Characterization of the functional role of the Cystein-rich with EGF-like domains (Creld) protein in mitochondrial quality control in *Drosophila melanogaster*

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

Marie Paradis

aus

Siegburg

Bonn

Januar 2019

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn

1. **Gutachter: PD Dr. Reinhard Bauer**

2. **Gutachter: Prof. Dr. Dr. h.c. Michael Hoch**

Tag der Promotion: 4. Juli 2019
Erscheinungsjahr: 2019

Table of Contents

1. Introduction

1.1 Cystein-rich with EGF-like domains – The Creld protein family

Cystein-rich with EGF-like domains (Creld) proteins belong to a protein family, evolutionary conserved throughout species. The mammalian genome contains two Creld genes – *Creld1* and *Creld2*. In *Drosophila*, however, there exists only one *Creld* gene which yields two transcript variants (Rupp *et al*., 2002).

The structure of mammalian Creld proteins comprises a signaling peptide at the N-terminus, a creld protein specific WE domain, which contains a unique nanopeptide and is considered to be involved in protein-protein interaction, two epithelial-growth factor (EGF) and calcium binding domains. Additionally, in the case of Creld1 two transmembrane domains which is absent in Creld2 (Rupp *et al*., 2002). The *Drosophila* Creld protein contains the same structural elements, the difference here is that only one EGF-like and Ca^{2+} binding domains are available and according to which transcript, one or two transmembrane domains. In the case of transcript RA there are two transmembrane domains predicted, RB contains only one (flybase.org).

Previous studies have shown that mammalian Creld1 and Creld2 are localized to the endoplasmic reticulum (ER) membrane (Oh-hashi *et al*., 2011, Mass *et al*., 2014). Also, our research group could show that the *Drosophila* Creld protein localizes there (Birgit Stümpges, PhD Thesis).

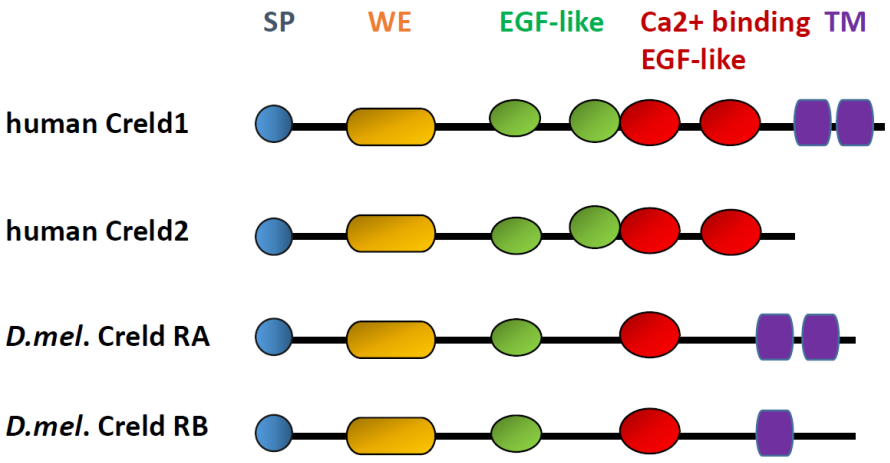

Figure 1-1: Predicted primary protein structure of human and fly Creld proteins. The proteins share a common primary structure. Every protein contains a N-terminal signalling peptide (SP, depicted in blue), the Creld-specific WE domain (containing a conserved nano peptide). Mammalian Creld proteins contain two epidermal growth factor (EGF)-like domains, whereas in Drosophila only one EGF-like domain is existing (shown in green). The same is true for the Calcium-binding EGF like domains (in red). Mammalian Creld1 contains two transmembrane domains (TM, depicted in magenta), which are lacking in Creld2 proteins. In *Drosophila* the Creld RA transcript is predicted to have two TM, whereas the transcript RB is supposed to have only one. Figure modified from Elvira Mass, 2014 PhD Thesis.

Mammalian Creld1 is considered as a regulator of endocardial cushion formation during embryogenesis by interacting directly with Calcineurin. Calcineurin consists of a regulatory subunit Calcineurin subunit B (CnB) and a catalytic subunit Calcineurin A (can). It is a Ca2+/Calmodulin dependent serine/threonine phosphatase that upon activation dephosphorylates a variety of target proteins. The activation occurs through proteins of the so-called Regulator of the Calcineurin (RCAN) family. But Calcineurin can also be regulated through Ca2+ concentrations. Creld1 was shown to interact with the regulatory subunit CnB directly and to consequently activate it. It is suggested that the unique WE domain is necessary for this interaction (Mass *et al*., 2014).

Creld2 however, is hypothesized to be involved in the mammalian Endoplasmic reticulum (ER) stress response. Previous studies could show that upon induction of ER stress Creld2 expression is increased (Oh-hashi et al., 2011). Oh-hashi *et al*., could show that the induction of *Creld2* expression is mediated via an ER stress response element in the promoter of *Creld2*, which is activated via the ER stress response gene activating transcription factor 6 (ATF6) and can even be used as a urinary ER stress marker in mammals (Kim *et al*., 2017). So far, no investigations of the *Drosophila* Creld protein and its role in ER stress response have been made.

1.2 *Drosophila* heart morphology

In previous studies it was shown that in the mammalian system Creld1 is involved in proper heart development: Creld1 is needed for Calcineurin/NFATc1 dependent heart valve formation. Creld1 knockout mice die between embryonic stage E11 and E11.5, the point of time when the four-chambered heart is formed (Mass *et al.*, 2014).

Between mammalian and fly hearts there are obvious morphological differences: Mammals and animals with high metabolic rates contain a closed circulatory, high hydrostatic pressure system, in which the blood is restricted to the network of capillaries, arteries and veins. The heart itself is comprised of a four chambered structure (Rotstein and Paululat, 2016).

In contrast to that, insects, including flies, have an open circulatory system, where the fluid is pumped from the posterior to the anterior part of the body cavity via a longitudinal tube (Ocorr *et al.*, 2015), which is called dorsal vessel. The morphological differences between mammalian and fly hearts can be seen in Figure 1-2.

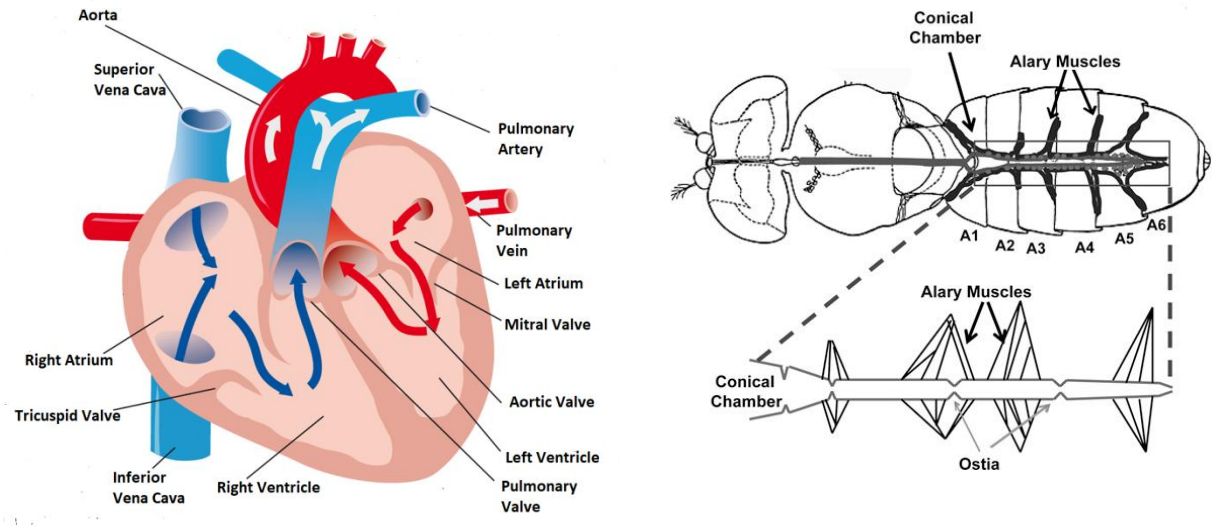

Figure 1-2: Morphological differences between a mammalian and a fly heart. Shown are the schematic drawings of a mammalian heart (left) and the dorsal vessel of Drosophila (right). The mammalian heart shows the typical four chambered morphology where the blood enters the heart and flows unidirectional, coordinated by valves. In contrast to that the insect heart is a longitudinal structure with the conical chamber, the main "heart", located at segment A1. The insects heart contractility is mediated though alary muscles and ostial cells show valve-like function. Figure modified from "Georg Thieme Verlag, New York" and Wolf and Rockman, 2011.

Even though the general structure is different, the fly heart also contains valves and heart-like cells. Additionally, the insects "blood" is more similar to interstitial fluid as it is not needed for oxygen transport (it lacks oxygen-transporting blood cells) but supplies organs with nutrients, signaling peptides and other metabolites. The gas-exchange is achieved through a tubular network of trachea instead (Rotstein and Paululat, 2016). The simple methodology of studying a heart which is still intact, the advantage that oxygen supply and heart function are not coupled and a conserved mechanism of development and function make *Drosophila* a favorable organism to study heart development and function (Ocorr *et al.*, 2015).

1.3 Mitochondrial function and metabolism

1.3.1 Mitochondrial structure and function

Mitochondria are small organelles found in almost every eukaryotic cell and are commonly known as the "powerhouse" of the cell. They comprise the main site of the cell's energy production by the oxidation of molecules such as sugars, to produce high amounts of adenosine triphosphate (ATP) (Trait *et al*., 2012).

It is generally assumed that mitochondria were created a billion years ago by the engulfment of bacteria by the nucleated ancestral cell. This so-called "endosymbiont theory" is

supported by the double membrane structure of mitochondria and the fact that the organelles contain their own genome and machinery for protein biosynthesis, although the majority of mitochondrial genes were transferred to the nucleus over time (Martin *et al*., 2015). Mitochondria are present in almost every eukaryotic cell. However, their exact number greatly differs in tissues and cell types. For example, high energy demanding tissue as testis, muscle and brain are dependent on a relatively high number of mitochondria.

The mitochondrial structure is characterized by its double membrane. Whereas the outer mitochondrial membrane is porous, the inner mitochondrial membrane poses a strict barrier to all kinds of molecules. The membrane potential build up in the inner mitochondrial space is helping the electron transport chain localized in the inner membrane and will be discussed in detail below. The two membranes are the reason for the two distinct mitochondrial compartments: The large inner lumen is called matrix space, containing a unique set of enzymes needed for oxidation of fatty acids for the citric acid cycle. Between the inner and outer mitochondrial membrane, the intermembrane space is formed (Sherratt, 1991).

Mitochondria rarely are generated *de novo* but they originate from growth and division of existing mitochondria. The events leading to fusion and fission are complex and will be discussed in more detail.

1.3.2 Mitochondrial dynamics

Since mitochondria are detrimental for energy production, it is crucial to maintain their integrity and function. This is ensured by the so-called mitochondrial dynamics (Twig and Shirihai, 2011).

Mitochondrial dynamics describe the event of a repetitive cycle of mitochondrial fusion and fission (Chen and Chan, 2011) and are summarized in Figure 1-3. Mitochondrial fission helps to segregate damaged or dysfunctional mitochondria from the healthy network whereas its opposing event, the mitochondrial fusion, is described as content mixing of healthy mitochondrial components, including mitochondrial DNA. This is especially important as mitochondria have their own genome which encodes proteins involved in mitochondrial metabolism (Knott *et al*, 2008). As occasionally mutations such as insertions or deletions occur in the mitochondrial genome, mixing of mutated DNA with wild type mitochondrial DNA is crucial to mitigate arising defects and to maintain mitochondrial function. The mixing is ensured by mitochondrial fusion (Youle *et al*., 2012).

1. Introduction

Mitochondria fusion is conserved throughout species. In mammals, the fusion of both outer (OMM) and inner (IMM) mitochondrial membranes is orchestrated by Dynamin-like GTPases called Mitofusins (Mfn1 and Mfn2, Marf in *Drosophila*) and Optic atrophy 1 (Opa1) (Santel and Frank, 2009). The initial tethering of two mitochondria is mediated by Mfn1 and Mfn2 at the OMMs. They anchor two adjacent mitochondria via interactions of the cytosol-facing C-terminal membrane binding-domains. Their N-terminal GTPase domain is crucial for the process, since the fusion reaction is dependent on GTP hydrolysis (Ishihara et al., 2014). The second step of mitochondrial fusion is dependent on Opa1 proteins which accumulate in the intermembrane space and mediate physical interactions between IMMs. IMM fusion requires proteolytic processing of the Opa1 protein producing many isoforms. The phenomenon of proteolytic processing of Opa1 is hardly understood. What is known is that Opa1 precursors are imported into mitochondria where, after removing the mitochondrial targeting sequence, the long isoform is imbedded into the IMM. Cleavage at S1 and S2 sites by diverse proteases creates short isoforms which are no longer bound to the membrane. For proper IMM fusion a combination of both long and short isoforms of Opa1 is needed (Mishra *et al*., 2014)**.** Actual fusion of the two IMMs is stabilized by physical interaction of the soluble Opa1 isoforms located at the intermembrane space, which allows the interaction of the long isoforms bound to the membrane of the fusing mitochondria.

As an opposing event to fusion, mitochondrial fission sits at the other side of the dynamic balance. The key player in mitochondrial fission is the large GTPase Dynamin-related-protein 1 (Drp1). It undergoes different posttranslational modifications which regulate its shuttling between the cytosol and mitochondria. During normal cellular processes the majority of Drp1 proteins localizes as oligomers in the cytosol and need covalent modification to get activated and to translocate to mitochondria (Youle *et al*., 2012). The Drp1 protein contains phosphorylation sites. Phosphorylation describes the modulation of protein function via protein kinases and is widely studied at the Drp1 protein. Serine616 (Ser616) was shown to be phosphorylated by the kinase complex Cdk1/CyclinB, promoting mitochondrial fission and segregation of mitochondrial DNA during mitosis. Another prominent phosphorylation site of Drp1 is Ser637. It is phosphorylated by the protein Kinase A (PKA) and Calcium/calmodulin-dependent protein kinase I (CaMKI). Phosphorylation at this site was reported to decrease Drp1 translocation to mitochondria. In contrast to that, the dephosphorylation via

Calcineurin at this site promotes mitochondrial fission (Chang and Blackstone, 2009). This aspect is of great interest to this study and will be described later in detail.

Other posttranslational modifications of the Drp1 protein include sumoylation and ubiquitination. The functional role of attachment of sumo to Drp1 by, for example, the sumo E3 ligase MAPL is not clear yet. It seems not to be required for Drp1 translocation but it is suggested, that it is involved within the interaction of Drp1 with the mitochondrial membrane. A similar process, the ubiquitination of the Drp1 protein, was shown to help Drp1 translocation to mitochondria (Chang and Blackstone, 2010).

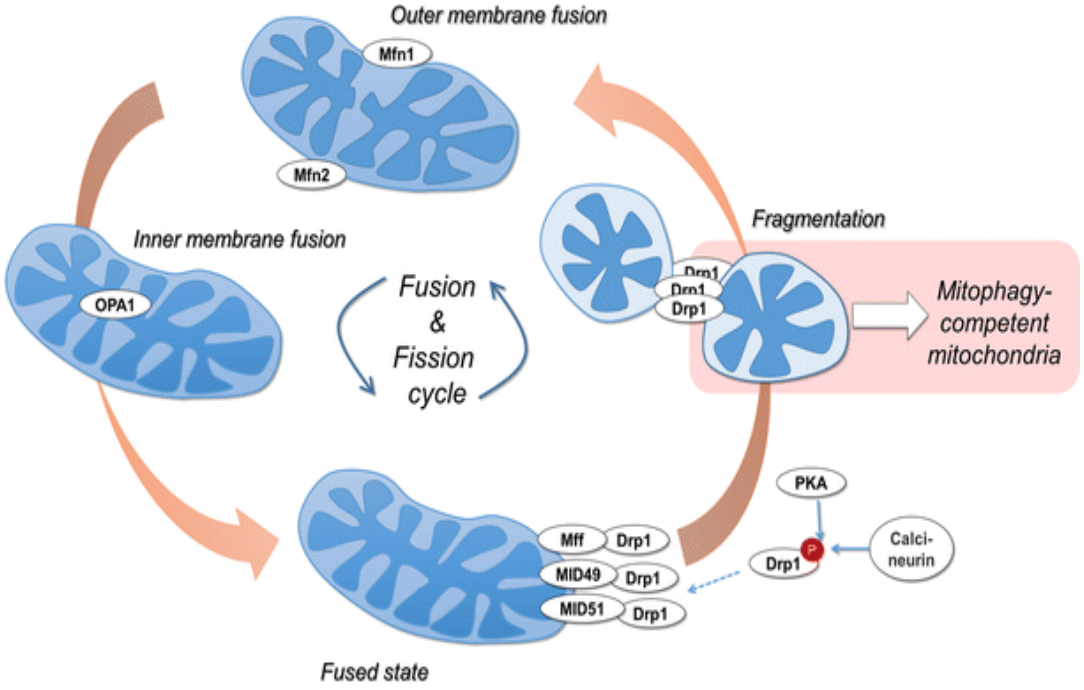

Figure 1-3: Mitochondrial Dynamics. The pool of mitochondria is regulated via constant cycle of fusion and fission events. The key proteins in fusion are Opa1 (optic atrophy 1) and Mfn1 and Mnf2 (Mitofusin 1 and 2, Marf in *Drosophila*). Pro-fission protein Drp1 (dynamin related protein 1) is regulated by post-translational modifications. PKA (protein kinase A) mediated phosphorylation keeps the protein in the cytoplasm. Upon dephosoprylation by Calcineurin, Drp1 travels to mitochondria where it forms multimeric structures and initiates fission of mitochondria. Fragmented mitochondria can efficiently be removed by mitophagic degradation. Figure modified from Hamacher-Brady et al., 2016.

Upon activation, Drp1 proteins get recruited to the mitochondrion and most likely use mitochondrial fission protein 1 (Fis1) as an adaptor protein, which resides at the outer mitochondrial membrane. Upon translocation Drp1 self-assembles into large multimeric structures and forms a spiral-like ring around the mitochondrion to constrict the two membranes destined for fission (Parrone *et al.*, 2008).

Following the procedure outlined above, the cell ends up with two daughter mitochondria. Usually one contains the healthy content whereas the other one bears the damaged parts

and is destined for degradation. For the destruction of damaged mitochondria, a mechanism of selective autophagy comes into play.

1.3.3 Mitochondrial quality control via mitophagy

Autophagy comprises the main degradation system in the cell. In general, autophagy is the process used to degrade and recycle bulk intracellular material, like damaged organelles, macromolecules and even invading bacteria. The process of autophagy begins with the engulfment of damaged cellular material by a double membrane building up an autophagosome, which eventually fused with lysosomes for content degradation (Kuma and Muzushima, 2010). The different steps of the autophagic process are orchestrated by various proteins, with the autophagy related gene (ATG) proteins acting as key regulators.

Different proteins and protein complexes are involved in autophagy induction. Under nutrient-rich conditions, autophagy is repressed via the Target of Rapamycin complex 1 (TORC1), which functionally inhibits Atg1, a protein needed for the initiation of formation of the autophagosomal membrane. However, TORC1 inhibition under prolonged conditions of starvation initiates autophagy to supply the cell with nutrients derived from the breakdown of macromolecules. TORC1 inhibition results in the activation of Atg1, which binds its substrates Atg13 and Atg17 to initiate the early steps of phagophore formation. Therefore, Atg1 can be used as a marker for autophagy induction (Hansen and Johansen, 2011).

During energetic stress another sensor is able to modulate autophagy regulation, which is the AMP activated kinase (AMPK). It can activate the autophagic process by repressing TORC1 or directly by phosphorylation and therefore the direct activation of Atg1. The regulation of autophagy via AMPK is of special importance as it controls the metabolic homeostasis (Gowans *et al*., 2014).

Autophagy can occur very selectively, only targeting specific organelle structures. Mitochondrial degradation through the autophagic pathway is commonly known as mitophagy (Youle and Narendra, 2012). As damaged mitochondria (result of age, usage or disease) might produce a high amount of toxic reactive oxygen species, the cell is dependent on mechanisms controlling the removal of dysfunctional mitochondria. Mitophagy occurs on a basal level but can be upregulated by different stimuli when the cell is in need. Mitophagy is controlled through two proteins called PTEN Induced Putative Kinase 1 (Pink1) and Parkin. Pink1 is localized on the outer surface of the mitochondrial membrane and healthy mitochondria are able to degrade Pink1. When mitochondria become dysfunctional, they are

no longer able to degrade Pink1 which leads to a passive accumulation of the protein. This accumulation recruits Parkin, an E3 ubiquitin-ligase, to the mitochondria and activates it. Parkin is then able to ubiquinate outer proteins on the mitochondrium, thereby marking it for degradation by recruiting the core autophagic machinery (Pickles *et al*., 2018).

It is widely accepted that mitophagy is dependent on mitochondrial fission. The engulfment of damaged mitochondria by the autophagosomal membrane is facilitated by a reduced mitochondrial size, which is achieved by mitochondrial fission (Kageyama *et al*., 2014).

1.4 Cellular respiration

For a cell to keep up its function it needs a constant supply of energy. Energy is gained from food that gets digested and broken down via a three step process to generate energy in the form of ATP which is needed for biosynthetic and other energy demanding cellular processes. Step one is mainly carried out in the intestinal tract and involves enzymes that break down macromolecules into simple building blocks: proteins to amino acids, polysaccharides to monosaccharides (glucose) and lipids to fatty acids and glycerol (Lam and Ravussin, 2016). The main metabolic pathways are summarized in Figure 1- 4.

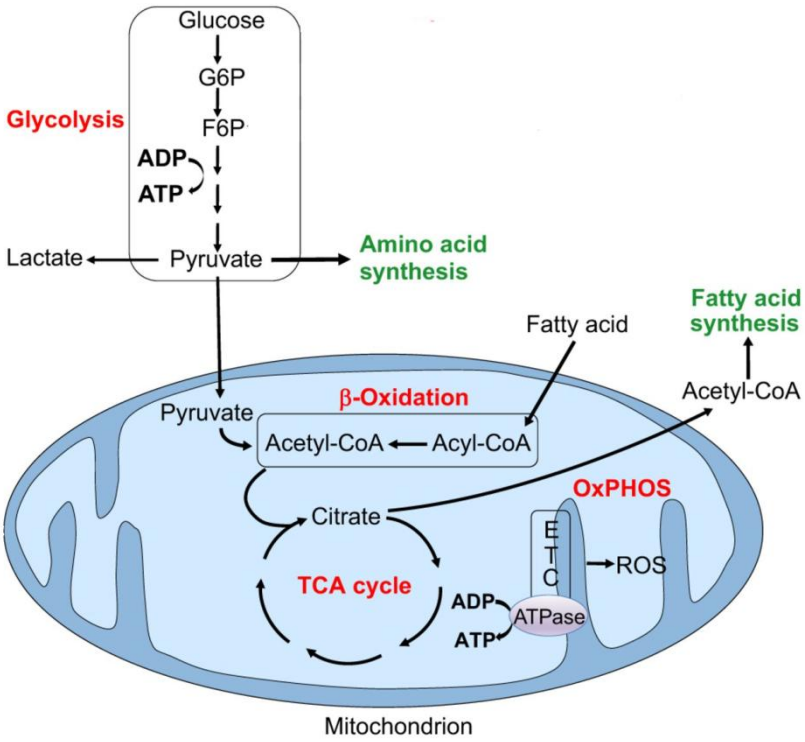

Figure 1-4: Summary of major metabolic pathways. Glycolysis, ß-oxidation, TCA cycle and oxidative phosphorylation (OXPHOS) generate metabolites and produce ATP for energy-requiring cellular processes. Figure modified from Mathieu and Ruahola-Baker, 2017.

On a cellular level these building blocks are further converted into acetyl-Coenzyme A (acetyl-CoA) and a small amount of ATP and NADH. The last step involves the complete oxidation of acetyl CoA to H_20 and Co_2, yielding high amounts of ATP. The energy-yielding chemical intermediates, mainly acetyl-CoAs, are provided to the mitochondria by feeding into the tricarboxylic acid cycle (TCA) via two metabolic pathways, namely glycolysis and fatty acid oxidation (Rajan and Perrimon, 2013).

1.4.1 Glycolysis

Carbohydrates are broken down to monosaccharides/glucose. Glucose serves as the general energy fuel. It can be directly catabolized to gain ATP, or stored in glycogen stores. Glucose molecules get catabolized in three distinct steps. The first step in glucose utilization is happening in the cytoplasm in a process called glycolysis. In this process 10 different enzymatic reactions break the six-carbon glucose down to finally two three-carbon atoms containing pyruvates and two ATP and NADH. After being actively pumped into the mitochondrial matrix, the acetyl groups of pyruvate are further oxidized by the pyruvate dehydrogenase (PDH) complex to generate acetyl CoA which is fed into the TCA cycle (also called citric acid cycle or Krebs cycle) (Sica *et al*., 2017).

Therefore glycolysis can be seen as a prelude step before TCA cycle and oxidative phosphorylation. However, anaerobic organisms or tissue that functions under aerobic conditions (like skeletal muscle), glycolysis can become the major source of energy production. Additionally, for example cancer cells switch their main metabolism towards gylcolysis, rather than producing ATP through oxidative phosphorylation. This might be due to the anaerobic tumor-environment or the damage to mitochondria in cancer tissues. Also, it might be possible that tumor cells turn down mitochondrial metabolism in general, to avoid the apoptotic programs mitochondria play key roles in. This phenomenon, in which cells switch from the more favorable mechanism of oxidative phosphorylation to glycolysis to produce ATP, is called the Warburg effect (Vander Heiden *et al*., 2009).

1.4.2 Fatty acid ß-oxidation

The process of fatty acid oxidation begins in the cytoplasm, where the enzyme acetyl-CoA-synthase activates free fatty acids by the conjugation of coenzyme A. The acyl group of the fatty acid will covalently be attached to coenzyme A: This activation is necessary because free fatty acids cannot pass membranes due to their negative charge. Further modification with carnitin produces fatty acyl carnitine which is transported through the membrane into the mitochondria matrix. Once inside the mitochondrial matrix ß-oxidation is carried out via a series of steps mediated through many enzymes, cleaving two carbons every cycle from the fatty acetyl-CoA, generating one acetyl-CoA molecule each cycle. The newly formed acetyl CoA enters the TCA cycle and is used to produce ATP in the same way as acetyl CoA derived from pyruvate (Wanders *et al*., 2010).

1.4.3 The TCA cycle

The carbon atoms of acetyl-CoA molecule, either coming from pyruvate or oxidation of fatty acids, get completely oxidized and converted to CO_2 in the process known as tricarboxyclic acid cycle (TCA, also called Krebs Cycle or citric acid cycle) to generate high-energy electron carrier NADH, which will be passed along the electron transport chain to generate ATP.

The circle starts with the formation of acetyl-CoA to citrate by a compound called oxalacetate. The formed citrate undergoes rounds of reactions with eight distinct steps, closing to a cycle because the oxalacetate needed in step one, is regenerated in the last step. Even though one ATP molecule is produced in the process of TCA cycle, its main function is to generate high-energy electrons in form of NADH which can be used in the electron transport chain (White, 2015).

1.4.4 Oxidative phosphorylation via the electron transport chain

The last step in the oxidation of food molecules to generate energy is mediated via the electron transport chain (ETC).

The ETC consists of four enzyme complexes localized in the inner mitochondrial membrane (simplified shown in Figure 1-5). The high-energy form NADH is passed along the electron transport chain, thereby falling to a lower energy state and the losing energy is used by the chain complexes to pump H^+ ions from the matrix into the intermembrane space. The accumulating H+ ions will follow their electrochemical gradient back into the matrix, this

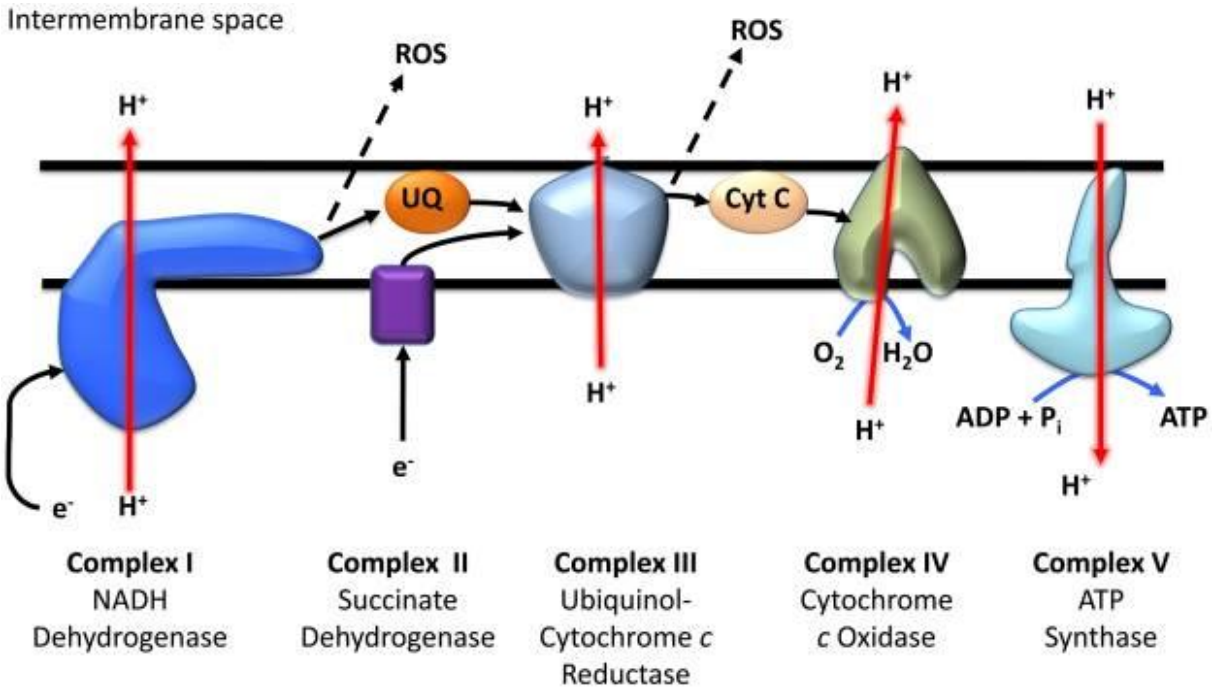

Figure 1-5: Electron transport chain inside mitochondrial matrix. The electron transport chain contains five multi-subunit complexes which reside in the inner mitochondrial membrane. By transferring electrons from donor to acceptor molecules, protons are transferred across the membrane and establish a proton gradient. This gradient is used by the the last complex, ATP synthase to generate ATP. (Ghouleh *et al.*, 2011).

phenomenon is called chemiosmosis, producing an electrochemical gradient. This gradient is responsible for the reflux of protons, which leads to the addition of a phosphate group to ADP, generating ATP, by an enzyme called ATP synthase (or complex V). In total 32 ATP molecules are produced in the process of oxidative phosphorylation (Lodish *et al*., 2000).

1.4.5 Energy storage and mobilization

In order to guarantee a continuous energy supply even in situations where nutrients get scarce, organisms have established regulation mechanisms to answer metabolic needs. In times where there are excessive nutrients available, the energy that is not used directly can be stored and mobilized when needed (Hansen and Johansen, 2011). Here the processes of catabolic and anabolic metabolism will be explained in more detail.

As described before, many cellular processes are dependent on a constant supply of glucose to generate ATP via glycolysis and subsequent oxidative phosphorylation. Glucose can be stored as branched polysaccharide glycogen in the cytoplasm. Glycogen can be broken down to glucose-6-phosphate which can be directly used in glycolysis. The breakdown of glycogen is inhibited when ATP is available, generating a control feedback loop.

An energy-requiring reaction can convert pyruvate back to glucose in the process of gluconeogenesis. Also other non-carbohydrate metabolites, as amino acids or lactate (which is produced as a byproduct in glycolysis) can be converted to glucose. The enzyme controlling the switch between glycolysis and gluconeogenesis is called phosphofructokinase (PFK). In mammals, gluconeogenesis is mainly carried out in the liver and kidney to keep up glucose blood levels (Aronoff *et al*., 2012).

More favorable storing material presents fat. In contrast to glycogen, lipid breakdown generates far more ATP molecules. In fact, 30% of the total energy expenditure is based on fatty acids which are hydrolyzed from TAG storages (Watt and Steinberg, 2008). Lipid metabolism and homeostasis is similar in fly and human: Not only the basic metabolic signaling pathways are conserved, also anatomy-wise *Drosophila* fat body cells, which store fats in form of lipid droplets, resemble mammalian adipocytes and functions of liver.

Animals have to make sure that their energy supply is constantly guaranteed even in periods of reduced feeding. Therefore in times of excessive nutrient availability, carbohydrates and lipids are stored and can be mobilized in times of reduced feeding or increasing energy demands. The fat body is the main site of storage for glycogen and lipids. They are stored as triacylglycerols (TAG, composed of three molecules of fatty acids esteryfied to a glycerol backbone) in so called lipid droplets. TAG can be mobilized to match acute energy needs, and this process known as lipolysis is in a tight balance with TAG re-esteryfication, or lipogenesis (Cohen *et al.,* 2015).

Lipolysis is mediated by a variety of lipases, with the rate-limiting enzyme called adipokinetic hormone (AKH), which resembles mammalian glucagon. TAG gets broken down to diacylglycerol (DAG), which is broken down to monoacylglycerol (MAG) eventually. In situations of energy demand (during exercise or fasting) fatty acids are broken down and loaded onto lipophorins into circulation to reach the peripheral demanding tissue where the oxidation of fatty acids is carried out to generate ATP via ß-oxidation as described above (Wanders *et al*., 2010).

1.5 Aim of this thesis

Despite investigations made before, it was not entirely possible to study the function of Creld proteins in the mammalian system. As outlined above, *Drosophila* only bears one *Creld* gene which makes it easy to analyze its function. Earlier studies show that *Creld* mutant flies presumably have an energy deficit (B. Stümpges, PhD Thesis). Therefore, this study is contributing to unravel the function of the Creld protein in *Drosophila* and proposes a link between energy metabolism and Creld function.

2. Materials and Methods

2.1 Materials

2.1.1 Devices

List of equipment and devices used:

Device	Company
Autoclave H+P Dampfsterilisator EP-2	H+P Labortechnik
Balance KERN ew 4200-2NM	Kern & Sohn
Binocular Zeiss Stemi 2000	Carl Zeiss
Camera HDC-SD40 Full HD	Panasonic
Centrifuge Allegra X-15R	Beckman Coulter
Centrifuges Eppendorf 5415 R; 5424 R	Eppendorf
Cover slips 24x40 mm; 24x60 mm	Menzel
Developer machine Curix 60	AGFA
Glass beads	Roth
Incubators (18 °C, 25 °C)	Rubarth Apparate GmbH
Light microscope DM-LB	Leica
LSM 710 Confocal microscope	Carl Zeiss
Oven	Binder
Photometer Nano Drop 2000	Peqlab
PVDF membrane	Merck Millipore
qRT-PCR Machine CFX96	Bio-Rad
qRT-PCR plastic plates 96 well	Bio-Rad
qRT-PCR plate plastic cover film	Bio-Rad
Speed-Vac SPD111 Savant	Thermo Fisher Scientific
Thermomixer	Eppendorf
Western Blot Equipment	Bio-Rad
X-Ray Film Fuji Medical Super RX	Fujifilm

2.1.2 Kits

Following kits have been used:

Kit	Company
cDNA using the reverse transcription Kit	Qiagen
FITC Annexin V/ Dead cell apoptosis kit	Molecular probes, life technologies
ECL Western Blotting Substrate Pierce SuperSignal West Femto	Thermo Fisher Scientific
go taq qRT master mix	Promega
Hydrogen peroxide kit	Abcam
RNA purification kit	Zymo Research

2.1.3 Solutions and Buffers

If not noted otherwise, all buffers and solutions were made with double distilled water (ddH_2O)

Buffer	Ingredients
Artificial hemolymph	108mM NaCl2, 5mM KCL2, 2mM CaCl2 2xH20, 8mM MgCl2 6xH20, 1mM NaH2PO4, 4mM NaHCO3, 15mM HEPES, 1mM Sachharose, 0.5mM Trehalose , pH 7.1
Laemmli buffer 5x	100 mM Tris-HCl, 3 % SDS, 10 % Glycerol, 0.01 % Bromophenol Blue, 5% β-Mercaptoethanol, pH 6.8
Mitochondrial isolation buffer	250 mM sucrose, 10 mM Tris, pH 7.4, and 0.15 mM $MgCl_2$
PBS (phosphate buffered saline)	130 mM KCl, 7mM Na2HPO4; 3 mM NaH2PO4 (pH 7,4)
PBST	PBS + 0.1% Triton X 100
SDS-gel running buffer 10x	250 mM Tris, 192 mM Glycine, 1% SDS
Transfer buffer 10x	250 mM Tris, 1.5 M Glycine

2.1.4 Antibodies

Antibody, dilution	Company
GFP (Chicken) 1:600	Abcam
phosphoAMPK Thr 172(mouse) 1:1000	Cell Cignaling Technology
α-tubulin (mouse) 1:500	DSHB
HA (mouse) 1:400	Roche

2.1.5 Fly food

Apple juice agar plates: For apple juice agar plates 43.5g agar was dissolved in 1.5 L distilled water, 500mL apple juice and 50g sugar was added and the mixture was autoclaved. After the solution had cooled down to 60°C 30mL Nipagin was added. The agar war poured into petri dishes and stored at 4°C. For fly experiments a drop of wet yeast paste was added to the plates.

Standard yeast food: 130g yarn agar, 248g Baker's yeast, 1223g Cornmeal and 1.5L sugar beet syrup were heated up in 20L distilled water. Nipagin (10% in 70% Ethanol) was added after cooling down to 60°C. The food was stored at 4°C.

Longevity food: 37.5g Baker's yeast and 10g Kobe agar were dissolved in 300mL VE-water. After autoclavation the mixture was cooled down to 60°C and 37.5g glucose and 10% Nipagin (in 70% Ethanol) were added.

PBS agar plates: 2% agar was dissolved in PBS and cooked. For starvation nothing was added. For low sugar feeding a drop of wet yeast paste was added to the plates.

Jazzmix (JM): Instant JZ food was prepared according to manufacturer's manual (Fisherbrand^{Tm}).

2.2 Methods

2.2.1 Handling of flies

2.2.1.1 General keeping and crossings

All flies were kept on standard fly food (see section 2.2.2) in food vials at room temperature, if not stated otherwise. For experimental procedure flies were raised in incubators with a steady day-night cycle at 25°C. Individual crossings were performed on standard food at 25°C.

For egg collection flies were held in a cage on apple juice agar plates plus yeast (recipe: section 2.1) on which they were allowed to lay eggs for a defined time period between 2h and 24h at 25°C.

Crossing of flies was done with freshly hatched virgin females of the desired phenotypes and individual crosses were kept on 25°C.

Following is a table of fly lines which were used for this thesis. The mutant *Creld* $^{\Delta 51}$ was generated by Birgit Stümpges, UAS Creld RNAi (BL 51741) and UAS Drp1-HA (BL 42208) was obtained from the Bloomington *Drosophila* Stock center. The other fly lines were present in the laboratory.

Fly line	**Genotype**
w- (control flies)	$\frac{w-}{+/y}; \frac{+}{+}; \frac{+}{+}$
Oregon R (control flies)	$\frac{+}{+/y}; \frac{+}{+}; \frac{+}{+}$
Creld$^{\Delta 51}$	$\frac{+}{+/y}; \frac{Creld\ \Delta 51}{Creld\ \Delta 51}; \frac{+}{+}$
UAS Creld RNAi	$\frac{+}{+/y}; \frac{+}{+}; \frac{\mathrm{CG11377_{GLC01799}}+}{Tb}$
UAS Drp1-HA	$\frac{+}{+/y}; \frac{+}{+}; \frac{Flash-HA-Drp1,Ki}{Flash-HA-Drp1,Ki}$
Tubulin Gal4 driver	$\frac{+}{+/y}; \frac{+}{+}; \frac{tubG4}{Tb}$

2.2.1.2 GAL4/UAS System

Using the Gal4/UAS system (Elliot and Brand, 2008), specific genes can be differentially expressed in a time and tissue dependent manner.

Flies carrying a Gal4-driver (tubulin Gal4 as a ubiquitous driver) were crossed with flies carrying the desired UAS (upstream activating sequence) target gene. In the offspring of these flies the Gal4 specifically binds to the UAS, activating the transcription of the target gene the UAS.

2.2.2 Feeding experiments

Different chemical supplements were added to the fly food. The concentration and brand can be found in table 2. The drugs were added to Jazzmix (JM, Fisherbrand^{Tm}) or into the yeast paste on apple juice agar. Flies were allowed to lay eggs in a defined time slot (2-24h) and L1 larvae were collected, placed and raised on the prepared food conditions.

Chemical, Brand	Concentration in food
MDIVI-1, Sigma	20µM/mL
Paraquat, Sigma	10µM/mL
Tunicamycin, Sigma	50µM/mL
Wortmannin, Sigma	10nM/mL

2.2.3 Crawling assay

L3 larvae raised on different food conditions were collected and placed on room temperature PBS-agar plates for crawling. Before starting the experiment, the larvae were allowed to acclimate for 5 minutes on the plates. Larval movements were recorded using a video camera (Panasonic, HDL-SD40, full HD) and a 20 second video was analyzed using "Shotcut" Videoanalysis software. Every 2 ms the position of the mouth hooks was determined and using image J software the total distance crawled was measured. In total the movement of 10 individual larvae was analyzed for each genotype and food condition.

2.2.4 Climbing assay

Adult flies were aged until the desired age and grouped into sex (10 males and females each) and tapped into glass vials. Before climbing was recorded, the flies were allowed to acclimate for 1h to recover from CO_2 anesthesia. All vials were tapped down simultaneously

and the climbing of the flies up the walls was recorded using a video camera (Panasonic, HDL-SD40, full HD). Videos were subsequently analyzed using the freeware “Shotcut” Video analysis software. Standing pictures were taken after 20s time and the distance climbed by the flies was measured using Image J software. From this the velocity can be calculated. For each genotype and food condition at least 10 individual flies were analyzed.

2.2.5 Starvation assay

Adult flies were aged to 10 days and transferred to vial containing starvation media (2% agar in PBS) and kept on room temperature. Every 12h the amount of dead flies was counted.

2.2.6 Fertilization assay

Flies of the same age were kept on cages and were allowed to lay eggs over 2h. From these plates 25 eggs were transferred to a fresh apple juice agar plate. 30h later the eggs where no larvae hatched from were counted. If the color was white they were counted as not fertilized, whereas brownish eggs were determined to be fertilized but not developing to larvae. The experiment was repeated 5 times for each genotype.

2.2.7 Stress sensitivity assays

For the ER stress sensitivity assay adult flies were aged to ten days on standard food. They were separated in groups of 10 and placed on food containing the ER stress-inducing drug Tunicamycin (Sigma): Tunicamyin was dissolved in DMSO and kept in stocks (5mM in DMSO). For the assay the stock was diluted to gain an endconcentration of 50µM/mL dissolved in Jazz Mix (JM) standard food. Flies were put on fresh ER-stress-inducing food and control food every second day. The control group was put on JM food containing the same amount of DMSO as a vehicle control. Surviving flies were counted every day. The experiment was repeated four times.

The oxidative stress sensitivity assay was done similar to the ER stress assay, with the only difference, that 5% hydrogen peroxide (Sigma) was added to the JM standard food as the stress-inducing drug in this assay.

2.2.8 Propodium iodide

For Propodium iodide (PI) staining 8-10 day old adult female flies were anesthesized and the brains were dissected in cold PBS. Propodium iodide staining was done following

manufacturer's instructions (Molecular probes): Briefly, after washing in PBS for one minute. A 100 µg/mL working solution of PI was prepared by diluting 5 µL of the 1 mg/mL PI stock solution in 45 µL 1X PBS. The dissected brains were transferred to 40 µL fresh PBS and 4 µL PI working solution was added. After 15 min incubation in dark at room temperature, the brains were washed one time 5 minutes in PBS and fixed in fresh 4% formaldehyde for 15 min and mounted in Flouromount-G (Invitrogen). Images were taken with the Zeiss LSM 710. For quantification of the staining the software Image J was used. The staining intensity of one optic lobe was quantified. In total 5 optic lobes of five different animals were imaged and statistically analyzed.

2.2.9 Microscopic imaging of Immunostainings

Immunoflourescent stainings were imaged using a Zeiss LSM 710 confocal microscope (Carl Zeiss, Jena). The used software to take images and imaging stacks were ZEN2012 and analyzed with ImageJ software.

2.2.10 quantitative RT PCR

2.2.10.1 Isolation of RNA and cDNA generation

Prior to RNA isolation whole L3 larvae or adult flies were collected in Precyllys tubes in TRiFast (ambion, life technology) and homogenized using a Precellys homogenizer at 3x 15s with 5000 rpm. RNA of larvae or adult flies was isolated using the Zymo RNA isolation kit following the manufacturer's instructions and kept on -20°C or -80°C for short and long term storage, respectively.

RNA concentration was measured with a Nano Drop Spectrophotometer. 500ng of RNA was further processed to cDNA using the reverse transcription Kit (Qiagen) and go taq qRT master mix (Promega) following the manufacturers descriptions and stored on -20°C.

2.2.10.2 Quantitative Real-Time PCR

SYBR green and Promega qRT PCR mix was used for qRT-analysis. The experiment was carried out in a clear 96-well plate and each well contained the following mixture:

cDNA	**0.75 µL**
ddH_2O	6 µL
Primer pair (5 pmol/µL)	0.75 µL
Promega SYBR mix	7.5 µL

The Biorad Thermocycler was used with the following protocol:

95°C for 3 min

95°C for 10 sec
59°C for 30 sec
72°C for 30 sec
} x 41

Primers used and their sequences are listed in the table below. Every value was normalized to the housekeeping gene rpl32. Data analysis was done with CFX Manager Software and Microsoft Excel.

All experiments were performed in at least four biological replicates as a technical duplicates or triplicates. As controls "–RT samples" (samples were no reverse transcriptase was added) and samples with just H2O were measured.

For statistical analysis the students T-Test was used for normal distributed samples, and for not normally distributed samples the Mann-Whitney-U-Test was applied. P-values are indicated by asterisks (*p<0.05, **p<0.01, *** p<0.001) and error bars show the standard deviation.

Primer name	Sequence
ACC	F: 5' GTGCAACTGTTGGCAGATCAGT 3' R: 5'TTTCTGATGACGACGCTGGAT 3'
ATF6	F: 5'GATCAGATATTTCTGCTTGCCA R: 5' TTTCGCTTCGTTCAGATCCT
Atg1	F: 5' TAAAGCCGTCGTCCAATGTG 3' R: 5' CAATAGTCCGCTGCCATTGAG 3'
Bmm	F: 5' AGATCTACGAAGGCTCTGTC R: 5' GGCATTACTTGTACTGATTCG
CreId	F: 5'TGAACACCTACGGACCAGAC R: 5' CACATTTGCACTTTCCGTTACC
FAS	F: 5' CCCCAGGAGGTGAACTCTATCA R: 5' GACTTGACCGATCCGATCAAC
Drp1	F: 5' GGACTGCCTGCCAGATCTTA 3' R. 5' CACAGTTCCGTGGTCTCGAT 3'
Hsc 70-3	F: 5'GCAGAAGAGCAAGAGATAAACC R: 5' CGAAGAACCCATTTATGAAGCC
Ire1	F: 5'TGGTGACAATCTGAAGCGAC R: 5' GATAATTCGGCTGTCCTCACTG
IsoDH	F: 5' CAACTGATTCTGCCCTTCCT R: 5' GGCGACTTCCACATCTTCTT
Lip3	F: 5' TGAGTACGGCAGCTACTTCCCT R: 5' TCAACTTGCGGACATCGCT
Opa1	F: 5' AACGGTGGAGCCAGTTCTCG R: 5'TGATCTCCGTCTGACGCGTC

Parkin	F: 5’ AGCCTCCAAGCCTCTAAATG R:5’ CACGGACTCTTTCTTCATCG
PEK	F: 5’ GACGATTTGGGCTTTAGTTTGG R: 5’ AATGCTATGACCACGATTTCC
Pink	F: 5’ GCTTTCCCCTACCCTCCAC R:5’ GCACTACATTGACCACCGAAT
rpl32	F:5'CGGATCGATATGCTAAGCTG3' R: 5' TGTTGGGCATCAGATACTGTC 3'
TFAM	F: 5’ ACGGAAATCTTATCGATCCACCT R: 5’ TATGTGATGGAAGAGGGCGG
TFB1	F: 5’ GCACACAGGATGGCCCA R: 5’ CGCTCGTCCATGAGGAAG
TFB2	F: 5’ CCCACGAAAGCGTTTACAGAT R: 5’ GAGATGTATGTATATGGGTG
Xbp1	F: 5’CGCCAGCGCAGGCGCTGAGG R: 5’ CTGCTCCGCCAGCAGACGCGC

2.2.11 Paraquat feeding

Wild type flies were exposed to the powerful oxidative stress producing drug paraquat (Sigma) for subsequent analysis via qRT PCR: 3-5 day old adult males were put on apple juice agar plates with yeast supplemented with 10µM Paraquat (dissolved in water) for 24h. For qRT PCR analysis the flies were handled as described before.

2.2.12 Semi-intact *Drosophila* heart preparation and digital high-speed movie analysis

All the dissection steps were done in artificial hemolymph (see section 2.1 Buffers). The flies were anesthetized with fly-nap, followed by transfer to a Petri dish coated with Vaseline for dissection. After the dissection, the submerged hearts were kept in oxygenated hemolymph for 15 min at room temperature for equilibration. The heart movements were recorded with a digital high-speed camera (connected to a Leica DM-LB microscope with a 10x water immersion lens) and Fire capture Red 1.2 software. Movie analysis of the heart activity is then carried using the MATLAB R2010a software. Statistical analysis was done using Microsoft Excel. Images of morphology of semi dissected fly hearts were taken by screenshots of the videos taken described above.

2.2.13 Longevity assay

For longevity assay 3 day old adult male and females were put on longevity food (see section 2.2.2). They were grouped into 20 flies per vial. Every second day the flies were put on fresh

food. Survival was counted every 24h. The analysis of survival rates was done using the Kapplan-Mayer method with Microsopft Excel and GraphPad Prism.

2.2.14 Western Blot

2.2.14.1 Generation of protein extracts

For protein separation Western blot analysis was chosen and the fly lysate and protein extraction was performed as follows: Flies (3-8 adults) were anesthetized and out into an Eppendorf tube. Per fly, 15 µL PBS was added and the whole flies were homogenized with a prestle. The samples were kept on ice and the protein concentration was measured using the Nanodrop. Then 1x Laemmli Buffer was added and the samples were boiled for 3 minutes at 99°C.

2.2.14.2 SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

For protein separation, SDS-PAGE was performed with the protein extracts made before.
30-60 µg protein was loaded in one lane. As a protein standard, 8µL precision blue protein standard (Bio-Rad) was loaded. The gel was run at 150V for approximately 45 minutes. The recipe of the 10% SDS gel can be found in the following table:

	15 mL Running Gel	**10 mL Stacking Gel**
H2O	7,15 mL	7,25 mL
40% AA	3,75 mL	1,25 mL
Tris-Hcl	3,8 mL (1,5 M pH 8,7)	1,25 mL (1,0 M pH 6,8)
10% SDS	150 µL	100 µL
10 % APS	150 µL	100 µL
TEMED	12 µL	10 µL

2.2.14.3 Blotting

In order to transfer the separated proteins from the gel onto a membrane, a PVDF membrane (Millipore) was activated in methanol. Blotting occurred in transfer buffer (850mL H2O + 50mL MeOH + 100mL 10X Transfer buffer) at 100V for 1 ½ hours together with cooling device and stirrer.

2.2.14.4 Antibody incubation and detection

After blotting after blotting the membrane was blocked in 5% milk (2,5 g milk powder in 50mL TBS-tween 20) for 1h.

After blocking the membrane was incubated with the primary antibody (pAMPK antibody 1:1000 in 5% milk/TBST) over night at 4°C on a rocking platform. On the next day the membrane was washed shortly in TBST and incubated with the secondary antibody (α-rabbit-HRP 1:7500 in 5% milk/TBST) for 2h in the dark at room temperature on rocking platform.

The membrane was washed in PBST three times for 5 minutes and one time for 10 minutes on a rocking platform.

For the detection the Femto ECL kit (Thermo Scientific) was used after the manufacturer's description. For the detection on X-ray films the X-ray developer Curix60 was used.

2.2.14.5 Procedure for the Drp1-HA Western blot

For the analysis of Drp1-HA tagged flies, the fly lysates had to be fractionated (into a cytoplasmic and a mitochondrial fraction) before loading onto the gel. Therefore, the flies were homogenized in 10 µL mitochondrial extraction buffer (recipe section 2.1.3) per fly. After homogenization with a prestle, the lysates were centrifuged at 800g for 5 min. The supernatant was collected and centrifuged at 11,000g for 10 min. The supernatant, which contained the cytoplasmic protein fraction and the pellet, containing the mitochondrial protein fraction were separated. The pellet was resuspended with the starting volume of mitochondrial extraction buffer. The protein content of both fractions was determined using the Bradford assay. To both samples, the mitochondrial and the cytoplasmic fraction, 1x Laemmli buffer was added and the samples were boiled at 99°C for 3 min.

Further procedure was done as decribed before (from section 2.2.14.2 onwards).

The quantification was done as follows: Band intensities of the blot were calculated using Image J. The calculated band intensities were put into relation to the protein content measured for the fraction.

2.2.15 Thin layer Chromatography to analyze TAG content

For each sample one adult male (10 days old) was collected and smashed in 50 µL Chlorophorm:Methanol (2:1). Afterwards the sample was centrifuged at 13.000g for 10 min and the liquid solution was transferred to a new Eppendorf cup. The sample was dried using a SpeedVac to completely dry out the Chlorophorm:Methanol solution.

Subsequently 50 µL of pure Chlorophorm was added and the samples were extensively vortex and sonicated for 10 min. Each sample was transferred to a silica plate and fan-dried afterwards before the plate was put into the first solution (containing CHCl3/triethylamine/ethanol/H20 35/35/40/9) for around 20 min.

After the incubation the plate was again thoroughly fan-dried and transferred to the second solution (containing Isohexane/ethyl acetate 5/1) for 45 min. After letting the plate dry completely, it was covered in a 20% sulfur solution and baked in an oven (Binder) for around 20 minutes at 160°C until the band became visible. Quantification was done using the software Image J and Microsoft Excel.

2.2.16 Measuring free non esterified fatty acids

Three L3 larvae were washed and weighted. Per 0.1mg larvae 1.83 µL Chloroform-Triton was added. Subsequently the larvae were homogenized and centrifuged for 10 min at 13.000g at 18°C. After centrifugation the Chlorophorm was evaporated in a 60°C heat block. The equal amount of phosphate buffer was added to the dried samples to dissolve lipids and samples were stored at -20°C.

The next day 500 µL of Chloroform:Heptane (4:3) was mixed with 25 µL of the lipid extract and vortexed 1 min followed by a 15 min incubation time at room temperature. This step ensures the extraction of lipids. 23mg of activated silic acid was added to the samples. With this step the unspecific background of phsopholipids was prevented. Then 300 µL of the Chlorophorm phase was taken and added to glass vials containing 250 µL of Copper-Triethanolamine (Cu-TEA), followed by additional shaking and centrifµgation. Of the organic phase 15 µL was transferred to a fresh cup. The remaining liquid was evaporated and lipids were dissolved in 100 µL of 100% ethanol.

Copper was detected by complexation with a mixture of dicarbazone–dicarbazide, and the color intensity was measured in a 96-well plate at 550 nm in a TECAN plate reader. The experiment was done with four biological replicates.

2.2.17 Transmission electron microscopy (TEM)

For ultrastructure images taken from the flies, TEM analysis was performed. Adult female flies were aged to 10 days on standard food. Heart tubes were dissected with the semi-dissection method described in section 2.2.9. For the imaging of indirect flight muscles, the thorax of the flies was cut open. Dissection was carried on in artificial hemolymph on ice. The tissue was fixed in TEM fixation buffer (2.0 % paraformaldehyde and 2.5% glutaraldehyde (both E.M. grade) in 0.1 M Na-Cacodylate buffer, pH 7.4) for 4h. The samples were put into tubes without air and send to the laboratory of Prof. Dr. Achim Paululat in Osnabrück, where TEM imaging was carried out.

2.2.18 H_20_2 production measurements

The Fluorimetric Hydrogen Peroxide Assay Kit from Sigma was used to measure hydrogen peroxide production in the fly. For that pre-wandering L3 larvae, grown on standard food, were collected. The assay was carried out following the manufacturer's manual.

2.2.19 mtDNA measurements

For the measurement genomic DNA was extracted from whole adult flies which were aged to 10 days. The DNA was extracted by homogenizing 3 adult flies in lysis buffer, followed by a proteinase K digestion (200µg/mL proteinase K into the lysis buffer) with incubation at 39°C for 45 min, followed by 5 min at 95°C. One volume of Chlorophorm:Phenol:Isoamylalcohol (Sigma) was added and the sample was vortexed. After centrifugation (5min at 13,000 rpm) the aqueous phase was taken and 150µL isopropanol was added. After additional centrifugation (5min at 10,000rpm) the liquid was removed and the pellet was washed once with 70% EtOH. The pellet was dried in a Speedvac and resuspendet in 20-50µL ddH_2O.

mtDNA measurement via qRT PCR was done as described before (Furda *et al.*, 2013). Sequences of the primers used can be found in tableBLABLA. The results were analyzed in Microsoft Excel.

Primer name	Sequences
nucDNA	F: 5' AGGCCCACZGAZCGZGAAGAA R: 5' TGTGCACCAGGAACTTCTTGAA
mtDNA	F: 5' AAAAAGATTGCGACCTCGAT R: 5' AAACCAACCTGGCTTACACC

2.2.20 Mitochondrial isolation

Female adult flies were aged to 10 days. The protocol was adapted from Rahman *et al.*: 10 whole animals were homogenized with manual strokes in 20mL of mitochondrial isolation buffer (section 2.1.3). The homogenate was filtered through cotton filled columns and centrifuged at 4,000g for 15 minutes at 4°C. The supernatant was taken and centrifuged again at 16,000g for 30 minutes at 4°C. The pellet, which contained the mitochondria, was resuspended in mitochondrial isolation buffer. The isolated mitochondria were immediately used for activity measurements or for Western blot analysis.

2.2.21 Mitochondrial activity measurements

Measurement of complex V activity

The measurement of mitochondrial complex V activity was done following the protocol of Rahman *et al.* In brief, 40µg of isolated mitochondria were added to wells containing Complex V reaction buffer (250 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 2 mM KCN, and 20 mM Tris-HCl, pH 7.5. Before the test, 0.25 mM NADH, 1 mM phosphoenol pyruvate, 2.5 U/ml lactate dehydrogenase, and 2 U/ml pyruvate kinase). The change in absorbance at 340nm was measured using a plate reader.

The principle of the assay relies on NADG oxidation via the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase and then pyruvate to lactate by lactate dehydrogenase, thereby measuring ATPase activity (Rahman *et al.*, 2014).

Measurements of citrate synthase activity

10 µg mitochondrial protein was incubated in citrate synthase activity buffer (0.1 M Tris-HCl, pH 8.0, 0.3 mM acetyl-CoA, 0.1 mM DTNB) for 10 min at room temperature. By adding 0.5mM oxaloacetate the reaction was started and the change in absorbance at 412nm was measured. The reduction in absorbance was due to the reduction of DTNB by citrate synthase (Rahman *et al.*, 2014).

2.2.22 Body weight protein to body weight

For body weight measurements, single 10 day old adult flies were weighted individually. The protein content was measured using a Bradford assay and normalized to body weight of the individual flies.

3. Results

3.1 Creld is ubiquitously expressed on a moderate level

According to Flybase data (flybase.org, FBgn0031217), *Creld* expression is ubiquitous on a moderate level (Figure 3-1). Peaks with high expression can be seen in larval salivary glands and adult male accessory glands.

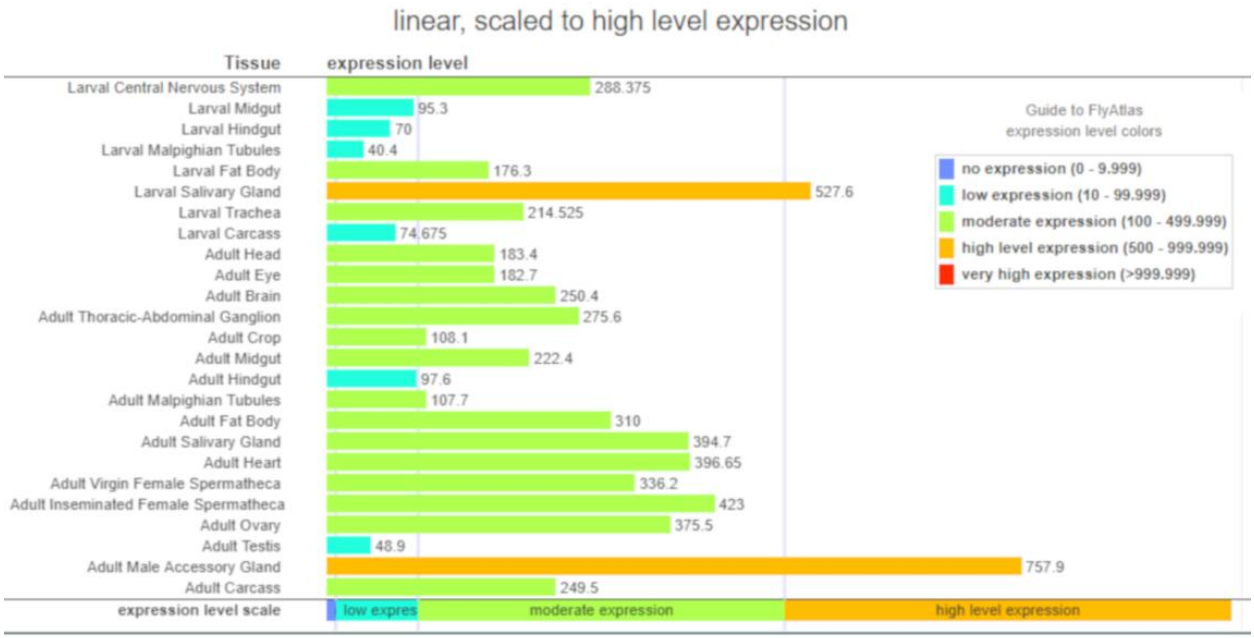

Figure 3-1: Expression data of Creld mRNA in Drosophila tissue. Highest expression (orange) can be found in larval salivary gland and adult male accessory gland. Nevertheless, Creld is expressed ubiquitously on a moderate level. Source: flybase.org

3.2 *Creld* mutant flies are semi-fertile but show no larval lethality

Creld mutant flies were generated by Birgit Stümpges, a former PhD Student, via homologous recombination, resulting in $Creld^{\Delta 51}$ mutant flies, from here on called *Creld* mutants (Birgit Stümpges, PhD thesis). She found that the Creld protein in *Drosophila* is localized subcellular to the endoplasmic reticulum (ER) and that adult *Creld* mutant flies show flight defects. She also stated that *Creld* mutants are semi-lethal, with a major lethality rate between embryonic and larval L1 stage. In contrast to these earlier findings, it could be shown that *Creld* mutants are not semi-lethal, but semi-sterile instead. As shown in Figure 3-2, only about 70% of the laid eggs are fertilized, whereas the wild typic flies lay about 90% fertilized eggs.

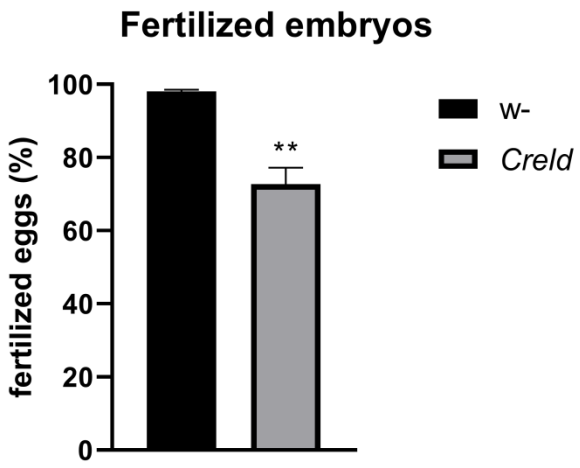

Figure 3-2: *Creld* mutants are semi-sterile. *Creld* mutants lay a significantly reduced amount of fertilized eggs compared to wild type control. Values are mean ± SD, n=4 (20 eggs each) **p<0.01 (t-test).

Indeed, once fertilized, the mutant embryos develop normally to larvae and adult flies. However, their morphogenesis is delayed by approximately 12 hours, showing that the lack of Creld proteins is not affecting survival rate but delays the development.

3.3 *Drosophila* Creld is not involved in ER stress resoponse

The mammalian genome contains two Creld genes, Creld1 and Creld 2. In previous studies it could be shown that the mammalian Creld proteins localize on the ER (Elvira Mass, PhD Thesis). This was true for the single *Drosophila* Creld protein as well (Birgit Stümpges, PhD Thesis). Murine Creld2 is considered to be involved in ER stress response (Oh-hashi *et al.*, 2009). ER stress is a consequence of accumulation of increased unfolded proteins, which leads to upregulation of specific chaperones to help decrease the protein load. Especially in tissues with increased protein turnover, the ER stress response can be increased (Xu *et al.*, 2005). Since the *Drosophila* Creld protein is highly expressed in salivary gland and male accessory gland, two organs with increased protein secretion and therefore prone to ER stress, the ER stress status in *Creld* mutant flies was assessed first. The answer of an organism to increased ER stress is the unfolded protein response (UPR). UPR is managed through three different routes: The ER transmembrane protein Ire1 senses misfolded proteins and as a response accumulates and dimerizes, leading to the splicing of mRNA of a transcription factor called Xbp1 (Ryoo, 2015). Once spliced and consequently activated, Xbp1 is able to translocate to the nucleus and initiate target gene expression of genes involved in ER stress combat.

Additional to Ire1, the PERK or PEK in *Drosophila* branch of the UPR gets simultaneously activated. Activated PEK phosphorylates and thereby inactivates the transcription factor eIF2α, which would normally charge the 40S ribosomal subunit with tRNAs. With the inactivation of eIF2α, the rate of general gene expression is decreased (Hampton, 2000). The third route of UPR is regulated via ATF6. Upon ER stress and accumulation of unfolded proteins, ATF6 is able to shuttle through the Golgi to the nucleus, where it initiates gene expression of ER quality genes, like chaperones (Ryoo, 2015). *Creld*2 was also shown to be upregulated in an ATF6 dependent manner in the mammalian system (Oh-hashi *et al.*, 2011). *Creld2* was shown to be upregulated in mammalian cells upon Tunicamycin (Tm) (Zhang *et al.*, 2013). Tm induces ER stress by inhibiting N-linked glycosylation, which leads to the accumulation of misfolded proteins in the ER (Girardot *et al.*, 2004). To induce ER stress and to see if *Creld* gene expression is upregulated, wild type flies were kept on food containing Tm and Creld expression was assessed via qRT PCR. The result can be seen in Figure 3-3 A: The ER stress marker PEK and Ire1 where upregulated, proving that the flies experienced ER stress. However, Creld was not significantly upregulated, indicating that in the fly, Creld is not increasingly transcribed as an ER stress response.

In order to observe the ER stress status in *Creld* mutant flies, their expression level of the UPR genes was measured using qRT PCR. The stress markers, PEK, Ire1 and ATF6 did not show altered expression in the mutant compared to wild type, which is shown in Figure 3-3 B. Also the chaperone Hsc70-3 (Heat shock chaperone 70-3), transcriptionally regulated and increasingly expressed upon ER stress, showed no upregulation when compared to wild type flies. Xbp1, which is activated upon ER stress through the Ire1 route, also did not show any differential expression pattern compared to wild type.

To exclude that the *Creld* mutant flies are not able to upregulate these genes as an ER stress response and that *Creld* mutants are able to combat ER stress, an ER stress sensitivity assay (Figure 3-3 C) was performed by feeding adult flies ER stressors Tm and counting survival. No significant difference of survival rates was observed when compared to wild type flies. Together, these results indicate that the fly *Creld* gene is not involved in ER stress response and UPR target genes are not increasingly expressed. The mutant flies also do not suffer from ER stress. Therefore, the investigation of ER stress in *Creld* mutant flies was not continued.

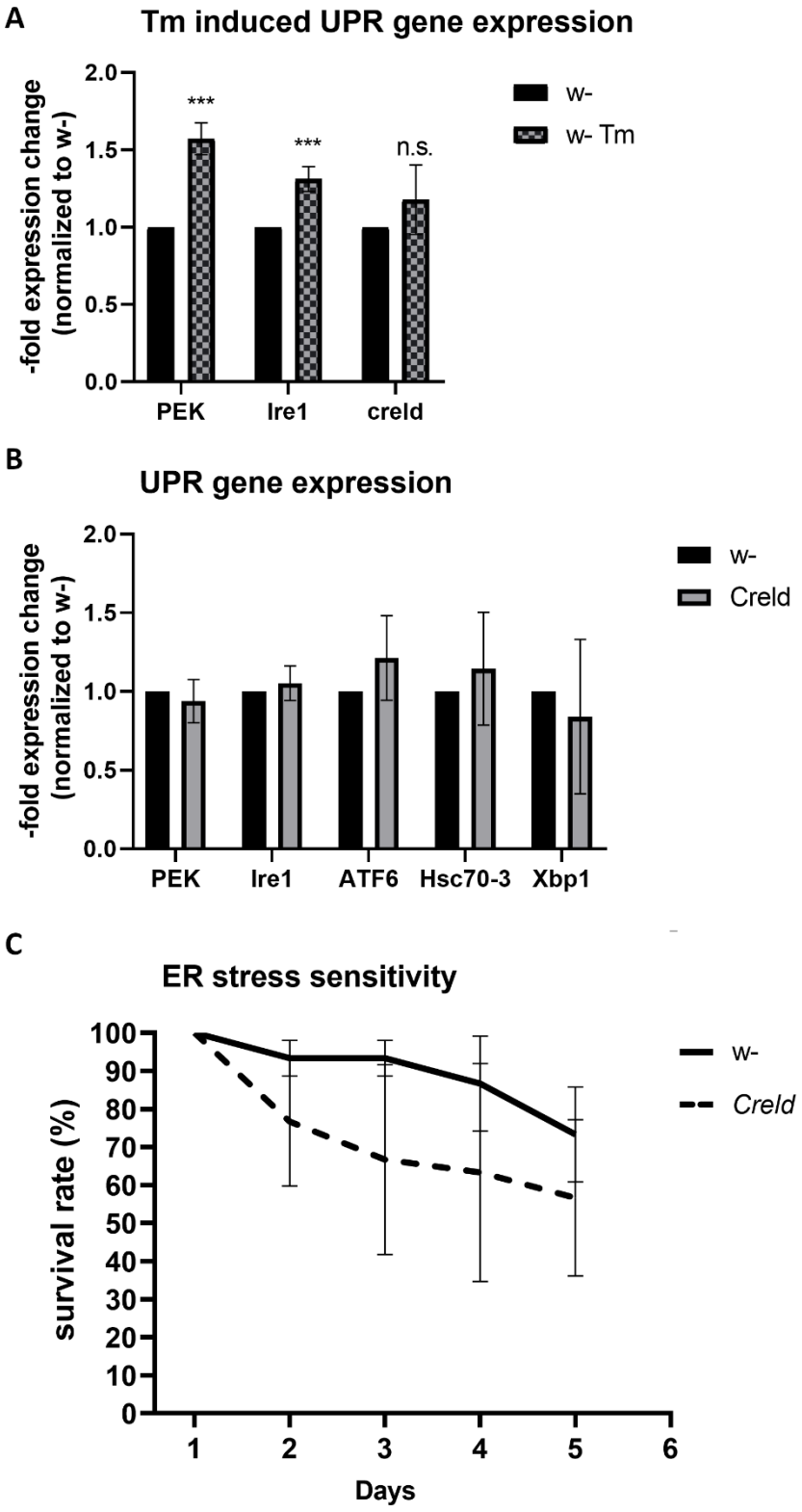

Figure 3-3: *Creld* is not involved in UPR and *Creld* mutant flies do not suffer from ER stress. (A) Real time analysis of genes involved in ER stress response in wild type w- flies fed with ER stress inducing drug Tunicamycin (Tm). Typical ER stress markers, PEK and Ire1, are upregulated in flies fed with Tm but *Creld* expression is not altered. Results are shown as mean±SD, n=5, ***p<0.001, n.s., not significant (t-test). (B) UPR response genes are not upregulated in *Creld* mutants as measured by qRT PCR, depicted as mean±SD, n=5. (C) 5 day old flies were subjected to ER stress, induced by the drug Tm. Loss of *Creld* does not increase ER stress sensitivity, as survival rates are not significantly different to wild type flies. Results shown asmean±SD, n=3 (10 flies each trial).

3.4 Creld deficient flies suffer from energy deficits

3.4.1 Creld mutant flies show heart rate disturbances

Even though morphological differences between mammalian and fly hearts exist, *Drosophila* still is a favorable model organism to study heart development and function (Ocorr *et al.*, 2015). As stated in the introduction in detail, an involvement of Creld1 in proper heart development could be shown before (Mass *et al.*, 2014). Mice lacking Creld1 fail to form proper endocardial cushions and die in embryonic stages. Embryonic lethality is rarely seen in *Creld* mutant *Drosophila* (see section 3.2), however, the functionality of the heart in larval and adult stages might still be disrupted in the mutants.

Taking into account this background information *Drosophila Creld* mutant heart function was analyzed. Between *Creld* mutant larvae and wild types, the morphological structure seems to be unaltered. The larval heart contains valve-like structures, which ensures that the hemolymph is not pumped backwards (Ocorr *et al.*, 2015). These valve-like structures can clearly be identified in Figure 3-4, marked by red arrows. They are also able to fully close and mediate contact with each other, meaning that they are functional. Also the pericardial cells,

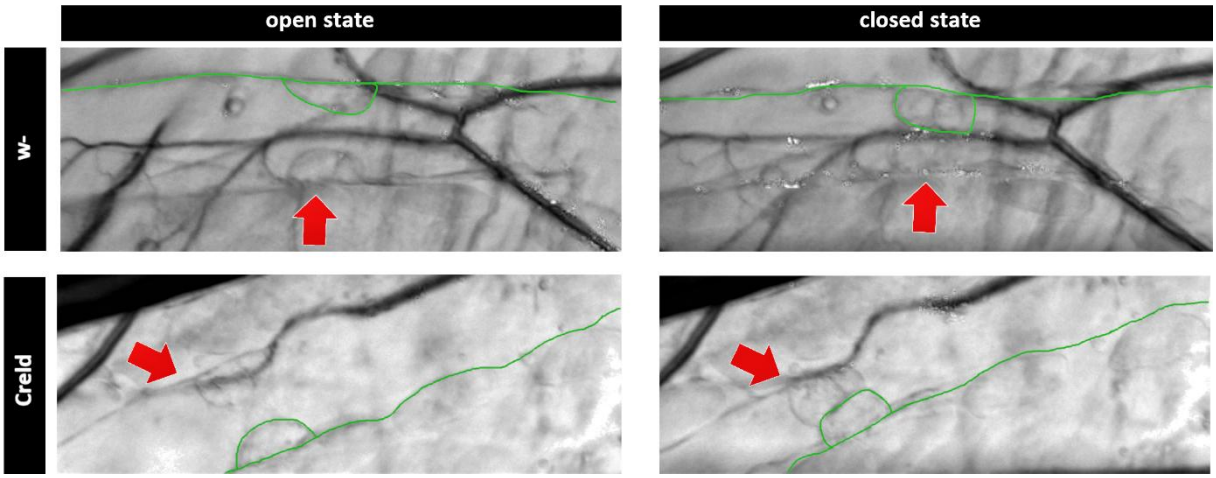

Figure 3-4: Morphology of valve-like ostial cells in L3 larvae. Valve-like ostial cells can be seen in semi-dissected L3 larvae, marked by red arrows. For better visualization the opposite tube wall and ostial cells are highlighted in green. No difference can be observed between mutant and control flies. The images were taken as screen shots from video analysis. It is visible that the cells are able to mediate contact between each other, meaning they are functional.

which surround the heart tube, are visible in both mutant and wild type.

In cooperation with the group of Achim Paululat, University of Osnabrück, heart function was analyzed. Adult flies have a steadier heartbeat, which makes them more favorable when studying differences concerning the heart beat pattern (Kai Lemmers, personal

communication). Therefore, the heart movement of semi-dissected adult flies was recorded in order to attain an overall insight of the cardiac performance. In the M-Mode analysis (Figure 3-5 A, B), which traces the movement of a single pixel throughout several heart periods, it is already possible to detect a difference in the movement pattern between mutant and wild type hearts. However, no irregular heart beat pattern could be observed. Marked in red are the visualized heart period (HP), the diastolic interval (DI) and the systolic interval (SI). The image shows that there is a difference in length of these parameters between wild type and *Creld* mutant flies.

The software used to analyze the heart function reads out the systolic and diastolic interval as well as the whole heart period. When quantified, the total heart period is significantly longer in mutant flies than in wild type, with a difference of around 100ms per period (Figure 3-5 C). The systolic interval, the time the heart needs for contraction is not different in *Creld* mutants as seen in Figure 3-5 F. However, the resting phase, and the diastolic interval in the mutants (Figure 3-5 E) take around 150ms longer, which explains the prolonged total heart period.

An estimate to analyze the contractility of the heart provides the percentage of fractional shortening, which uses the heart tube diameter in contracted and relaxed state. Indirectly, the strength of cardiac contractility, meaning the strength of muscle contraction, can be calculated using the fractional shortening (Occor and Vogler, 2014). As seen in Figure 3-4 D, there is no difference in the percentage of fractional shortening between mutant and wild type hearts. Therefore, it can be assumed that the contractility is not disturbed, but the duration of resting phase between the contractions is prolonged. In the literature, prolonged heart rates and especially increased diastolic intervals are often associated with aging and disturbances in energy supply (Kass *et al*., 2004). As a high energy demanding tissue, the heart especially depends on the mitochondrial function to gain a sufficient amount of ATP. As no morphological differences in the heart structure could be observed, it can be assumed that the hearts of *Creld* mutant are not supplied with sufficient ATP.

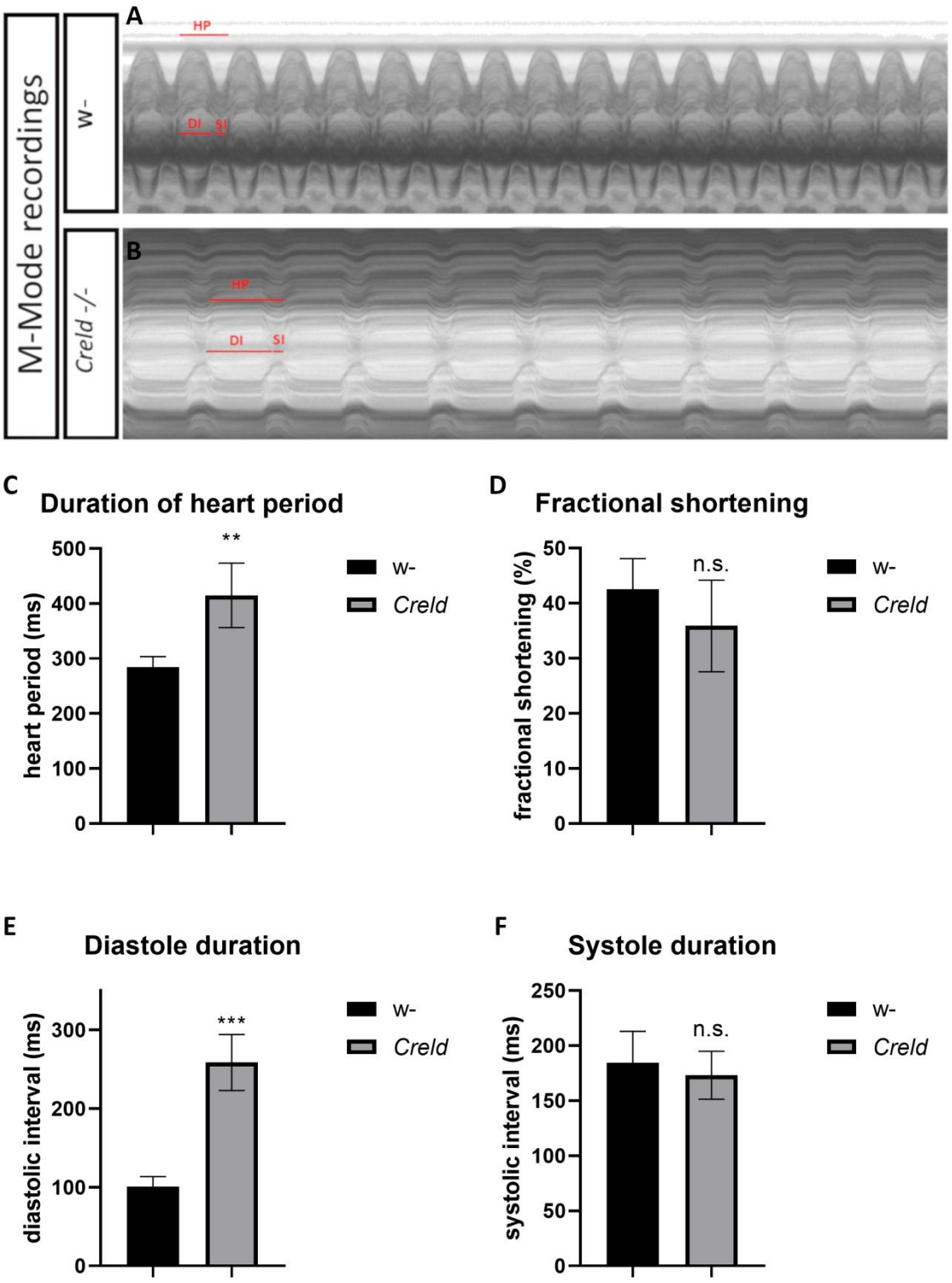

Figure 3-5: Analysis of heart function in *Creld* mutant flies. (A,B) Representative M-mode traces of semi dissected *Drosophila* hearts of 5 day old adults, describing the movement of hearts in time. *Creld* mutants show a different pattern compared to wild type flies. Heart period (HP), diastolic interval (DI) and systolic interval (SI) are marked in red. (C) Heart period is prolonged in *Creld* mutant flies. (D) Fractional shortening, depicting the contractile ability of heart tube, is not significantly changed between w- and *Creld* mutants. (E) Diastolic interval length is much longer in *Creld* mutant hearts compared to wild type. (F) Systole duration is not different in wild type and *Creld*-deficient flies. All results are depicted as mean±SD, 30 second interval, w- n=9, *Creld* mutants n=11, **p<0.01, ***p<0.001, n.s. not significant, (t-test).

3.4.2 Creld mutants show reduced lifespan

Creld mutant flies showed a reduction in lifespan. Figure 3-6 shows that flies lacking the Creld protein survive around twenty days less than the wild type control flies. Reduction in lifespan is often accompanied with locomotor defects and neuronal disorders (Pareek *et al.*,

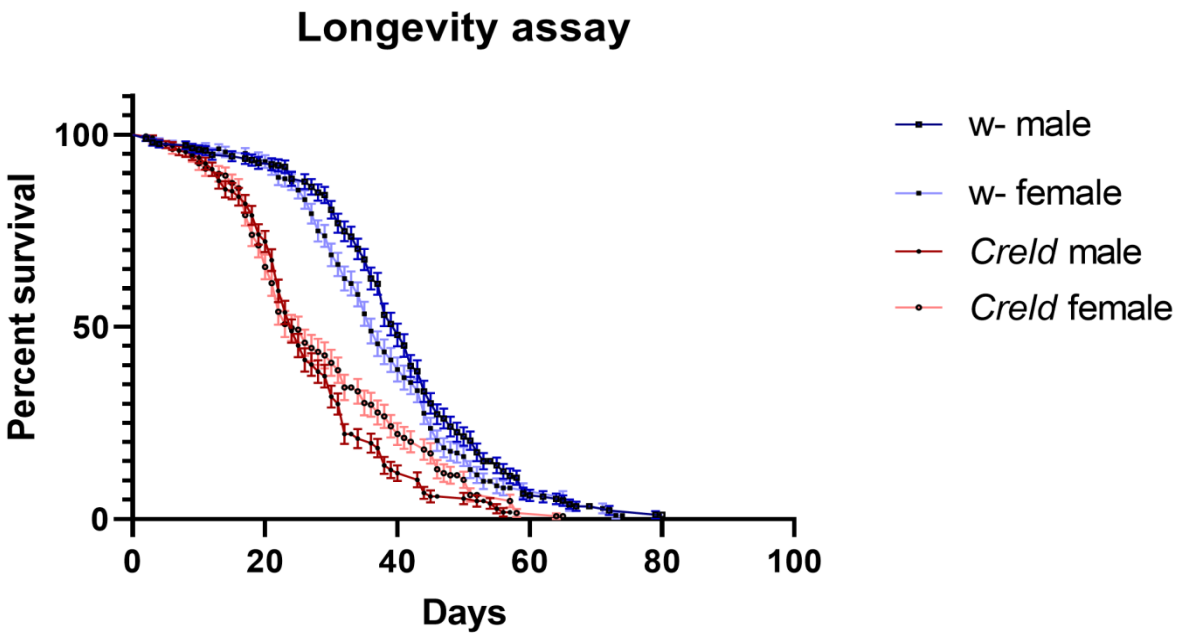

Figure 3-6: ***Creld*** **mutant flies have a reduced lifespan.** Longevity measurements of adult flies show a shorter lifespan in *Creld* mutant flies compared to control flies. Error bars depict SD, n=<13, difference between survival curves significant ****p<0.0001 calculated with Log-rank Mantel-Cox test.

2018), therefore, the locomotion ability of *Creld* mutant flies was assessed next.

3.4.3 *Creld* mutants display locomotor deficits and have increased number of dead cells in the brain

One of the most intrinsic behaviors in larvae, besides feeding, is the forward locomotion. Larval locomotion is mediated by neuronal circuits, and can be controlled by a variety of external stimuli such as food, light and temperature. It is characterized by peristaltic contraction of the larval body wall muscles from the posterior to the anterior part (Clark *et al.*, 2018). When observing *Creld* mutant flies, the most obvious phenotype they exhibit is their reduced locomotion. When tracking their movement over a period of time, they show a reduction in crawling speed when compared to age matched control larvae. In a defined time frame of 20 seconds, wild type larvae crawl around 3cm, whereas *Creld* mutants pass a distance of 1.5 cm (Figure 3-7 A).

Furthermore, adults also suffer from locomotion deficits already in young age, as seen in the negative geotaxis assay (Figure 3-7 B). Adult flies have an intrinsic behavior to crawl up the wall when tapped down in a vial, which can be analyzed quantitatively. Wild type and mutant flies differ significantly in their climbing success rate: *Creld* mutants show a reduction in climbing success of over 60% compared to wild types, which score a climbing success of 100%. Additionally, the climbing success of mutant female flies is worse than mutant male flies. The climbing phenotype is even stronger than these numbers suggest: The experimental setup in this climbing assay was chosen easy enough so that about half of the *Creld* mutants have climbing success, which was not the case with standard conditions for the assay. The threshold, the flies had to climb up were only 2 cm within 2 minutes, which is not a large distance to overcome. Consequently, there is hardly any deviation in wild type climbing. These conditions were chosen to be able to perform genetic and pharmacological assays that modify this phenotype in both directions (amelioration and exacerbation). These results are in agreement with previous locomotion assays performed by Birgit Stümpges. She could show that flying ability of *Creld* mutants is disturbed as well (B. Stümpges, PhD Thesis).

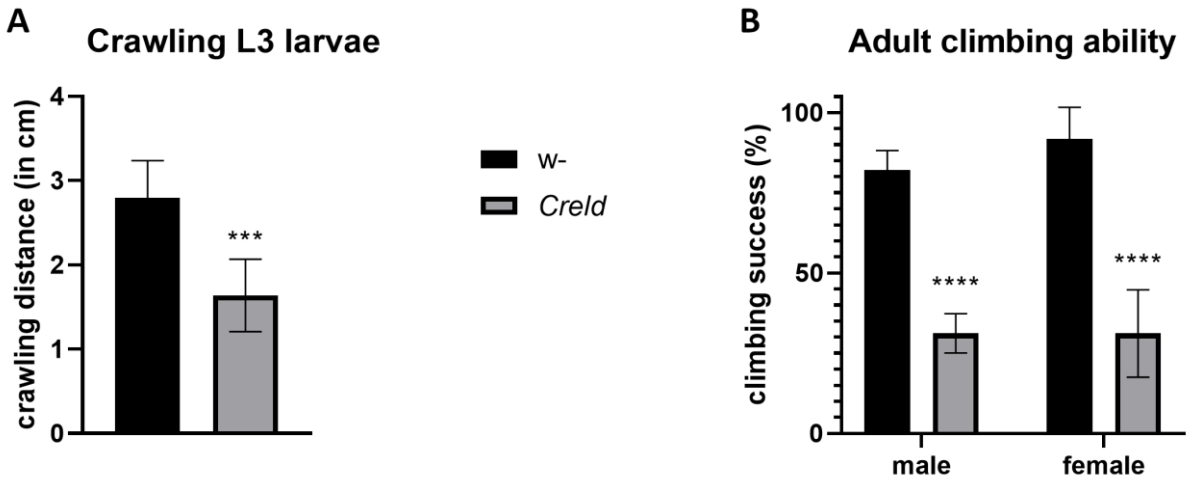

Figure 3-7: Locomotor ability of *Creld* mutants is reduced (A) Crawling capability of *Creld* mutant L3 larvae is significantly reduced, depicted as mean±SD, n=10, ***p>0.001 (t-test) (B) Climbing ability of adult mutant flies is reduced after 10 days. Result shown as mean±SD, n=10, ****p>0.0001 (t-test).

It is generally accepted that locomotor defects in flies correlate with neuronal disorders (Günther *et al*., 2016). Not only in the fly but also in humans, neurodegenerative diseases are a major cause for locomotive disorders, like for example in Parkinson's disease, amyotrophic lateral sclerosis or hereditary spastic paraplegia (Madabattula *et al.*, 2015). To analyze neurodegeneration in *Creld* mutants, their brains were stained with the necrotic

marker propodium iodide (PI) which marks dead cells (Yang *et al.*, 2013). Figure 3-8 A-D shows the PI staining of 10-day old female wild type and *Creld* mutant fly brains. *Creld* mutant brains show a dramatic and obvious increase of dead cells compared to the few PI positive cells in the wild type control. When quantified, the difference is even more ovious (Figure 3-8 E): When compared to wild type control, *Creld* mutants have an increased number of dead cells of about thirteen-fold.

The control flies used in this study where white^{1118} (w^-), as the *Creld* mutants were generated in this genetic background (Birgit Stümges, PhD Thesis). However, the *white* mutation is considered to be able to enhance neurodegenerative phenotypes (Ambegarokar *et al.*, 2010). In order to rule out that the increase of necrotic cells seen in *Creld* mutant brains is enhanced by the missing *white* gene, the same experiment was repeated with red-eyed flies. For this, the *Creld* mutation was crossed into Oregon R flies to gain a red-eyed *Creld* mutant with an intact *white* gene, and the Oregon R wild type flies were used as control.

As seen in Figure 3-8 C, D and E, the red-eyed mutant also shows a massive increase in PI positive cells compared to Oregon R flies. Quantification shows that these red-eyed *Creld* mutants show even significantly higher amount of PI positive cells than the white-eyed *Creld* mutants. Therefore, the necrotic cells are not the result of the missing *white* gene..

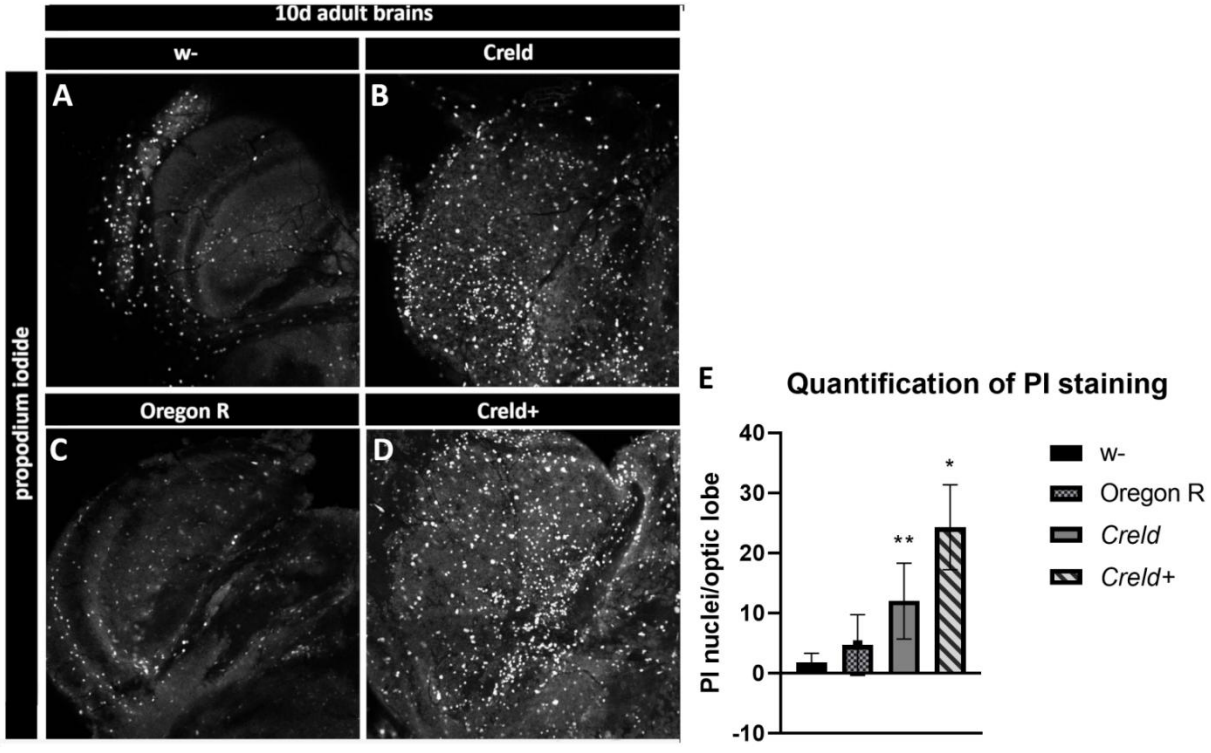

Figure 3-8: *Creld* mutant flies show symptoms of neurodegeneration. (A- D) Popodium iodide (PI) staining of 10-day old adult brains show an increased amount of PI positive cells in *Creld* and *Creld+* mutant flies compared to control. (E) Quantification of PI staining in optic lobe, results are shown as mean±SD, n=5, *p<0.05, p<0.01 (t-test).

3.4.4 *Creld* mutants suffer from an overall energy deficit

Many of the observed phenotypes could be explained with the lack of energy in *Creld* mutants. Locomotion and the observed heart phenotype are both consistent with energy deficits. Furthermore, the brain is one of the most sensitive tissues with respect to energy metabolism: even though it makes up only a small portion of the whole body mass, it belongs to the group of tissues with the highest energy demand (Bélanger *et al*., 2011). Neurodegeneration is therefore also consistent with a lack of energy/ ATP. As pointed out in the introduction, cells rely on energy provided in form of ATP. If the cell experiences a drop in ATP levels, either through excessive energy consumption or defective energy producing pathways, the ratio of ADP:ATP increases since more ATP is hydrolyzed to ADP than is reproduced. For an organism, it is important to maintain the balance between ATP production and consumption, which is monitored mainly by the AMP-activated protein kinase (AMPK). AMPK acts as a metabolic sensor by binding of AMP (adenosine mono phosphate) as an activating ligand: AMP is derived from conversion of ADP to AMP and is therefore an indirect measure for the ADP:ATP ratio in the call. AMPK activation as a result of drop in cellular energy subsequently leads to changes in the transcription of genes concerning cell growth and metabolism (Gowans and Hardie, 2014).

AMPK phosphorylation as a measure of the overall energy status in *Creld* mutants was measured in pre-pupae (to avoid developmental delay discrepancies) using Western blot analysis (Figure 3-9). Indeed, increased presence of phosphorylated AMPK in *Creld* mutants by almost 1.5 fold was observed, indicating an energy crisis in *Creld* mutants.

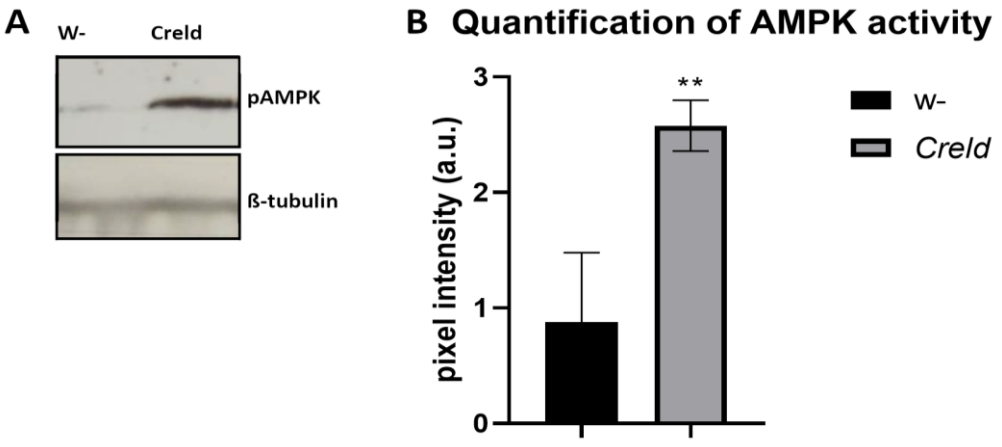

Figure 3-9: Western blot analysis of phosphorylated AMPK (pAMPK). (A) Representative image from Western blot analysis of 10d old adult extracts with pAMPK antibody shows increased band in *Creld*-deficient sample, ß-tubulin was used as a loading control (B) Quantification of measurements reveals increased band intensity in *Creld* mutants, represented as mean±SD, n=4, **p<0.01 (Mann-Whitney-U-test).

3.5 Analysis of mitochondria in *Creld* mutants

The observed energy crisis in *Creld* mutants could occur due to various reasons. However, as discussed in the introduction in detail, mitochondria are the cell's main site for energy production in the form of ATP. Therefore, *Creld* mutants were analyzed with respect to mitochondrial function and morphology.

3.5.1 Mitochondrial biogenesis is increased in *Creld* mutants

To gain an overall insight into mitochondrial function in *Creld* mutants, the amount of mitochondria in different tissues was assessed first. As stated above, the heart is an organ with high energetic demands. Therefore, transmission electron micrographs (TEM) (in cooperation with Christian Mayer, Paululat Lab) were taken from the heart.

Figure 3-10 shows electron micrographs of the heart in two magnifications. On the lower magnification (Figure 3-10 A, B) it is observable that the amount of mitochondria seems to be increased drastically in *Creld* myocardial cells. When using a higher magnification, it is also possible to detect differences in morphology as the mutant mitochondria seem to have elongated structures (shown in Figure 3-10 C and D). The morphology will be analyzed in detail in the following chapter of this thesis.

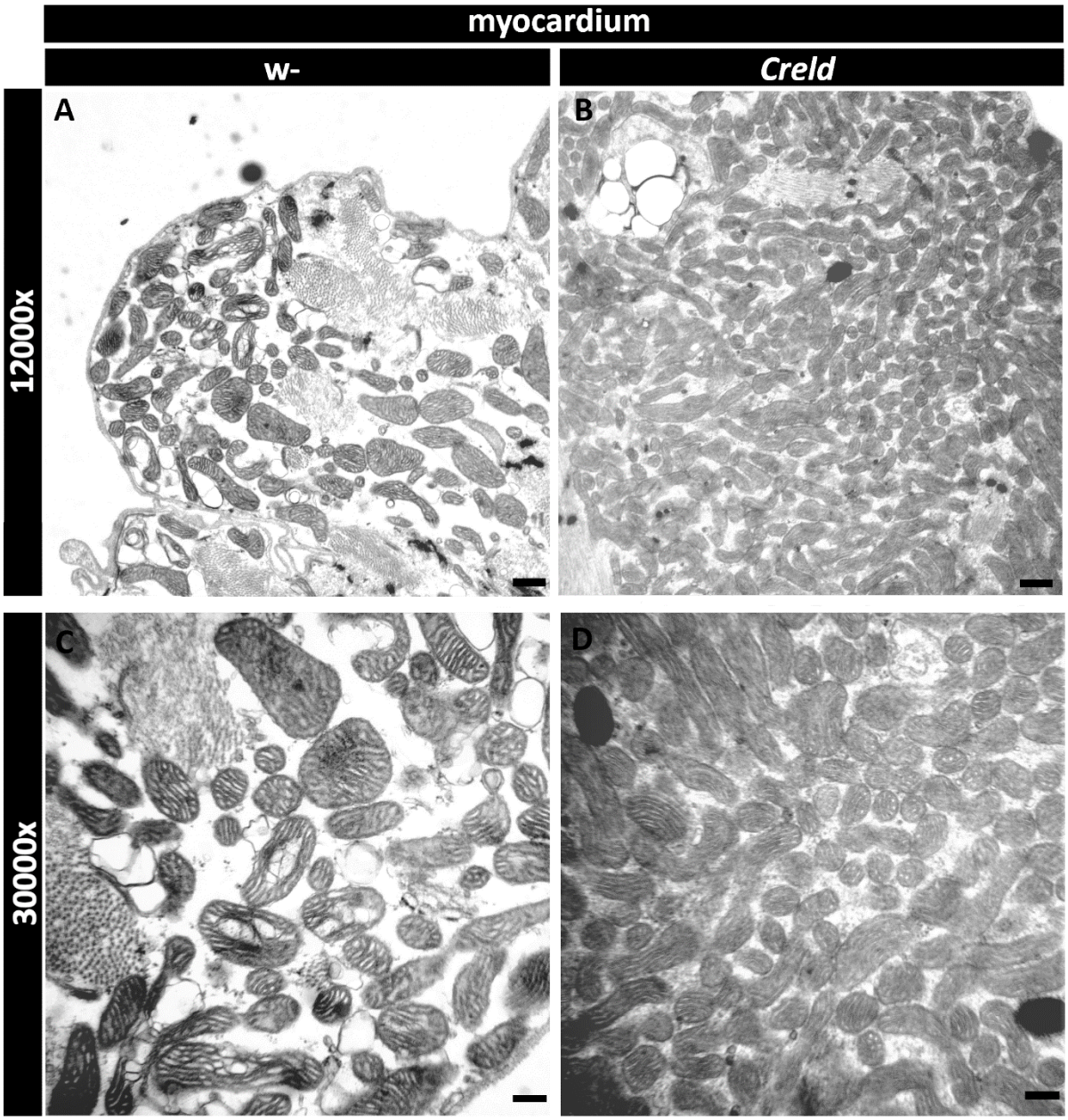

Figure 3-10: Ultrastructual analysis of myocardial cells shows an increased number of mitochondria in *Creld* mutant tissue**.** (A) displays w-, and (B) *Creld* mutant tissue. The amount of mitochondria is increased in *Creld* mutant myocardial cells. C and D show the same cell with a higher magnification to see that in *Creld* mutant tissue mitochondria are enlarged compared to w-. Scale bars: 500nm (A ,B) and 250nm (C, D).

One direct measure for mitochondrial abundance is the amount of mitochondrial DNA (mtDNA), as mitochondria contain their own genome, which is maternally inherited.

However, mtDNA amounts are not always correlating with mitochondrial mass, as failure of maintenance of integrity of mtDNA is associated with a variety of diseases, including neurodegenerative diseases (Parone *et al*., 2008). The amount of mtDNA was assessed in *Creld* mutants via qRT PCR as a ratio to nuclear DNA. Compared to wild type flies, *Creld* mutants show a reduction of mtDNA of around 35% (figure 3-11 A). This could hint at either

a decrease in mitochondrial mass or a defective mtDNA integrity and mitochondrial maintainance.

Mitochondrial biogenesis is controlled by an overall regulator called Spargel (the *Drosophila* homolog of mammalian *PGC1α*) (Tiefenböck *et al.*, 2010). To investigate whether the increased amount of mitochondria seen in pericardial cells (Figure 3-10) is because of an increased mitochondrial biogenesis, the expression of the gene coding for Spargel was assessed via qRT PCR. As can be seen in Figure 3-11 B, Spargel seems to be upregulated in *Creld* mutants. However, the deviation between the different measurements was too high to show a significant result. Spargel activity is not mainly regulated on a transcriptional level (Tiefenböck *et al*., 2010). To have a better read out of Spargel activity, Spargel target genes were analysed via qRT PCR. As can be seen in Figure 3-11 B also the transcription of its target gene *Isocitrate dehydrogenase* (IsoDH) is also not significantly misregulated in *Creld* mutants.

mtDNA integrity is guaranteed by different transcription factors. The three main transcription factors regulating mtDNA replication, transcription and translation, are TFAM (mitochondrial transcription factor A), TFB1 (transcription factor B1) and mitochondrial transcription factor subunit 2 (TFB2) (Matsushima *et al.*, 2004). Mice lacking any of these factors show a reduced number of mtDNA (Alonso-Montes *et al.*, 2008). The transcription level of these genes was assessed in *Creld* mutants, shown in Figure 3-11 C. All three genes were increasingly expressed in *Creld*-mutant tissue. AT first, this result seems to be in contrast to the reduced mtDNA amount, however, it could be possible that *Creld* mutants try to compensate the loss of mtDNA by upregulating TFAM, TFB1 and TFB2 transcription to re-establish their mtDNA integrity and homeostasis.

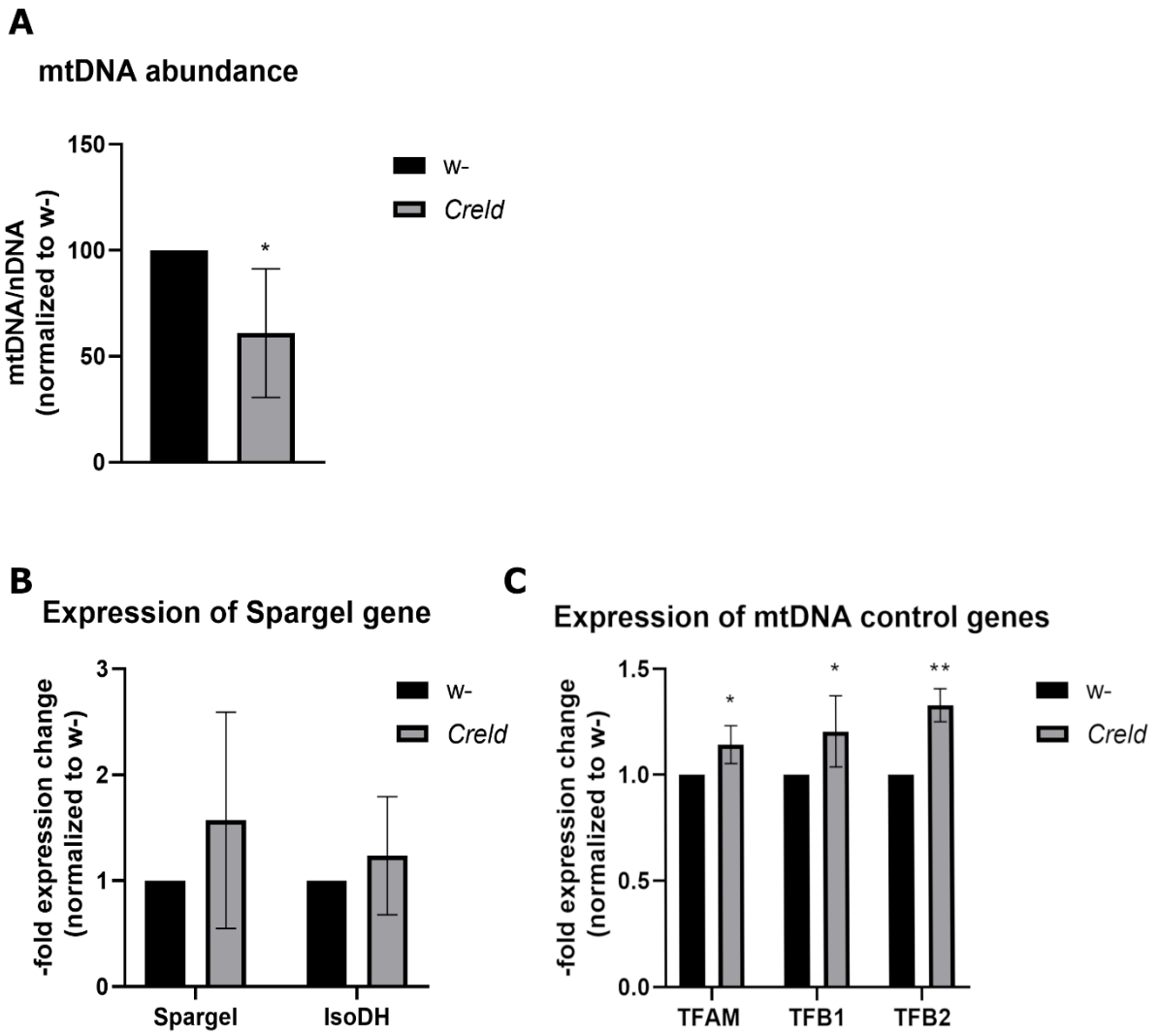

Figure 3-11: *Creld* mutant flies have increased mitochondria abundance. (A) Mitochondrial DNA (mtDNA) of 10 d old adult flies was measured by qRT PCR and set into relation to nuclear DNA (nDNA). *Creld* mutants show decreased amount of mtDNA compared to wild type flies. Results shown as mean±SD, n=5, *p<0.01 (t-test) (B,C) Expression of genes involved in mitochondrial abundance analyzed by qRT PCR. Spargel gene and Spargel target gene IsoDH (B), involved in mitochondrial biogenesis are not differentially expressed, but genes involved in mtDNA replication and maintainance(C) are upregulated in *Creld* mutants. Results are displayed as mean as mean±SD, n=5, *p<0.05, **p<0.01 (t-test).

3.5.2 ROS production is not enhanced by *Creld* deficiency

Mitochondrial problems often lead to oxidative stress, which is mainly induced through the increased production of reactive oxygen species (ROS), as a by-product inside mitochondria during the normal production of mitochondrial ATP. The main sites of ROS leakage are complex I and complex III (Ghouleh *et al*., 2011). Accumulation of ROS can induce oxidative damage to macromolecules like nucleic acids, lipids and proteins, and it is often correlated with neurodegeneration. Therefore, the oxidative stress status of *Creld* mutant flies was assessed.

First, the expression level of the *Creld* gene was analyzed in wild type flies fed with the drug Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) to induce oxidative stress via ROS

generation through mitochondria. The proposed mechanism of action of how Paraquat mediates the production of ROS is through interfering with the electron transport chain (ETC) in mitochondria, most likely by blocking the transfer of electrons from complex III (Castello *et al*., 2011).

Creld expression was increased around 1.8 fold compared to flies fed with control food, indicating that Creld might be involved in the oxidative stress response (Figure 3-12 A).

As part of the response to oxidative stress, organisms express genes coding for enzymes like superoxide dismutase (SOD), and catalases, which act as ROS scavenger, as they catalyze the conversion of superoxide into oxygen and hydrogen peroxide (Wang *et al.*, 2018). In *Drosophila* three different SOD genes (SOD1-3) are present and their expression levels were assessed via qRT PCR. No differential expression of SOD genes could be found in *Creld* mutants and control flies, as seen in Figure 3-12 B. This result could either indicate that *Creld* mutants do not suffer from oxidative stress, or that the oxidative stress response Is defective. Flies which are unable to induce ROS protective pathways show sensitivity to cellular stress (Wang *et al.*, 2018). To follow up if *Creld* mutants can cope with oxidative stress, an oxidative stress-sensitivity assay with adult flies was performed. For this assay adult flies were kept on food containing a high amount of H_2O_2, which is itself a form of ROS, and survival rates were checked every day. As seen in Figure 3-14 C, there is no obvious difference in survival of mutant flies compared to wild type flies, indicating no enhanced vulnerability when exposed to oxidative stress. These results indicate that the machinery to activate ROS protective pathways is still functional in *Creld* mutants, and suggests that *Creld* mutants on normal food do not suffer from oxidative stress as concluded from normal expression of SOD 1-3.

In order to follow up if the formation of reactive oxygen species is indeed normal in *Creld* mutants, the amount of hydrogen peroxide (H_20_2) was measured in *Creld* mutant lysates. The results show that *Creld* mutant flies produce even a significantly lower amount of H_20_2 compared to control flies (Figure 3-12 D).

Taken together, mitochondria show increased abundance, defective mtDNA integrity with reduced amounts and decreased ROS production. Together with the fact that ATP amounts seem to be reduced as inferred from increased AMPK phosphorylation, it was assumed that mitochondria in *Creld* mutants are generally less active.

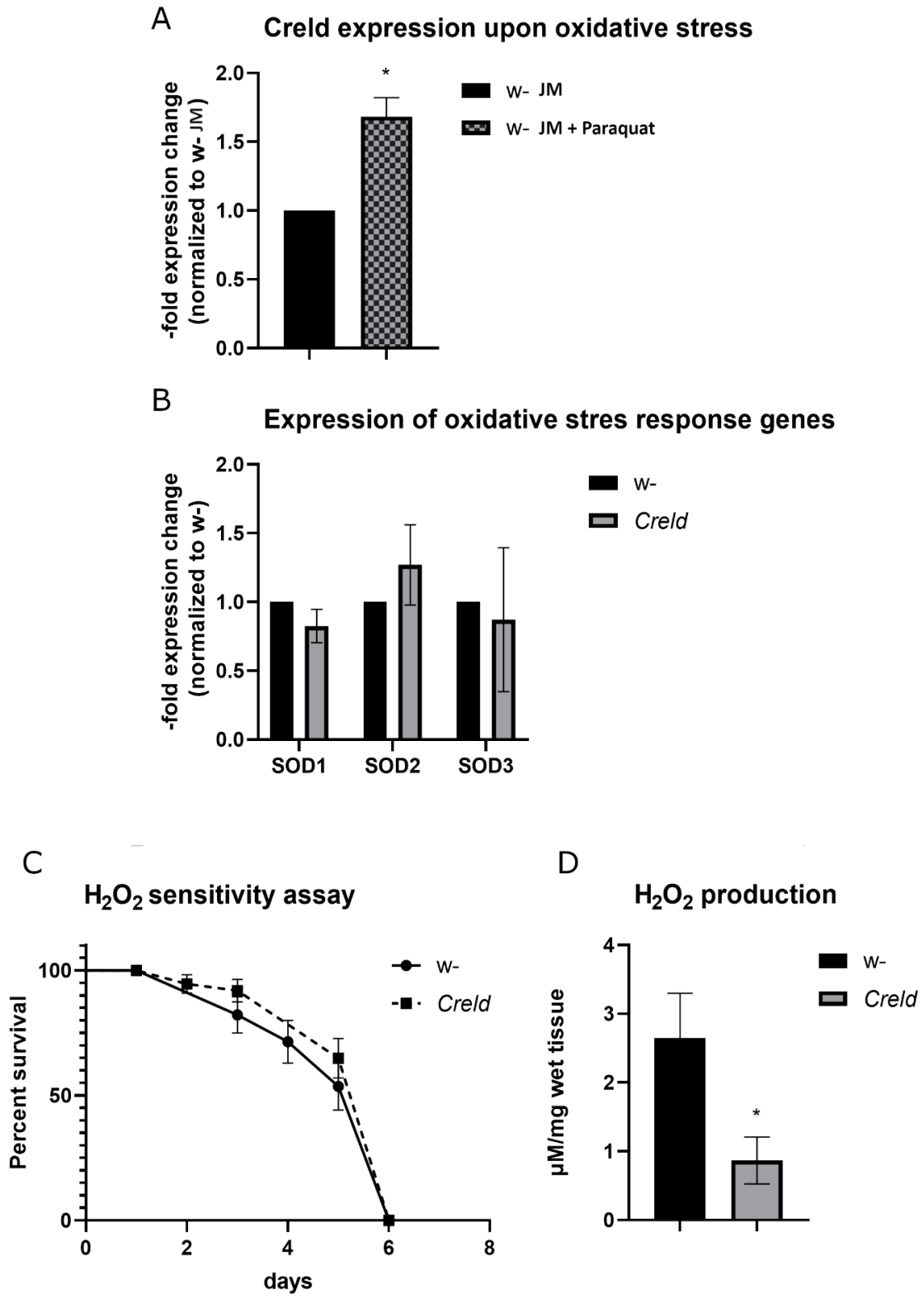

Figure 3-12: *CreId* mutants are not suffering from oxidative stress and produce less reactive oxygen species (ROS). (A) Wild type flies fed on oxidative stress inducing drug paraquat for 24h are significantly upregulating creld gene expression as analyzed by qRT PCR, results depicted as mean±SD, n=3, *p<0.05 (t-test). (B) Oxidative stress response genes (SOD1-3) are not upregulated in *CreId* mutant flies, indicating that they are not suffering from oxidative stress. Results measured by qRT PCR and shown as mean±SD, n=5, n.s. (t-test). (C) Treatment with 5% hydrogen peroxide to induce oxidative stress. Death rate of *CreId* mutants is not different to wild type flies. Results shown as mean±SD, n=3 in groups of 10. (D) H_2O_2 production of *CreId* mutant and wild type larvae. *CreId* mutants produce less H_2O_2 than w-, depicted as mean±SD, n=3, *p<0.05 (t-test).

3.5.3 Mitochondrial function in respect to ATP production is reduced upon lack of CreId

To confirm if ATP production by mitochondria is indeed reduced, the activity of complex V of the electron transport chain (ETC) in mitochondria was assessed in *CreId* mutants using a chemiluminescent assay. This complex produces ATP by utilizing the membrane potential generated via the electron transport chain (ETC) (Lodish *et al*., 2014). The result presented in Figure 3-13 B shows that complex V activity (normalized to Citrate synthase acitivity a mitochondrial marker) in *CreId* mutants seems to be decreased, consistent with decreased ATP production (Figure 3-13 C). However, instable results gained from the wild type control hamper a clear interpretation of the data, and as a consequence, the difference is much smaller than expected from the dramatically increased amount of phosphorylated AMPK.

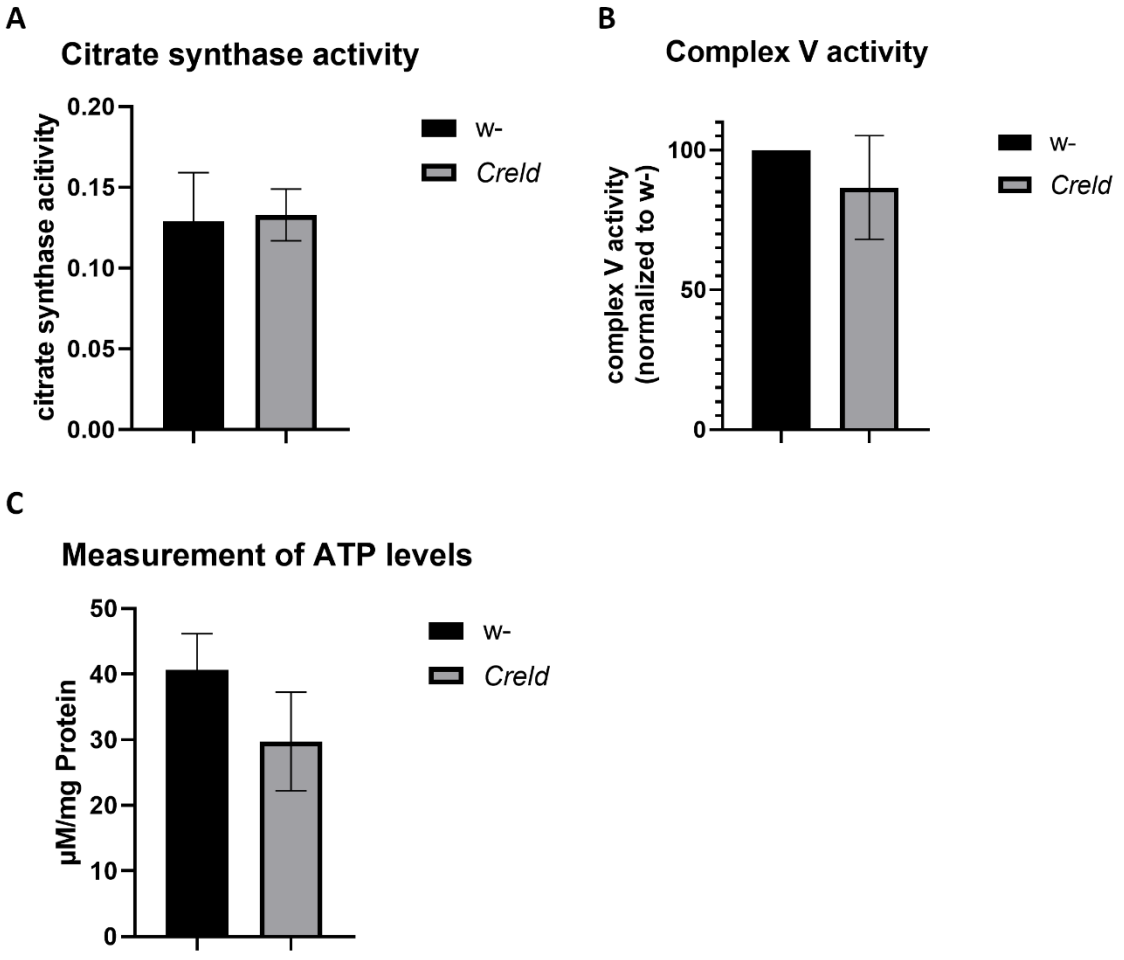

Figure 3-13: *CreId* mutants show decreased activity of OXPHOS subunit complex V .(A) shows citrate synthase (CS) activity alone and reveals that CS activity is not altered in C*reId* mutants. (B) Complex V activity was measured in 10d old adult flies, activity, normalized to citrate synthase activity, w- was set to 100%, *CreId* mutants show reduced activity All results shown as mean±SD, n=3. (C) ATP measurement show reduced levels of amount of ATP in *CreId*-deficient flies, results depicted as mean±SD, n=2.

Nevertheless, the trend points towards decreased activity of complex V, and thereby decreased ATP production via mitochondria, in *Creld* mutants. However, it is important to mention that this experiment should be repeated with another control genotype, maybe with another genetic background than w- flies.

As expected the functionality of mitochondria in *Creld* mutant flies seems to be reduced. Complex V or ATP synthase is the fifth oxidative phosphorylation complex and comprises the last step in the electron transport chain to generate energy. It does so by using the electron gradient caused by the electron transport chain to synthesize ATP from ADP (Jonckheere *et al*., 2012).

As the previous result shows a reduction in Complex V activity, it is reasonable to expect a decline in ATP levels. Therefore, the amount of ATP in whole adult flies was measured. As expected, a lower amount of ATP was measureable in *Creld*-deficient flies when compared to wild types (Figure 3-13 C). Although the result is not significantly different to the measurements in wild types, the amount of ATP is still visibly reduced in *Creld* mutant flies.

Taken together, these results show that *Creld*-deficient flies suffer from energy deficits in the form of reduced ATP production probably via oxidative phosphorylation in mitochondria.

3.5.4 Mitochondrial morphology is different in *Creld* mutant flies

TEM analysis revealed that mitochondria are more tubular in Creld mutants (section 3.4.1, Figure 3-10), which is an indication for altered mitochondrial dynamics, which could also explain the discrepancy between increased mitochondrial abundance and decreased amount of mtDNA. As explained in the introduction the fission of damaged mitochondria and the subsequent fusion with healthy ones is contributing to a healthy mitochondrial population, in combination with the segregation and autophagic degradation of damaged parts. Tissues with a high demand for ATP tend to have more mitochondria, as they provide the major amount of ATP (Pareek *et al*., 2018). Therefore, mitochondrial morphology was analyzed in muscle tissue, more precisely in adult indirect flight muscles (IFM).

Indeed, a difference in wild type and mutant tissue could be observed. As already suspected, by the tubular structure seen in myocardial cells (see Figure 3-10), an increased number of tubular mitochondria could also be observed in cross-sections of IFM in *Creld* mutants, marked in Figure 3-14 A by green asterisks. Whereas in the wild type control the

mitochondria are separated and barely extend around a muscle fiber, this can be seen quite often in the mutant sample.

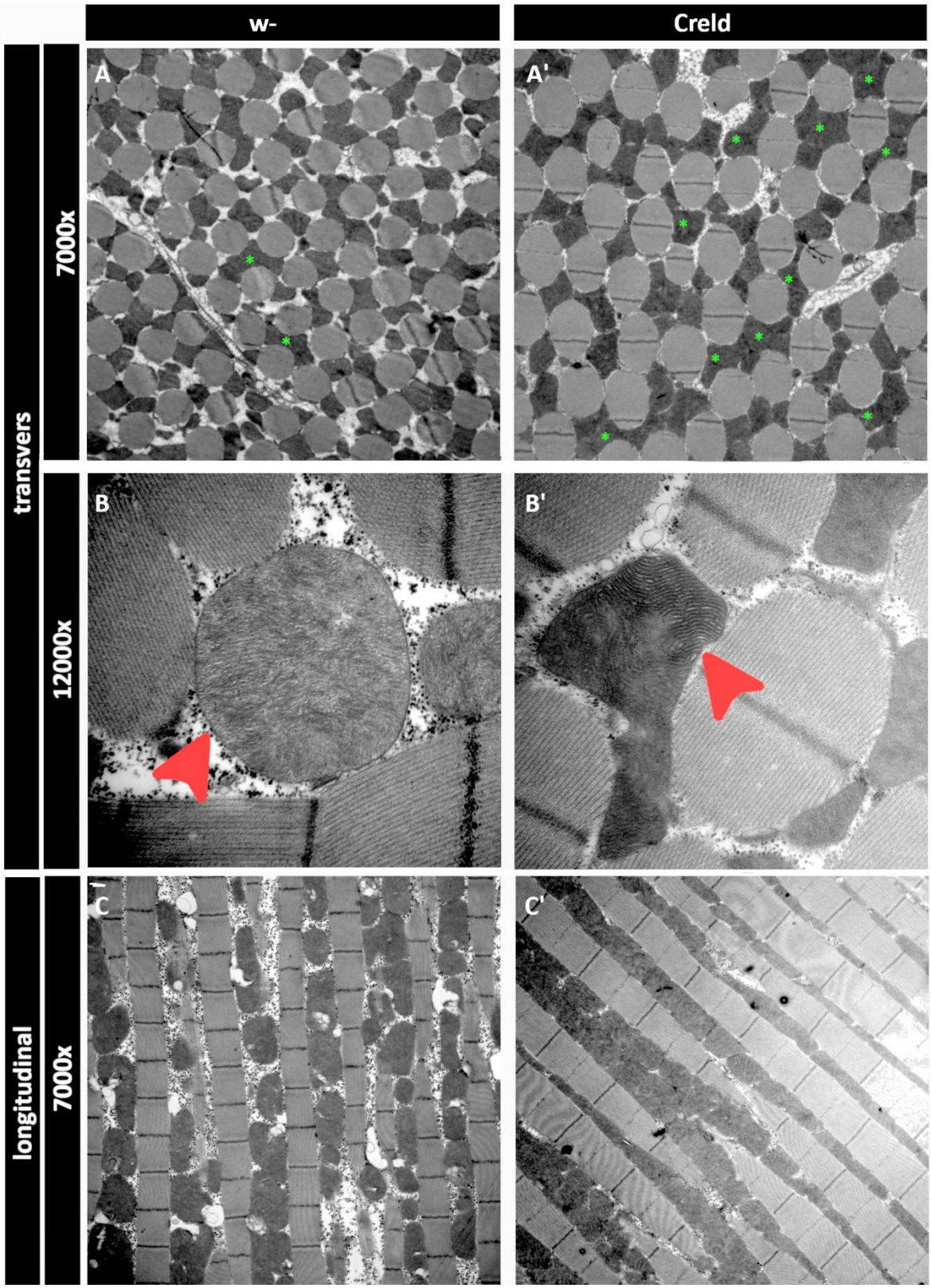

Figure 3-14: *CreId* deficiency causes increased mitochondrial mass and elongation in muscle. Ultrastructure analysis of indirect flight muscle (IFM) of 10d old adult flies. (A, A') overview of cross section of IFM shows increase in mitochondria in *CreId* mutant tissue. Elongated structures are more abundant in mutant flies as marked with green asterisks. (B, B') higher magnification reveals no difference in mitochondrial cistae structure (shown by red arrow). Elongated and more mitochondria are also seen in longitudinal sections (C, C') in *CreId* mutants.

Almost every mitochondria in mutant IFM is extending between more than one muscle fiber. The ultrastructure of mitochondrial cistae (Figure 3-14 B, marked by red arrows) was however not disturbed in *Creld* mutants, but this increased magnification could also show the differences in morphology (roundish shaped in wild type and longer in the mutant tissue). The longitudinal sections of the IFM also showed that the amount and size of mitochondria in the mutant tissue is clearly increased. Summarized it can be said, that *Creld* mutant muscle tissue contains a higher number of elongated mitochondria.

Since muscle is among the group of tissues that are using a lot of ATP to function, flies reduce muscle mass when nutritional input gets limited. By doing so, they reduce energy consuming processes (Kemppainen *et al.*, 2016). The muscle mass in *Creld* mutant tissue was measured from TEM pictures and quantified (Figure 3-15). In contrast to the expectations, the muscle mass, measured by the fiber diameter length, seems to be increased by around 10% compared to control.

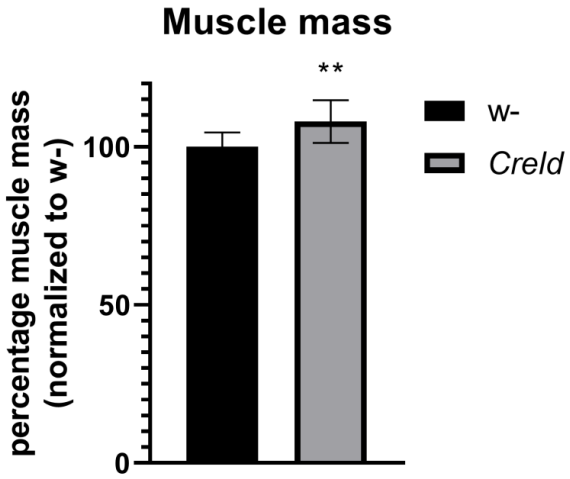

Figure 3-15: *Creld*-deficient flies have more muscles than wild types. Muscle mass was analyzed by measuring muscle fiber diameter in TEM pictures. Data are shown as mean±SD, n=10, **p<0.01 (t-test).

3.5.5 Mitochondrial dynamics are disturbed in flies lacking CreId function

In addition to the morphological analysis, the expression of genes functioning as key players in orchestrating mitochondrial dynamics was observed. A functional homeostasis in mitochondrial dynamics is crucial to maintain healthy mitochondria (Chen and Chang, 2009). While a reduction in mtDNA content was measured, an increase in mitochondrial abundance could be observed simultaneously. Normally, mitochondria bearing DNA with defects get degraded via fission-induced mitophagy. As another means, the damaged mitochondrion can fuse with a healthy one for content mixing. In this case the mitochondrial mass increases, as fused mitochondria show an enlarged tubular structure, while the amount of healthy mtDNA is reduced (Youle and Narrenda, 2011).

To get an overall picture of mitochondrial dynamics, the expression of proteins acting as key regulators was analyzed first. Indeed, the expression of pro-fusion gene *Opa1* was significantly increased in *Creld* mutants, shown in Figure 3-16.

Expression of *Drp1*, which is necessary to induce mitochondrial fission, was not significantly altered compared to wild type flies.

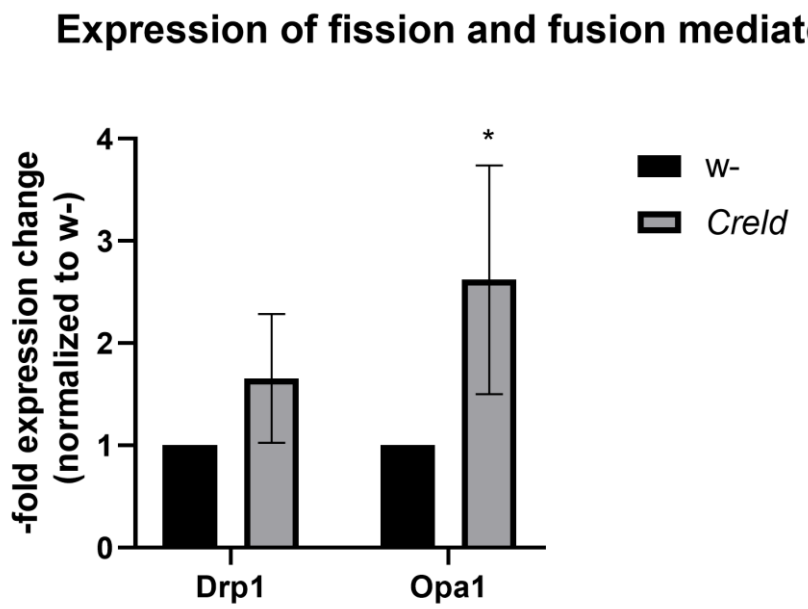

Figure 3-16: Gene expression of mitochondrial dynamics regulators is disturbed in *Creld* mutants. qRT PCR analysis of gene expression of pro-fission gene *Drp1* and pro-fusion gene *Opa1* shows increased expression of both genes in flies lacking *Creld*. *Drp1* expression increase however is not significant. Results are shown as mean±SD, n=5, *p<0.05 (t-test).

As stated in the introduction, the main regulation of Drp1 activity occurs through posttranslational modifications and not on mRNA level. Taken together, these results point towards a misregulation in mitochondrial dynamics.

In order to establish whether pharmacological Drp1 inhibition is harmful to *Creld* mutants, flies were raised on food containing the Drp1-inhibitor MDIVI-1 (figure 3-17). Survival rates did not change in any genotype, however, the climbing phenotype of *Creld* mutants was enhanced slightly when fed with MDIVI-1. At the same time, wild type flies were not hampered by the drug, which indicates that *Creld* mutant flies are susceptible to Drp1 inhibition and the resulting decreased mitochondrial fission.

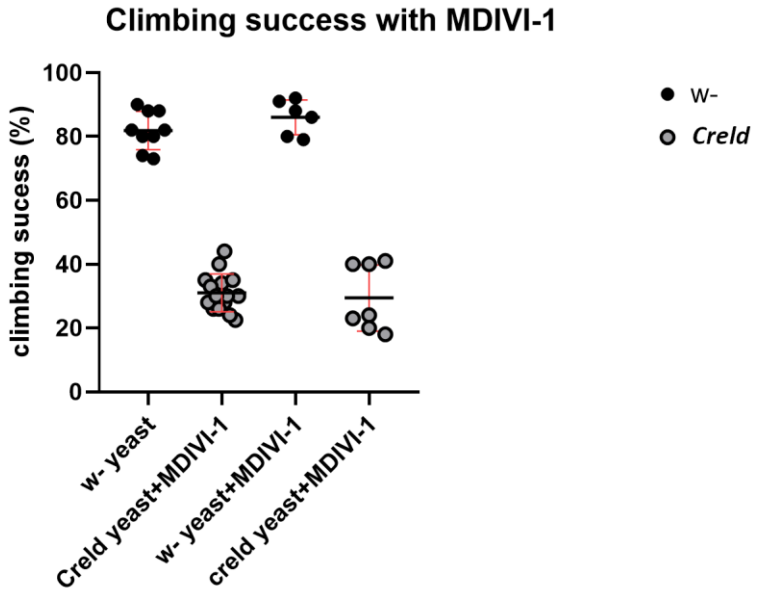

Figure 3-17: Climbing success of *Creld* mutant flies is tends to be reduced when treated with Drp1 inhibitor MDIVI-1. Climbing assay of 10d old adult flies, raised on food containing MDIVI1. *Creld* mutants show no significant reduction of climbing ability but a decreased trend is visible. Flies had to cross a 10cm mark in 2 min. Each dot shows the climbing success of one individual fly. Red error bars mark the mean of all flies.

Since Drp1 fission activity is regulated on the protein level, and the protein changes localization from cytoplasm to mitochondria upon activation, Drp1 subcellular localization was analyzed. A Drp1-HA-tagged version was therefore introduced into the *Creld* mutant background and the localization of the Drp1-HA protein was followed up via Western blot analysis. The used construct does not influence the mitochondrial phenotype by itself (REF). Cytoplasmic and mitochondrial fractions from whole adult animals were isolated and the amount of Drp1-HA tagged protein in the fractions was observed by Western blot. In addition, the total protein content of each fraction was measured. At first look it seems that

the Drp1 band intensity is equally distributed to the mitochondrial and cytoplasmic fractions of *Creld* mutant and control samples (Figure 3-18 A). However, one has to keep in mind that *Creld* mutant flies have a higher mitochondrial mass, which is reflected in a higher ratio of protein content of the mitochondrial fraction to cytoplasmic fraction in *Creld* mutants compared to control sample (Figure 3-18 B). Therefore, the localization of Drp1 protein has to be normalized to the total protein content of the fractions.

As expected, when quantified (Figure 3-18 C) and normalized to total protein amount in the cytosolic and mitochondrial fractions, less relative Drp1-HA content was found in the mitochondrial fraction in *Creld* mutants compared to control flies, while cytoplasmic and overall (sum of cytoplasmic and mitochondrial fraction values) Drp1-HA content in Creld mutants was increased (Figure 3-18 C). This suggests that Drp1 activation and subsequent translocation to mitochondria is reduced in *Creld* mutant flies, which would explain why mitochondria are elongated in *Creld* mutants as a result of reduced fission through Drp1 activity. It would also explain why MDIVI-1 mediated Drp1 inhibition exacerbates the phenotype, as Drp1 activity is already too low in *Creld* mutants.

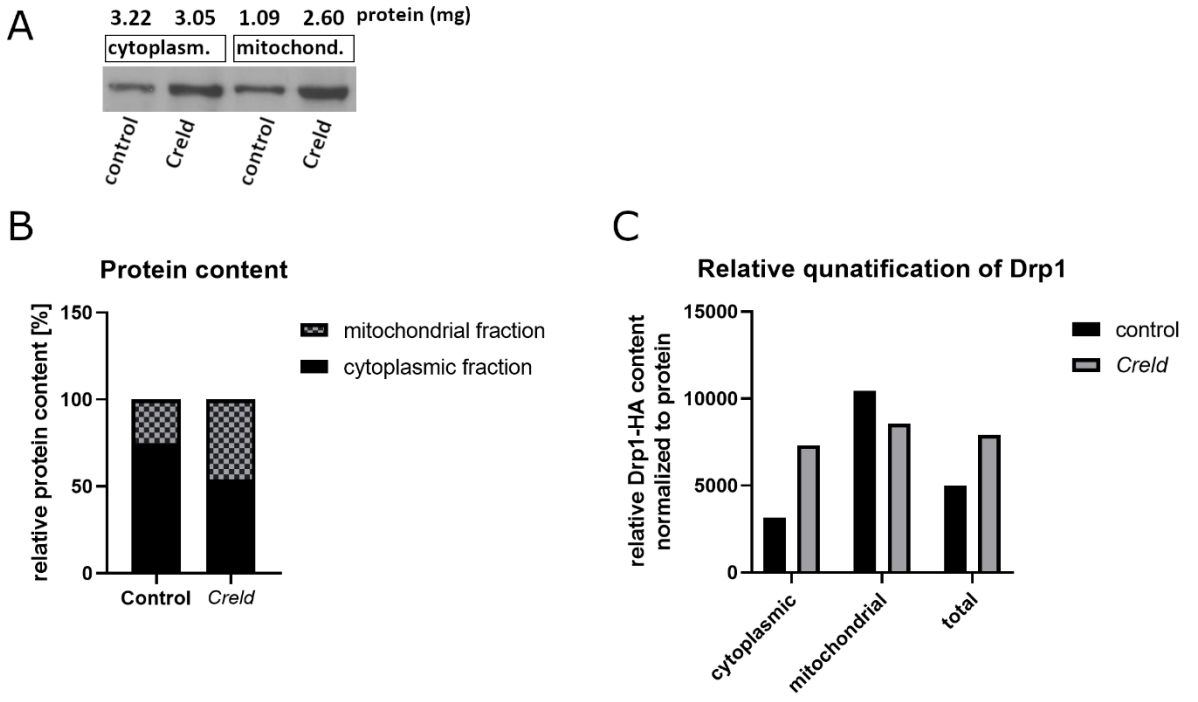

Figure 3-18: Drp1 expression levels analysed via Western blot. (A) Fly lysates from flies containing a Drp1-HA tagged version were fractionated into mitochondrial and cytoplasmic fraction and blotted with antibodies against HA. Band intensities suggest that the same amount of Drp1-HA is detected in the cytoplasmic and mitochondrial fraction in the control samples as well as creld mutant samples. (B) Creld mutant sample have a higher ratio of protein in the mitochondrial fraction to cytoplasmic fraction when compared to control samples, indicating a higher mitochondrial mass in the mutant sample. (C) When quantified it is clearly visible that the Drp1 content is over-represented in the cytoplasmic fraction in *Creld* mutants. N=1 (in groups of three).

Among the activity-regulating posttranslational modifications of Drp1 is the dephosphorylation via the phosphatase Calcineurin, which is needed to activate its mitochondrial fission function and for localization to mitochondria (Cereghetti *et al.*, 2008). Previously, it was shown that mammalian Creld1 is indeed needed for Calcineurin activation by forming a complex with Calcineurin subunit B (CanB) (Mass *et al.*, 2014). Assuming that this mechanism might be also true in *Drosophila*, it was tried to phenocopy the locomotor dysabilities of *Creld* mutants using a chemical approach to suppress Calcineurin. Wild type larvae were fed with strong Calcineurin inhibitor Cyclosporin A (CsA) (Martinez-Martinez & Redondo, 2004). The main characteristic phenotype for *Creld* mutants is their reduced locomotion. Therefore, a crawling assay with L3 larvae grown on standard JM food supplemented with CsA was conducted (Figure 3-18). Compared to control flies, which were fed standard JM food, a reduction of crawling activity in these larvae could be observed (Figure 3-19).

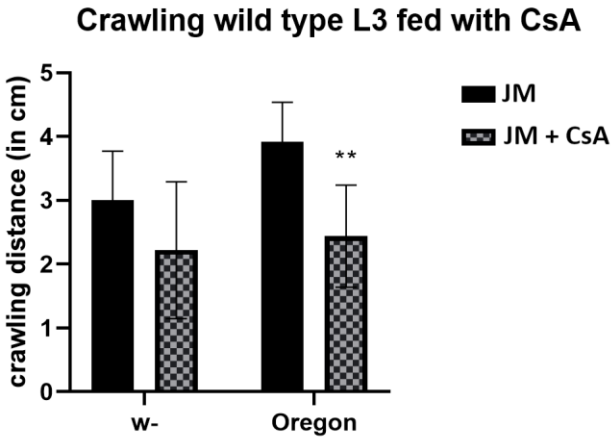

Figure 3-19 Crawling ability of wild type flies (w- and Oregon R) when fed with Calcineurin-inhibitor Cyclosporin A (CsA). Crawling is reduced in both genotypes compared to control fed flies. Result is shown as mean±SD, n=10, **p<0.01 (t-test).

3.5.6 Mitophagic turnover is impaired in *Creld* mutant flies

In order to remove bulk damaged material from the cytoplasm, like unfolded proteins damaged organelles, the cell uses a mechanism called autophagy (Hansen *et al.*, 2011). As stated in the introduction, damaged and dysfunctional mitochondria are removed and recycled by the autolysosomal machinery via a selective autophagic process known as mitophagy. Mitophagic removal of dysfunctional mitochondria necessary to protect the cell from oxidative damage, and defects of the process can have deleterious consequences and are often implicated in severe diseases. One prominent example is the Parkinson's disease,

which is characterized by neurodegeneration as a result of damaged mitochondria (Pickles *et al*., 2018).

Research on Parkinson's diseases revealed a role of the proteins Pink1 and Parkin in the regulation of mitophagy. Roughly summarized, mitophagy is initiated by the recruitment of Parkin from the cytoplams to damaged mitochondria. This recruitment is mediated via Pink1. It is unclear so far how Pink1, which is present in all mitochondria, is able to distinguish between healthy mitochondria and the mitochondria destined for degradation. However, it was suggested, that in healthy mitochondria Pink1 gets proteolytically turned over so that Parkin recruitment is not fast enough. Parkin not only marks damaged mitochondria for degradation via the autophagic pathway, but also inhibits pro-fusion proteins Opa1 and Marf1, which means that damaged mitchondria are not able to fuse with healthy ones (Youle and Narenda, 2016).

Beside Pink and Parkin induced mitophagy, Drp1 mediated mitophagy exists as well. How the process takes place in detail is not explored yet, but it is believed that Drp1 mediated fission helps to generate small mitochondria which easily can be engulfed by the autophagic membrane (Kageyama *et al.*, 2014). Additonally, it was observed that Drp1 interacts directly with autophagy-mediator LCIII (Atg8a in *Drosophila*) (Rana *et al*., 2017)

Since a reduced activity of Drp1 was observed in *Creld* mutants, together with the fact that mitochondrial mass was increased and mtDNA decreased, the question arose if mitochondrial turnover and quality control are affected. Therefore, the functionality of mitophagy was assessed in *Creld* mutant flies.

The first step in autophagy is the generation of the autophagosomal membrane, which is needed to engulf the damaged cellular component. This step is orchestrated by a variety of proteins, which form a functional complex (Hansen *et al*., 2011). One of these proteins is called autophagy related gene 1 (Atg1), which was shown to be overexpressed when autophagy is induced. To assess the expression of this protein, real time experiments with whole adults were performed. Figure 3-20 A shows that the gene expression of Atg1 is reduced in *Creld* mutant tissue, when compared to control flies. This indicates reduced autophagy induction in mutant flies. If autophagy or mitophagy in general is indeed suppressed in *Creld* mutant tissue, further inhibition of the process should enhance the phenotype. To test this, flies were fed with the drug Wortmannin (WT), a compound inhibiting autophagy induction irreversible via PI3K.

As shown in Figure 3-20 C, these flies stopped their climbing completely, which confirms that pharmacological autophagy repression indeed exacerbates the *Creld* phenotype.
Additionally, the expression profiles of Pink and Parkin were analyzed using qRT PCR. As seen in Figure 3-20 B, the expression of the two genes is increased in *Creld* mutants, with a

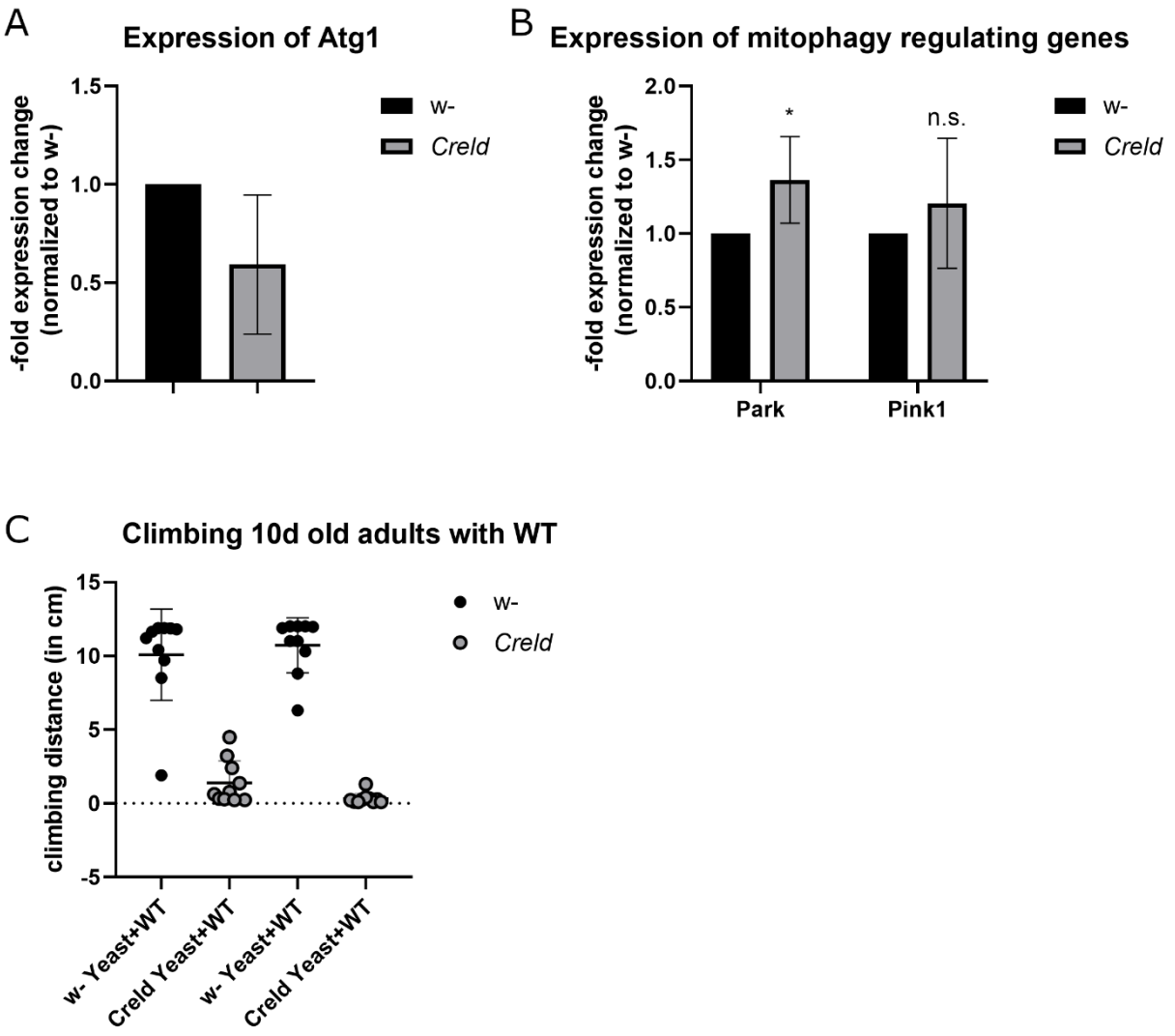

Figure 3-20: Autophagy and mitophagy is misregulated in *Creld* deficient flies. (A,B) Gene expression of autophagy and mitophagy regulating genes is measured by qRT PCR and reveals that Atg1 expression tends to be down regulated in mutant adult flies (A), whereas expression of mitophagy inducing genes Parkin (Park) and Pink1 are upregulated. All results shown as mean±SD, n=5, *p<0.05 (t-test). (C) *Creld* mutant flies fed with autophagy inhibiting drug Wortmannin (WT) reveal a drastic decline in climbing ability compared to control flies. Dots displays the climbing distance each individual fly was climbing in 10 seconds, error bars represent the SD.

significant increase of Parkin. As stated above, these two genes mainly exhibit their activation not through transcriptional control but through posttranslational modifications. Nevertheless, these results are consistent with an attempt of the cell to activate mitophagy though.

3.6 Metabolic state

3.6.1 Increased Starvation sensitivity of flies with reduced Creld function

Under normal conditions autophagy is kept at a normal level, mainly to degrade damaged cellular material such as organelles and long-lived proteins. In starving situations, when nutrients are limited, the autophagic machinery gets activated via the insulin signaling pathway to supply the organism with internal nutrient reservoirs (Kuma *et al*., 2011). Autophagy seems to be misregulated in *Creld* mutants (section 3.5.6). The fact that the locomotor phenotype of *Creld* mutants is enhanced when fed with Wortmannin, which mimics the effects of starvation by inhibiting PI3K, suggests that *Creld* mutants might be sensitive to starvation. In order to establish whether the mutant flies are capable of surviving under nutrient stress, a starvation assay with 10d old adult flies was performed (Figure 3-21). As expected, *Creld* mutant flies have a dramatic decrease in survival rate under starvation conditions compared to age-matched w- flies. Whereas wild type flies are able to survive up to one week without nutrient supply, *Creld* mutants all die within the first 4 days, giving a strong indication that they are starvation sensitive.

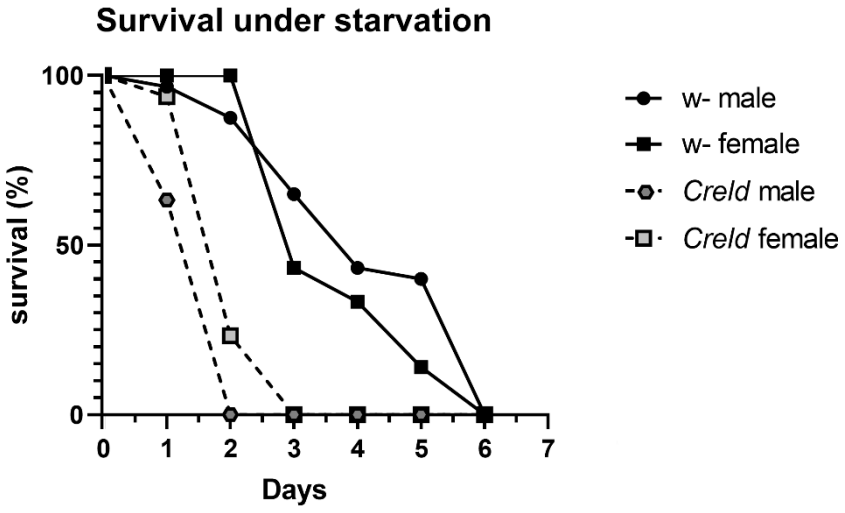

Figure 3-21: *Creld* mutant adult flies are starvation sensitive. Survival under starvation was observed in 5d old adult flies. *Creld* mutant flies show a reduced survival compared to wild type. Male survival was worse than female survival in both genotypes. Experiment was carried out 3 times with 10-20 flies per genotype. Difference between survival curves significant **p<0.01 calculated with Log-rank Mantel-Cox test.

3.6.2 Reduced fat storage and mobilization in *Creld* mutants

In the nutrient starvation response, several signaling pathways are involved. If no nutrients are available insulin signaling is inactive. Therefore, PI3K is inhibited, which results in the inactivation of Akt. Akt usually inhibits the transcription activator FoxO directly. When nutrients are limited, FoxO is consequently no longer repressed and is able to induce target gene expression (Gershman *et al*., 2007). One target of FoxO is the eIF4E-binding protein (4E-BP or Thor in *Drosophila*). The expression level of the gene encoding 4EBP/Thor was assessed in *Creld* mutant flies. In Figure 3-22 it can be seen that Thor expression is indeed upregulated compared to control flies, although not significant, indicating that *Creld* mutants under normal food conditions already show signs of starvation, which fits to their increased

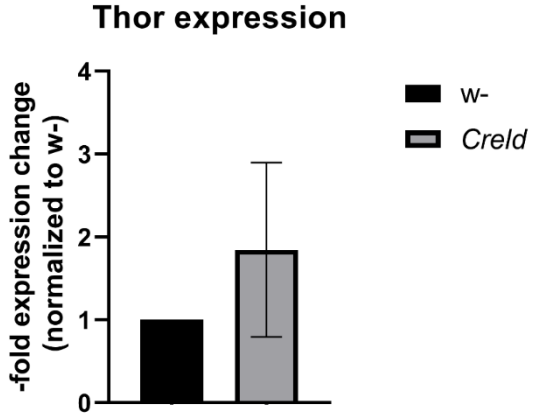

Figure 3-22 Thor expression in *Creld* mutants not significantly different when compared to w-. The expression of Thor gene was assessed using qRT PCR, depicted as mean±SD, n=5, not significant (t-test).

sensitivity to actual food deprivation.

All animals evolved a mechanism to survive times of nutrient deprivation: in times of nutrient availability, excess nutrients get stored as reservoirs in the tissue. Glucose is stored as glycogen and fats are stored in triacylglycerol (TAG) in the fatbody. In starvation periods these stores are mobilized and enter the catabolic processes to generate ATP: glycogen will eventually be catabolized in glycolysis and TAG gets broken down to free fatty acids which are fed into the TCA cycle. TAG is the preferred way of energy storage as the oxidation of fatty acids has a greater ATP outcome than glycolysis, and the ration of energy gain vs. weight and volume is higher than for carbohydrates (Rambold *et al*., 2015).

In order to identify possible causes for the observed starvation sensitivity, the amount of TAG was measured in whole adult animals. Compared to wild type, the amount of TAG is

indeed drastically reduced in *Creld* mutants compared to wild type, which is outlined in the thin layer chromatography and its quantification in Figure 3-23 A and B.

TAG gets mobilized in the process of lipolysis to generate free fatty acids and a glycerol molecule. This is achieved through many different enzymes, collectively known as lipases, which are extensively expressed under starvation signaling (Rambold *et al*., 2015). In order to analyze if the TAG stores of *Creld* mutant flies are empty because of an increase in TAG mobilization by lipases, the expression of three different lipases was examined via qRT PCR. Surprisingly, the expression of all lipases was significantly downregulated in *Creld* mutants (figure 3-23C). The most dramatic decrease was seen in the expression of lipase 3 (lip3).

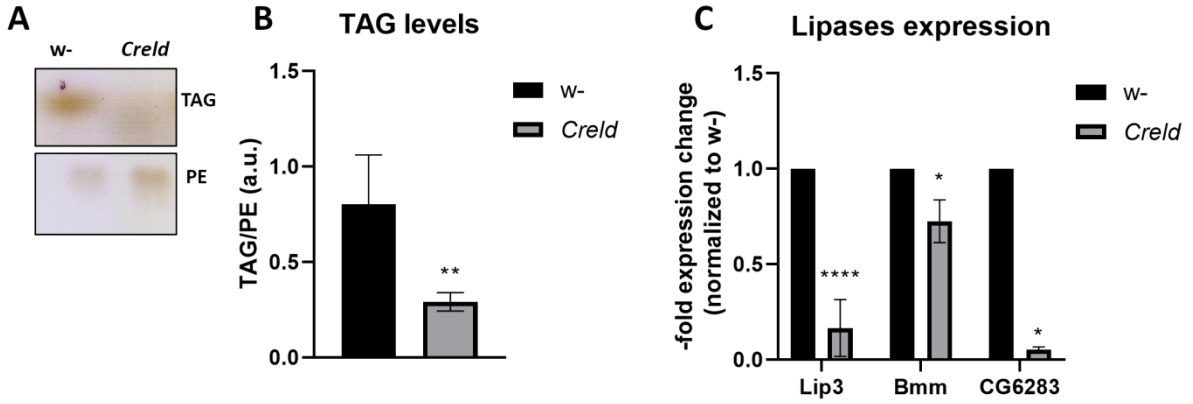

Figure 3-23: Fat storage is reduced in *Creld* mutants while they show a reduction in lipase expression. Triacylglycerol (TAG) content of whole 10d old adult flies was measured by thin layer chromatography, A) shows a representative sample. (B) Quantification of TAG levels measured via TLC reveals a decreased TAG storage in *Creld* mutant flies, shown as mean±SD, n=4, **p<0.01 (t-test). (C) Lipase expression of three genes encoding different lipases as measured by qRT PCR, shows decrease in *Creld* mutant flies compared to w-. Results shown as mean±SD, n=5, *p<0.05, ****p<0.0001 (t-test).

TAGs are hydrolyzed to produce free fatty acids, also called non-esterified free fatty acids (NEFAs) because they are no longer bound to glycogen backbone via an ester bond (Rambold *et al*., 2015). As *Creld* mutants show reduced TAG levels, the amount of free fatty acids or NEFAS, should be increased. Contrary to the expectations, this was not observable in the mutants: the amount of NEFAs measured in whole adult tissues showed a slight decrease in *Creld* mutant flies, as shown in figure 3-24 A.

Since TAGs do not seem to be reduced as a result of lipolysis, reduced lipogenesis might be and alternative explanation for reduced NEFA and TAGs. Lipids can be synthesized from carbohydrate precursors in the process of *de novo* lipogenesis (Stable *et al.*, 2010). During glycolysis in the cytoplasm, pyruvate is produced and shuttled into mitochondria where it is converted to acetyl-CoA before entering the TCA cycle to produce ATP. Another product of

the TCA cycle is citrate, which can be shuttled out of the mitochondrion into the cytoplasm where the enzyme ATP-citrate lyase (ACL) transfers the acetyl group of the citrate to coenzyme A, thereby producing acetyl CoA. Acetyl CoA is a key component of lipid metabolism, and it is the substrate for the first step in *de novo* lipogenesis, catalyzed by acetyl-CoA-carboxylase (ACC) to generate malonyl-CoA (Wang *et al.*, 2016). Finally, the enzyme Fatty acid synthase (FAS) generates the fatty acid palmitate with malonyl CoA as educt (Solinas *et al.*, 2015). The feeding of anabolic pathways, like *de novo* lipogenesis, from the TCA cycle is known as cataplerosis.

In order to assess the expression level of lipogenesis pathway components, ACC and FAS transcription were measured via qRT PCR. Both genes show a massive increase in transcription, suggesting that *Creld* mutants try to upregulate the *de novo* synthesis of fatty

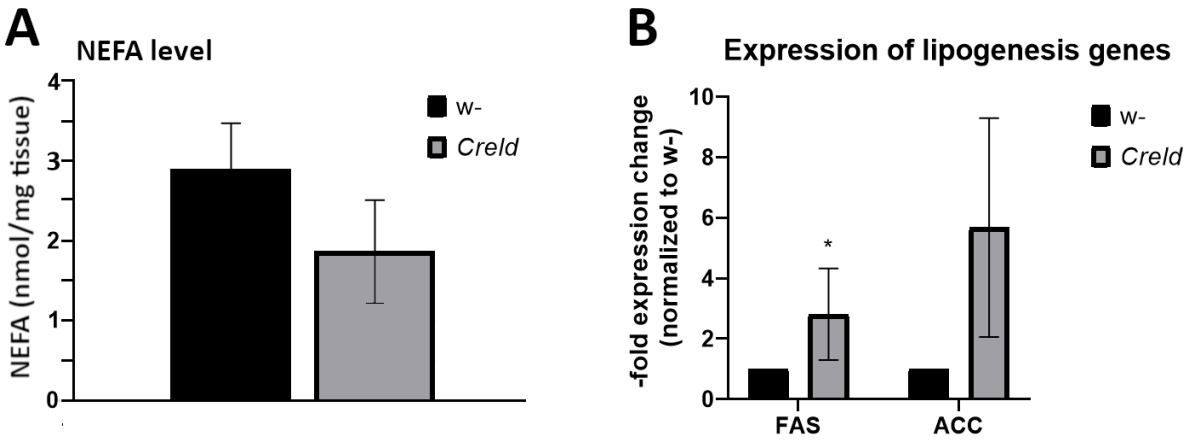

Figure 3-24: Amount of non-esterified fatty acids (NEFAs) is reduced in *Creld* mutants whereas genes involved in *de novo* lipogenesis are increasingly expressed. (A) Colorimetric measurement of NEFAs in 10d old adults reveals a reduced amount in *Creld* deficient flies, shown as mean±SD, n=5. (B) Gene expression of key players in de novo lipogenesis are upregulated as measured by qRT PCR and shown as mean±SD, n=5, *p<0.05 (t-test).

acids through lipogenesis (Figure 3-24 B).

As stated before, acetyl CoA can be regarded as a crucial intermediate in metabolism. ACC converts acetyl CoA from citrate coming from the TCA cycle. As the TCA cycle occurs in mitochondria, and the previous results show decreased mitochondrial metabolism in *Creld* mutant flies, it is reasonable to expect that the amount of acetyl CoA is generally reduced in *Creld* deficient flies.

The main route of acetyl-CoA generation for the TCA cycle is through the hydrolysis of fatty acids via ß-oxidation. This process takes place in mitochondria and the previous results allow the assumption that *Creld* mutants suffer from mitochondrial dysfunction and reduced ß-

oxidation because the amount of acety-CoA is reduced as a feed forward loop. As the main fuel (acetyl CoA) is missing, it can be suspected that the TCA cycle is not sufficiently producing citrate (White *et al*., 2015).

Taken together, decreased amounts of TAG and NEFA, together with reduced amounts of mitochondrial ROS production as indicator for low β-oxidation activity, suggest that *de novo* lipogenesis in decreased, not increased in *Creld* mutants. The induction of ACC and FAS could be interpreted as a countermeasure as a result of decreased amounts of lipids, which is not successful in the absence of substrates from the TCA cycle (cataplerosis defect).

The results presented so far demonstrate a lipid storage imbalance. Since changes in lipid composition are prone to influence weight changes, the total body weight of adult *Creld* mutants was measured. Surprisingly no difference to wild type animals could be observed. Also the protein to bodyweight ratio is not significantly altered, though there is a trend towards reduction (Figure 3-25 A and B).

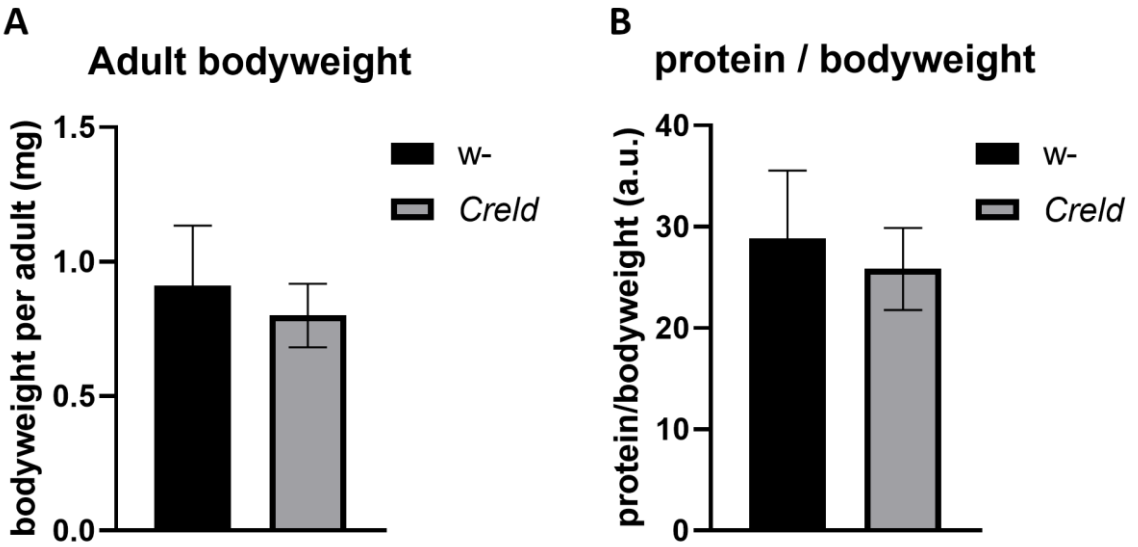

Figure 3-25: *Creld*-deficient flies do not exhibit bodyweight or protein level changes. Bodyweight was measured in whole adult females (A) but no significant change could be seen. Also protein to body weight (B) seems to be unaltered. Data are shown as mean±SD, n=5 (in groups of three), n.s. (t-test).

3.6.3 Creld-deficient flies are dependent on sugar

Creld mutant flies seem to suffer from mitochondrial dysfunction and reduced ATP levels, which is consistent with a lack in mitochondrial ATP production.

ATP cannot only be produced by the mitochondria but also through glycolysis (see section 1.3.2). As the name implies, the starting material for glycolysis is glucose. In order to see whether glucose variation in food is changing the observed phenotypes, *Creld* mutant and

control larvae were grown on a medium containing different sugar concentrations. Larval crawling ability was chosen as a read out (Figure 3-26). When grown on food lacking a sugar source (PBS agar plus yeast, depicted as low sugar), the crawling phenotype of *Creld* larvae is enhanced. Although the difference to *Creld* larvae fed with control food is not significant, the overall trend of crawling distance is reduced on low sugar diet. The crawling distance of w- larvae is not changed under sugar withdrawal.

On the other hand, under conditions of increased sugar concentration, wild type crawling performance drops to a level that is no longer significantly different to the mutant larvae, whereas the *Creld* mutants even increase the crawling distance compared to control flies.

In summary, *Creld* mutants are sensitive to sugar withdrawal, while they are resistant to the negative effects of a high sugar diet. These observations are consistent with increased glycolytic activity in *Creld* mutants as a countermeasure to reduced mitochondrial activity.

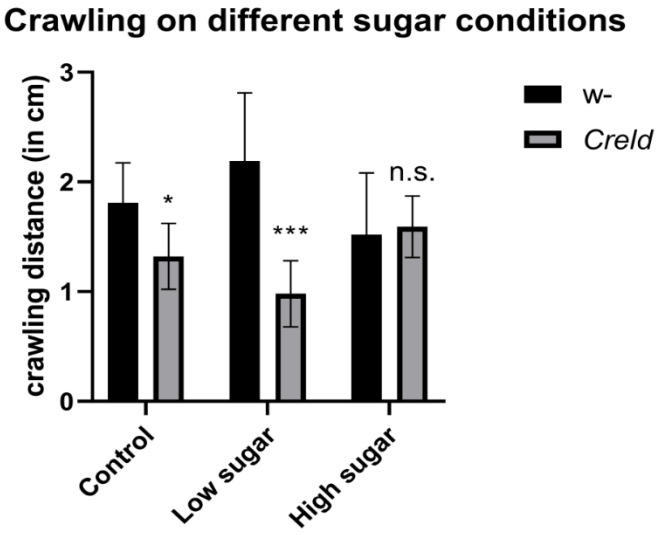

Figure 3-26: *Creld* mutants are dependent on sugar. Crawling assay of L3 larvae raised on food with different sugar concentrations reveals that *Creld* mutant larvae show decreased crawling ability when fed with low sugar food (PBS agar plus yeast) compared to control (fed with JM), whereas their crawling speed increases when raised on high sugar (10% sugar in JM). All results shown as mean±SD, n=10, *p<0.05, ***p<0.001 (t-test).

4. Discussion

In the past different investigations were done to unravel the function of proteins belonging to the Creld protein family. The studies concentrated on the mammalian Creld proteins, namely Creld1 and Creld2. Especially the role of Creld2 could not be fully understood so far.
Drosophila's genome contains only one *Creld* gene, which reduces the potential genetic redundancy between different members of the Creld family, allowing the characterization of the key functions. *Creld* mutation gives rise to a complex phenotype, whose main hallmarks are locomotor deficiencies and susceptibility to nutrient limitations.
This study was able to present a functional role of the *Drosophila* Creld orthologue in controlling mitochondrial metabolism and helped to further understand the importance of Creld proteins.

4.1 Creld proteins are not involved in ER stress response

Mammalian Creld2 has been shown to be upregulated upon ER stress (Oh-hashi *et al*., 2009). Although on a basal level, Creld2 is not ubiquitously expressed, the expression can be induced upon ER stress via the activating transcription factor 6 (ATF6). ATF6 is released from the ER as a response to ER stress and travels to the Golgi where it is activated via cleavage. The activated form is able to travel to the nucleus and start expression of genes involved in unfolded protein response (UPR), including *Creld2*. The *Drosophila* genome contains an ATF6 homolog, however, the functional resembling is still not validated yet (Ryoo, 2015).
In this thesis no involvement of *Drosophila* Creld in ER stress combat could be observed. Neither the transcription of the *Creld* gene was increased upon ER stress, nor any of the ER stress markers were expressed differentially in *Creld*-lacking flies. The fact that *Creld* mutants are able to survive upon induction of ER stress over a defined period of time leads to the suggestion that Creld is not needed for the combat of ER stress in *Drosophila*.

4.2 Creld is not needed for embryonic heart development or metamorphosis

Various studies showed an involvement of mammalian Creld1 in embryonic development. It is commonly accepted that Creld1 is needed for normal heart valve formation: *Creld1* mutant mice embryos die in embryonic stage E11.5, as they fail to develop endocardial cushions, which are the precursors of heart valves (Mass *et al.*, 2014). Endocardial cushion

defects and atrioventricular septal defects (ASVD) occur in over 40% of Down Syndrome patients (Asim *et al.*, 2017), and Creld1 was found to be mutated in most of these cases, emphasizing its role in proper heart formation.

Drosophila is widely used to study heart function, even though the morphology is different to the mammalian heart. The linear fly heart is divided into four compartments by the so called ostial cells, which have valve-like function to allow a one-directional fluid flow. The separation of the oxygen transporting tracheal system from the heart itself, allows manipulation of the heart function without directly affecting the viability of the animal (Na *et al.*, 2013).

The advantage *Drosophila* has as a model organism to study heart formation is obvious: despite the handling difficulties caused by the small heart size, dissection methods have been evolved that allow the analysis of heart function *in vivo,* preserving its myogenic function (Vogler and Occor, 2009). Several studies aided in overcoming the prejudice that due to its different structure it cannot be used to gain useful information that can be transferred to the mammalian system (Bier and Bodmer, 2004, Occor *et al.*, 2007, Wolf *et al.*, 2006).

In this project, the involvement of Creld in the development of the heart could not be observed. The heart valve formation seems to be undisturbed in *Creld* mutant flies. Additionally, no embryonic or larval lethality is provoked by the loss of Creld, indicating a normal heart development. However, further studies concerning heart morphogenesis should be undertaken in the future to rule out that other heart structures, apart from the ostial cells (functioning as heart valves), are influenced by *Creld* dysfunction.

4.3 Cardiac activity is impaired in *Creld* mutants

Despite lacking morphological differences, a clear functional discrepancy could be demonstrated in *Creld* mutant flies. Contractile dynamics can efficiently be studied in semi-dissected hearts of *Drosophila*. In this study, a clear increase in heart period could be observed, which was caused predominantly by a prolonged diastolic interval, whereas the systolic interval did not change. A prolonged diastole, which determines the resting phase between two contractive intervals, is termed as bradycardia and belongs to one of many myopathies, which also exist in humans. With aging, fly hearts exhibit longer diastolic intervals due to muscle stiffness (Cannon *et al.*, 2017). *Creld*-deficient flies show no

abnormalities in the contractile power and volume of hemolymph pumped through the heart, as the fractional shortening showed no difference when compared to control flies. Taking this into account, it can be ruled out that the delayed diastolic interval is because of muscle stiffness. Cardiac arrhythmias also increase with age (Cannon *et al.*, 2017). Flies lacking *Creld* are not susceptible to arrhythmic pattern, seen by the small standard error of the measurements of heart parameters. Taken together, it can be said that *Creld* dysfunction increases heart rate due to dilated diastolic intervals, without showing other age-related cardiac dysfunctions.

The impairment of diastolic function with simultaneous non-changed systolic interval and fractional shortening is known as the disease called heart failure with preserved ejection fraction (HFpEF) and is found in around 40% of patients suffering from heart failure. The molecular mechanisms of pathophysiology of HFpEF are currently unknown (Pouleur, 1990). However, one of the suggested potential mechanisms is the abnormality in energy metabolism in cardiomyocytes, including limited ATP production (Klassen *et al.*, 2017).

The heart is one of the major ATP consuming organs in the body. Therefore, the supply of ATP is obviously a prerequisite for proper heart functioning. The increased sensitivity of aging hearts towards stress is believed to be caused by mitochondrial dysfunction (Lesnefsky *et al.*, 2016).

Since the results in this thesis could show a general decrease in energy supply, probably caused by mitochondrial dysfunction, this is most likely also the reason for the myopathic phentopye observed in *Creld* mutant flies.

4.4 Creld regulates mitochondrial dynamics via Drp1 activity

Mitochondrial function goes far beyond ATP production, as it is involved in regulating apoptosis, immune responses and calcium signaling (Tilokani *et al.*, 2018). Mitochondrial dysfunction is the cause of a variety of diseases and occurs in 1 of 5000 people (Suárez-Rivero *et al.*, 2017). It is therefore important for cellular survival to preserve normal mitochondrial function. Mitochondrial quality control is mainly mediated by the so-called mitochondrial dynamics, involving fusion and fission events and degradation of mitochondria. Under physiological condition mitochondrial dynamics are in a tightly controlled balance. Mitochondrial fusion, mainly orchestrated by GTPase proteins Opa1 and Marf, ensures mtDNA quality control, by content mixing and distribution of mitochondrial

material over a wider department (Pernas *et al.*, 2017). It also allows the protection of mitochondria to prevent degradation via the autophagic machinery, as fused and elongated mitochondria cannot get engulfed efficiently by the phagophore membrane (Suárez-Rivero *et al.*, 2017).

On the other hand, mitochondrial fission is required to create new mitochondria by the segregation of damaged ones, and helps furthermore to induce mitophagic removal of dysfunctional mitochondria. Fission is orchestrated by Drp1, which is activated via different posttranslational modifications.

If the balance of mitochondrial dynamics is disturbed, different kinds of diseases which potentially affect almost all tissues of the body can be the consequence (Suárez-Rivero *et al.*, 2017). The main diseases characterized by mitochondrial dysfunction are neurodegenerative disorders. For example, the pathology of the second most common neurodegenerative disease, Parkinson's disease (PD), involves the impaired function of Pink and Parkin (Wang *et al.*, 2011). Pink and Parkin are regulators of mitochondrial quality control by mediating the turnover of damaged mitochondria via induction of mitophagy. PD is characterized by the progressive loss of dopaminergic neurons and concurrent motor impairment of the patients. Among others this can be caused by excessive accumulation of ROS producing defective mitochondria which are not efficiently cleared by the mitophagic pathway (Chen and Chang, 2009).

The alteration in fusion and fission events are generally linked to a decline in ATP levels due to the accumulation of dysfunctional mitochondria, which are no longer able to fulfill cellular energy demands (Suárez-Rivero *et al.*, 2017). Mitochondrial fusion however, is believed to be beneficial to the cellular physiology (Westermann 2016). The advantage lies in the spreading of metabolites, counteracting against the accumulation of mtDNA defects and general content mixing to avoid mitochondrial dysfunction.

Creld mutant flies show a clear shift towards mitochondrial fusion, most likely provoked by reduced activity of the pro-fusion gene *Drp1*. Mitochondrial fission is regulated by the GTPase Drp1, and its activity is mainly regulated post-transcriptionally. Under normal conditions, Drp1 is localized in the cytoplasm and its activity is inhibited by the phosphorylation at Ser 637 through PKA. Mitochondrial fission is induced when Drp1 proteins accumulate on the mitochondrium, thereby forming a ring like structure and constricting the mitochondrial membranes (Santel and Frank, 2008). It could be shown that

Drp1 localization to mitochondria is hampered in *Creld* mutants and therefore indeed have reduced mitochondrial fission. Nevertheless, the total amount of Drp1 protein is increased in *Creld* mutants (as the cytoplasmic Drp1 pool is higher compared to control flies). This might be explained by the fact that Drp1 degradation is somehow impaired in the mutants. Normally, Drp1 proteins get degraded via the proteosome-dependent degradation system: The E3 ligase Parkin, which ubiquinates Drp1 proteins and marks it for degradation (Wang *et al*., 2011). If the proteosomal-dependent degradation pathway is indeed insufficiently working in *Creld* mutant flies would be of interest for future analysis.

Despite the benefits caused by mitochondrial fusion described in the literature (Westermann 2016), the *Creld* mutant flies suffer from energy deprivation and show symptoms of neurodegeneration, which is seen in increased cells tested positive for Propodium iodide (PI) staining. The amount of dead cells was even higher in red eyed Oregon R control and mutant flies. The increased level of dead cells might be explained by the fact that the white eyed w- flies are blind (Krstic *et al.*, 2013) and therefore the cells in the optic lobe are not as active as the ones in red eyed Oregon R. The red eyed flies might have a higher activity of neurons in the optic lobe which renders them more dependent on mitochondrial function.

Additionally, mitochondrial fusion is thought to diminish aging-phenotypes, like reduced life span and cardiac performance. The opposite is observable in *Creld*-deficient flies: they exhibit significantly reduced longevity and show clearly disturbed heart rates. This result, which is rather contradictory, may be the case because of the reduced amount of mtDNA measured in the mutants. It has been shown that *Drp1* lacking mice possess a decreased amount of mtDNA and the fact that *Creld* mutants show increased mitochondrial mass as well as a reduction of mtDNA at the same time points towards discrepancies in mitochondrial dynamics.

Mitochondria contain their own genome and many proteins belonging to subunits of the electron transport chain are encoded over mitochondrial DNA (mtDNA) instead of nuclear DNA (Westermann 2016). Drp1 might directly or indirectly affect mtDNA replication and segregation via mitochondrial fission and that is considered to interfere with proteins involved in mtDNA maintenance (Parone *et al.*, 2008). *Creld* mutant flies not only show a decrease in mtDNA compared to control flies, they also upregulate gene expression of proteins involved in mtDNA quality control, like the mitochondrial transcription factor TFAM.

Increasing the factors involved in mtDNA replication might be a compensatory measure to rescue the reduced amount of mtDNA.

Further supporting the hypothesis that *Creld*-deficient flies suffer from decreased mitochondrial fission is the fact that they produce a reduced amount of reactive oxygen species in the form of H_2O_2 and are able to survive under oxidative stress situations, just as wild type flies. Links between ROS/RNS production and mitochondrial morphology has been described before: fragmented mitochondria produce a higher amount of ROS than fused ones (Szabo *et al.*, 2018). Also, it has been shown that *Opa1*-deficient mice are susceptible to oxidative stress, which indicates that tubular mitochondrial networks might be protective against oxidative stress. Higher ROS levels induce Drp1 mediated mitochondrial fission, resulting in fragmented, nonfunctional mitochondria (Willems *et al.*, 2015). A likely explanation is that *Creld*-deficient flies are protected against oxidative stress because they are not able to mediate Drp1-induced fission events.

Damaged and dysfunctional mitochondria are degraded via selective autophagy, known as mitophagy. As explained above, mitophagy is regulated by Pink and Parkin and is disturbed in diseases like Parkinsons's Disease. Mitophagy removes damaged mitochondria via the lysosomal degradation route. The process depends on the depolarization of the mitochondrial membrane. Depolarization occurs as a direct result of mitochondrial fission (Twig and Shirihai, 2011).

Furthermore, it is thought that fused mitochondria are simply too big to be engulfed by the autophagic membrane and that the size reduction by mitochondrial fission is another prerequisite for mitophagy (Twig *et al.*, 2008). These findings are in agreement with this study, where it could be outlined that *Creld*-deficient flies show reduced mitophagy induction (shown by the reduced expression of autophagy regulating gene ATG1), although they are suffering from energy deficits which usually would upregulate the autophagic machinery. However, an induction of gene expression of Parkin could be observed in *Creld* mutants. Studies in *Drosophila* showed a direct physical interaction of Drp1 and Parkin (Buhlman *et al.*, 2014). It is further hypothesized that Parkin is able to ubiquinate Drp1 with a non-degradable ubiquitin form to promote Drp1 localization to mitochondria. Another assumption is that Parkin is able to inhibit proteins involved in the fusion machinery via the proteosomal degradation pathway (Poole *et al.*, 2008). This might explain why Parkin is

increasingly expressed in *Creld* mutant flies, to promote fission via Drp1 or decrease fusion events.

The main question that needs to be answered is why Drp1 activity is reduced in *Creld* mutant flies. *Drp1* is not differentially expressed in *Creld* mutants. Its expression is, whilst not significant when compared to wild type, actually increased. However, as stated above, the activity is regulated via posttranslational modifications. Drp1 gets inhibited by PKA, preventing its accumulation on mitochondrial membranes. One of the activating modifications is mediated via Calcineurin, a Ca^{2+} activated serine/threonine phosphatase (Santel and Frank, 2008). It is suggested that increasing cellular calcium concentrations activate Calcineurin which in turn dephosphorylates Drp1, promoting the recruitment of the cytosolic pool onto mitochondria (Cereghetti *et al.*, 2008). In *Creld* mutant flies only around one third of the Drp1 protein population is located at mitochondria, whereas in control flies half of the Drp1 proteins localize to mitochondria. This result demonstrates that the recruitment of Drp1 to mitochondria (and thereby initiate mitochondrial fission) is hampered in *Creld* mutant flies.

Previous studies have proven a direct interaction of Creld1 with Calcineurin in mammals (Mass *et al.*, 2014). Creld1 thereby activates the regulatory subunit of Calcineurin CnB. Therefore, the current working model for the present study is that due to lack of Creld, the activation of Calcineurin is reduced. As a result, Calcineurin mediated dephosphorylation of Drp1 is not sufficiently achieved and by this the inhibitory action of PKA on Drp1 might be augmented. One hint in this direction could be achieved by the phenocopy of the main phenotype of *Creld* mutants by inhibiting Calcineurin chemically in wild type flies. However, further studies are necessary to see if the phenotypes observed in *Creld*-deficient flies are actually caused by decreased Calcineurin activation. One possible experiment might be the inhibition of PKA to see if this could partly rescue *Creld* mutant flies. Another experiment could include the rescue of *Creld* mutants by the overexpression of *Creld* via the UAS/Gal4 system in the mutant background. By simultaneous RNAi mediated knockdown of Calcineurin the rescue of Drp1 activation should not be successful. This experiment would prove that the Creld protein is regulating Drp1 activity through Calcineurin.

The findings from this study presented here suggest that Creld can have an effect on mitochondrial fission control, by regulating Calcineurin. The mitochondrial phenotypes, like the elongated morphology and decreased ROS production, give evidence that the

mitochondrial dynamic is imbalanced. Supporting this idea is that *Creld* mutants resemble the phenotypes reported for *Calcienurin*-deficient flies. Pfluger *et al*. outlined that flies lacking Calcineurin show mitochondrial elongation and reduced TAG levels, which is in line with the results reported here. They argued, that the reduced amount of fat levels is caused by an enhanced metabolic rate (Pfluger *et al*., 2015). The data presented here indicates, that the reduced TAG amounts are caused by defects in cataplerosis, which is caused by the dysfunction of mitochondria provoked by loss of Creld.

4.5 *Creld*-deficiency provokes a shift in energy metabolism

Starvation is one of the most prevalent environmental stressors animals have to encounter. To survive under limited time of nutrient deprivation they evolved different strategies by adapting to changes in nutrient availability. The key in overcoming times of scarce food availability is the mobilization of energy stores. These energy stores are built up during excessive nutrient availability: carbohydrates are stored as glycogen whereas fat is converted to triacylglycerol (TAG) and stored in lipid droplets in fat body (or adipose tissue in mammals).

TAG get hydrolyzed under acute energy demanding situation, such as starvation or increased muscle exercise, which means that it is broken down to yield free fatty acids which then can be converted and used as fuel substrate for TCA cycle and ATP production (Grönke *et al.*, 2005). The main route for TAG biosynthesis is the Kennedy pathway, which generates TAG in a four step enzymatic chain mainly catalyzed by the enzyme Acetyl transferase using acetyl-CoA as substrate. Metabolic accessibility is ensured in the process of lipolysis- the hydrolysis of TAG by lipases. The lipase Brummer (the homolog of mammalian Adipocyte-triglyceride lipase, ATGL) is the key enzyme in this process (Kühnlein, 2012).

Creld-deficient flies are sensitive to starvation, which is most obvious in male flies, as they hardly survive one day without nutrients. This led to the false assumption that they fail to mobilize their energy stores and cannot meet the energy demands to keep the organism alive. However, during the investigation of the metabolic state in *Creld*-deficient flies, a general change in lipid profile could be observed. *Creld* mutants show a dramatic decrease of fat storage in form of TAG while the expression of lipases is down regulated and the amount of non-esterified free fatty acids (NEFAs) is not increased. This implies that not a defect in lipid mobilization is the cause of starvation sensitivity but the general lack of storage fat in

these animals. This opens the question of why the fat storage is depleted even under non-starvation conditions.

What follows is a short description of how fats are build up in general. Apart from taken up by the diet and being hydrolyzed from TAG, fatty acids can also be synthesized from non-lipid precursors in the process of *de novo* lipogenesis. In this process, which is summarized in Figure 4-1, pyruvate (which is produced from glucose in glycolysis) is entering the TCA cycle. Here, citrate is formed and transported out of the mitochondria into the cytosol. The enzyme ATP citrate lyase converts the citrate to acetyl-CoA, which is converted via different enzymatic steps to fatty acids. These fatty acids are subsequently stored as TAGs.

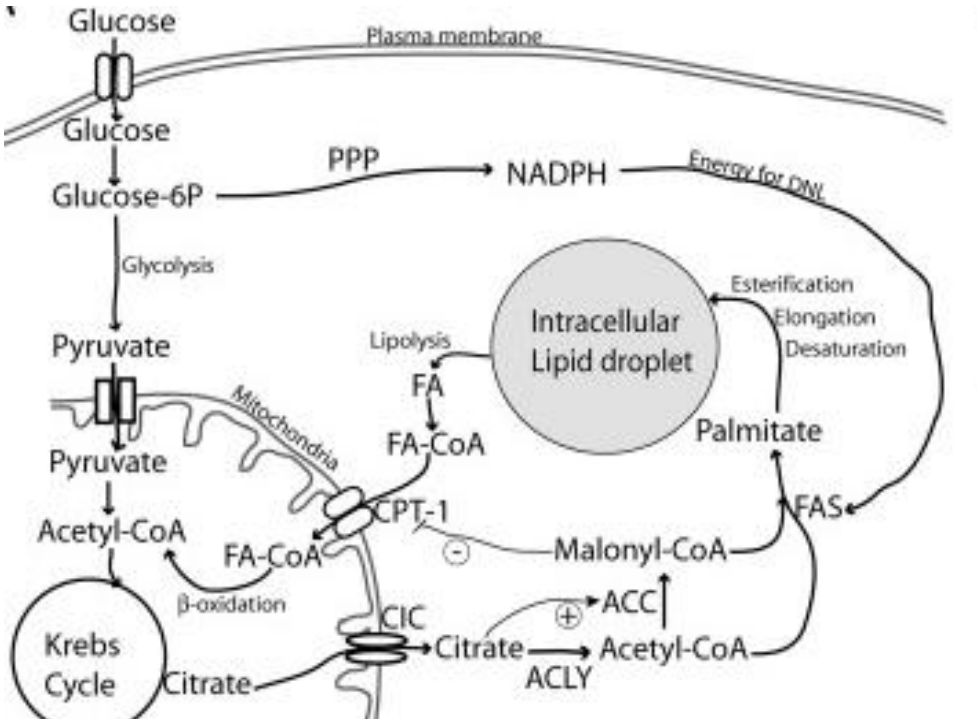

Figure 4-1: Scematic view of Lipid metabolsim. Glucose enters the cytoplasm via specific glucose transporters and gets converted to pyruvate in Glycolysis. Pyruvate is shuttled into the mitochondrium where it is converted to acetyl-CoA which is fed into the TCA (or Krebs) cycle. Citrate is shuttled out of the TCA cycle and transportet into the cytoplasm where it serves as a substrate for de novo lipogenesis. Acetyl-CoA carboxylase (ACC) converts citrate to malonyl-CoA which is converted by Faty acid synthase (FAS) to the fatty acid palmitate. Produced palmitate can be esterified and stored in lipid droplets as triacylglyerol (TAG). Breakdown of TAG occurs by the action of lipases to produce free fatty acids that can be oxidized inside mitochondria to produce acetyl-CoA which serves as material for the TCA cycle and subsequent oxidative phosphorylation to produce ATP. Image modified from Solinas *et al.*, 2015.

Acetyl-CoA carboxylase (ACC) and Fatty acid synthase (FAS) are the key enzymes in lipogenesis and were highly upregulated in *Creld* mutant flies, nevertheless no increase in the amount of fat could be observed. It is important to highlight that citrate, which comes from the TCA cycle and is needed as starting material for lipogenesis, is produced in mitochondria. Cataplerosis describes the removal of TCA cycle intermediates (Owen *et al.*,

2012). Obviously, *Creld* mutant flies suffer from defects in cataplerotic pathways, as they are not able to produce a sufficient amount of substrates for the lipogenesis pathway. As mitochondrial dysfunction could be observed in *Creld*-deficient flies, it might be possible that the TCA cycle is working insufficiently and therefore the substrate for lipogenesis is limited. It was previously shown, that cancer cells with defective mitochondrial metabolism use an alternative route to partially compensate these effects in such a way that they use glutamine-dependent carboxylation for citrate formation (Mullen *et al.*, 2012). In the process of glutaminolysis the TCA cycle can produce citrate without the need of acetyl-CoA as "starting material" (Metallo *et al.*, 2012). It might be worth to find out if Creld mutant flies could be rescued by feeding them glutamine and thereby circumventing the need of acetyl-Co.

Cataplerosis is only working properly when the opposing reaction, namely anaplerosis, is normal (White 2012). This means that when the pool of TCA cycle intermediates, most important acetyl-CoAs as carbon donors, are reduced, the TCA is not able to work efficiently and produces less intermediate substrates.

Acetyl-CoAs are produced inside the mitochondria, either through conversion of pyruvate, produced via glycolysis, or by fatty acid ß-oxidation (detailed description can be found in the introduction of this thesis). *Creld* deficiency has been associated with mitochondrial dysfunction. This suggests that the acetyl-CoA pool is most likely built from pyruvate instead of ß-oxidation. Therefore, there seems to be a definite need for sugar in mutants to generate acetyl-CoA but also to produce ATP via the glycolytic pathway. Indeed, compared to wild types the crawling defect of *Creld* mutant larvae was enhanced by sugar withdrawal. On the other hand, complementing the diet with high amounts of sugar eliminated the crawling differences between wild type and *Creld* mutant flies. The main mediator of intracellular glucose sensing is the complex built of the transcription facor ChREBP, MondoA and their bining partner Mlx (Havula *et al.*, 2013). The complex mediates transcription responses to intracellular glucose by regulating genes involved in glycolysis and lipogenesis. Mutants for MondoA/Mlx are not able to utilize circulating sugar, which is shown by their incapacity to survive on elevated dietary sugar levels (Havula *et al.*, 2013). This is not true for *Creld* mutants. As they are able to survive well when fed with high sugar concentrations, this points out that they are still able to adapt to elevated sugar levels.

The data reported here appears to support the assumption that *Creld* mutant flies shift their metabolism from mitochondrial oxidative phosphorylation to glycolysis to gain ATP. Defined as the Warburg effect, the shift to glycolysis as the main route to produce ATP is often seen in cancer cells. They switch from mitochondrial respiration to cytoplasmic glycolysis because they often contain defective mitochondria. Moreover, by reducing mitochondrial respiration cancer cells also reduce the amount of ROS production which could possibly harm their proliferation (Liberti and Locosale, 2016). Reduced ROS production is also observed in *Creld* mutant flies, further supporting the hypothesis that these flies have a reduction in mitochondrial respiration, which renders them more dependent on glycolysis. In this context, it would be worth to study if the glycolytic pathway is disturbed in the mutants. For example, this could be achieved by measuring the expression of genes involved in glycolysis. Hexokinase C is the initiating enzyme in glycolysis and its activity can be measured via qRT PCR experiments. Additionally, it is possible to manipulate the glycolytic pathway by feeding the flies the glucose analog 2-Deoxyglucose, which cannot be metabolized and thereby blocks glycolysis (Zhang *et al.*, 2014). It would be interesting to see how *Creld* mutant flies are able to cope with inhibited glycolysis.

So far, this thesis has argued that Creld is involved in mitochondrial fission regulation. However, the function of Creld has to include more than just regulating Drp1 activity through Calcineurin and thereby altering the mitochondrial respiration capacity. The phenotypes observed here go beyond the ones described for mutants with oxidative phosphorylation defects. For example, the fly mutant technical knockout (tko, mutant for mitochondrial protein S 12), shares the main phenotypes but they upregulate enzymes involved in lipid mobilization (Fernández-Ayala *et al.*, 2010). *Creld* mutants show a decrease in TAG stores just as *tko* deficient flies, however they show a dramatic down regulation of lipase expression, implying that the limitation of TAG or lipids in general is not caused by increased lipid mobilization seen in *tko* mutants. Moreover, these mutants reduce their muscle biosynthesis for energy-sparing reasons (Kemppainen *et al.*, 2015). Muscle is one of the main energy-consuming tissues. In situations where it is necessary to save energy because of ATP depletion, organisms reduce their muscle mass and store fat as energy reserves instead. In contrast to the theory, *Creld* mutants have increased muscle mass. This rather contradictory result may be because of the defects in cataplerosis seen in *Creld* mutants. It is possible that they cannot store energy in the form of fat for later catabolism

due to limited substrates for lipogenesis. Because of that, they store biomass in form of muscle tissue. This would also explain why *Creld* mutants have no reduced weight compared to wild type, even though their lipid stores are empty.

4.6 Future prospective and conclusion

Although this study gives insights into a novel function of the Creld protein, further investigations concerning the role of Creld in metabolism should be made. One of the most important experiments missing is the rescue of *Creld* mutants by bringing back a functional Creld protein. This can be done by using the UAS/Gal4 system. Additionally, the connection between Creld and Calcineurin is poorly understood in *Drosophila*.

Concerning the metabolic situation of *Creld* mutants, it might be useful to try to analyze if other pathways are involved in the occurring phenotypes.

Despite the still lacking experiments, a further step in understanding the biological function of the Creld protein in *Drosophila melanogaster* could be made here. The new insights that Creld might control mitochondrial dynamics via regulating Calcineurin-mediated Drp1 activity, is new and this link was not done before. The arising metabolic changes, caused by mitochondrial dysfunction, help to establish a novel link between Creld and the regulation of metabolism.

5. Summary

Cystein-rich with EGF-like domains (Creld) proteins belong to a protein family conserved between species. The mammalian genome contains two *Creld* genes. Unraveling the function of mammalian Creld proteins has been tried before. It is thought that Creld1 is involved in heart development during embryogenesis and Creld2 is believed to be involved in ER stress response. The genome of *Drosophila* contains only one *Creld* gene, which simplifies the analysis of Creld function. Therefore, a *Creld* mutant was generated and in this project the phenotypes were analyzed in order to unravel the main function of the Creld protein in *Drosophila*.

Creld mutant flies suffer from an overall energy deficit, which could be proven by determining the phosphorylation status of the overall energy sensor AMPK. The energy deficits are accompanied with an increased number of apoptotic cells in the brain and severe locomotor deficiencies. Additionally, the heart function of *Creld* mutant flies is disturbed.

Mitochondria are known as the power house of the cell, as they account for the major cellular ATP production via oxidative phosphorylation. By analyzing mitochondrial abundance, morphology and function in *Creld* mutants, it could be shown that the overall function of mitochondria is reduced whereas mitochondrial mass and abundance is increased. The morphology of mitochondria was assessed and revealed an elongated form compared to wild type tissue. Together, these results could demonstrate a shift of mitochondrial dynamics towards increased fusion and decreased fission.

The resulting dysfunction of mitochondria caused a disturbance in the overall cellular metabolism including altered lipid homeostasis.

Taken together, this thesis could identify a novel role for the Creld protein in *Drosophila* by regulating mitochondrial maintenance and thereby controlling cellular energy and lipid metabolism.

Zusammenfassung

Cystein-rich with EGF-like Domains (Creld) Proteine gehören zu einer zwischen Spezies konservierten Proteinfamilie. Das Säugergenom enthält zwei *Creld* Gene. Die Funktion der Creld Proteine im Säuger wurde zuvor in verschiedenen Studien untersucht. Es wird angenommen, dass Creld1 während der Embryogenese an der Herzentwicklung beteiligt ist und Creld2 vermutlich an der ER-Stressreaktion eine Rolle spielt. Das Genom der Taufliege *Drosophila melanogaster* enthält nur ein *Creld* Gen, welches die Analyse der Creld-Funktion vereinfacht. Daher wurde eine *Creld* Mutante generiert und die Phänotypen in diesem Projekt analysiert, um die Hauptfunktion des Creld Proteins in Drosophila zu entschlüsseln.

Creld mutierte Fliegen leiden unter einem Energiedefizit, welches durch die Bestimmung des Phosphorylierungsstatus des Energiesensors AMPK nachgewiesen werden konnte. Die Energiedefizite gehen einher mit der erhöhten Anzahl apoptotischer Zellen im Gehirn und daraus resultierenden Lokomotordefiziten. Zusätzlich ist die Herzfunktion von *Creld*-mutierten Fliegen gestört.

Mitochondrien sind als das Kraftwerk der Zelle bekannt, da sie für die Hauptproduktion von ATP durch oxidative Phosphorylierung verantwortlich sind. Es konnte gezeigt werden, dass die Funktion der Mitochondrien reduziert ist, während die mitochondriale Masse erhöht ist. Außerdem wurde eine veränderte Morphologie der Mitochondrien in verschiedenen Geweben der *Creld* Mutante gezeigt. Die elongierten Mitochondrien deuten auf eine Verschiebung der mitochondrialen Dynamik hin. Eingehende Untersuchungen konnten zeigen, dass die Spaltung der Mitochondrien in *Creld* Mutanten vermindert abläuft.

Die daraus resultierende Dysfunktion der Mitochondrien verursachte eine Störung des gesamten Zellstoffwechsels. Es wird vermutet, dass *Creld* Mutanten aufgrund von mitochondrialer Dysfunktion für die ATP-Produktion auf Glykolyse angewiesen sind und eine Fehlregulation des Lipidstoffwechsels stattfindet.

Zusammengefasst konnte diese Arbeit eine neue Rolle des Creld-Protein in *Drosophila* zeigen, und nachweisen, dass es den mitochondrialen Stoffwechsel reguliert und dadurch den zellulären Energie- und Fettstoffwechsel steuert.

6. Appendix

6.1 List of abbreviations

°C	degrees Celsius
µ	micro
ACC	Acetylcarbocylase
ADP	Adenosine diphosphate
ATF6	activating transcription factor 6
ATG	autophagy related gene
ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
AMPK	5' AMP-activated protein kinase
Bmm	Brummer
Ca2+	Calcium
Cdk1	Cyclin-dependent kinase 1
cDNA	complementary DN
CO2	Carbon Dioxide
CoA	Coenzyme A
CnA and CnB	Calcineurin subunit A and B
CreId	Cystein-rich with EGF-like domains
CsA	Cyclosporin A
CS	Citrate synthase
DAG	Diacylglycerol
ddH2O	double distilled water
DI	Diastolic interval
DMSO	Dimethyl sulfoxid

DNA	Deoxyribonucleic acid
Drp1	Dynamin-related-protein 1
ER	endoplasmic reticulum
ETC	Electron transport chain
FAS	Fatty acid synthase
g	gram
Gal4	Yeast transcription factor
GTP	Guanosine-5'-triphosphate
h	hours
H+	Hydrogen peroxide
H202	Hydrogen peroxide
HP	Heart period
Hsc 70-3	Heat shock chaperone 70-3
IMM	Inner mitochondrial membrane
Ire1	Inositol-requiring enzyme 1
IFM	Indirect flight muscle
L	liter
L (1, 2,3)	Larval stage (1,2,3)
Lip3	Lipase 3
LSM	Laser scanning microscope
M	Molar
m	milli
MAPK	Mitogen-activated protein kinase
MAG	Monoacylglycerol
MDIVI-1	Mitochondrial Division Inhibitor 1
min	minutes
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA

n	nano
NADH	Nicotinamide adenine dinucleotide
NFATc1	Nuclear Factor Of Activated T Cells 1
nm	nanometers
OMM	Outer mitochondrial membrane
Opa1	Optic atrophy 1
p	pico
P	p-value (statistics, probability value)
PCR	Polymerase chain reaction
PKA	Protein kinase A
PI	Propodium iodide
Pink1	PTEN-induced kinase 1
PEK	Pancreatic eIF-2α kinase
qRT PCR	Quantitative reverse transcriptase polymerase chain reaction
RNAi	ribonucleic acid interference
ROS	Reactive oxygen species
rpm	Rounds per minute
rpl32	Ribosomal Protein L32
sec	seconds
sd	Standard derivation
SI	Systolic interval
SOD	Superoxide dismutase
TAG	Triacylglycerols
TCA	Tricarboxyclic acid cycle
TEM	Transmission electron microscopy
TFAM	Mitochondrial transcription factor
TFB1/2	Mitochondrial transcription factor B1 /B2

TORC1	target of rapamycin complex 1
w-	White minus, white1118
WT	Wortmannin
Xbp1	X-Box Binding Protein 1
YM	Yazz mix

6.2 List of figures

6. Appendix

6.2 References

Alonso-Montes, Cristina; Castro, Monica G.; Reguero, Julian R.; Perrot, Andreas; Ozcelik, Cemil; Geier, Christian et al. (2008): Mitochondrial transcription factors TFA, TFB1 and TFB2: a search for DNA variants/haplotypes and the risk of cardiac hypertrophy. In: *Disease markers* 25 (3), S. 131–139.

Aronoff, S. L.; Berkowitz, K.; Shreiner, B.; Want, L. (2004): Glucose Metabolism and Regulation: Beyond Insulin and Glucagon. In: *Diabetes Spectrum* 17 (3), S. 183–190. DOI: 10.2337/diaspect.17.3.183.

Asim, Ambreen; Agarwal, Sarita; Panigrahi, Inusha; Sarangi, Aditya Narayan; Muthuswamy, Srinivasan; Kapoor, Aditya (2018): CRELD1 gene variants and atrioventricular septal defects in Down syndrome. In: *Gene* 641, S. 180–185. DOI: 10.1016/j.gene.2017.10.044.

Asim, Ambreen; Agarwal, Sarita; Panigrahi, Inusha; Sarangi, Aditya Narayan; Muthuswamy, Srinivasan; Kapoor, Aditya (2018): CRELD1 gene variants and atrioventricular septal defects in Down syndrome. In: *Gene* 641, S. 180–185. DOI: 10.1016/j.gene.2017.10.044.

Bélanger, Mireille; Allaman, Igor; Magistretti, Pierre J. (2011): Brain Energy Metabolism: Focus on Astrocyte-Neuron Metabolic Cooperation. In: *Cell metabolism* 14 (6), S. 724–738. DOI: 10.1016/j.cmet.2011.08.016.

Bier, Ethan; Bodmer, Rolf (2004): Drosophila, an emerging model for cardiac disease. In: *Gene* 342 (1), S. 1–11. DOI: 10.1016/j.gene.2004.07.018.

Bonini, Nancy M.; Fortini, Mark E. (2003): Human neurodegenerative disease modeling using Drosophila. In: *Annual review of neuroscience* 26, S. 627–656. DOI: 10.1146/annurev.neuro.26.041002.131425.

Britton, Jessica S.; Lockwood, Wendy K.; Li, Ling; Cohen, Stephen M.; Edgar, Bruce A. (2002): Drosophila's insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. In: *Developmental cell* 2 (2), S. 239–249.

Buhlman, Lori; Damiano, Maria; Bertolin, Giulia; Ferrando-Miguel, Rosa; Lombès, Anne; Brice, Alexis; Corti, Olga (2014): Functional interplay between Parkin and Drp1 in mitochondrial fission and clearance. In: *Biochimica et biophysica acta* 1843 (9), S. 2012–2026. DOI: 10.1016/j.bbamcr.2014.05.012.

Cannon, Leah; Zambon, Alexander C.; Cammarato, Anthony; Zhang, Zhi; Vogler, Georg; Munoz, Matthew et al. (2017): Expression patterns of cardiac aging in Drosophila. In: *Aging Cell* 16 (1), S. 82–92. DOI: 10.1111/acel.12559.

Castello, Pablo R.; Drechsel, Derek A.; Patel, Manisha (2007): Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain. In: *The Journal of biological chemistry* 282 (19), S. 14186–14193. DOI: 10.1074/jbc.M700827200.

Cereghetti, G. M.; Stangherlin, A.; Brito, O. Martins de; Chang, C. R.; Blackstone, C.; Bernardi, P.; Scorrano, L. (2008): Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria. In: *Proceedings of the National Academy of Sciences of the United States of America* 105 (41), S. 15803–15808. DOI: 10.1073/pnas.0808249105.

Chang, Chuang-Rung; Blackstone, Craig (2010): Dynamic regulation of mitochondrial fission through modification of the dynamin-related protein Drp1. In: *Annals of the New York Academy of Sciences* 1201, S. 34–39. DOI: 10.1111/j.1749-6632.2010.05629.x.

Chen, Hsiuchen; Chan, David C. (2009): Mitochondrial dynamics–fusion, fission, movement, and mitophagy–in neurodegenerative diseases. In: *Human Molecular Genetics* 18 (R2), R169-76. DOI: 10.1093/hmg/ddp326.

Clark, Matthew Q.; Zarin, Aref Arzan; Carreira-Rosario, Arnaldo; Doe, Chris Q. (2018): Neural circuits driving larval locomotion in Drosophila. In: *Neural development* 13 (1), S. 6. DOI: 10.1186/s13064-018-0103-z.

Crowley, Lisa C.; Christensen, Melinda E.; Waterhouse, Nigel J. (2016): Measuring Mitochondrial Transmembrane Potential by TMRE Staining. In: *Cold Spring Harbor protocols* 2016 (12). DOI: 10.1101/pdb.prot087361.

Elliott, David A.; Brand, Andrea H. (2008): The GAL4 system : a versatile system for the expression of genes. In: *Methods in molecular biology (Clifton, N.J.)* 420, S. 79–95. DOI: 10.1007/978-1-59745-583-1_5.

Fendt, Sarah-Maria; Bell, Eric L.; Keibler, Mark A.; Olenchock, Benjamin A.; Mayers, Jared R.; Wasylenko, Thomas M. et al. (2013): Reductive glutamine metabolism is a function of the α-ketoglutarate to citrate ratio in cells. In: *Nature Communications* 4, 2236 EP -. DOI: 10.1038/ncomms3236.

Fernández-Ayala, Daniel J. M.; Chen, Shanjun; Kemppainen, Esko; O'Dell, Kevin M. C.; Jacobs, Howard T. (2010): Gene expression in a Drosophila model of mitochondrial disease. In: *PLoS ONE* 5 (1), e8549. DOI: 10.1371/journal.pone.0008549.

Fink, Martin; Callol-Massot, Carles; Chu, Angela; Ruiz-Lozano, Pilar; Izpisua Belmonte, Juan Carlos; Giles, Wayne et al. (2009): A new method for detection and quantification of heartbeat parameters in Drosophila, zebrafish, and embryonic mouse hearts. In: *BioTechniques* 46 (2), S. 101–113. DOI: 10.2144/000113078.

Galluzzi, Lorenzo; Bravo-San Pedro, José Manuel; Kroemer, Guido (Hg.) (2017): Methods in Enzymology : Molecular Characterization of Autophagic Responses, Part B: Academic Press.

Galluzzi, Lorenzo; Bravo-San Pedro, José Manuel; Kroemer, Guido (Hg.) (2017): Methods in Enzymology : Molecular Characterization of Autophagic Responses, Part B: Academic Press.

Gershman, Boris; Puig, Oscar; Hang, Lilian; Peitzsch, Robert M.; Tatar, Marc; Garofalo, Robert S. (2007): High-resolution dynamics of the transcriptional response to nutrition in Drosophila: a key role for dFOXO. In: *Physiological genomics* 29 (1), S. 24–34. DOI: 10.1152/physiolgenomics.00061.2006.

Ghouleh, Imad Al; Khoo, Nicholas K.H.; Knaus, Ulla G.; Griendling, Kathy K.; Touyz, Rhian M.; Thannickal, Victor J. et al. (2011): Oxidases and Peroxidases in Cardiovascular and Lung Disease: New Concepts in Reactive Oxygen Species Signaling. In: *Free radical biology & medicine* 51 (7), S. 1271–1288. DOI: 10.1016/j.freeradbiomed.2011.06.011.

Gowans, Graeme J.; Hardie, D. Grahame (2014): AMPK: a cellular energy sensor primarily regulated by AMP. In: *Biochemical Society transactions* 42 (1), S. 71–75. DOI: 10.1042/BST20130244.

Grewal, Savraj S. (2009): Insulin/TOR signaling in growth and homeostasis: a view from the fly world. In: *The international journal of biochemistry & cell biology* 41 (5), S. 1006–1010. DOI: 10.1016/j.biocel.2008.10.010.

Günther, Maximilian N.; Nettesheim, Guilherme; Shubeita, George T.: Quantifying and predicting *Drosophila* larvae crawling phenotypes. In: *Scientific Reports* 6, S. 27972. DOI: 10.1038/srep27972.

Hamacher-Brady, Anne; Brady, Nathan Ryan (2016): Mitophagy programs: mechanisms and physiological implications of mitochondrial targeting by autophagy. In: *Cellular and Molecular Life Sciences* 73 (4), S. 775–795. DOI: 10.1007/s00018-015-2087-8.

Hampton, R. Y. (2000): ER stress response: getting the UPR hand on misfolded proteins. In: *Current biology : CB* 10 (14), R518-21.

Hansen, Tom Egil; Johansen, Terje (2011): Following autophagy step by step. In: *BMC biology* 9, S. 39. DOI: 10.1186/1741-7007-9-39.

Havula, Essi; Teesalu, Mari; Hyötyläinen, Tuulia; Seppälä, Heini; Hasygar, Kiran; Auvinen, Petri et al. (2013): Mondo/ChREBP-Mlx-Regulated Transcriptional Network Is Essential for Dietary Sugar Tolerance in Drosophila. In: *PLoS Genetics* 9 (4). DOI: 10.1371/journal.pgen.1003438.

Ishihara, Naotada; Eura, Yuka; Mihara, Katsuyoshi (2004): Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity. In: *Journal of cell science* 117 (Pt 26), S. 6535–6546. DOI: 10.1242/jcs.01565.

Kageyama, Yusuke; Hoshijima, Masahiko; Seo, Kinya; Bedja, Djahida; Sysa-Shah, Polina; Andrabi, Shaida A. et al. (2014): Parkin-independent mitophagy requires Drp1 and maintains the integrity of mammalian heart and brain. In: *The EMBO journal* 33 (23), S. 2798–2813. DOI: 10.15252/embj.201488658.

Kass, David A.; Bronzwaer, Jean G. F.; Paulus, Walter J. (2004): What mechanisms underlie diastolic dysfunction in heart failure? In: *Circulation research* 94 (12), S. 1533–1542. DOI: 10.1161/01.RES.0000129254.25507.d6.

Kemppainen, Esko; George, Jack; Garipler, Görkem; Tuomela, Tea; Kiviranta, Essi; Soga, Tomoyoshi et al. (2016): Mitochondrial Dysfunction Plus High-Sugar Diet Provokes a Metabolic Crisis That Inhibits Growth. In: *PLoS ONE* 11 (1), e0145836. DOI: 10.1371/journal.pone.0145836.

Kim, Yeawon; Park, Sun-Ji; Manson, Scott R.; Molina, Carlos Af; Kidd, Kendrah; Thiessen-Philbrook, Heather et al. (2017): Elevated urinary CRELD2 is associated with endoplasmic reticulum stress-mediated kidney disease. In: *JCI insight* 2 (23). DOI: 10.1172/jci.insight.92896.

Knott, Andrew B.; Bossy-Wetzel, Ella (2008): Impairing the Mitochondrial Fission and Fusion Balance: A New Mechanism of Neurodegeneration. In: *Annals of the New York Academy of Sciences* 1147, S. 283–292. DOI: 10.1196/annals.1427.030.

Krstic, Dimitrije; Boll, Werner; Noll, Markus (2013): Influence of the White Locus on the Courtship Behavior of Drosophila Males. In: *PLoS ONE* 8 (10). DOI: 10.1371/journal.pone.0077904.

Kühnlein, Ronald P. (2012): Thematic review series: Lipid droplet synthesis and metabolism: from yeast to man. Lipid droplet-based storage fat metabolism in Drosophila. In: *Journal of lipid research* 53 (8), S. 1430–1436. DOI: 10.1194/jlr.R024299.

Kuma, Akiko; Mizushima, Noboru (2010): Physiological role of autophagy as an intracellular recycling system: with an emphasis on nutrient metabolism. In: *Seminars in cell & developmental biology* 21 (7), S. 683–690. DOI: 10.1016/j.semcdb.2010.03.002.

Lam, Yan Y.; Ravussin, Eric (2016): Analysis of energy metabolism in humans: A review of methodologies. In: *Molecular Metabolism* 5 (11), S. 1057–1071. DOI: 10.1016/j.molmet.2016.09.005.

Lee, Kwang Pum; Jang, Taehwan (2014): Exploring the nutritional basis of starvation resistance in Drosophila melanogaster. In: *Funct Ecol* 28 (5), S. 1144–1155. DOI: 10.1111/1365-2435.12247.

Lesnefsky, Edward J.; Chen, Qun; Hoppel, Charles L. (2016): Mitochondrial Metabolism in Aging Heart. In: *Circulation research* 118 (10), S. 1593–1611. DOI: 10.1161/CIRCRESAHA.116.307505.

Liberti, Maria V.; Locasale, Jason W. (2016): The Warburg Effect: How Does it Benefit Cancer Cells? In: *Trends in biochemical sciences* 41 (3), S. 211–218. DOI: 10.1016/j.tibs.2015.12.001.

Lodish H, Berk A, Zipursky SL, et al. (Hg.) (2000): Molecular Cell Biology. 4th edition. New York: W. H. Freeman; 2000. Section 16.2, Electron Transport and Oxidative Phosphorylation. Available from: https://www.ncbi.nlm.nih.gov/books/NBK21528/. New York.

Madabattula, Surya T.; Strautman, Joel C.; Bysice, Andrew M.; O'Sullivan, Julia A.; Androschuk, Alaura; Rosenfelt, Cory et al. (2015): Quantitative Analysis of Climbing Defects in a Drosophila Model of Neurodegenerative Disorders. In: *Journal of visualized experiments : JoVE* (100), e52741. DOI: 10.3791/52741.

Martin, William F.; Garg, Sriram; Zimorski, Verena (2015): Endosymbiotic theories for eukaryote origin. In: *Philosophical Transactions of the Royal Society B: Biological Sciences* 370 (1678). DOI: 10.1098/rstb.2014.0330.

Mass, Elvira; Wachten, Dagmar; Aschenbrenner, Anna C.; Voelzmann, Andre; Hoch, Michael (2014): Murine Creld1 controls cardiac development through activation of calcineurin/NFATc1 signaling. In: *Developmental cell* 28 (6), S. 711–726. DOI: 10.1016/j.devcel.2014.02.012.

Matsushima, Yuichi; Garesse, Rafael; Kaguni, Laurie S. (2004): Drosophila mitochondrial transcription factor B2 regulates mitochondrial DNA copy number and transcription in schneider cells. In: *The Journal of biological chemistry* 279 (26), S. 26900–26905. DOI: 10.1074/jbc.M401643200.

Metallo, Christian M.; Gameiro, Paulo A.; Bell, Eric L.; Mattaini, Katherine R.; Yang, Juanjuan; Hiller, Karsten et al. (2011): Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. In: *Nature* 481 (7381), S. 380–384. DOI: 10.1038/nature10602.

Mishra, Prashant; Carelli, Valerio; Manfredi, Giovanni; Chan, David C. (2014): Proteolytic cleavage of Opa1 stimulates mitochondrial inner membrane fusion and couples fusion to oxidative phosphorylation. In: *Cell metabolism* 19 (4), S. 630–641. DOI: 10.1016/j.cmet.2014.03.011.

Mullen, Andrew R.; Wheaton, William W.; Jin, Eunsook S.; Chen, Pei-Hsuan; Sullivan, Lucas B.; Cheng, Tzuling et al. (2011): Reductive carboxylation supports growth in tumour cells with defective mitochondria. In: *Nature* 481 (7381), S. 385–388. DOI: 10.1038/nature10642.

Na, Jianbo; Musselman, Laura Palanker; Pendse, Jay; Baranski, Thomas J.; Bodmer, Rolf; Ocorr, Karen; Cagan, Ross (2013): A Drosophila Model of High Sugar Diet-Induced Cardiomyopathy. In: *PLoS Genetics* 9 (1). DOI: 10.1371/journal.pgen.1003175.

Ocorr, Karen; Akasaka, Takeshi; Bodmer, Rolf (2007): Age-related cardiac disease model of Drosophila. In: *Mechanisms of ageing and development* 128 (1), S. 112–116. DOI: 10.1016/j.mad.2006.11.023.

Ocorr, Karen; Perrin, Laurent; Lim, Hui-Ying; Qian, Li; Wu, Xiushan; Bodmer, Rolf (2007): Genetic control of heart function and aging in Drosophila. In: *Trends in cardiovascular medicine* 17 (5), S. 177–182. DOI: 10.1016/j.tcm.2007.04.001.

Ocorr, Karen; Vogler, Georg; Bodmer, Rolf (2014): Methods to assess Drosophila heart development, function and aging. In: *Methods (San Diego, Calif.)* 68 (1), S. 265–272. DOI: 10.1016/j.ymeth.2014.03.031.

Oh-hashi, Kentaro; Fujimura, Keito; Norisada, Junpei; Hirata, Yoko (2018): Expression analysis and functional characterization of the mouse cysteine-rich with EGF-like domains 2. In: *Scientific Reports* 8. DOI: 10.1038/s41598-018-30362-4.

Oh-hashi, Kentaro; Kunieda, Ryosuke; Hirata, Yoko; Kiuchi, Kazutoshi (2011): Biosynthesis and secretion of mouse cysteine-rich with EGF-like domains 2. In: *FEBS letters* 585 (15), S. 2481–2487. DOI: 10.1016/j.febslet.2011.06.029.

Owen, Oliver E.; Kalhan, Satish C.; Hanson, Richard W. (2002): The key role of anaplerosis and cataplerosis for citric acid cycle function. In: *The Journal of biological chemistry* 277 (34), S. 30409–30412. DOI: 10.1074/jbc.R200006200.

Pareek, Gautam; Thomas, Ruth E.; Pallanck, Leo J. (2018): Loss of the Drosophila m-AAA mitochondrial protease paraplegin results in mitochondrial dysfunction, shortened lifespan, and neuronal and muscular degeneration. In: *Cell death & disease* 9 (3), S. 304. DOI: 10.1038/s41419-018-0365-8.

Parone, Philippe A.; Da Cruz, Sandrine; Tondera, Daniel; Mattenberger, Yves; James, Dominic I.; Maechler, Pierre et al. (2008): Preventing Mitochondrial Fission Impairs Mitochondrial Function and Leads to Loss of Mitochondrial DNA. In: *PLoS ONE* 3 (9). DOI: 10.1371/journal.pone.0003257.

Pernas, Lena; Scorrano, Luca (2016): Mito-Morphosis: Mitochondrial Fusion, Fission, and Cristae Remodeling as Key Mediators of Cellular Function. In: *Annual review of physiology* 78, S. 505–531. DOI: 10.1146/annurev-physiol-021115-105011.

Pfluger, Paul T.; Kabra, Dhiraj G.; Aichler, Michaela; Schriever, Sonja C.; Pfuhlmann, Katrin; Garcia, Veronica Casquero et al. (2015): Calcineurin Links Mitochondrial Elongation with Energy Metabolism. In: *Cell metabolism* 22 (5), S. 838–850. DOI: 10.1016/j.cmet.2015.08.022.

Pickles, Sarah; Vigié, Pierre; Youle, Richard J. (2018): Mitophagy and Quality Control Mechanisms in Mitochondrial Maintenance. In: *Current biology : CB* 28 (4), R170-R185. DOI: 10.1016/j.cub.2018.01.004.

Poole, Angela C.; Thomas, Ruth E.; Andrews, Laurie A.; McBride, Heidi M.; Whitworth, Alexander J.; Pallanck, Leo J. (2008): The PINK1/Parkin pathway regulates mitochondrial morphology. In: *Proceedings of the National Academy of Sciences of the United States of America* 105 (5), S. 1638–1643. DOI: 10.1073/pnas.0709336105.

Rahman, Motiur; Nirala, Niraj K.; Singh, Alka; Zhu, Lihua Julie; Taguchi, Kaori; Bamba, Takeshi et al. (2014): Drosophila Sirt2/mammalian SIRT3 deacetylates ATP synthase β and regulates complex V activity. In: *The Journal of Cell Biology* 206 (2), S. 289–305. DOI: 10.1083/jcb.201404118.

Rajan, Akhila; Perrimon, Norbert (2013): Of flies and men: insights on organismal metabolism from fruit flies. In: *BMC biology* 11, S. 38. DOI: 10.1186/1741-7007-11-38.

Rambold, Angelika S.; Cohen, Sarah; Lippincott-Schwartz, Jennifer (2015): Fatty acid trafficking in starved cells: regulation by lipid droplet lipolysis, autophagy, and mitochondrial fusion dynamics. In: *Developmental cell* 32 (6), S. 678–692. DOI: 10.1016/j.devcel.2015.01.029.

Rana, Anil; Oliveira, Matheus P.; Khamoui, Andy V.; Aparicio, Ricardo; Rera, Michael; Rossiter, Harry B.; Walker, David W.: Promoting Drp1-mediated mitochondrial fission in midlife prolongs healthy lifespan of Drosophila melanogaster. In: *Nature Communications* 8 (1), S. 448. DOI: 10.1038/s41467-017-00525-4.

Rotstein, Barbara; Paululat, Achim (2016): On the Morphology of the Drosophila Heart. In: *Journal of cardiovascular development and disease* 3 (2). DOI: 10.3390/jcdd3020015.

Rupp, Paul A.; Fouad, Gameil T.; Egelston, Carley A.; Reifsteck, Carol A.; Olson, Susan B.; Knosp, Wendy M. et al. (2002): Identification, genomic organization and mRNA expression of CRELD1, the founding member of a unique family of matricellular proteins. In: *Gene* 293 (1), S. 47–57. DOI: 10.1016/S0378-1119(02)00696-0.

Ryoo, Hyung Don (2015): Drosophila as a model for unfolded protein response research. In: *BMB reports* 48 (8), S. 445–453.

Santel, Ansgar; Frank, Stephan (2008): Shaping mitochondria: The complex posttranslational regulation of the mitochondrial fission protein DRP1. In: *IUBMB life* 60 (7), S. 448–455. DOI: 10.1002/iub.71.

Sherratt, H. S. (1991): Mitochondria: structure and function. In: *Revue neurologique* 147 (6-7), S. 417–430.

Sica, V.; Bravo-San Pedro, J. M.; Pietrocola, F.; Izzo, V.; Maiuri, M. C.; Kroemer, G.; Galluzzi, L. (2017): Chapter Nine - Assessment of Glycolytic Flux and Mitochondrial Respiration in the Course of Autophagic Responses. In: Lorenzo Galluzzi, José Manuel Bravo-San Pedro und Guido Kroemer (Hg.): Methods in Enzymology : Molecular Characterization of Autophagic Responses, Part B, Bd. 588: Academic Press, S. 155–170. Online verfügbar unter http://www.sciencedirect.com/science/article/pii/S0076687916303524.

Sica, V.; Bravo-San Pedro, J. M.; Pietrocola, F.; Izzo, V.; Maiuri, M. C.; Kroemer, G.; Galluzzi, L. (2017): Chapter Nine - Assessment of Glycolytic Flux and Mitochondrial Respiration in the Course of Autophagic Responses. In: Lorenzo Galluzzi, José Manuel Bravo-San Pedro und Guido Kroemer (Hg.): Methods in Enzymology : Molecular Characterization of Autophagic Responses, Part B, Bd. 588: Academic Press, S. 155–170. Online verfügbar unter http://www.sciencedirect.com/science/article/pii/S0076687916303524.

Solinas, Giovanni; Borén, Jan; Dulloo, Abdul G. (2015): De novo lipogenesis in metabolic homeostasis: More friend than foe? In: *Molecular Metabolism* 4 (5), S. 367–377. DOI: 10.1016/j.molmet.2015.03.004.

Solinas, Giovanni; Borén, Jan; Dulloo, Abdul G. (2015): De novo lipogenesis in metabolic homeostasis: More friend than foe? In: *Molecular Metabolism* 4 (5), S. 367–377. DOI: 10.1016/j.molmet.2015.03.004.

Strable, Maggie S.; Ntambi, James M. (2010): Genetic control of de novo lipogenesis: role in diet-induced obesity. In: *Critical reviews in biochemistry and molecular biology* 45 (3), S. 199–214. DOI: 10.3109/10409231003667500.

Szabo, Aliz; Sumegi, Katalin; Fekete, Katalin; Hocsak, Eniko; Debreceni, Balazs; Setalo, Gyorgy et al. (2018): Activation of mitochondrial fusion provides a new treatment for mitochondria-related diseases. In: *Biochemical pharmacology* 150, S. 86–96. DOI: 10.1016/j.bcp.2018.01.038.

Taghli-Lamallem, Ouarda; Plantié, Emilie; Jagla, Krzysztof (2016): Drosophila in the Heart of Understanding Cardiac Diseases: Modeling Channelopathies and Cardiomyopathies in the

Fruitfly. In: *Journal of cardiovascular development and disease* 3 (1). DOI: 10.3390/jcdd3010007.

Tait, Stephen W. G.; Green, Douglas R. (2012): Mitochondria and cell signalling. In: *Journal of cell science* 125 (Pt 4), S. 807–815. DOI: 10.1242/jcs.099234.

Teleman, Aurelio A. (2009): Molecular mechanisms of metabolic regulation by insulin in Drosophila. In: *The Biochemical journal* 425 (1), S. 13–26. DOI: 10.1042/BJ20091181.

Tilokani, Lisa; Nagashima, Shun; Paupe, Vincent; Prudent, Julien (2018): Mitochondrial dynamics: overview of molecular mechanisms. In: *Essays in biochemistry* 62 (3), S. 341–360. DOI: 10.1042/EBC20170104.

Twig, Gilad; Elorza, Alvaro; Molina, Anthony J. A.; Mohamed, Hibo; Wikstrom, Jakob D.; Walzer, Gil et al. (2008): Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. In: *The EMBO journal* 27 (2), S. 433–446. DOI: 10.1038/sj.emboj.7601963.

Twig, Gilad; Shirihai, Orian S. (2011): The Interplay Between Mitochondrial Dynamics and Mitophagy. In: *Antioxidants & Redox Signaling* 14 (10), S. 1939–1951. DOI: 10.1089/ars.2010.3779.

Vander Heiden, Matthew G.; Cantley, Lewis C.; Thompson, Craig B. (2009): Understanding the Warburg effect: the metabolic requirements of cell proliferation. In: *Science (New York, N.Y.)* 324 (5930), S. 1029–1033. DOI: 10.1126/science.1160809.

Vogler, Georg; Ocorr, Karen (2009): Visualizing the Beating Heart in Drosophila. In: *Journal of visualized experiments : JoVE* (31). DOI: 10.3791/1425.

Wanders, Ronald J. A.; Ruiter, Jos P. N.; IJlst, Lodewijk; Waterham, Hans R.; Houten, Sander M. (2010): The enzymology of mitochondrial fatty acid beta-oxidation and its application to follow-up analysis of positive neonatal screening results. In: *Journal of Inherited Metabolic Disease* 33 (5), S. 479–494. DOI: 10.1007/s10545-010-9104-8.

Wang, Hongxia; Song, Pingping; Du, Lei; Tian, Weili; Yue, Wen; Liu, Min et al. (2011): Parkin Ubiquitinates Drp1 for Proteasome-dependent Degradation: IMPLICATION OF DYSREGULATED MITOCHONDRIAL DYNAMICS IN PARKINSON DISEASE*. In: *The Journal of biological chemistry* 286 (13), S. 11649–11658. DOI: 10.1074/jbc.M110.144238.

Wang, Yuhui; Viscarra, Jose; Kim, Sun-Joong; Sul, Hei Sook (2015): Transcriptional regulation of hepatic lipogenesis. In: *Nature reviews. Molecular cell biology* 16 (11), S. 678–689. DOI: 10.1038/nrm4074.

Westermann, Benedikt (2012): Bioenergetic role of mitochondrial fusion and fission. In: *Biochimica et biophysica acta* 1817 (10), S. 1833–1838. DOI: 10.1016/j.bbabio.2012.02.033.

White, Heather M. (2015): The Role of TCA Cycle Anaplerosis in Ketosis and Fatty Liver in Periparturient Dairy Cows. In: *Animals : an Open Access Journal from MDPI* 5 (3), S. 793–802. DOI: 10.3390/ani5030384.

Willems, Peter H. G. M.; Rossignol, Rodrigue; Dieteren, Cindy E. J.; Murphy, Michael P.; Koopman, Werner J. H. (2015): Redox Homeostasis and Mitochondrial Dynamics. In: *Cell metabolism* 22 (2), S. 207–218. DOI: 10.1016/j.cmet.2015.06.006.

Wolf, Matthew J.; Amrein, Hubert; Izatt, Joseph A.; Choma, Michael A.; Reedy, Mary C.; Rockman, Howard A. (2006): Drosophila as a model for the identification of genes causing adult human heart disease. In: *Proceedings of the National Academy of Sciences of the United States of America* 103 (5), S. 1394–1399. DOI: 10.1073/pnas.0507359103.

Wolf, Matthew J.; Rockman, Howard A. (2011): Drosophila, genetic screens, and cardiac function. In: *Circulation research* 109 (7), S. 794–806. DOI: 10.1161/CIRCRESAHA.111.244897.

Xu, Chunyan; Bailly-Maitre, Beatrice; Reed, John C. (2005): Endoplasmic reticulum stress: cell life and death decisions. In: *Journal of Clinical Investigation* 115 (10), S. 2656–2664. DOI: 10.1172/JCI26373.

Youle, Richard J.; Narendra, Derek P. (2010): Mechanisms of mitophagy. In: *Nature Reviews Molecular Cell Biology* 12, 9 EP -. DOI: 10.1038/nrm3028.

Youle, Richard J.; van der Bliek, Alexander M (2012): Mitochondrial fission, fusion, and stress. In: *Science (New York, N.Y.)* 337 (6098), S. 1062–1065. DOI: 10.1126/science.1219855.

Zhang, Dongsheng; Li, Juan; Wang, Fengzhen; Hu, Jun; Wang, Shuwei; Sun, Yueming (2014): 2-Deoxy-D-glucose targeting of glucose metabolism in cancer cells as a potential therapy. In: *Cancer letters* 355 (2), S. 176–183. DOI: 10.1016/j.canlet.2014.09.003.

6. 4 Danksagungen

Als erstes möchte ich mich bei meinen „zwei Doktorvätern“ PD Dr. Reinhard Bauer und Prof. Dr. Dr. h.c. Michael Hoch dafür bedanken, dass ich in ihrem Labor meine Doktorarbeit anfertigen durfte und mir bei ihnen immer Rat holen konnte.

Mein besonderer Dank geht an Dr. Julia Sellin, die mir nicht nur jederzeit mit Rat und Tat zur Seite stand, sondern auch eine gute Freundin und ein Vorbild für mich geworden ist.

Weiterhin danke ich der gesamten AG Bauer/Hoch für die tolle Zeit – mit euch wurde es nie langweilig. Vor allem danke ich Nicole, die eine super Studentin war und mir sehr geholfen hat.

Prof. Dr. Achim Paululat möchte ich für die Kooperation bei der Erstellung der TEM Aufnahmen danken und dafür, dass ich in seinem Labor die Herzmessungen durchführen konnte.

Ein Großer Dank geht an alle meine Freunde, die vor allem in schweren Zeiten immer für mich da waren und mit denen man neben wissenschaftlichen Themen auch mal Unsinn bereden konnte.

Außerdem möchte ich mich bei Dr. Gabi Gilles für die tolle Unterstützung in all den Jahren bedanken.

Jim Walker danke ich sehr für die Geduld und das Aushalten mancher Launen.

Meiner Mutter danke ich von ganzem Herzen für den großen Rückhalt in jeder Hinsicht. Ohne dich hätte ich dieses Ziel gar nicht erreichen können!

…..Mischief managed!