insignificant changes in cytochrome c levels were observed (Fig 3.4.2 C-D), suggesting cristae morphology to be unaffected. Before subscribing to this inference, one must take into account that cytochrome c is an indirect readout of cristae disruption. It is plausible certain diseased cristae could sequester cytochrome c normally. On the other hand, hyperfusion rescued the observed increase in ROS (Fig 3.4.2 A-B). Since hyperfusion does not rescue the differentiation defect, this result suggests Yme1L to be regulating NBII differentiation through a ROS-independent mechanism.

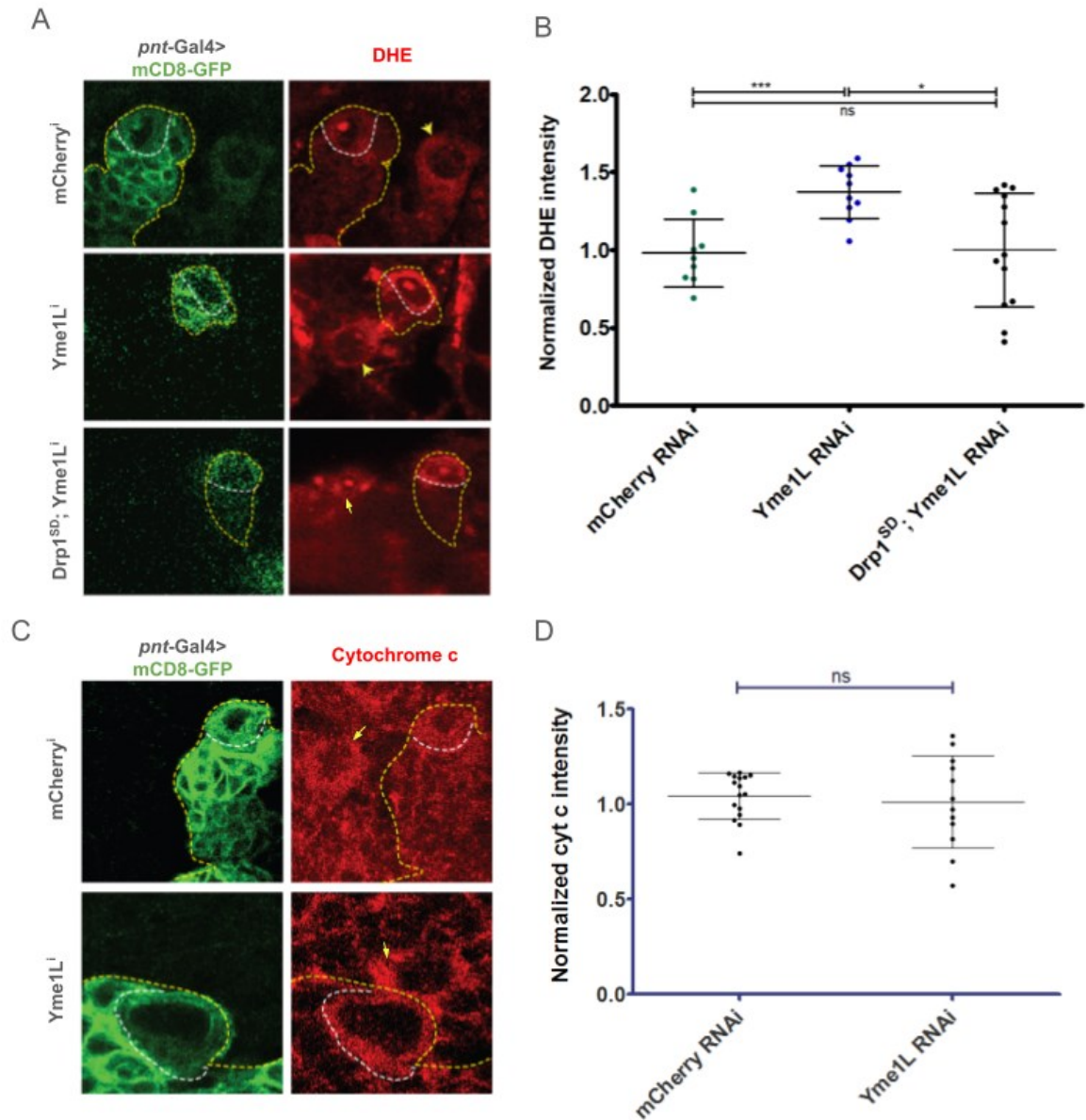


Fig 3.4.2: Analysis of ROS levels and cytochrome c levels with Yme1L depletion and expression of Drp1^{SD} in NBII. A. Confocal microscopy images representing type II neuroblast lineages showing DHE intensity with mCherry RNAi (reused images), Yme1LRNAi and Drp1SD; Yme1L RNAi (3 brains per genotype). The type II neuroblasts are demarcated by white dashed outline while the lineage is demarcated by yellow dashed outline. The yellow arrows indicate type I neuroblast. B. Quantification of normalized DHE intensity for the three genotypes C. Representative confocal images of type II neuroblast lineages showing cytochrome c intensity with mCherry RNAi (reused images), Yme1LRNAi and Drp1SD; Yme1L RNAi (3 brains per genotype). The type II neuroblasts are demarcated by white dashed outline while the lineage is demarcated by yellow dashed outline. The yellow arrows indicate type I neuroblast. D. Quantification of normalized DHE intensity for the three genotypes (For details, see Materials and Methods). Non-parametric Mann-Whitney test was used for statistical analysis. ns-non-significant ($p > 0.05$)

3.5 Increased ROS in NBII causes a decreased number of lineage cells

ROS species such as H₂O₂ (peroxide) act as a secondary signalling molecule, participating in cell signalling by precisely modulating the oxidation state of active cysteine residue containing proteins, thereby regulating their activity downstream. Dedicated machinery comprising ROS scavengers such as superoxide dismutases (SODs), catalase, and glutathione peroxidases exist to tightly regulate the levels and localisation of ROS within the cell. Superoxide dismutases convert superoxides into

more stable peroxides, with SOD1 dealing with cytoplasmic superoxide radicals and SOD2 dealing with mitochondrial matrix superoxide radicals. In contrast, other enzymes like catalase further neutralise peroxides into water (Wang et al., 2018).

To further consolidate the role of ROS in NBII differentiation, I increased the cellular ROS by depleting SOD2, independent of any direct genetic manipulation of mitochondrial morphology or ultrastructure (Fig 3.5a A-B). By increasing the ROS levels, I could recapitulate the differentiation defect, observing a decrease in the number of mINPs, GMCs (Fig 3.5a C-E), and neurons (Fig 3.5b), suggesting ROS to be downstream of cristae morphology disruption and sufficient to cause the differentiation defect.

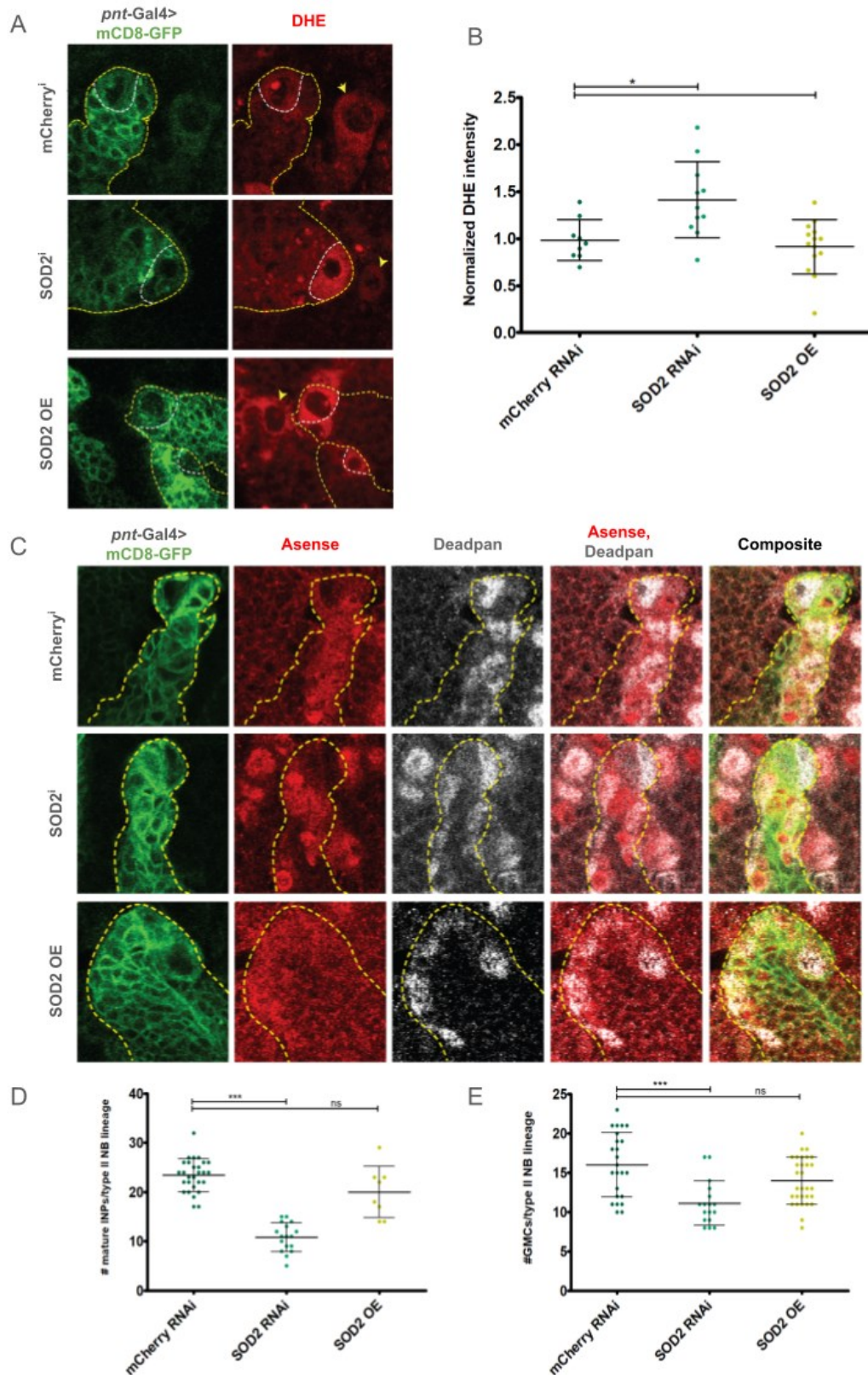


Fig 3.5a: Increased ROS in NBII causes a decreased number of lineage cells. **A.** Confocal microscopy images representing type II neuroblast lineages showing DHE intensity with mCherry RNAi, SOD2 RNAi and UAS-SOD2 (3 brains per genotype). The type II neuroblasts are demarcated by white dashed outline while the lineage is demarcated by yellow dashed outline. The yellow arrows indicate type I neuroblast. **B.** Quantification of normalized DHE intensity for the three genotypes (For details, see Materials and Methods). **C.** Representative confocal images of type II neuroblast lineage showing in Dpn and Ase positive cells in mCherry RNAi (n=3), UAS-SOD2 RNAi (5), and UAS-SOD2 (4) each expressed with *pnt-Gal4*, *mCD8-GFP*. **D.** Quantification of number of mature INPs per lineage in mCherry RNAi (n=3), UAS-SOD2 RNAi (5), and UAS-SOD2 (4). **E.** Quantification of number of GMCs per lineage in mCherry RNAi (n=3), UAS-SOD2 RNAi (5), and UAS-SOD2 (4). Non-parametric Mann-Whitney test was used for statistical analysis. ns-non-significant ($p > 0.05$).

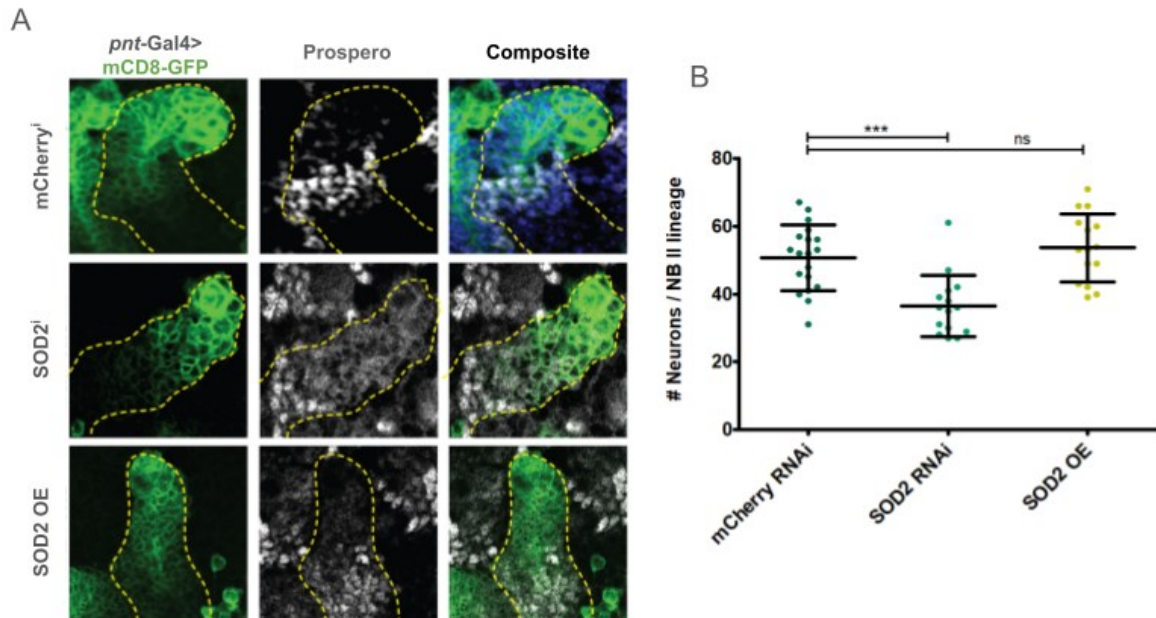
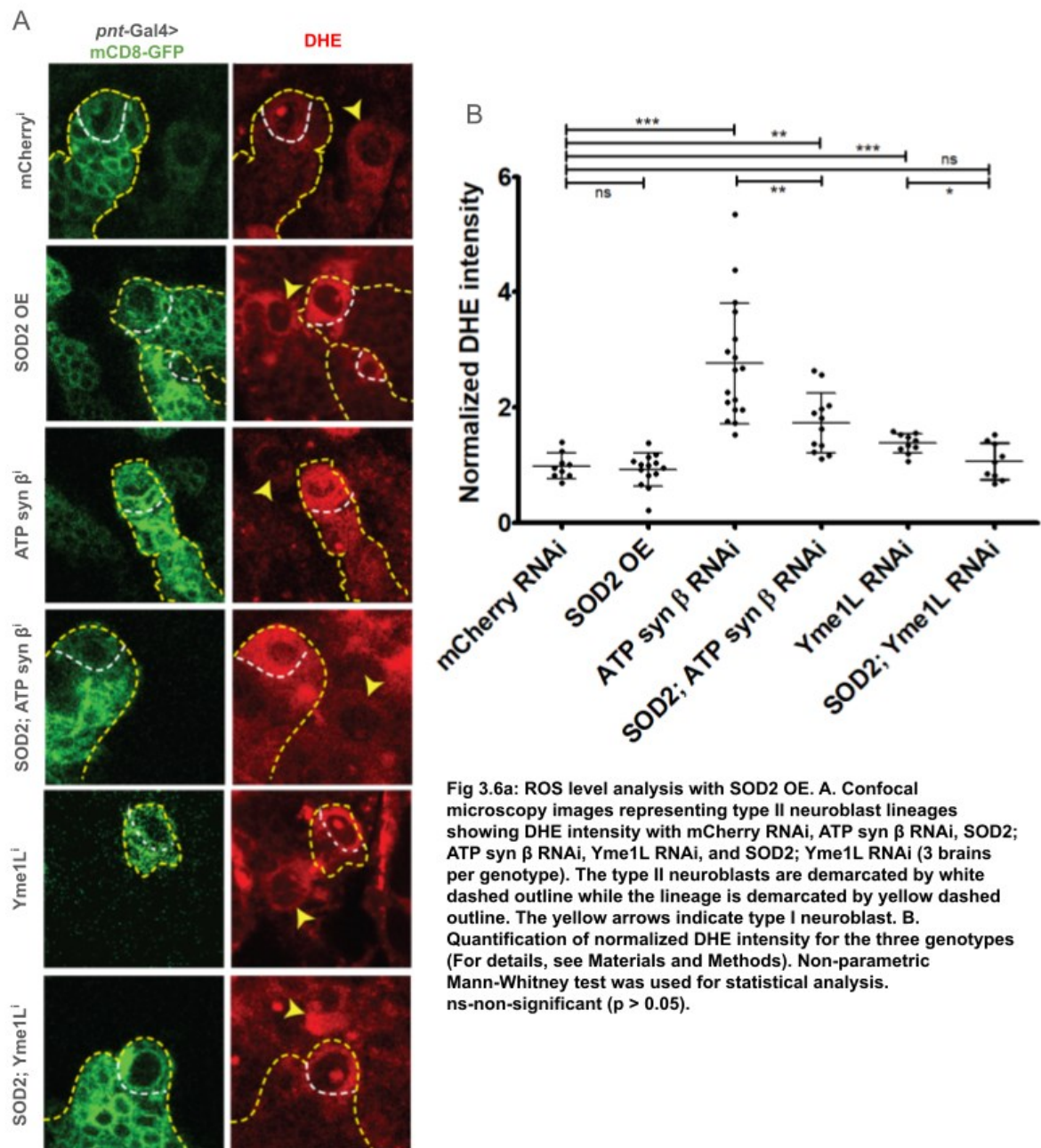


Fig 3.5b: Increased ROS in NBII causes a decreased number of lineage cells. A. Confocal microscopy images representing type II neuroblast lineage showing in Pros positive cells in mCherry RNAi (n=3, reused images), UAS-SOD2 RNAi (5), and UAS-SOD2 (4) each expressed with pnt-Gal4, mCD8-GFP. B. Quantification of number of neurons in mCherry RNAi (n=3), UAS-SOD2 RNAi (5), and UAS-SOD2 (4). Non-parametric Mann-Whitney test was used for statistical analysis. ns-non-significant (p > 0.05)

3.6 ROS causally links cristae morphology and NBII differentiation

To ascertain the role of the observed ROS trends with cristae defects, I attempted to rescue the observed differentiation defect by rescuing the ROS levels. By combining ATP synthase β RNAi and Yme1L RNAi with SOD2 overexpression, I was able to partially rescue the ROS defect with ATP synthase β RNAi and completely rescue it in the case of Yme1L RNAi (Fig 3.6a). The ROS rescue ATP synthase β RNAi with was observed to insufficient to rescue the differentiation defect (Fig 3.6b&c). On the other hand, a partial rescue of the differentiation defect was observed with Yme1L RNAi (Fig 3.6b&c). The observations further consolidate the position of cristae morphology upstream of ROS in the regulation of NBII.



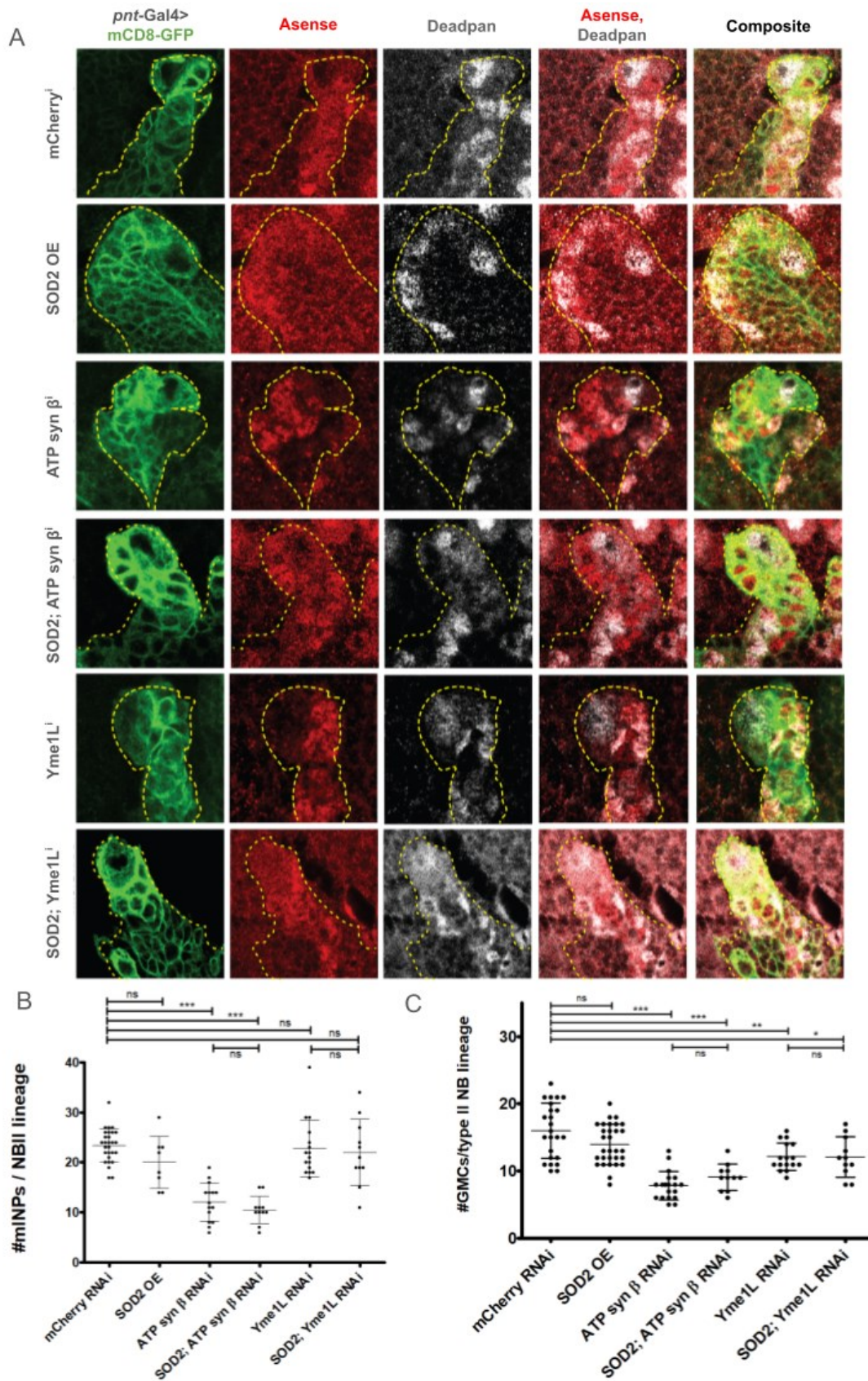
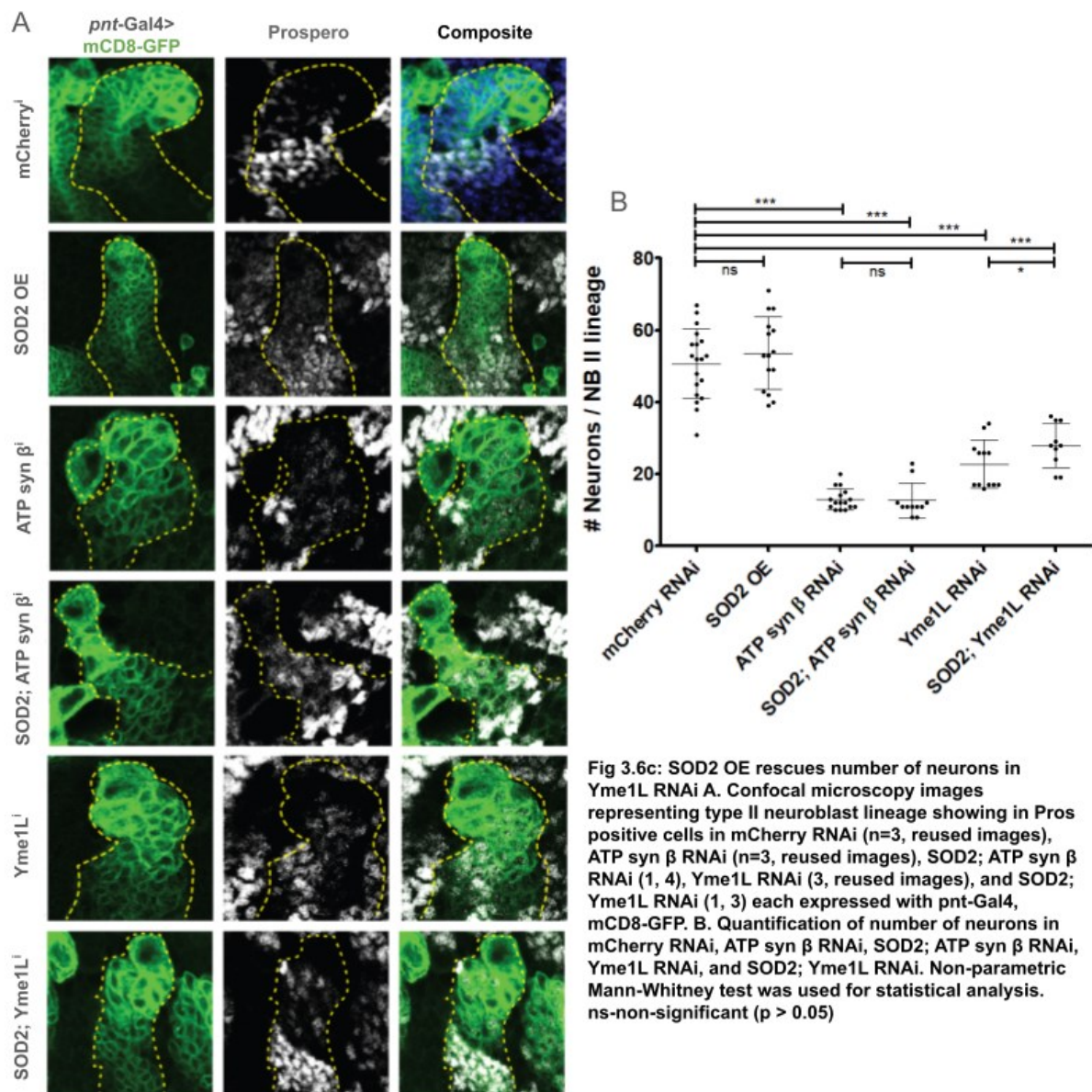


Fig 3.6b: SOD2 OE rescues number of mINPs and GMCs in Yme1L RNAi. **A.** Confocal microscopy images representing type II neuroblast lineage showing Dpn and Ase positive cells in mCherry RNAi ($n=3$, reused images), ATP syn β RNAi ($n=3$, reused images), SOD2; ATP syn β RNAi (1, 4), Yme1L RNAi (3, reused images), and SOD2; Yme1L RNAi (1, 3) each expressed with *pnt-Gal4*, *mCD8-GFP*. **B.** Quantification of number of mature INPs per lineage in mCherry RNAi ($n=3$, reused images), ATP syn β RNAi ($n=3$, reused images), SOD2; ATP syn β RNAi (1, 4), Yme1L RNAi (3, reused images), and SOD2; Yme1L RNAi (1, 3). **C.** Quantification of number of GMCs per lineage in mCherry RNAi ($n=3$, reused images), ATP syn β RNAi ($n=3$, reused images), SOD2; ATP

syn β RNAi (1, 4), Yme1L RNAi (3, reused images), and SOD2; Yme1L RNAi (1, 3). Non-parametric Mann-Whitney test was used for statistical analysis. ns-non-significant ($p > 0.05$).



Chapter 4 Discussion

This study indicates mitochondrial ultrastructure regulates type-II neuroblast proliferation and differentiation in *Drosophila melanogaster* larval brains. More specifically, disrupting cristae morphology by modulating the proportions of L- and S-OPA1 or hampering ATP synthase dimerisation in NBII causes a significant decrease in the number of differentiated lineage cells. Though Dubal et al. (2022) have previously observed such a defect with mitochondrial fragmentation and not mitochondrial fusion, I have observed differentiation defects to accompany fused mitochondria with faulty cristae morphology, demonstrating the defect to be more causally proximal to mitochondrial ultrastructure than mitochondrial morphology. Furthermore, I established ROS as a causal link between cristae morphology and NB differentiation. Ultimately, the findings suggest that cristae morphology mediates the interaction between mitochondrial morphology and ETC in the co-regulation of NB proliferation and differentiation.

Though the results can be clearly inferred from the data, a few exceptional observations remain to be smoothened over. For the instance, the apparent rescue of ROS levels and number of GMCs by mitochondrial hyperfusion in the case of Yme1L RNAi does not match the rest of the observations. Such inconsistencies need to be verified by repeating the experiment/quantification. Furthermore, the cause of lethality with MICOS subunit depletion is unexplained since no differentiation defects were observed. By producing double knockdowns of MICOS subunits, the role or the lack thereof of MICOS subunits in NBII should be verified. Furthermore, to verify the role of ROS in linking ATP synthase β knockdown with NBII differentiation, the RNAi line should be combined with a stronger antioxidant overexpression lines, to produce a sufficient rescue of ROS. Though Dubal et al. (2022) have established mitochondrial hyperfusion to not be affecting NBII differentiation, it remains to be verified, and should be included as a control in the rescue experiments.

4.1 Mitochondrial ultrastructure dynamics

Hu et al. (2020) have observed OPA1, Yme1L, MICOS, and ATAD3a differentially regulating cristae dynamics in HeLa cells. While OPA1 KO is associated with a

decrease in cristae elongation, shortening, merging dynamics and an increased loss of cristae junction, Yme1L KO was associated with unchanged cristae elongation, merging dynamics but decreased shortening and cristae junction loss. On the other hand, MICOS KO is associated with a severe loss of cristae dynamics, and ATAD3a KO has been associated with an increase in cristae dynamics except for cristae elongation. Since I have not observed a differentiation defect with MIC60/19 KD in NBII, it is plausible that certain aspects of cristae dynamics play a greater role in differentiation than others.

Though the biological significance of cristae dynamics is unclear, decreased ADP/ATP exchange has been observed to perturb cristae dynamics independent of mitochondrial morphology and membrane potential, suggesting cristae dynamics to be significant to bioenergetics (Golombek et al., 2023). Furthermore, individual cristae have been observed to have distinct membrane potential (Wolf et al., 2019), suggesting cristae merging and splitting events to be coupled with the reorganisation of ETC complexes. Further analysis of the role of cristae dynamics in NB differentiation is necessary.

4.2 ETC supercomplex formation

An avenue of exploration remains the junction between ETC activity and cristae morphology. ETC complexes through blue native PAGE analysis have been observed to exist in the mitochondrial inner membrane as supercomplexes with variable stoichiometry, with the extent of super-complexation reflecting the cell's OXPHOS requirements (Schägger et al., 2000). Such an observation conflicted with the dominant understanding of ETC as disarranged complexes in the inner membrane, with cyt c and CoQ diffusing, carrying electrons between them. The super-assembly of respiratory complexes suggests the plausibility of “respiratory strings,” the dynamic direct physical association between the ETC complexes producing highly efficient self-sufficient respiration units as and when required, adapting to the environmental demands (Cogliati et al., 2021).

Cristae morphology plays a critical role in modulating mitochondrial energetics by facilitating or hampering supercomplex formation. OPA1 depletion has been shown to disrupt cristae morphology, supercomplex formation, and reduce respiration and respiratory growth (Cogliati et al., 2013; Fig 4.2). Furthermore, starvation has been

demonstrated to activate the protein kinase R-like ER kinase mediated pathway (also called the PERK-mediated pathway), which downstream enhances respiration by increasing cristae density and supercomplexation (Balsa et al., 2019).

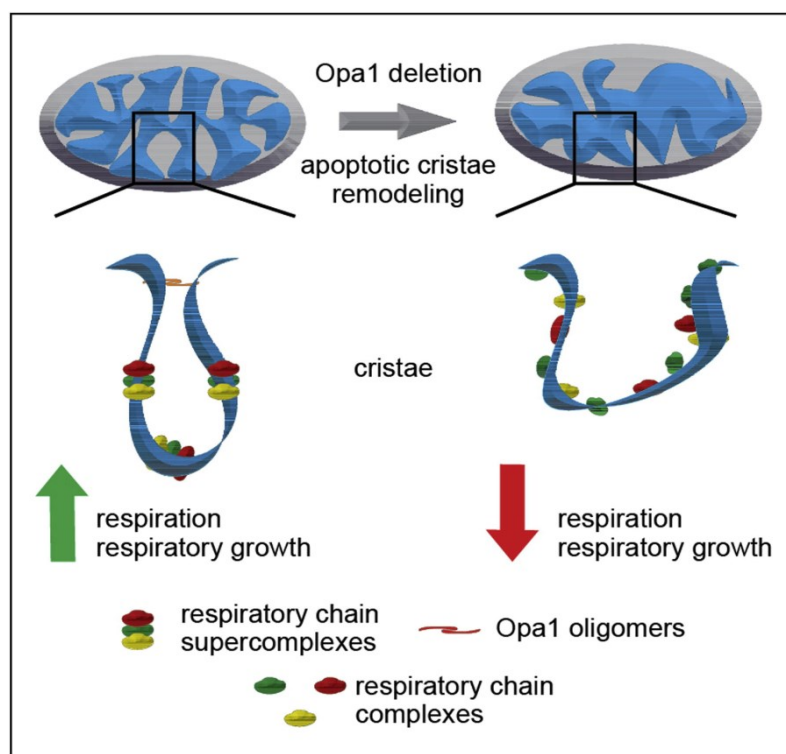


Fig 4.2: Schematic representation of the role of cristae morphology in ETC supercomplex formation (Cogliati et al., 2013)

The proposed hypothesis behind hyperfusion-mediated rescue of differentiation defect – hyperfusion enhancing ETC activity by ameliorating cristae morphology and supercomplexation – is well-substantiated by previous literature. However, the verification of the hypothesis using BN-PAGE is mandatory.

4.3 Downstream of ROS

ROS signalling has been observed to be regulating stem cell proliferation and differentiation in a variety of systems. For instance, Nox4-dependent H_2O_2 signalling is crucial for the development of smooth muscle cells from mouse embryonic stem cells (Xiao et al., 2008). Similarly, in *Drosophila* intestinal stem cells, the knockdown of complex I subunits have been shown to increase cellular ROS and intestinal stem cell proliferation. Furthermore, decreasing the ROS levels by overexpression of antioxidants has been shown to cause underproliferation, consolidating the position

of ROS signalling in stem cell proliferation (Hochmuth et al., 2011). However, the cell signalling pathway downstream of ROS in stem cells is underexplored.

Redox-sensitive signalling molecules such as HIFs, p38 and p53 have been implicated to be acting downstream of ROS in stem cells, with their oxidisable active cysteine residue identified and experimentally verified (Wang et al., 2013). The activation of p38 MAPK causes underproliferation in human mesenchymal stem cells (Bhandari et al., 2010). Similarly, p53 activation has been associated with underproliferation in murine neural stem cells (Motoshi et al., 2008).

However, to decipher the signalling pathway downstream of ROS in NBs, a proteome-wide mass spectrometric analysis for oxidised active cysteine residue analysis could be performed (van der Reest et al., 2018). Such an analysis would yield the exact cell-signalling pathways affected by redox potential regulating NB differentiation, a severely unexplored territory.

4.4 ROS-mitochondrial morphology interplay

The influence of mitochondrial morphology on ROS in NBII has been analysed previously. Though similar levels of mitochondrial fragmentation have been observed with OPA1 depletion and Marf depletion, a significant increase in ROS has been observed only with OPA1 depletion. Unexpectedly, Drp1^{SD}-mediated mitochondrial hyperfusion has also been observed to cause a significant increase in ROS, suggesting ROS to not play a role in NBII differentiation (Dubal et al., 2022). My analysis contradicts this study demonstrating ROS to be causally linking mitochondria with NBII differentiation, with hyperfusion-mediated rescue of both ROS levels and differentiation defect.

Therefore, we propose cristae disruption to hamper ETC supercomplex formation, diminish their efficiency, and cause increased ROS production. Mitochondrial hyperfusion rescues cristae morphology and, therefore, ROS downstream.

On the other hand, the role of ROS in regulating mitochondrial morphology in NBII is unexplored. In *Drosophila* ovarian follicle cells, increasing ROS causes mitochondrial fragmentation, suggesting a crosstalk between ROS and mitochondrial morphology (Uttekar et al., 2024). Various signalling pathways have been implicated in connecting ROS with mitochondrial dynamics by affecting the activity of fusion

proteins and fission proteins (as reviewed by Ježek et al., 2018; Fig 4.4). A similar mitochondrial morphology analysis in NBII would deepen our understanding of ROS-mitochondrial morphology crosstalk, further compounding our proposed model, implicating not just the interaction between ETC activity and mitochondrial morphology but their intersection with redox stress to be regulating NBII differentiation.

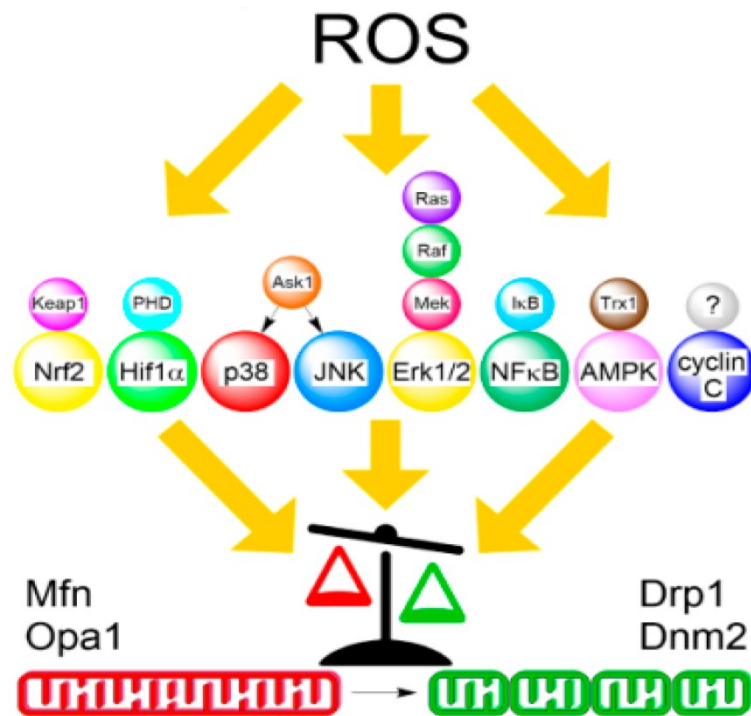


Fig 4.4: Schematic representation of ROS-mediated signaling pathways regulating mitochondrial morphology (Ježek et al., 2018)

4.5 Mitophagy

The significance of membrane depolarization on ATP synthase depletion in NBII has not been explored. Mitochondrial membrane depolarization has been observed to cause delay G1-to-S transition in yeast, independent of other factors such as genetic stress (Gorospe et al., 2023). Furthermore, an intimate relationship exists between mitochondrial morphology, membrane potential and mitophagy. Membrane depolarization acts as a readout of mitochondrial defect and marks it using Parkin and PTEN-induced putative kinase 1 (Pink1) for mitophagy (Elmore et al., 2001; Priault et al., 2005; Narendra et al., 2008; Narendra et al, 2010). Twig and Shrihail (2011) pointed out mitochondrial fission, and not spontaneous loss of membrane potential, to be the depolarization event predominantly preceding mitophagy.

Furthermore, they pointed out that Drp1-mediated fusion rescues the healthy fissioned mitochondria from mitophagy, differentiating the healthy from the defective one by membrane potential. Therefore, it is plausible that mitophagy could rescue the observed differentiation defect in NBII by the removal of defective mitochondria. Mitochondrial hyperfusion by OPA1 overexpression decreases the levels of mitophagy (Twig et al., 2008). Thus, Drp1^{SD}-mediated hyperfusion could act as an antagonistic mechanism, rescuing the mitochondria from mitophagy, by rescuing their membrane potential.

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