

UCLA

UCLA Electronic Theses and Dissertations

Title

Functional Characterization of Kans12 and Its Role in Mitochondrial DNA Repair and Maintenance

Permalink

<https://escholarship.org/uc/item/77h8c5jh>

Author

Liau, Wei-Siang

Publication Date

2012

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Functional Characterization of Kansl2 and Its Role in Mitochondrial DNA

Repair and Maintenance

A dissertation submitted in satisfaction of the requirements for the degree Doctoral of
Philosophy in Biochemistry and Molecular Biology

by

Wei-Siang Liao

2012

ABSTRACT OF THE DISSERTATION

Functional Characterization of Kansl2 and Its Role in Mitochondrial DNA Repair and Maintenance

by

Wei-Siang Liao

Doctor of Philosophy in Biochemistry and Molecular Biology

University of California, Los Angeles, 2012

Professor Carla M. Koehler, Chair

Mitochondrial DNA repair is a highly regulated, stress-responsive event that is crucial for maintaining mtDNA integrity. Oxidative damage and the mutation load of mtDNA are greater than nuclear DNA. The base excision repair (BER) machinery is important for repair of mtDNA as well as nuclear DNA; however, the pathway has not been as well characterized in mitochondria compared to the nucleus. Recent studies have shown that several repair factors are localized to mitochondria and form stable complex with mtDNA. In this study, I characterize a newly identified mtDNA protein, Kansl2, and its role in mtDNA repair. Kansl2 is conserved from flies to vertebrates, expressed ubiquitously in developing zebrafish and localizes to the mitochondrial matrix. Inactivation of Kansl2 in developing zebrafish caused abnormal heart and muscle development, with eventual death because Kansl2 is essential. Knockdown of Kansl2 enhanced apoptosis. Loss of Kansl2 resulted in aberrant mitochondrial cristae and compromised

complex II and complex IV activity. Kansl2 binds to the mtDNA promiscuously via its Rad51-like domain. More importantly, I found that Kansl2 assembles in a 300 kDa molecular weight complex with uracil DNA glycosylase (Ung) in zebrafish mitochondria. I further characterized the molecular interaction of Kansl2 and Ung by determining the effect of Kansl2 on Ung activity *in-vitro*. These results affirm the hypothesis that Kansl2 serves as a scaffold for Ung and the interaction enhances the removal of uracil from the mtDNA by Ung. This study employs western blot analysis, animal studies, knockdown technology, native PAGE analysis and electron microscopy.

The dissertation of Wei-Siang Liao is approved.

Catherine F. Clarke

Jau-Nian Chen

Carla M. Koehler, Committee Chair

University of California, Los Angeles

2012

TABLE OF CONTENTS

	<u>PAGE</u>
ABSTRACT.....	ii
COMMITTEE PAGE.....	iv
TABLE OF CONTENTS.....	v
LIST OF TABLES.....	vi
LIST OF FIGURES.....	vii
ACKNOWLEDGMENTS.....	viii
VITA.....	ix
CHAPTER 1: INTRODUCTION.....	1
1.1 <i>DANIO RERIO</i> AS THE MODEL ORGANISM.....	2
1.2 THE MITOCHONDRION.....	3
1.3 THE MITOCHONDRIAL DNA.....	7
1.4 MTDNA REPLICATION.....	8
1.5 MTDNA REPAIR.....	9
1.6 MITOCHONDRIAL BASE EXCISION REPAIR.....	10
1.7 REFERENCES/BIBLIOGRAPHY.....	12
CHAPTER 2: KANSL2 STABILIZES UNG IN THE MITOCHONDRIAL MATRIX.....	20
2.1 INTRODUCTION.....	20
2.2 EXPERIMENTAL PROCEDURES.....	22
2.3 RESULTS.....	28
2.4 DISCUSSION.....	34
2.5 REFERENCES/BIBLIOGRAPHY.....	49
CHAPTER 3: SUMMARY.....	55

LIST OF TABLES

<u>TABLE</u>		<u>PAGE</u>
1.1	Phenotypic analysis of morpholino injection and rescue by co-injection of the <i>kansl2</i> RNA.....	36

LIST OF FIGURES

<u>FIGURE</u>		<u>PAGE</u>
1.1	Electron micrography image showing zebrafish muscle mitochondria.....	6
2.1	The enhancer trap screen identified <i>kansl2</i>	37
2.2	Protein sequence alignment of the human, zebrafish and Drosophila (dgt1) orthologs using the ClustalW and Boxshade programs.....	38
2.3	Loss of Kansl2 results in cardiac and somite defects.....	39
2.4	The <i>kansl2</i> transcript is ubiquitously expressed.....	40
2.5	<i>kansl2</i> mutant exhibits abnormal patterning in the heart and somites.....	41
2.6	Kansl2 localizes to the mitochondrial matrix.....	42
2.7	Kansl2 requires the MLS for mitochondria import.....	43
2.8	Loss of Kansl2 perturbs mitochondrial ultrastructure and respiration.....	44
2.9	Purification and circular dichroism analysis of Kansl2 Rad51 N-terminal like domain.....	45
2.10	The Kansl2 Rad51 N-terminal like domain binds to DNA.....	46
2.11	Kansl2 and Ung are partner proteins.....	47
2.12	Purification of Kansl2 and Ung.....	48

ACKNOWLEDGMENTS

The scientific advances that I have made these past few years are owned to the intense efforts of my mentor, Carla M. Koehler. My progress toward becoming an independent, critical thinker would be impossible without her patience and support. I also wish to thank Dr. Chen, Dr. Clarke, Dr. Van der Bliet and Dr. Quilan for agreeing to serve on my dissertation committee, and for their advice and guidance. I have indebted to Dr. Krauss and Dr. Harris for their immense efforts in generating mutant line for our laboratory. Dr. McCaffery has been a great help in generating electron micrograph images for our studies.

On a personal level, I would like to acknowledge Dr. LaMunyon, my previous mentor and a great friend, for his help, support and advice. Without his encouragement, I would not have considered pursuing a career in science. Finally, I would like to thank present and past members of the Koehler laboratory and many friends around the world for their help, support, guidance and patience.

VITA

2004	B. S. California State Polytechnic University, Pomona
2004	Internship N-terminus Research Laboratory
2004-2005	Research Technician/Research Assistant California State Polytechnic University, Pomona
2005-2007	Teaching Assistant California State Polytechnic University, Pomona
2007	M. S. California State Polytechnic University, Pomona
2007-2008	Teaching Assistant University of California, Los Angeles
2008-present	Graduate Student Assistant University of California, Los Angeles

Awards

2006	The Dr. Edward T. Roche Research Assistant Award Scholarship California State Polytechnic University, Pomona
2008	Ralph and Shirley Shapiro International Fellowship University of California, Los Angeles

Publications

1. Austin M. U., Liao, W.-S., Balamurugan K., Ashokkumar B., Said, H. M., LaMunyon C. W. "Knockout of the folate transporter *folT-1* causes germline and somatic defects in *C. elegans*." *BMC Developmental Biology*, 10:46, 2010.
2. Lewis S.C., Dyal L.A., Hilburn C.F., Weitz S., Liao W.-S., LaMunyon C.W., Denver D.R. "Molecular evolution in *Panagrolaimus* nematodes: origins of parthenogenesis, hermaphroditism and the Antarctic species *P. davidi*." *BMC Evolutionary Biology*, 9:15, 2009.
3. Gosney, R., Liao W.-S., and LaMunyon C.W. "A novel function for the presenilin family member *spe-4*: inhibition of spermatid activation in *Caenorhabditis elegans*." *BMC Developmental Biology*, 8:44, 2008.

4. Liao, W.-S., Deshombres C., Chin K., Gonzalez-Serricchio A., and LaMunyon C.W. “A persistent mitochondrial deletion reduces fitness and sperm performance in heteroplasmic populations of *C. elegans*.” *BMC Genetics*, 8:8, 2007.

Presentations

1. Allan C. M., Hill S., Morvaridi S., Saiki R., Liao W.-S., Hirano K., Kawashima T., Clarke C. F. “Characterization of CC1736, a conserved START domain protein and putative coenzyme Q-binding protein.” The LIPID MAPS Annual Meeting, University of California San Diego’s Scripps Institution of Oceanography, May 2-3, 2011.
2. Liao W.-S., Krauss J., Levesque M., Koehler C.M. “An Enhancer Trap Line Associated with Mitochondrial DNA Repair Gene in Zebrafish.” Ninth International Conference on Zebrafish Development and Genetics, University of Wisconsin, Madison, June 16-20, 2010.
3. Liao, W.-S., Gonzalez-Serricchio A., Muhlrud P., LaMunyon C.W. “*hc197*, a Checkpoint-like Gene that Inhibits Spermiogenesis in *C. elegans*.” Genetic Analysis: Model Organisms to Human Biology Meeting, San Diego, California, USA, January 5-7, 2006.
4. Liao, W.-S., Gonzalez-Serricchio A., Muhlrud P., LaMunyon C.W. “Mapping and characterization of allele *hc197*, a suppressor of *spe-27*.” Fifteenth International *C. elegans* Meeting, University of California, Los Angeles, USA, June 25-29, 2005.

CHAPTER 1

INTRODUCTION

Cells are constantly exposed to damaging substances – the reactive oxygen species (ROS) generated from the oxidative phosphorylation as by-products, and genotoxic stressors from surroundings. Many of these substances are potential sources of DNA damage. To prevent DNA damage, cells have developed means to eliminate these harmful chemical substances. For example, there are enzymes that convert ROS to a less reactive form (McCord and Fridovich, 1988). To maintain genome integrity, cells possess elaborate systems to repair DNA damage or lesion. DNA repair factors such as glycosylases, endonucleases, polymerases and ligases function to recognize and eliminate various lesions in genomes. Under various cellular conditions, cells can react by modulating these factors to the appropriate lesion site. The control of flux of these factors into various DNA-containing compartments is also crucial to prevent accumulation of detrimental mutations and ensure cellular survival.

Two types of genomes exist in a cell – the nuclear and the mitochondrial genome. Mitochondrial genome resides in a specific organelle called mitochondrion, an organelle that provides energy to the cell. Because mitochondrion contains its own genome that encodes proteins required for energy production, maintaining the integrity of the mitochondrial genome is vital for cellular function. A decrease in the mitochondrial repair capacity can lead to mutations, compromised metabolism and cellular death. In response to cellular stress, repair factors are targeted to the mitochondrion in a tightly controlled fashion. A picture of the complex cellular signaling mediating this targeting process is emerging (Boesch et al., 2010; Swartzlander et al.,

2010). Although the mechanism regulating the import of these repair factors into the mitochondrion is not clear, a set of post-translational regulatory themes is clear. These include protein phosphorylation, sumoylation, and protein-protein interactions through homo and heterotypic binding domains.

Aside from post-translational modification, transcriptional regulation is a common strategy for deploying proteins in organelles. Some mitochondrial repair factors are generated through alternative splicing or the utilization of different regulatory elements. These mitochondrial repair factors typically contain a N-terminus mitochondrial targeting signal (MTS) required for mitochondrial matrix localization. However, it is still not clear how cells turn on these regulatory elements in response to DNA damage and cellular stress.

1.1 *DANIO RERIO* AS THE MODEL ORGANISM

Danio rerio or zebrafish is a small (~2.5 inches long) tropical freshwater fish that feeds on zooplankton, insects, insect larvae and phytoplankton. Adult zebrafish can grow up to 2 inches and thrive in temperature between 22°C and 30°C. Zebrafish are dioecious and oviparous vertebrate. Adult male has a torpedo-shaped body and are typically smaller than females. Females have larger whitish belly, and spawn in two to three days, laying about 100 to 200 eggs in a single clutch. Although adult zebrafish can be easily distinguished, its sex determination is still not clearly understood.

In the early 1980s, George Streisinger pioneered the used of zebrafish as the model system for elucidating molecular and genetic basis of development. Zebrafish has many advantages over other model systems because embryos are optically clear and can be produced in large quantity. Due to its relatively small size, it is relatively easy to culture zebrafish in the

laboratory. The average generation time for zebrafish is 3 months. All these attributes make zebrafish ideal for screening a large number of mutant traits with a dissecting microscope. *in-vitro* fertilization can be performed on unfertilized eggs and sperm can be frozen for long-term storage.

Comparative genomics have detected an apparent genome-wide duplication in zebrafish (Postlethwait et al., 2000). Despite this duplication event, zebrafish has roughly the same chromosomes as humans (25 pairs). Because the genome is sequenced, genes can be mapped in a relatively short amount of time. The genome of zebrafish can be manipulated genetically by mutagens, transgenic overexpression, transgenic insertion, targeted knockouts and gene knockdown (Draper et al., 2001; Galbiati et al., 2000; Haffter et al., 1996; Nasevicius and Ekker, 2000; Solnica-Krezel et al., 1994; Wienholds et al., 2003). For example, Tol2 transposomal element has been used to generate both knockout and transgenic animals with high efficiency (Nagayoshi et al., 2008). Because a large number of embryos can be produced *ex vivo*, zebrafish is ideal for *in-vivo* drug screens (Zon and Peterson, 2005). In addition, zebrafish has been widely used as model systems to study human diseases (Alexander et al., 1998; Bradbury, 2004; Chen et al., 1996; Dahme et al., 2009). In my dissertation work, I utilized zebrafish as my model organism to investigate how defects in mtDNA repair affects organ development in animals.

1.2 THE MITOCHONDRION

The mitochondrion is a complex and dynamic organelle with four major compartments: the outer membrane (OM), inner membrane (IM), intermembrane space (IMS) and matrix (Figure 1.1). Mitochondria can form a complex network within the cell and are transported to appropriate subcellular locations through specialized transport machineries (Detmer and Chan,

2007). Mitochondria are unique organelle because they can undergo fission and fusion (Detmer and Chan, 2007; Hoppins et al., 2007; Rube and van der Blik, 2004), and possess their own genome, the mitochondrial DNA (mtDNA). Several processes such as fatty acid beta-oxidation, Krebs cycle, urea cycle, and amino acid metabolism occur within mitochondria, but the most crucial role of mitochondria is the production of energy in the form of ATP through the action of electron-transport chain and oxidative phosphorylation system (OXPHOS) (DiMauro and Schon, 2003).

The mitochondrial respiratory chain (MRC) complexes reside in the inner membrane of mitochondria, encompasses five multimeric respiratory complexes: Complex I or reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase-ubiquinone oxidoreductase (46 subunits), Complex II or succinate dehydrogenase-ubiquinone oxidoreductase (4 subunits), Complex III or ubiquinone-cytochrome c oxidoreductase (11 subunits), complex IV or cytochrome c oxidase (13 subunits), and Complex V or ATP synthase (16 subunits) (DiMauro and Schon, 2003; Wallace, 2005). Electrons flow from NADH to Complex I or from succinate to Complex II and are subsequently passed to ubiquinone (coenzyme Q). Ubiquinone is reduced to ubisemiquinone (CoQH) and then to ubiquinol (CoQH₂). The electrons are further transferred from ubiquinol to Complex III, which then passes its electrons to cytochrome *c*. Cytochrome *c* donates its electrons to Complex IV and finally to oxygen to reduce it to water. The travel of electrons through the respiratory chain allow Complex I, III and IV to pump protons across the inner membrane from the matrix, thereby generating an electrochemical gradient (DiMauro and Schon, 2003; Wallace, 2001). The electrochemical gradient allows the protons to move back to the matrix via Complex V or ATP synthase to generate ATP. Protons can also travel back to the

mitochondrial matrix through the action of mitochondrial uncoupling proteins, by which electrons are uncoupled from ATP production (Nicholls et al., 1978).

The majority of proteins within mitochondria are encoded by nuclear DNA. Therefore, a rather complex machinery is required to transport proteins into this organelle (Koehler, 2004). Mitochondrial protein import employs the translocase of the OM (TOM) and translocase of the IM (TIM) complexes. The TOM complex mediates the transport of proteins across the OM of mitochondria, whereas the TIM complex transports proteins across the IM. In the IM, there are the TIM23 complex that mediates import of precursors with a N-terminal targeting presequence into the matrix, and the TIM22 complex which aides in the import of the IM proteins. Small TIM proteins within the IMS function to chaperone the precursor proteins to the TIM22 complex in the IM (Leuenberger et al., 2003; Roesch et al., 2002).

The unique properties of this organelle raised interests in understanding its biogenesis and function. Indeed, most recent studies on mitochondria are pointing toward clues on the basis of Alzheimer's disease (Wallace, 2005), dystonia (Roesch et al., 2002), Parkinson's disease (Gu et al., 1998) and myopathy (DiMauro and Schon, 2003).

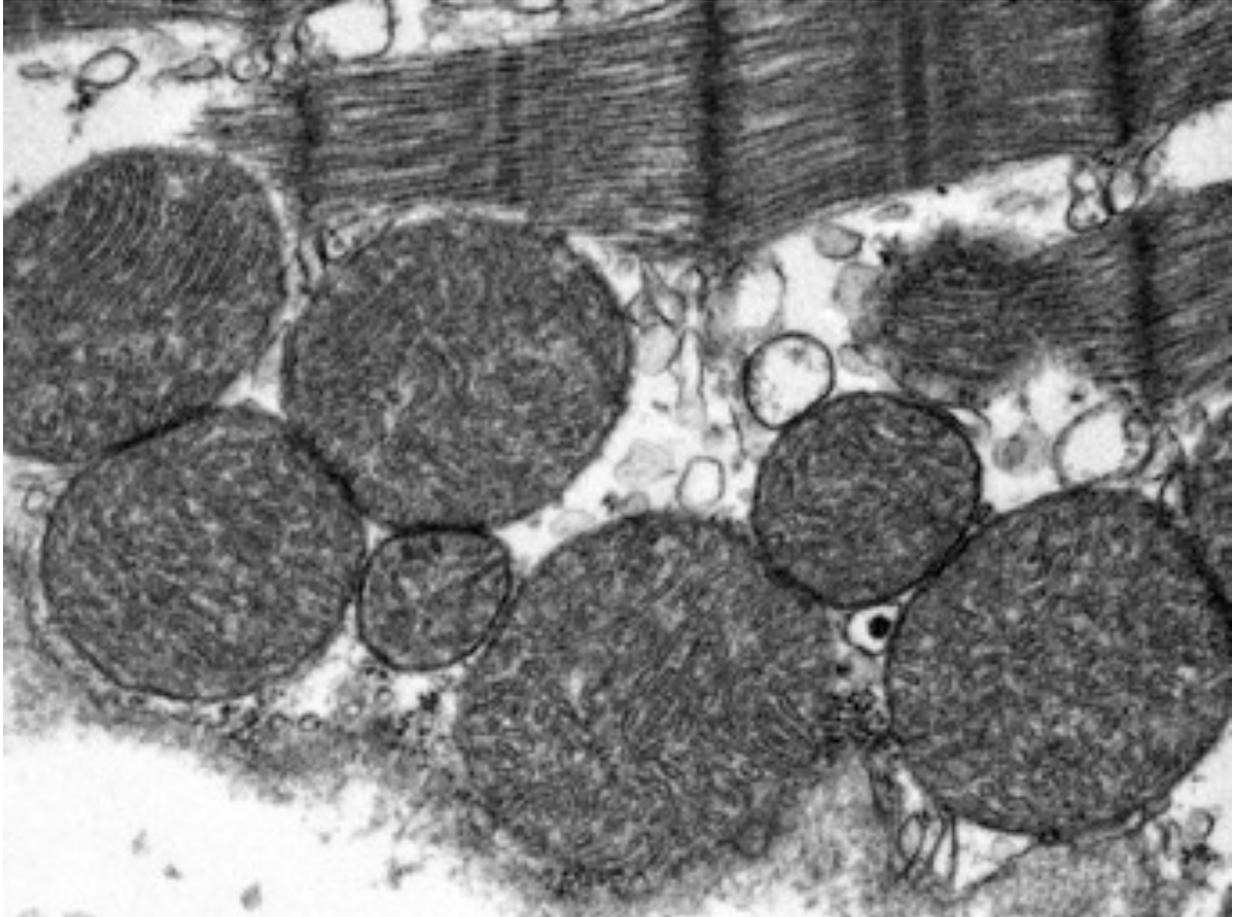


Figure 1.1: Electron micrograph image showing zebrafish muscle mitochondria. Scale bar = 2 μ m.

1.3 THE MITOCHONDRIAL DNA

Aside from the nucleus, the mitochondrion is another organelle in the mammalian cell that possesses its own DNA. Human mitochondrial DNA (mtDNA) is a small double-stranded circular molecule of 16,569 base pairs with no introns (DiMauro and Schon, 2003). The two strands of mtDNA differ from one another in the levels of guanine and thymine nucleotides, and can be separated into heavy (H) and light (L) strands by alkaline cesium chloride gradient centrifugation based on their densities. Each eukaryotic cell contains multiple mitochondria, and therefore, possesses from hundreds to thousands of copies of mtDNA.

mtDNA encodes 37 genes: 7 subunits of complex I, cytochrome *b*, 3 subunits of complex IV, 2 subunits of ATP synthase, 12S and 16S ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs). Each mitochondrial gene coding for a polypeptide is transcribed and translated in mitochondria by the mitochondrial protein synthesis machinery. Although these encoded genes do not possess introns, mtDNA has a functionally important non-coding region called the displacement (D)-loop. The D-loop contains the transcription initiation start sites for both H- and L- strand (HSP and LSP), binding sites for transcription factors, the H-strand origin of replications (O_H), conserved sequence blocks (CSB-1, CSB-2 and CSB-3) and termination associated sequences (TAS) (Foran et al., 1988; Shadel and Clayton, 1997). In addition, there is the L-strand origin of replications (O_L) located in the enriched tRNA genes region.

Mammalian mtDNA are maternally inherited, meaning that every single mtDNA molecule in the zygote is derived from the ovum. During cell division, mitochondria are randomly distributed resulting in a change in the proportion of mtDNA in different tissues and organs. In normal cells and tissues, all mtDNAs are identical, termed homoplasmy. Heteroplasmy can exist when the mitochondrion harbors both wildtype and mutant mtDNA.

Heteroplasmy can persist indefinitely in an organism and when a threshold value is reached, pathological symptoms such as myopathy prevail (Liau et al., 2007). Occasionally, mutant mtDNA can exist in a homoplasmic stage with detrimental consequences (Taylor et al., 2003). In the nucleus, nuclear DNA is wrapped around proteins called histones to form a compact structure called chromatin. In contrast, mtDNA is packaged into protein-DNA complexes called the nucleoid, a functional inheritance unit consists of one to five mitochondrial genomes and approximately twenty proteins (Kaufman et al., 2000; Kukat et al., 2011; Miyakawa et al., 1987). The most abundant protein in the mitochondrial nucleoid is the mitochondrial transcription factor A (TFAM) (Alam et al., 2003). TFAM is essential for mitochondrial transcription (Antoshechkin et al., 1997), and functions in packaging mtDNA (Garrido et al., 2003; Shen and Bogenhagen, 2001) and regulates mtDNA copy number (Larsson et al., 1998). mtDNA is coated with TFAM and estimated to have one molecule of TFAM every 10-20 bp (Ekstrand et al., 2004).

How does the mitochondrial nucleoid structure in mitochondria? ATAD3, a AAA+ protein family that binds mitochondrial D-loop, has been suggested to play a role in nucleoid formation and segregation (He et al., 2007). A mitochondrial IM protein, Prohibitin1 (PHB1), has been proposed to regulate mitochondrial nucleoid organization (Kasashima et al., 2008). Although a large number of proteins have also been suggested to be constituents of mitochondrial nucleoid, their role in packaging mtDNA remained to be determined.

1.4 MTDNA REPLICATION

Unlike the nucleus, mtDNA possesses rudimentary machineries for DNA replication. The main component of the mtDNA replisome is the mtDNA polymerase γ , a heterotrimeric protein consists of a catalytic subunit (Pol γ A) and a dimeric accessory subunit (Poly γ B) (Carrodeguas

et al., 2001; Kaguni, 2004). During mtDNA replication, the helicase, TWINKLE, unwinds the mtDNA, and DNA synthesis is initiated by Poly γ with the aid of a mitochondrial single-stranded binding protein (mtSSB) (Korhonen et al., 2003).

How does mtDNA replicate itself? Two models were proposed to explain how mtDNA replicates: the asymmetric replication model and the strand-coupled model. According to the asymmetric model, RNA primers made during mitochondrial transcription generate a RNA-DNA hybrid, initiating leading-strand synthesis at the O_H site. The synthesis of the lagging-strand commences at the O_L site when the leading-strand synthesis reached two-thirds of the mitochondrial genome (Clayton, 1991). In contrast to the asymmetric model, the strand-coupled model argues that mtDNA replication occurs by coupling leading- and lagging-strand synthesis (Holt et al., 2000). The latter model was formulated based on the assumption that L-strand synthesis starts at multiple sites rather at the O_L site. The exact mechanism underlies mtDNA replication is still controversial and awaits further investigation.

1.5 MTDNA REPAIR

The mitochondrial genome is mutated more readily than the nuclear genome (Hudson et al., 1998). Several factors can lead to mtDNA mutations. mtDNA mutations can arise when Pol γ is unsuccessful in removing mismatch nucleotides during mtDNA replication (Trifunovic et al., 2004). Because a large amount of ROS is generated through the action of mitochondrial respiratory chain in mitochondria, the accumulation of ROS can damage both the mtDNA and nucleotides in mitochondria. An aggressive form of ROS, the hydroxyl radical, can potentially react with DNA bases or the deoxyribose backbone, resulting in double-strand break and oxidized bases such as the 8-oxo-guanine (8-oxoG) and the 8-oxo-adenine (8-oxoA) (Dizdaroglu

et al., 2002). In addition to oxidative damaged DNA, oxidative damaged free nucleotides in mitochondria can be removed by deoxynucleotides triphosphatases such as MTH1 and DUT (Fujikawa et al., 1999; Ladner and Caradonna, 1997). Because mtDNA repair machineries are rudimentary, they are less effective in repairing certain types of DNA alterations such as pyrimidine dimers (Croteau et al., 1999). DNA lesions described above can affect the cell's ability to divide (Vascotto et al., 2011), and halt mtDNA replication and transcription (Boesch et al., 2010). But, the most prominent effect is the accumulation of mtDNA mutations. mtDNA mutations have been linked to neurodegenerative diseases (Gu et al., 1998; Weissman et al., 2007), mitochondrial myopathy (Nishigaki et al., 2003), cancer (Brandon et al., 2006) and aging (Boesch et al., 2010; Larsson, 2010).

For a long time, it was assumed that mitochondria devoid of DNA repair system. Recent research has shown that mitochondria possess means to repair mutant DNA (Boesch et al., 2010). The DNA replication enzyme, Pol γ , can detect and excise mispaired nucleotides from the mtDNA (Longley et al., 2001). Larsson et. al. has shown that the major constituent of the nucleoid, TFAM, is required for mtDNA maintenance (Larsson et al., 1998). Several other repair systems, such as the double-strand break repair, base excision repair and mismatch repair, have been discovered in mitochondria recently (Boesch et al., 2010).

1.6 MITOCHONDRIAL BASE EXCISION REPAIR (MTBER)

Perhaps the most well documented repair system in mitochondria is the base excision repair (BER) (Boesch et al., 2010; Van Houten et al., 2006). BER repairs single nucleotide damage caused by oxidation, alkylation, hydrolysis or deamination. The majority of the damage is most likely caused by oxidation due to elevated ROS in mitochondria. The initial step of BER

involved recognition and elimination of the modified nucleotide by DNA glycosylases that cleave the N-glycosidic bond between the purine/pyrimidine base and the deoxyribose sugar. This creates an apurinic/aprimidinic (AP) or abasic site in the DNA, which can be subsequently cleaved by AP endonucleases. Either a single nucleotide (short-patch) or a short sequence of nucleotides (2-10 nucleotides) (long-patch) can be inserted at the site of lesion by Pol γ . The newly synthesized nucleotides are further ligated by DNA ligase to complete the cycle.

The first mitochondrial DNA glycosylase identified was the uracil DNA glycosylase (Anderson and Friedberg, 1980). Uracil DNA glycosylase (UNG1) removes uracil, caused by cytosine deamination or misincorporation by Poly γ , from the mtDNA. Other mitochondrial glycosylases were discovered soon after. The 8-oxoG in mtDNA is eliminated exclusively by the 8-oxoG glycosylase, OGG1. Knockout of *OGG1* in mice results in the accumulation of 8-oxoG in the mtDNA (de Souza-Pinto et al., 2001). The MutY glycosylase, MYH, provides additional level of repair by excising adenines or guanines opposite to 8-oxoG in mtDNA (Parker et al., 2000). The mitochondrial thymine glycol is repaired by the type III endonuclease, NTH1. *NTH1* knockout mice lack thymine glycol incision activity (Karahalil et al., 2003). Another mitochondrial thymine glycol glycosylase, TGG1, was also reported in mammalian mitochondria (Takao et al., 2002). The AP site is further processed by the mammalian AP endonuclease, APE1, and the process creates a 3'-OH at the incision site for the incoming nucleotide (Mitra et al., 2007).

The presence of long-patch repair in mitochondria is still debated. During long-patch repair, the synthesis yields a “flap” sequence that can be cleaved by FEN1 (flap endonuclease 1) with the aid of the DNA2 helicase (Zheng et al., 2008). One report has clearly showed that FEN1 is localized to mitochondria and possesses a long-patch repair activity (Liu et al., 2008). UNG1

glycosylase also possesses both short-patch and long-patch repair activity (Akbari et al., 2008). Once the synthesis is completed, the nick is sealed by the mitochondrial LIG3. LIG3's activity is dispensable for the nuclear repair, but essential for mitochondrial BER (Simsek et al., 2011). How do BER factors localize to mitochondria? Because BER occurs in both the nucleus and mitochondria, the same enzyme can be present in both compartments. Furthermore, by utilizing alternative splicing or regulatory elements, different isoforms can be generated and targeted to the appropriate organelle. For example, the mitochondrial UNG1 is synthesized using a second promoter (Nilsen et al., 1997). Other repair factors are synthesized from a separate gene (Alseth et al., 1999) or undergo post-translational modification in response to cellular stress (Griffiths et al., 2009).

The functional organization of the BER machinery has been investigated in mammalian mitochondria. Several groups have shown that mtBER factors form a stable complex with the mtDNA (Gutman and Niyogi, 2009; Kamenisch et al., 2010) and are associated with the IM of mitochondria (Stuart et al., 2005). However, Akbari et. al. was unable to detect a stable Ung complex in mitochondria (Akbari et al., 2007). This dissertation describes in the following chapter a newly identified gene, *kansl2*, which may function to recruit and stabilize Ung in mitochondria. The functional study of *Kansl2* may enlighten us about the mtDNA repair and maintenance process in higher organisms, including humans. Such a repair phenomena is one of the most crucial processes in DNA maintenance in all species.

1.7 REFERENCES

1. **Akbari, M., Otterlei, M., Pena-Diaz, J. and Krokan, H. E.** (2007). Different organization of base excision repair of uracil in DNA in nuclei and mitochondria and selective upregulation of mitochondrial uracil-DNA glycosylase after oxidative stress. *Neuroscience* **145**, 1201-12.

2. **Akbari, M., Visnes, T., Krokan, H. E. and Otterlei, M.** (2008). Mitochondrial base excision repair of uracil and AP sites takes place by single-nucleotide insertion and long-patch DNA synthesis. *DNA Repair (Amst)* **7**, 605-16.
3. **Alam, T. I., Kanki, T., Muta, T., Ukaji, K., Abe, Y., Nakayama, H., Takio, K., Hamasaki, N. and Kang, D.** (2003). Human mitochondrial DNA is packaged with TFAM. *Nucleic Acids Res* **31**, 1640-5.
4. **Alexander, J., Stainier, D. Y. and Yelon, D.** (1998). Screening mosaic F1 females for mutations affecting zebrafish heart induction and patterning. *Dev Genet* **22**, 288-99.
5. **Alseth, I., Eide, L., Pirovano, M., Rognes, T., Seeberg, E. and Bjoras, M.** (1999). The *Saccharomyces cerevisiae* homologues of endonuclease III from *Escherichia coli*, Ntg1 and Ntg2, are both required for efficient repair of spontaneous and induced oxidative DNA damage in yeast. *Mol Cell Biol* **19**, 3779-87.
6. **Anderson, C. T. and Friedberg, E. C.** (1980). The presence of nuclear and mitochondrial uracil-DNA glycosylase in extracts of human KB cells. *Nucleic Acids Res* **8**, 875-88.
7. **Antoshechkin, I., Bogenhagen, D. F. and Mastrangelo, I. A.** (1997). The HMG-box mitochondrial transcription factor xl-mtTFA binds DNA as a tetramer to activate bidirectional transcription. *Embo J* **16**, 3198-206.
8. **Boesch, P., Weber-Lotfi, F., Ibrahim, N., Tarasenko, V., Cosset, A., Paulus, F., Lightowers, R. N. and Dietrich, A.** (2010). DNA repair in organelles: Pathways, organization, regulation, relevance in disease and aging. *Biochim Biophys Acta* **1813**, 186-200.
9. **Bradbury, J.** (2004). Small fish, big science. *PLoS Biol* **2**, E148.
10. **Brandon, M., Baldi, P. and Wallace, D. C.** (2006). Mitochondrial mutations in cancer. *Oncogene* **25**, 4647-62.
11. **Carrodeguas, J. A., Theis, K., Bogenhagen, D. F. and Kisker, C.** (2001). Crystal structure and deletion analysis show that the accessory subunit of mammalian DNA polymerase gamma, Pol gamma B, functions as a homodimer. *Mol Cell* **7**, 43-54.
12. **Chen, J. N., Haffter, P., Odenthal, J., Vogelsang, E., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Hammerschmidt, M., Heisenberg, C. P. et al.** (1996). Mutations affecting the cardiovascular system and other internal organs in zebrafish. *Development* **123**, 293-302.
13. **Clayton, D. A.** (1991). Replication and transcription of vertebrate mitochondrial DNA. *Annu Rev Cell Biol* **7**, 453-78.

14. **Croteau, D. L., Stierum, R. H. and Bohr, V. A.** (1999). Mitochondrial DNA repair pathways. *Mutat Res* **434**, 137-48.
15. **Dahme, T., Katus, H. A. and Rottbauer, W.** (2009). Fishing for the genetic basis of cardiovascular disease. *Dis Model Mech* **2**, 18-22.
16. **de Souza-Pinto, N. C., Eide, L., Hogue, B. A., Thybo, T., Stevnsner, T., Seeberg, E., Klungland, A. and Bohr, V. A.** (2001). Repair of 8-oxodeoxyguanosine lesions in mitochondrial dna depends on the oxoguanine dna glycosylase (OGG1) gene and 8-oxoguanine accumulates in the mitochondrial dna of OGG1-defective mice. *Cancer Res* **61**, 5378-81.
17. **Detmer, S. A. and Chan, D. C.** (2007). Functions and dysfunctions of mitochondrial dynamics. *Nat Rev Mol Cell Biol* **8**, 870-9.
18. **DiMauro, S. and Schon, E. A.** (2003). Mitochondrial respiratory-chain diseases. *N Engl J Med* **348**, 2656-68.
19. **Dizdaroglu, M., Jaruga, P., Birincioglu, M. and Rodriguez, H.** (2002). Free radical-induced damage to DNA: mechanisms and measurement. *Free Radic Biol Med* **32**, 1102-15.
20. **Draper, B. W., Morcos, P. A. and Kimmel, C. B.** (2001). Inhibition of zebrafish fgf8 pre-mRNA splicing with morpholino oligos: a quantifiable method for gene knockdown. *Genesis* **30**, 154-6.
21. **Ekstrand, M. I., Falkenberg, M., Rantanen, A., Park, C. B., Gaspari, M., Hultenby, K., Rustin, P., Gustafsson, C. M. and Larsson, N. G.** (2004). Mitochondrial transcription factor A regulates mtDNA copy number in mammals. *Hum Mol Genet* **13**, 935-44.
22. **Foran, D. R., Hixson, J. E. and Brown, W. M.** (1988). Comparisons of ape and human sequences that regulate mitochondrial DNA transcription and D-loop DNA synthesis. *Nucleic Acids Res* **16**, 5841-61.
23. **Fujikawa, K., Kamiya, H., Yakushiji, H., Fujii, Y., Nakabeppu, Y. and Kasai, H.** (1999). The oxidized forms of dATP are substrates for the human MutT homologue, the hMTH1 protein. *J Biol Chem* **274**, 18201-5.
24. **Galbiati, F., Volonte, D., Chu, J. B., Li, M., Fine, S. W., Fu, M., Bermudez, J., Pedemonte, M., Weidenheim, K. M., Pestell, R. G. et al.** (2000). Transgenic overexpression of caveolin-3 in skeletal muscle fibers induces a Duchenne-like muscular dystrophy phenotype. *Proc Natl Acad Sci U S A* **97**, 9689-94.

25. **Garrido, N., Griparic, L., Jokitalo, E., Wartiovaara, J., van der Blik, A. M. and Spelbrink, J. N.** (2003). Composition and dynamics of human mitochondrial nucleoids. *Mol Biol Cell* **14**, 1583-96.
26. **Griffiths, L. M., Swartzlander, D., Meadows, K. L., Wilkinson, K. D., Corbett, A. H. and Doetsch, P. W.** (2009). Dynamic compartmentalization of base excision repair proteins in response to nuclear and mitochondrial oxidative stress. *Mol Cell Biol* **29**, 794-807.
27. **Gu, M., Cooper, J. M., Taanman, J. W. and Schapira, A. H.** (1998). Mitochondrial DNA transmission of the mitochondrial defect in Parkinson's disease. *Ann Neurol* **44**, 177-86.
28. **Gutman, B. L. and Niyogi, K. K.** (2009). Evidence for base excision repair of oxidative DNA damage in chloroplasts of *Arabidopsis thaliana*. *J Biol Chem* **284**, 17006-12.
29. **Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., van Eeden, F. J., Jiang, Y. J., Heisenberg, C. P. et al.** (1996). The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development* **123**, 1-36.
30. **He, J., Mao, C. C., Reyes, A., Sembongi, H., Di Re, M., Granycome, C., Clippingdale, A. B., Fearnley, I. M., Harbour, M., Robinson, A. J. et al.** (2007). The AAA+ protein ATAD3 has displacement loop binding properties and is involved in mitochondrial nucleoid organization. *J Cell Biol* **176**, 141-6.
31. **Holt, I. J., Lorimer, H. E. and Jacobs, H. T.** (2000). Coupled leading- and lagging-strand synthesis of mammalian mitochondrial DNA. *Cell* **100**, 515-24.
32. **Hoppins, S., Lackner, L. and Nunnari, J.** (2007). The machines that divide and fuse mitochondria. *Annu Rev Biochem* **76**, 751-80.
33. **Hudson, E. K., Hogue, B. A., Souza-Pinto, N. C., Croteau, D. L., Anson, R. M., Bohr, V. A. and Hansford, R. G.** (1998). Age-associated change in mitochondrial DNA damage. *Free Radic Res* **29**, 573-9.
34. **Kaguni, L. S.** (2004). DNA polymerase gamma, the mitochondrial replicase. *Annu Rev Biochem* **73**, 293-320.
35. **Kamenisch, Y., Fousteri, M., Knoch, J., von Thaler, A. K., Fehrenbacher, B., Kato, H., Becker, T., Dolle, M. E., Kuiper, R., Majora, M. et al.** (2010). Proteins of nucleotide and base excision repair pathways interact in mitochondria to protect from loss of subcutaneous fat, a hallmark of aging. *J Exp Med* **207**, 379-90.

36. **Karahalil, B., de Souza-Pinto, N. C., Parsons, J. L., Elder, R. H. and Bohr, V. A.** (2003). Compromised incision of oxidized pyrimidines in liver mitochondria of mice deficient in NTH1 and OGG1 glycosylases. *J Biol Chem* **278**, 33701-7.
37. **Kasashima, K., Sumitani, M., Satoh, M. and Endo, H.** (2008). Human prohibitin 1 maintains the organization and stability of the mitochondrial nucleoids. *Exp Cell Res* **314**, 988-96.
38. **Kaufman, B. A., Newman, S. M., Hallberg, R. L., Slaughter, C. A., Perlman, P. S. and Butow, R. A.** (2000). In organello formaldehyde crosslinking of proteins to mtDNA: identification of bifunctional proteins. *Proc Natl Acad Sci U S A* **97**, 7772-7.
39. **Koehler, C. M.** (2004). New developments in mitochondrial assembly. *Annu Rev Cell Dev Biol* **20**, 309-35.
40. **Korhonen, J. A., Gaspari, M. and Falkenberg, M.** (2003). TWINKLE Has 5' -> 3' DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein. *J Biol Chem* **278**, 48627-32.
41. **Kukat, C., Wurm, C. A., Spahr, H., Falkenberg, M., Larsson, N. G. and Jakobs, S.** (2011). Super-resolution microscopy reveals that mammalian mitochondrial nucleoids have a uniform size and frequently contain a single copy of mtDNA. *Proc Natl Acad Sci U S A* **108**, 13534-9.
42. **Ladner, R. D. and Caradonna, S. J.** (1997). The human dUTPase gene encodes both nuclear and mitochondrial isoforms. Differential expression of the isoforms and characterization of a cDNA encoding the mitochondrial species. *J Biol Chem* **272**, 19072-80.
43. **Larsson, N. G.** (2010). Somatic mitochondrial DNA mutations in mammalian aging. *Annu Rev Biochem* **79**, 683-706.
44. **Larsson, N. G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G. S. and Clayton, D. A.** (1998). Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat Genet* **18**, 231-6.
45. **Leuenberger, D., Curran, S. P., Wong, D. and Koehler, C. M.** (2003). The role of Tim9p in the assembly of the TIM22 import complexes. *Traffic* **4**, 144-52.
46. **Liau, W. S., Gonzalez-Serricchio, A. S., Deshommès, C., Chin, K. and LaMunyon, C. W.** (2007). A persistent mitochondrial deletion reduces fitness and sperm performance in heteroplasmic populations of *C. elegans*. *BMC Genet* **8**, 8.
47. **Liu, P., Qian, L., Sung, J. S., de Souza-Pinto, N. C., Zheng, L., Bogenhagen, D. F., Bohr, V. A., Wilson, D. M., 3rd, Shen, B. and Demple, B.** (2008). Removal of

oxidative DNA damage via FEN1-dependent long-patch base excision repair in human cell mitochondria. *Mol Cell Biol* **28**, 4975-87.

48. **Longley, M. J., Nguyen, D., Kunkel, T. A. and Copeland, W. C.** (2001). The fidelity of human DNA polymerase gamma with and without exonucleolytic proofreading and the p55 accessory subunit. *J Biol Chem* **276**, 38555-62.
49. **McCord, J. M. and Fridovich, I.** (1988). Superoxide dismutase: the first twenty years (1968-1988). *Free Radic Biol Med* **5**, 363-9.
50. **Mitra, S., Izumi, T., Boldogh, I., Bhakat, K. K., Chattopadhyay, R. and Szczesny, B.** (2007). Intracellular trafficking and regulation of mammalian AP-endonuclease 1 (APE1), an essential DNA repair protein. *DNA Repair (Amst)* **6**, 461-9.
51. **Miyakawa, I., Sando, N., Kawano, S., Nakamura, S. and Kuroiwa, T.** (1987). Isolation of morphologically intact mitochondrial nucleoids from the yeast, *Saccharomyces cerevisiae*. *J Cell Sci* **88 (Pt 4)**, 431-9.
52. **Nagayoshi, S., Hayashi, E., Abe, G., Osato, N., Asakawa, K., Urasaki, A., Horikawa, K., Ikeo, K., Takeda, H. and Kawakami, K.** (2008). Insertional mutagenesis by the Tol2 transposon-mediated enhancer trap approach generated mutations in two developmental genes: *tcf7* and *synembryn*-like. *Development* **135**, 159-69.
53. **Nasevicius, A. and Ekker, S. C.** (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat Genet* **26**, 216-20.
54. **Nicholls, D. G., Bernson, V. S. and Heaton, G. M.** (1978). The identification of the component in the inner membrane of brown adipose tissue mitochondria responsible for regulating energy dissipation. *Experientia Suppl* **32**, 89-93.
55. **Nilsen, H., Otterlei, M., Haug, T., Solum, K., Nagelhus, T. A., Skorpen, F. and Krokan, H. E.** (1997). Nuclear and mitochondrial uracil-DNA glycosylases are generated by alternative splicing and transcription from different positions in the UNG gene. *Nucleic Acids Res* **25**, 750-5.
56. **Nishigaki, Y., Marti, R., Copeland, W. C. and Hirano, M.** (2003). Site-specific somatic mitochondrial DNA point mutations in patients with thymidine phosphorylase deficiency. *J Clin Invest* **111**, 1913-21.
57. **Parker, A., Gu, Y. and Lu, A. L.** (2000). Purification and characterization of a mammalian homolog of *Escherichia coli* MutY mismatch repair protein from calf liver mitochondria. *Nucleic Acids Res* **28**, 3206-15.
58. **Postlethwait, J. H., Woods, I. G., Ngo-Hazelett, P., Yan, Y. L., Kelly, P. D., Chu, F., Huang, H., Hill-Force, A. and Talbot, W. S.** (2000). Zebrafish comparative genomics and the origins of vertebrate chromosomes. *Genome Res* **10**, 1890-902.

59. **Roesch, K., Curran, S. P., Tranebjaerg, L. and Koehler, C. M.** (2002). Human deafness dystonia syndrome is caused by a defect in assembly of the DDP1/TIMM8a-TIMM13 complex. *Hum Mol Genet* **11**, 477-86.
60. **Rube, D. A. and van der Blik, A. M.** (2004). Mitochondrial morphology is dynamic and varied. *Mol Cell Biochem* **256-257**, 331-9.
61. **Shadel, G. S. and Clayton, D. A.** (1997). Mitochondrial DNA maintenance in vertebrates. *Annu Rev Biochem* **66**, 409-35.
62. **Shen, E. L. and Bogenhagen, D. F.** (2001). Developmentally-regulated packaging of mitochondrial DNA by the HMG-box protein mtTFA during *Xenopus* oogenesis. *Nucleic Acids Res* **29**, 2822-8.
63. **Simsek, D., Furda, A., Gao, Y., Artus, J., Brunet, E., Hadjantonakis, A. K., Van Houten, B., Shuman, S., McKinnon, P. J. and Jasin, M.** (2011). Crucial role for DNA ligase III in mitochondria but not in Xrcc1-dependent repair. *Nature* **471**, 245-8.
64. **Solnica-Krezel, L., Schier, A. F. and Driever, W.** (1994). Efficient recovery of ENU-induced mutations from the zebrafish germline. *Genetics* **136**, 1401-20.
65. **Stuart, J. A., Mayard, S., Hashiguchi, K., Souza-Pinto, N. C. and Bohr, V. A.** (2005). Localization of mitochondrial DNA base excision repair to an inner membrane-associated particulate fraction. *Nucleic Acids Res* **33**, 3722-32.
66. **Swartzlander, D. B., Griffiths, L. M., Lee, J., Degtyareva, N. P., Doetsch, P. W. and Corbett, A. H.** (2010). Regulation of base excision repair: Ntg1 nuclear and mitochondrial dynamic localization in response to genotoxic stress. *Nucleic Acids Res* **38**, 3963-74.
67. **Takao, M., Kanno, S., Shiromoto, T., Hasegawa, R., Ide, H., Ikeda, S., Sarker, A. H., Seki, S., Xing, J. Z., Le, X. C. et al.** (2002). Novel nuclear and mitochondrial glycosylases revealed by disruption of the mouse Nth1 gene encoding an endonuclease III homolog for repair of thymine glycols. *Embo J* **21**, 3486-93.
68. **Taylor, R. W., Giordano, C., Davidson, M. M., d'Amati, G., Bain, H., Hayes, C. M., Leonard, H., Barron, M. J., Casali, C., Santorelli, F. M. et al.** (2003). A homoplasmic mitochondrial transfer ribonucleic acid mutation as a cause of maternally inherited hypertrophic cardiomyopathy. *J Am Coll Cardiol* **41**, 1786-96.
69. **Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J. N., Rovio, A. T., Bruder, C. E., Bohlooly, Y. M., Gidlof, S., Oldfors, A., Wibom, R. et al.** (2004). Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* **429**, 417-23.

70. **Van Houten, B., Woshner, V. and Santos, J. H.** (2006). Role of mitochondrial DNA in toxic responses to oxidative stress. *DNA Repair (Amst)* **5**, 145-52.
71. **Vascotto, C., Bisetto, E., Li, M., Zeef, L. A., D'Ambrosio, C., Domenis, R., Comelli, M., Delneri, D., Scaloni, A., Altieri, F. et al.** (2011). Knock-in reconstitution studies reveal an unexpected role of Cys-65 in regulating APE1/Ref-1 subcellular trafficking and function. *Mol Biol Cell* **22**, 3887-901.
72. **Wallace, D. C.** (2001). Mouse models for mitochondrial disease. *Am J Med Genet* **106**, 71-93.
73. **Wallace, D. C.** (2005). A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu Rev Genet* **39**, 359-407.
74. **Weissman, L., Jo, D. G., Sorensen, M. M., de Souza-Pinto, N. C., Markesbery, W. R., Mattson, M. P. and Bohr, V. A.** (2007). Defective DNA base excision repair in brain from individuals with Alzheimer's disease and amnesic mild cognitive impairment. *Nucleic Acids Res* **35**, 5545-55.
75. **Wienholds, E., van Eeden, F., Kusters, M., Mudde, J., Plasterk, R. H. and Cuppen, E.** (2003). Efficient target-selected mutagenesis in zebrafish. *Genome Res* **13**, 2700-7.
76. **Zheng, L., Zhou, M., Guo, Z., Lu, H., Qian, L., Dai, H., Qiu, J., Yakubovskaya, E., Bogenhagen, D. F., Demple, B. et al.** (2008). Human DNA2 is a mitochondrial nuclease/helicase for efficient processing of DNA replication and repair intermediates. *Mol Cell* **32**, 325-36.
77. **Zon, L. I. and Peterson, R. T.** (2005). In vivo drug discovery in the zebrafish. *Nat Rev Drug Discov* **4**, 35-44.

CHAPTER 2

KANSL2 STABILIZES URACIL DNA GLYCOSYLASE IN THE MITOCHONDRIAL MATRIX

INTRODUCTION

Mitochondria are vital organelles that provide cellular energy to tissues via oxidative phosphorylation (OXPHOS). The mitochondrion contains a mitochondrial genome (mtDNA) that encodes a conserved subset of 13 proteins for OXPHOS components as well as ribosomal RNA and tRNAs for mitochondrial-based transcription. However, the proteins required for mtDNA replication, transcription and repair are encoded in the nuclear genome and imported into mitochondria from the cytosol. A cell contains multiple copies of the mitochondrial genome, with the number varying widely by cell type (Bogenhagen and Clayton, 1974; Robin and Wong, 1988). Unlike the nucleus, mitochondria lack histones to organize the mtDNA. Instead, the organizational unit of the mtDNA is the nucleoid that contains proteins that associate with mtDNA and links the genome to the inner membrane (Bogenhagen, 2011). Each nucleoid holds approximately 3 to 8 genomes (Brown et al., 2011). A diverse list of proteins in the nucleoid has been identified. The most abundant is the HMG-box protein transcription factor A (TFAM) that binds to the duplex DNA to package the mtDNA, and mitochondrial single-stranded DNA binding protein (mtSSB) that binds to single-stranded mtDNA (Fisher and Clayton, 1988; Parisi and Clayton, 1991; Van Tuyle and Pavco, 1985). Additional components include chaperones and

quality control proteases, mitochondrial ribosomal proteins and enzymes of lipid metabolism (Bogehagen, 2011).

Like the nucleus, mitochondria also have DNA repair pathways; however, they may not be as efficient as the nuclear system. Indeed, oxidative damage and the mutation load are higher for mtDNA than the nuclear genome (Hamilton et al., 2001; Michikawa et al., 1999). Elevated reactive oxygen species (ROS), potentially from OXPHOS, can lead to mtDNA lesions such as modified bases, abasic sites (AP, apurinic and apyrimidinic site) and double-strand breaks (Dizdaroglu et al., 2002; Evans et al., 2004). Oxidative stress-induced mtDNA lesions have been implicated in a myriad of mitochondrial diseases (Tuppen et al., 2010), neurodegenerative diseases (Swerdlow and Khan, 2009), cancer (Ishikawa et al., 2008) and aging (Gredilla et al., 2010).

The majority of oxidative lesions in mtDNA are repaired via the BER pathway. The initial step in BER is catalyzed by a DNA glycosylase to remove the damaged nucleotide; DNA glycosylases include uracil DNA glycosylase UNG1 (Boesch et al., 2009), 8-oxo-dG DNA glycosylase OGG1 (de Souza-Pinto et al., 2001) and endonuclease III NTH1 (Karahalil et al., 2003). The remaining abasic nucleotide (AP site) is also eliminated by a variety of lyases and AP endonucleases (Mitra et al., 2007). Insertion of a nucleotide (short-patch BER) or short patches of nucleotides (long-patch BER) by DNA polymerase gamma repairs the mismatch (Pinz and Bogehagen, 2000). For long-patch BER, the flap sequences are further removed by the flap endonuclease with the aid of a helicase (Copeland and Longley, 2008; Liu et al., 2008). Finally, DNA ligase III ligates the newly synthesized nucleotides to the remaining DNA, thereby completing the cycle (Lakshmiathy and Campbell, 2001).

How different factors are recruited to the damaged site is currently unknown. In addition, estimating the breadth of the repair factors localized to mitochondria has been difficult because the conditions to recruit repair factors may be specific, factors share dual localization with the nucleus, and factors may be low in abundance in the mitochondrion (Boesch et al., 2010). As an example, a single gene in humans encodes for the uracil DNA glycosylase, but alternative splicing driven from separate promoters generates the mitochondrial form UNG1 and the nuclear form UNG2 (Haug et al., 1998). As a result, human UNG1 is preferentially expressed in skeletal muscle, heart and testis, whereas UNG2 is enriched in proliferating tissues such as testis, placenta, colon and small intestine (Haug et al., 1998). Furthermore, how a defective mtDNA repair system impacts the development and maintenance of tissues remains largely unexplored. Here, we identify a new mtBER component, *kansl2*, from an enhancer trap screen in zebrafish. *Kansl2* localizes to the mitochondrial matrix and binds to Ung as well as mtDNA, *Kansl2* likely functions as a scaffold for Ung, because Ung failed to accumulate in mitochondria from the *kansl2* mutants. Because *kansl2* is essential for embryonic development, the BER pathway plays a critical role in tissue maintenance.

2.1 EXPERIMENTAL PROCEDURES

Lines and reagents – The insertion cassette that was expressed from the *edar* minimal promoter (Harris et al., 2008) and identification of TDL13 from a screen has been described (Asakawa et al., 2008; Levesque et al., 2012). The integration site was analyzed by inverse PCR and was further confirmed by PCR using the following primer set: sense, 5'-CTTCGGCGCACATAGTTGTTG; antisense, 5'-ATGAGTAAAGGAGAAGAA. The transgenic line described here was designated as *kansl2*^{TDL13Et}. The zebrafish colony was

maintained as previously described (Levesque et al., 2012). Lines included AB and Tuebingen. A mitochondrial targeted DsRed (designated MLSDsRed) was placed under control of the *cmhc2* and *α -actin1* promoters. The *kansl2* cDNA was amplified from EST clone (BC083416) (Open Biosystems) using the following primers: Sense, 5'-TCCCCGCGGTGTTGGTGCATTTTCGTTGG; antisense, 5'-TCCCCGCGGTACATCACTGAATTCCAG, and cloned into pcGlobin2 or PQCXIP-10XHisPC vector. PQCXIP-10XHisPC contains a Protein C epitope tag and 10XHis tag downstream of the multi-cloning site and was used for stable cell line establishment and protein purification (Claypool et al., 2008). HEK293 and HeLa cells were maintained in DMEM supplemented with 4mM glutamine, 10% fetal bovine serum, 50 U/ml of penicillin, 50 μ g/ml of streptomycin. PQCXIP-Kansl2-PC-10XHis stable cell line was generated as previously described (Wang et al., 2010).

To inhibit *kansl2* translation, morpholinos antisense oligonucleotides (Gene Tools) were synthesized for the ATG site (ATG-MO, 5'-GATCCTGTTTCATCACTCAGTTCTTC, complementary to the translation initiation region) and the splice site between exon 4 and 5 (Sp-MO, 5'-CATGCCTATAGAATCAAACCACACT, complementary to the exon 5 splicing acceptor region). 8 ng of Sp-MO and 0.8 ng of ATG-MO were injected as described (Draper et al., 2001). Total RNA or protein isolated from embryos at 3 dpf was subjected to either RT-PCR or immunoblot analysis to determine the MO efficacy. Primers used for RT-PCR are: *kansl2*, sense, 5'-GATGAGAGTTGGAGTGAAGAAGAGC; antisense, 5'-TTGGCTGCTGTCTTGGTAAATG; *β -actin1*, sense, 5'-CCCAGACATCAGGGAGTGAT; antisense, 5'-CACCGATCCAGACGGAGTAT. For immunoblotting, mitochondria from

morphants were isolated and quantified with a BCA protein assay kit (Thermo Scientific) prior to loading.

Acridine orange staining – Morphants were stained for 10-15 minutes in 0.5 µg/ml of acridine orange (Molecular Probes) in E3 buffer (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄), washed three to four times in E3, mounted and observed on a fluorescence microscope.

In-situ hybridization – WT embryos at 1 dpf were collected and fixed in 4% paraformaldehyde overnight at 4°C. Whole mount *in-situ* hybridization was performed on fixed embryos as described previously (Thisse et al., 1993). Full-length *kansl2* RNA sense and anti-sense probes were labeled with DIC (Roche) according to the manufacturer's protocols.

Protein purification – The Kansl2 Rad51-like domain (149-245), Kansl2 (43-407), and Ung was cloned into pET28a with the following primes: Kansl2 Rad51-like domain, Sense, 5'-ACGCGTCGACGCGGCTACAGTCGGCCTGAA, Antisense, 5'-AAGGAAAAAAGCGGCCGCTCTTCGTTCTCTGAGCTG; Kansl2 (43-407), Sense 5'-ACGCGTCGACCGCTGGAGTTCTGCATCAA, Antisense, 5'-AAGGAAAAAAGCGGCCGCTACATCACTGAATTCCAG; Ung, Sense, 5'-ACGCGTCGACGCATGATCGGACAGAAAAGC, Antisense, 5'-AAGGAAAAAAGCGGCCGCCAGAGCTTTCCAGTCAAT. Constructs were transformed into BL21 (DE3) and grown in LB supplemented with 50 µg/ml kanamycin. Protein expression was induced with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C for 2 hours. The protein was purified using Ni²⁺-NTA agarose (Qiagen) under native conditions as described by the manufacturer. The purified Kansl2 Rad51 domain was dialyzed in 1 mM sodium phosphate,

50 mM sodium fluoride, pH 6.8 and the secondary structure of the Kansl2 Rad51-like domain was characterized by circular dichroism analysis (Tienson et al., 2009).

Antibodies – A polyclonal antibody (Pacific Immunology) against the Kansl2 Rad51-like domain was generated in this study. Polyclonal antibodies against Tomm20 (SC-11415, Santa Cruz Biotechnology), cytochrome *c* (SC-13156, Santa Cruz Biotechnology), uracil DNA glycosylase (AB-13668, Abcam) and monoclonal Protein C antibody (11814508001, Roche Applied Science) were purchased.

Imaging – Images were taken using a Leica S8APO dissecting stereoscope and Leica MZ16F fluorescence microscope equipped with a Leica DFC280 color digital camera or Leica DFC360 FX high- resolution monochrome digital camera. Leica Firecam or Leica LAS software was used for image recording and manipulation. HeLa cells were observed using a Zeiss Axiovert 200M inverted microscope equipped with Hamamatsu ORCA-ER digital camera. Axiovision software was used for image capturing and manipulation. Images were assembled using Adobe Photoshop with only linear adjustments in brightness and contrast.

Mitochondria isolation – Yeast mitochondria were isolated as previously described (Glick and Pon, 1995). HEK293 or HeLa cells were collected and resuspended in mitochondria isolation buffer (20 mM HEPES, pH 7.5, 250 mM sucrose, 1 mM EDTA and 2.5 mM MgCl₂) supplemented with complete protease inhibitor cocktail (Roche). Suspended cells were dounced 40 times using a Teflon-glass dounce homogenizer. Unbroken cells and cellular debris were removed by centrifugation at 1000 x g for 10 min. To collect the mitochondrial fraction, the supernatant was subsequently pelleted at 20,000 x g for 10 min. Zebrafish mitochondria were obtained from approximately 100 4-6 dpf embryos. Embryos were resuspended in mitochondria isolation buffer and purified as described for cultured cells.

Mitochondrial import assays – Detailed methodology for mitochondrial import assays is essentially as previously described (Koehler et al., 1998a).

Electron microscopy – Zebrafish embryos were processed essentially as previously described. Briefly, the embryos were isolated and fixed in 3% formaldehyde, 1.5% glutaraldehyde contained in 0.1 M sodium cacodylate, pH 7.4, 5 mM CaCl₂, 5 mM MgCl₂, and 2.5% sucrose for 1 hour at 22°C with gentle agitation. The specific organs/tissue were trimmed into small blocks (~1-2mm in square). The samples were then post-fixed in Palade's OsO₄ for 1hr at 4°C; en bloc stained in Kellenberger's uranyl acetate (UA) overnight; dehydrated through a graded series of ethanol; and subsequently embedded in Epon resin. Sections were cut on a Leica Ultracut UCT ultramicrotome; post-stained with UA and lead citrate, and observed on an FEI Tecnai 12 at 100kV. Images were recorded with a Soft Imaging System Megaview III digital camera, and figures were assembled in Adobe Photoshop 7.0 with only linear adjustments in brightness and contrast.

Cytochrome oxidase and succinate dehydrogenase staining – To detect Complex IV (cytochrome oxidase) activity, embryos were incubated in buffer (5 mM potassium phosphate, 0.1% 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.1% cytochrome *c*, 0.02% catalase, pH 7.4) overnight at 4°C and a control reaction with inhibitor (10 mM KCN) was included. For Complex II (succinate dehydrogenase), the embryos were incubated in buffer (5 mM potassium phosphate, 5 mM EDTA, 1 mM KCN, 0.2 mM phenazine methasulfate, 20 mM sodium succinate, 200 µg/ml nitro blue tetrazolium (NBT), pH 7.6) overnight at 4°C and a control reaction with inhibitors (20 mM malonate and 20 mM malate) was included.

DNA gel shift assay – Juveniles mitochondria were incubated in DNA extraction buffer (10 mM Tris, pH 8.2, 10 mM EDTA, 200 mM NaCl, 0.5% SDS) in the presence of 200 µg/ml of

proteinase K at 50°C overnight. The DNA was subsequently precipitated with ethanol, washed, dried and resuspended in sterile deionized water. 0.1-0.5µg DNA was incubated with the recombinant protein at various concentrations in the DNA gel shift buffer (1 mM DTT, 1 mM EDTA, 100 mM MgCl₂, 40 mM HEPES, pH 7.5) overnight at room temperature. The mix was loaded onto 0.8-1% ethidium bromide agarose gels and visualized in an ultraviolet transilluminator chamber (Alpha Innotech Corporation).

2D clear native PAGE (CNPAGE)/SDS-PAGE – Mitochondria isolated from 4-5 dpf embryos were solubilized with 2% digitonin on ice for 30 min. Solubilized fraction was pre-cleared by centrifuging at 20,000 x g for 10 min. Pre-cleared total mitochondrial extract was quantified by BCA assay, diluted in CNPAGE buffer (5% glycerol, 0.01% Ponceau) and loaded onto a 3-12% non-denaturing polyacrylamide gradient gel as previously described (Schagger et al., 1994). The native molecular marker (GE Healthcare) was loaded along with the samples to mark the size of each native complex. Gel was run overnight at 4°C at 100 constant voltage. Gel slice corresponded to the protein lane was excised and subjected to a second dimension 12% denaturing SDS-PAGE. Protein bands were resolved using western blot with indicated antibodies.

Uracil incision assay – The cleavage of a 30-mer 5'-[P32]-end labeled oligonucleotide duplex containing a single uracil residue was assessed essentially as described (Maynard et al., 2010). Approximately 0.8 µg of purified Kans12 and Ung were incubated with the substrate at various time points at 37°C. Reaction products were ran on a 15% (w/v) denaturing polyacrylamide gel (7 M urea, 1 X TBE), followed by autoradiography.

2.2 RESULTS

The enhancer trap mutagenesis screen identified the zebrafish gene, kansl2 – From an enhancer trap mutagenesis screen using a GFP reporter (Fig. 1A) that has been described in detail by Levesque et. al. (Levesque et al., 2012), we identified a zebrafish line, designated *TDL13* (Tuebingen Driver Line), showing strong GFP expression in the muscle and heart (Fig. 1B). *TDL13* fish exhibited developmental defects that appeared in a Mendelian frequency, suggesting that a single genetic locus with essential roles in development was disrupted by the insertion. Through inverse PCR and subsequent genotyping of carriers and mutants, we identified an insertion site linked with the mutant phenotype (Fig. 1C,D). Cloning the flanking sequence allowed us to identify that *TDL13* harbored a single-copy insertion of the enhancer trap cassette in the 5' UTR of *kansl2*, and the integration site was 22 nucleotides upstream of exon 1, as verified by PCR and sequencing (Fig. 1C,D).

kansl2 (NSL2/FLJ20436) was first identified in pull-down/mass spectrometry experiments as one of 9 subunits in the lysine acyltransferase 8 (Kat8/MOF/MYST1) non-specific lethal (NSL) regulatory complex in *Drosophila* (Cai et al., 2010; Raja et al., 2010). *kansl2* encodes a 506 amino acid protein with a predicted mitochondrial targeting sequence (MTS) at its N-terminus (Claros and Vincens, 1996) and amino acids 169-226 have weak similarity to the Rad51 N-terminal domain-like fold (Fig. 1E) (Gough et al., 2001). In zebrafish, two isoforms are reported; the second isoform possesses a truncated C-terminus (Fig. 1E). The protein is conserved across species from flies to vertebrates and shares 65% identity to its human homolog (Fig. S1); however, Kansl2 activity remains to be characterized, including roles in mitochondrial function and in regulating development.

Loss of Kansl2 leads to heart and muscle defects in developing zebrafish – The developmental phenotype of the TDL insertion in *kansl2* suggests that it may have a specific role in regulating development through its function in mitochondria. The phenotype segregation correlated perfectly with the presence of the GFP transgene, with marked defects in cardiac and muscle development (Fig. 2A). Incrossing *TDL13* resulted in mutants with an average lifespan of 5.9 ± 0.2 days (n=24), indicating the insertion resulted in lethality. However, it is not clear if the *TDL13* insertion abrogated *kansl2* expression or another gene linked to the locus. RT-PCR analysis confirmed that the *kansl2* cDNA transcript was absent in the affected embryos, but present in the unaffected siblings (Fig. 2B) These findings were also supported by our analysis of Kansl2 protein in mutants showing the absence of a protein product specifically in the *TDL13* mutants (Fig. 2C). Thus, the *TDL13* insertional mutant lacks Kansl2.

To confirm that inhibition of *kansl2* expression underlies phenotypes observed in the enhancer trap line, we specifically knocked down *kansl2* translation by injection of translational-blocking (ATG-MO) and splicing-blocking (Sp-MO) morpholinos. As expected, the Sp-MO blocks removal of intron 4, generating a transcript that is 79 bp longer (Fig. 2D,E); additionally, the Kansl2 protein was not detected in morphants treated with ATG-MO (Fig. 2D,F). Both ATG-MO (218/241; 90%) and Sp-MO (53/61; 87%) injections yielded mutants with heart and somite defects, similar to the *TDL13* insertional line (Fig. 2D, Table 1); however, the Sp-MO was less efficient. The phenotype of the ATG-MO morphants was rescued by co-injection of the zebrafish *kansl2* mRNA in half of the embryos (Table 1). *kansl2* showed ubiquitous expression during embryonic development (Fig. S2A,C) and in adult tissues, including brain, eye, heart and muscle (Fig. S2B). Because the *TDL13* line and the morphants failed to survive, the ATG-MO morphants (n=4) were stained with acridine orange at 1 dpf to assess cell death (Fig. 2G).

Depletion of *Kansl2* resulted in an increased load of apoptotic cells as shown by more intense staining with acridine orange. Therefore, MO injection phenocopies the *TDL13* insertional mutant and co-injection of the *kansl2* mRNA rescues the morphant phenotype. *TDL13* is thus an allele of *kansl2* (*kansl2*^{*TDL13Et*}).

Because *Kansl2* contained a putative MLS, we postulated that the developmental defects in *kansl2*^{*TDL13Et*} may be due to an essential role in mitochondrial function. The *kansl2*^{*TDL13Et*} mutant shows prominent hydrocephaly, edema and defects in the formation of the heart and muscles along the body axis (Fig. 2A). As the phenotypes seen in *kansl2*^{*TDL13Et*} were predominantly seen in the muscle and heart of larva, we looked at the mitochondrial phenotypes using transgenic lines in which DsRed is targeted to the mitochondria and expressed from tissue-specific promoters for heart (Tg(*cmlc2:MLSDsRed*)) and muscle (Tg(α -*actin1:MLSDsRed*)) (Higashijima et al., 1997; Mably et al., 2003). In the Tg(α -*actin1:MLSDsRed*) line, organization of the hypoxial myotome region was obviously disrupted in *kansl2*^{*TDL13Et*} mutant compared to unaffected siblings (n=6) (Fig. 3A). In the Tg(*cmlc2:MLSDsRed*) line, the expression highlights patterning defects in the ventricle and extension that is associated with cardiac edema (n=8) (Fig. 3B). Both of these phenotypes are consistent with developmental delay and general growth defects (Haffter et al., 1996; Kimmel et al., 1995).

Kansl2 localizes to the mitochondrial matrix – To investigate the potential role of *Kansl2* in mitochondria, we appended GFP to the C-terminus of *Kansl2* and transiently transfected HeLa cells to test if *Kansl2* localizes to mitochondria; a MitoDsRed construct was included as a control for localization (Griparic et al., 2007). Overexpression of tagged *Kansl2* localized to mitochondria that overlapped with MLSDsRed (Fig. 4A). We also tagged *Kansl2* at the C-terminus with 10XHis-Protein C (HisPC) to allow sequential purification over Ni²⁺ agarose and

protein C resin (Claypool et al., 2008); stable HEK293 cell lines expressing Kansl2-HisPC were generated. This cell line was fractionated crudely into mitochondria (M) and post-mitochondrial supernatant (S) fractions followed by immunoblotting with an antibody against the PC tag and control cytochrome *c* (cyt *c*). Kansl2 and cyt *c* were detected in the mitochondrial fraction (Fig. 4B). Because positively-charged arginine and lysine residues in the MLS are critical for mitochondrial targeting (Habib et al., 2007), the arginine residues were changed to alanine residues. Radiolabeled precursors were imported into isolated yeast mitochondria, because import pathways are highly conserved across species (Hoogenraad et al., 2002). Whereas Kansl2 translocated into mitochondria in a membrane potential-dependent manner, mutation of the arginine residues subsequently abolished import (Fig. S3). Thus, Kansl2 is a mitochondrial protein that is imported via its N-terminal targeting sequence.

We also localized Kansl2 within yeast mitochondria using *in organello* import assay with additional manipulations. After import of Kansl2, the isolated mitochondria were subjected to osmotic shock to selectively disrupt the outer membrane, thereby generating mitoplasts that contain the intact matrix (Koehler et al., 1998b). Kansl2 was imported into mitochondria (M) and purified with the mitoplast (MP) fraction (Fig. 4C). When the mitoplasts were treated with protease, Kansl2 and matrix control Hsp60 were protected, supporting residency in the matrix; in contrast, the intermembrane space control Mia40 was degraded. As Kansl2 may be an integral membrane protein, the import assays were followed by carbonate extraction (Fujiki et al., 1982) and membrane proteins were recovered in the pellet fraction (Fig. 4D). Kansl2 and soluble Hsp60 were recovered in the supernatant (S) in contrast to membrane anchored Mia40 that was recovered in the pellet fraction (P). Thus, Kansl2 is a soluble mitochondrial matrix protein.

Kansl2 deficiency affects mitochondrial morphology and respiration – To investigate mitochondrial ultrastructure, we performed electron microscopy (EM) analysis on 4 dpf embryos that were fixed and sectioned. Mitochondria in the *kansl2*^{TDL13Et} mutant were rounded and swollen with less ordered cristae in contrast to the typical ordered cristae structure of mitochondria in unaffected siblings (Fig. 5A). As *Kansl2* resides in the mitochondrial matrix, we investigated the bioenergetics of the *Kansl2* mutant. Histochemical analysis of Complex IV (cytochrome oxidase) and Complex II (succinate dehydrogenase) activity was investigated in 4 dpf embryos (Tanji et al., 2008). Complex IV-positive cells stained brown in unaffected siblings, but activity was markedly decreased in the *kansl2*^{TDL13Et} mutant (n=10) (Fig. 5B). Similar data was found for Complex II activity (n=5) (Fig 5C). Thus, *Kansl2* deficiency impairs mitochondrial ultrastructure and respiratory function.

Kansl2 possesses a unique DNA binding domain and resides in the same complex as *Ung* in mitochondria – Other than copurifying with a histone acetyltransferase in flies (Cai et al., 2010), an obvious function has not been assigned to *Kansl2*. In addition, the *Drosophila* *Kansl2* lacks a typical mitochondrial targeting sequence (Fig. S1) and may therefore reside in a different location and have a different function. Because the Rad51 N-terminal domain-like fold binds to DNA (Aihara et al., 1999), we tested if this domain (amino acids 169-226) of *Kansl2* has DNA binding properties *in vitro*. Recombinant Rad51-like domain was purified under native conditions (Fig. S4A,B) and was mostly alpha-helical as assessed with circular dichroism analysis (Fig. S4C). We performed a series of DNA mobility shift experiments with various DNA substrates, a 1kb DNA ladder, the Displacement (D)-loop region of mtDNA, and isolated wildtype fish mtDNA. The Rad51 N-terminal domain-like fold bound to all substrates in a dose-dependent fashion, retarding the mobility of the DNA in the agarose gel (Fig. 6). In contrast,

negative control BSA showed no DNA binding properties. Thus, Kansl2 possesses general DNA binding properties.

Because Kansl2 associates with mtDNA, we used a candidate approach to identify partner proteins by co-immunoprecipitation. A potential candidate, mitochondrial DNA polymerase gamma, did not co-immunoprecipitate with Kansl2 (data not shown). We also investigated association with the BER protein Ung because the human UNG1 shares an expression profile with Kansl2 (Haug et al., 1998). Ung co-immunoprecipitated with Kansl2 in both zebrafish (Fig. 7A) and HEK293 cells (data not shown). As a negative control, Tomm20 did not purify with Kansl2. Moreover, in *kansl2*^{TDL13Et} mutants that lack Kansl2, Ung was not detectable, indicating that Ung may require Kansl2 for translocation or stability in mitochondria (Fig. 7B). Using colorless-native PAGE in the first dimension followed by SDS-PAGE in the second dimension and immunoblot analysis, we probed the size of Kansl2 and Ung complexes in mitochondria. Both Ung and Kansl2 co-migrated in a similar-size complex of 300 kDa (Fig. 7C).

As Kansl2 and Ung are partner proteins, we tested whether Ung activity may require Kansl2. The uracil incision activity with a double-stranded oligonucleotide containing a single uracil mismatch was assayed *in vitro* with recombinant Ung and Kansl2 (Fig. S5) (Maynard et al., 2010). Ung cleaved the oligonucleotide in a time-dependent manner (Fig. 7D). Whereas Kansl2 did not contain uracil incision activity, Kansl2 addition to Ung enhanced the rate at which Ung cleaved the substrate (Fig. 7D). Collectively, these data support that Kansl2 and Ung form a complex in the mitochondrial matrix and that Kansl2 enhances the activity of Ung in catalyzing the removal of uracil in mismatched DNA.

2.3 DISCUSSION

Using zebrafish as a model, we identify a gene that is required for normal development. Insertion of a transgene in the 5' UTR of *kansl2* leads to developmental defects that correlate with the lack of Kansl2 protein expression. As both morpholino-mediated knockdown and the insertion lead to similar phenotypes, we feel that the observed defects are specific to Kansl2 depletion.

Mitochondria possess elaborate BER machinery for maintaining mitochondrial genome integrity (Boesch et al., 2010). Several groups have suggested a functional organization of the BER machinery in the mitochondrial inner membrane (Akbari et al., 2007; Stuart et al., 2005). Previously, Akbari et al. (Akbari et al., 2007) was unsuccessful in identifying a stable Ung complex in the mitochondria. Using biochemical approach, we showed that Kansl2 resides in a 300 kDa complex with Ung and may function as a scaffold protein. In addition, loss of Kansl2 resulted in marked decrease in Ung in mitochondria and the two proteins co-immunoprecipitated. Given that Kansl2 contains a typical domain that binds DNA, we confirmed that Kansl2 indeed displays general DNA binding properties. Moreover, Kansl2 increases the activity of Ung in an *in vitro* uracil incision assay, suggesting that Kansl2 may also function in BER. Together, this analysis supports a role for Kansl2 as a scaffold for Ung in BER. Additional factors in the repair pathway may also bind to Kansl2 but await further investigation.

Kansl2 was first identified by *Drosophila* with a likely localization in the nucleus (Cai et al., 2010; Raja et al., 2010). Given that numerous DNA repair enzymes show dual localization to nucleus and mitochondria, we cannot exclude a role for Kansl2 in the nucleus. However, fractionation studies favor a major role for Kansl2 in the mitochondrion. Kansl2 depletion in zebrafish leads to lethality by 6 dpf. The mutant was first selected for the strong GFP expression

in muscle. However, general development seems impaired with notable defects in muscle, heart and nervous system. This likely reflects the essential function for mitochondria in all tissues. Not unexpected, Kansl2, like the typical cast of mitochondrial proteins, may be important for maintenance of tissues with high energetic requirements, such as heart, muscle and neural system (Wallace, 1999).

The specific function of Kansl2 requires additional biochemical investigation, which is limited in zebrafish because mitochondrial manipulations are difficult. Mitochondrial energetic and morphology were compromised in the homozygous mutant, but this is likely a secondary defect associated with the essential function of Kansl2 in mitochondria, as this scenario typically occurs when essential mitochondrial proteins are impaired (Chen et al., 2006). Surprisingly, mtDNA damage was not detected in the *kansl2*^{TDL13Et} mutant (data not shown). However, this may not be unexpected, because mice lacking UNG1 and OGG1 are viable and mutations in mtDNA were difficult to detect (Klungland et al., 1999; Nilsen et al., 2000). In addition, Ung abundance in the *kansl2*^{TDL13Et} mutant may be present at a very low threshold that is adequate for mtDNA repair. Alternatively, Kansl2 may have additional function required for mitochondrial function. In summary, our genetic approach has revealed a new mitochondrial protein Kansl2 that partners with Ung, potentially increasing Ung activity in the BER pathway *in vivo*. Importantly, our analysis indicates that zebrafish embryos are potential model for future investigations to dissect the BER pathway in mitochondria.

TABLE 1. Phenotypic analysis of morpholino injection and rescue by co-injection of the *kansl2* RNA.

Phenotype	Control	ATG-MO	ATG-MO Rescue	Sp-MO	Sp-MO Rescue
Normal	351	23	62	8	N.D. ^b
Mutant Phenotype ^a	28	218	45	53	N.D. ^b

^aThe mutant phenotype represents embryos with heart and somites defects.

^bN.D., not determined.

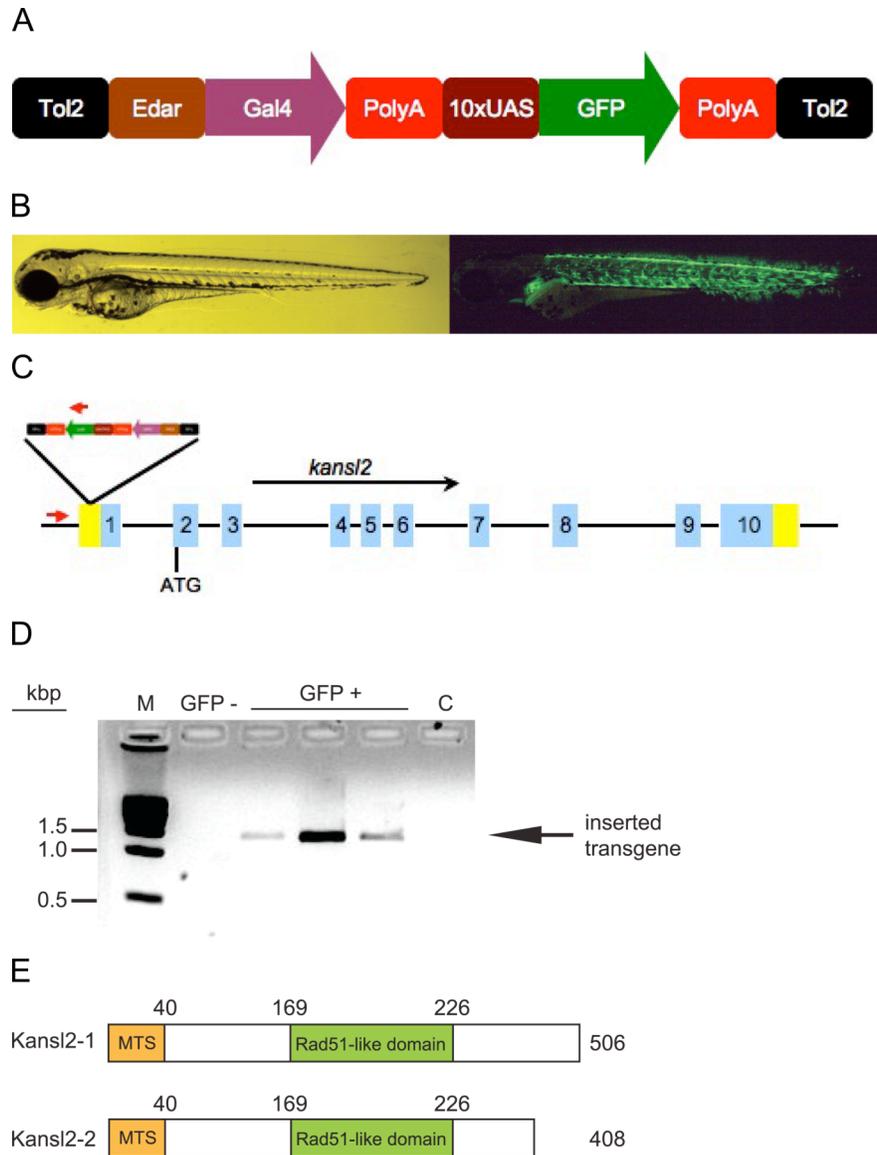


Figure 2.1. The enhancer trap screen identified *kansl2*. A. Schematic representation of the enhancer trap cassette. B. The enhancer trap line, *TDL13*, has strong GFP expression in muscle and heart at 4 dpf. Left: bright field image; right: GFP fluorescence image C. *TDL13* carries a single-copy insertion within the 5' UTR of the *kansl2* (arrow). ATG start site, exons (blue boxes), introns (solid lines) and UTRs (yellow boxes) are shown. The primer set used for genotyping is indicated with red arrows. All annotations are based on Ensembl (<http://www.ensembl.org/index.html>). D. Genotypic analysis on single embryos by PCR with the primer set shown in 'C'. Larvae carrying the insertion displayed a 1.3 kbp band (Lane 3-6). Lane 2 shows WT embryos that were negative for GFP staining (GFP-). A PCR control (C) lacking DNA is included. 'M' is the 1 kb DNA marker (lane 1). E. Schematic representation of zebrafish Kansl2 isoforms. Mitochondrial targeting sequence (MTS) marked in yellow and the Rad51 N-terminal domain-like fold is marked with green. The size of each isoforms is indicated.

```

hKans12      1  ---MNRIRIHVLPFNRRGRITPVPRSQEP-----LSCAFTHRPCS
zKans12      1  ---MNRIRIHVLPSSRGRVTQAARAQEP-----QTCSYTORPCS
dgt1         1  MSSFSPNQLRQCARTFTGTPCTPDKEHQLRREQIHSELVKIKYCANPTYECS

hKans12     37  HPRLEGQEFCKIHILEDKNAPFKQCSYVSTKNGKRCFNAAFKPEKDD---
zKans12     37  QPRLEGLEFCIKHILEDKNAPYRQCSYVSNKNGKRCFNAAFKPEKKE---
dgt1        51  LMRTEGYEYCI RHILRDPRSNFRQCSHVYS-NGRKCINAVAKYDNKKELI

hKans12     84  GVSFCAEHVRRNALAL-HAQMKKTNP-GPVGETLLCOLSSYA-----
zKans12     84  SVSFCAEHARRNALVQ-QAQMRKASASGPSPEVLLSQLSGYS-----
dgt1       100  LTTLCFEHNROTQLQKTHMNVGRIRSRVETNETLLHSLSSHINVEDIKPE

hKans12    124  -----KTELGSQTPESSRSEASRILDEDSWSDGEOEPITVD
zKans12    125  -----RPEPCAHSQEG-HSEASRILDEDSWSEEQDPVVLDD
dgt1       150  VKKIERDDDEIDVVSEHVTPFVTQDHRNSIGHVVESSRKKRRRILDYASDS

hKans12    160  QTWRGDP-----DSEADSIDSDQEDPLKHAGVYTAEVALIM
zKans12    160  QTWRGDP-----DSEAESIDSDHEDPLKHAGVYTAEVALIT
dgt1       200  SSNDADPPCLRNTAQDI EFYESDHESIDSEDDDPLKHAGVFTRAEAVKIA

hKans12    197  REKLIRLQSLYIDQFKRLQHLKKEKRRYLHNRRKVEHEALGSSLLTGPEG
zKans12    197  REKLIRLQSLYIDQFKRLQHLKKEKRRYLHSRKIEHETIGSSLLTGPEG
dgt1       250  ETKLIRLQGLYREQISYLVQNVLRQRRRYLHDIRRERELYCS--IHDQLK

hKans12    247  LAKERENLKRRLKCLRRYRQRYGVEALLHRQLKERRMLATDGAAQQAHTT
zKans12    247  LSMKERENLKRRLKALRRYRRRYGVEALLHRQLRERRQATEGGPTQA--M
dgt1       298  DTPHERKLYEQLKALNSYHRRQGVEAVLYKKLKEKRARDALYAAKSS---

hKans12    297  RSSORCLAFVDDVRCASNQSLPMTRECLTHICQDTNQVLFKCCQGSEE-VP
zKans12    295  RLGQRCISFVEGTRCSNQCLPMTRHCVSHIYQDSSQVLFKLCPLGLKD-VP
dgt1       345  -SNPKCVFTEGGVRCGERTLPCCRHRKHILEDKRVLFRACGVRSRGV

hKans12    346  CNKPVVVSLSSEDFCCPLHFOLPPQNYKPEQVLSVPDDLEAGPMDLYLSAA
zKans12    344  CDRPVHMGQSEEPKRCPLHLSLPPQNYQPEQESLAPEQLASAPTDMYLSAA
dgt1       394  CQEPVVAS-ILEDSSCVLHLTVAPAKRQYIQKKCESETEBEETQSTSLIST

hKans12    396  ELQPTESLPLEFSDDDLVDVGDGMQCPPSPLLFDPSLTLEDHLLVKEIAEDP
zKans12    394  ELQPTENLPLEFSDDDLVEDEGLQCPPSPLLFDTALALEDOTIREIAEAP
dgt1       443  IKKEEDP-----SDKLYTSQALNV

hKans12    446  VDILGOMQMGDGCRSQGSRNSEKASAPLSQS-----GLATANG
zKans12    444  MDILTGAEQGDLDASAQDAELSERDEVSVEDSVDTEILEAVEQAIPMQNS
dgt1       463  TTAHGEDNKGDVKPREYAPNRS-----

hKans12    485  KPEPTSIS-----
zKans12    494  SKDSDTKTTDALS-----
dgt1

```

Figure 2.2. Protein sequence alignment of the human, zebrafish and Drosophila (dgt1) orthologs. Sequence alignment was obtained using the ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and Boxshade (http://www.ch.embnet.org/software/BOX_form.html) programs. The mitochondrial targeting sequence of human and zebrafish (red) and the Rad51 N-terminus domain-like fold (green) are underlined. Highly conserved residues are highlighted.

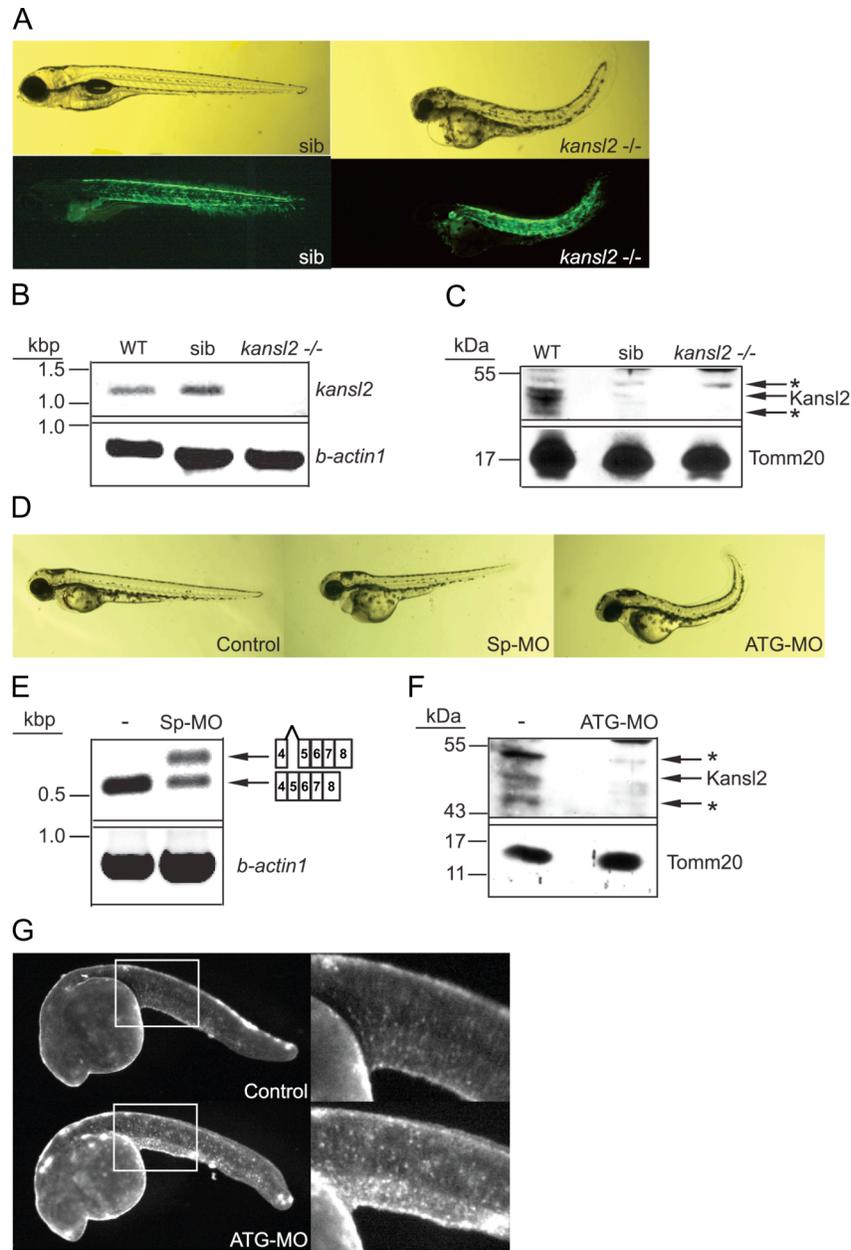


Figure 2.3. Loss of Kansl2 results in cardiac and somite defects. A. Analysis of 5 dpf embryos from an incross of *kansl2*^{TDL13Et} line. Bright field (top) and GFP fluorescence images (bottom) are shown. B. RT-PCR analysis with primers for the *kansl2* cDNA in wildtype siblings (WT), siblings (sib) and *kansl2* ^{-/-} mutant embryos. *β-actin1* is included as a control. C. Immunoblot analysis with a polyclonal antibody against Kansl2 in WT, sib and *kansl2* ^{-/-} embryos. *β-actin1* is included as a control. D. Phenotypic analysis of embryos injected with 8 ng Sp-MO (n=61) or 0.8 ng ATG-MO (n=241). Morphants were imaged at 3 dpf. E. RT-PCR analysis of the transcript generated by Sp-MO injection from single embryos. F. As in ‘C’, immunoblot analysis was used to detect the Kansl2 protein in ATG-MO morphants. G. Acridine orange staining of 1 dpf ATG-MO morphants (n=4). The right panel shows the enlarged boxed regions. Punctate staining marks apoptotic cells.

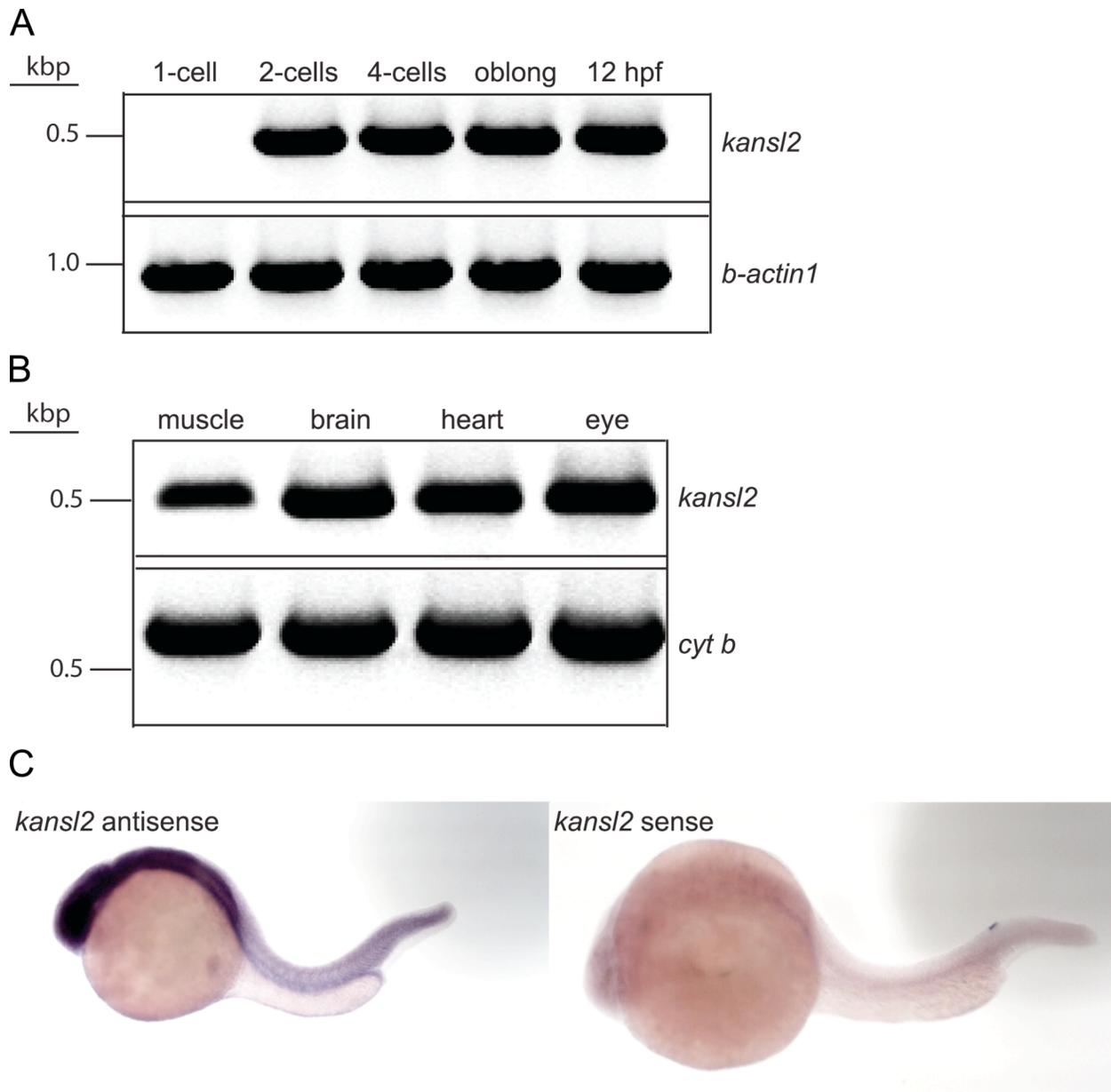


Figure 2.4. The *kansl2* transcript is ubiquitously expressed. A. RT-PCR analysis of the *kansl2* transcript in WT embryos at various stages of development (1-cell, 2-cell, 4-cell, oblong and 12-hpf). *β-actin1* was used as control. B. RT-PCR analysis with the primer set as in ‘A’ for tissues from adult WT fish. *Cytochrome b* (*cyt b*) was included as control. C. In-situ hybridization with a 1.3 kbp *kansl2* RNA probe at 1 dpf (n=5). Right panel is the negative control with the sense RNA probe.

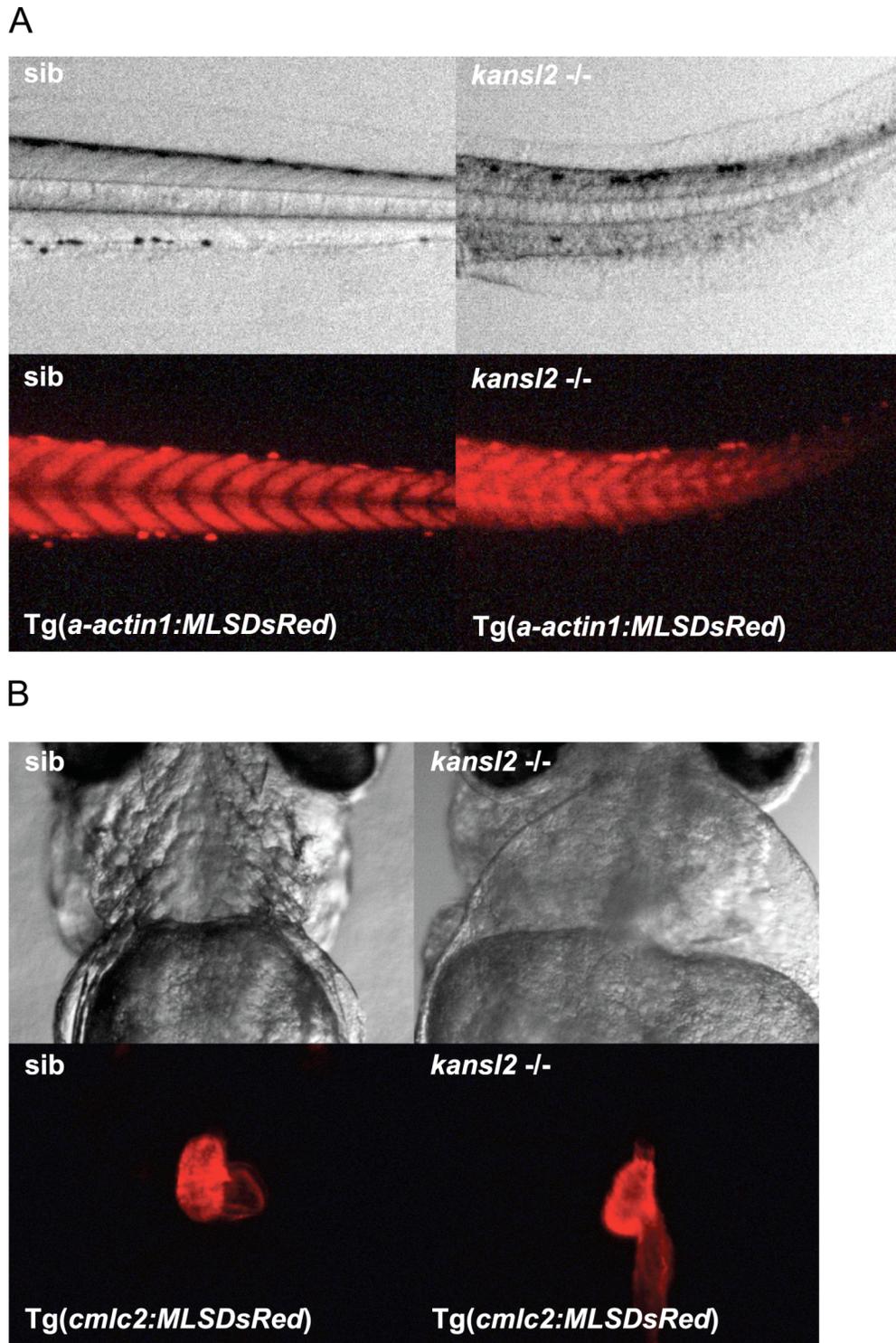


Figure 2.5. *kansl2* mutant exhibits abnormal patterning in the heart and somites. Phenotypic analysis of 4 dpf sib and *kansl2*^{-/-} embryos expressing transgenes A. *cmlc2:MLSDsRed* or B. *a-actin1:MLSDsRed*. MLSDsRed is a mitochondrial-targeted DsRed. Top: brightfield image; bottom: fluorescent image.

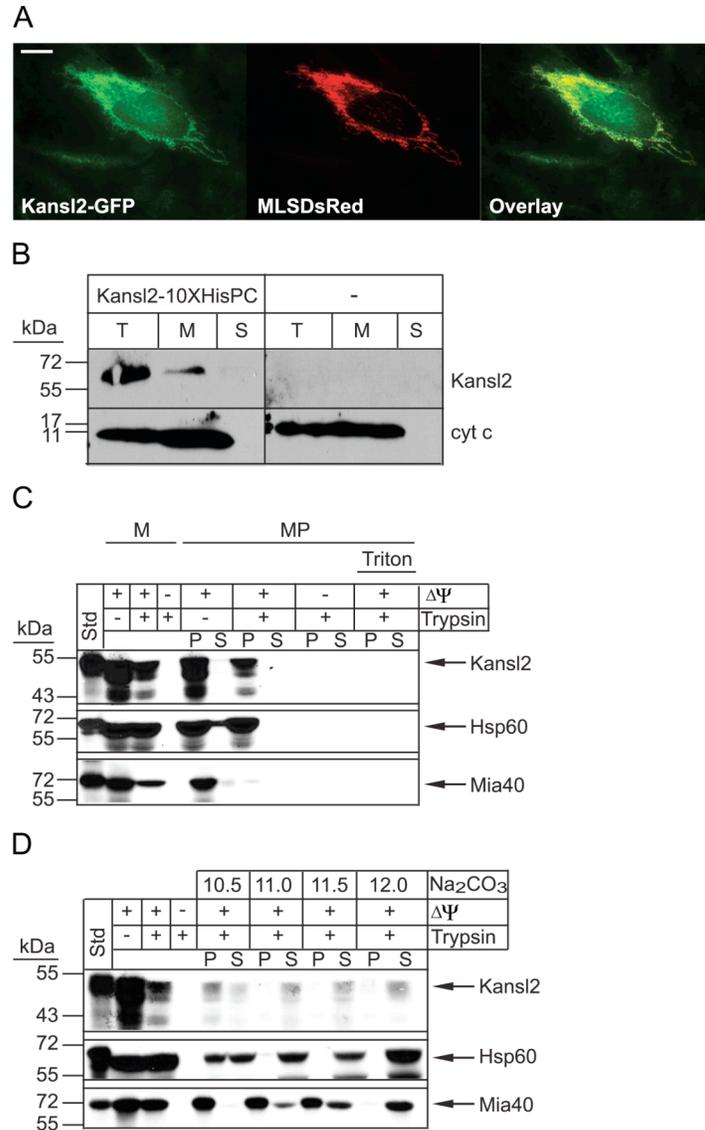


Figure 2.6. Kansl2 localizes to the mitochondrial matrix. A. Fluorescent image of a HeLa cell transfected with both Kansl2-GFP and mitochondrial-targeted DsRed (MLSDsRed). Overlay represents the merged image. Scale bar: 10 μm . B. Immunoblot of HisPC-tagged Kansl2 expressed in HEK293 cells. Cells were fractionated into total (T), mitochondria (M) and cytosolic (S) fractions. Cyt c was included as the control and '-' is a negative control of untransfected cells. Kansl2-HisPC was blotted with a monoclonal antibody against the Protein C tag. C. Radiolabeled Kansl2 was imported into yeast mitochondria (M) in the presence or absence of a membrane potential ($\Delta\Psi$) and mitoplasts (MP) were generated by osmotic shock, which were recovered by centrifugation in the pellet (P); the supernatant (S) contained the soluble intermembrane space fraction. Trypsin treatment removed IMS-facing proteins. Triton X-100 addition verified that proteins were trypsin-sensitive. Controls include matrix Hsp60 and intermembrane space Mia40. D. Kansl2 was imported as in 'C' and samples were subjected to carbonate extraction (Na_2CO_3) at varying pH, followed by centrifugation to separate the supernatant (S) and pellet (P) fractions.

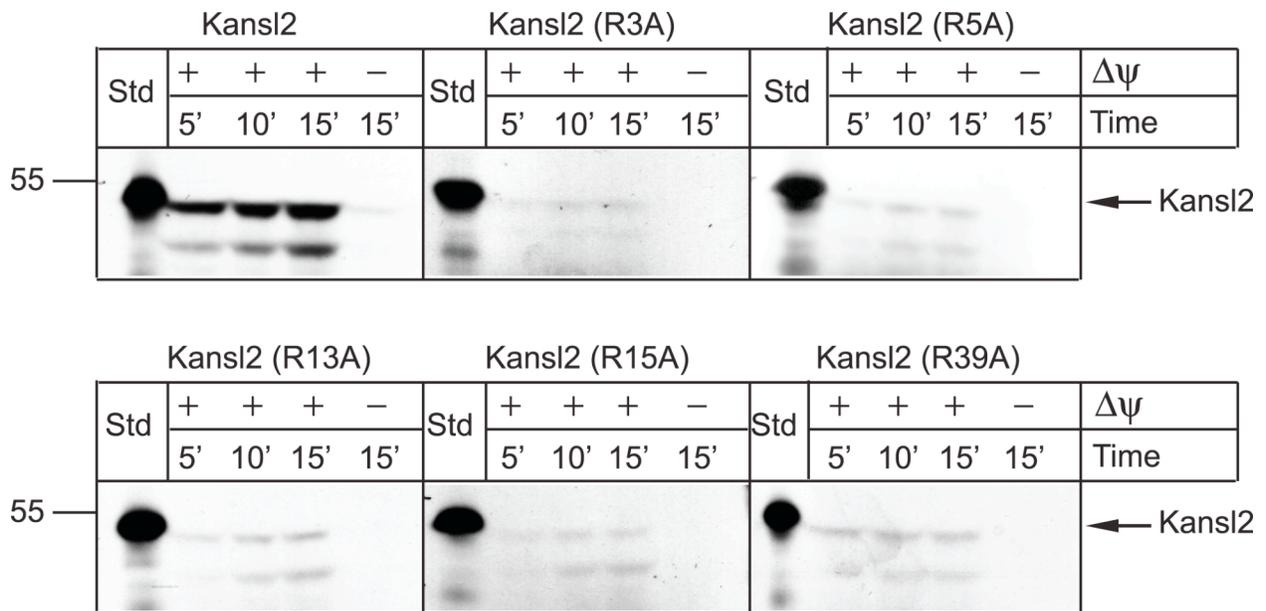
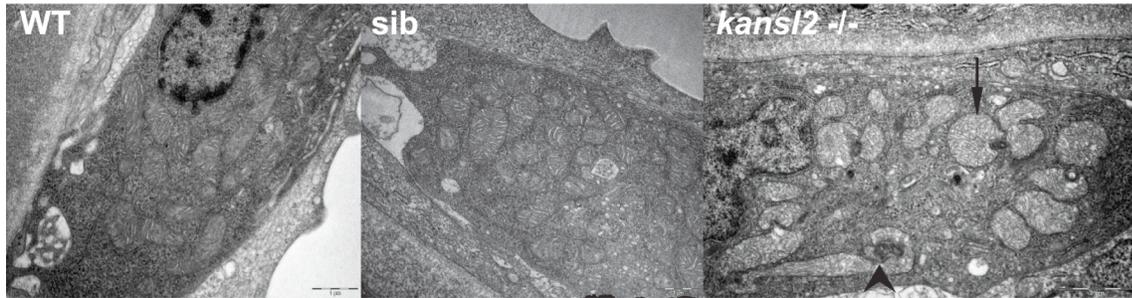


Figure 2.7. Kansl2 requires the MLS for mitochondria import. Radiolabeled Kansl2 and Kansl2 mutants in which key arginine residues in the MLS were changed to alanine residues were imported into isolated wildtype yeast mitochondria at indicated time. The membrane potential ($\Delta\psi$) was dissipated with uncouplers.

A



B



C



Figure 2.8. Loss of *Kansl2* perturbs mitochondrial ultrastructure and respiration. A. Electron micrographs showing mitochondria in the muscle region of WT, *sib* and *kansl2*^{-/-} line at 3 dpf. The black arrow and arrowhead highlight enlarged and elongated mitochondria respectively. B. WT, *sib* and *kansl2*^{-/-} embryos were stained for Complex IV activity at 4 dpf. C. As in 'B' but Complex II activity was stained.

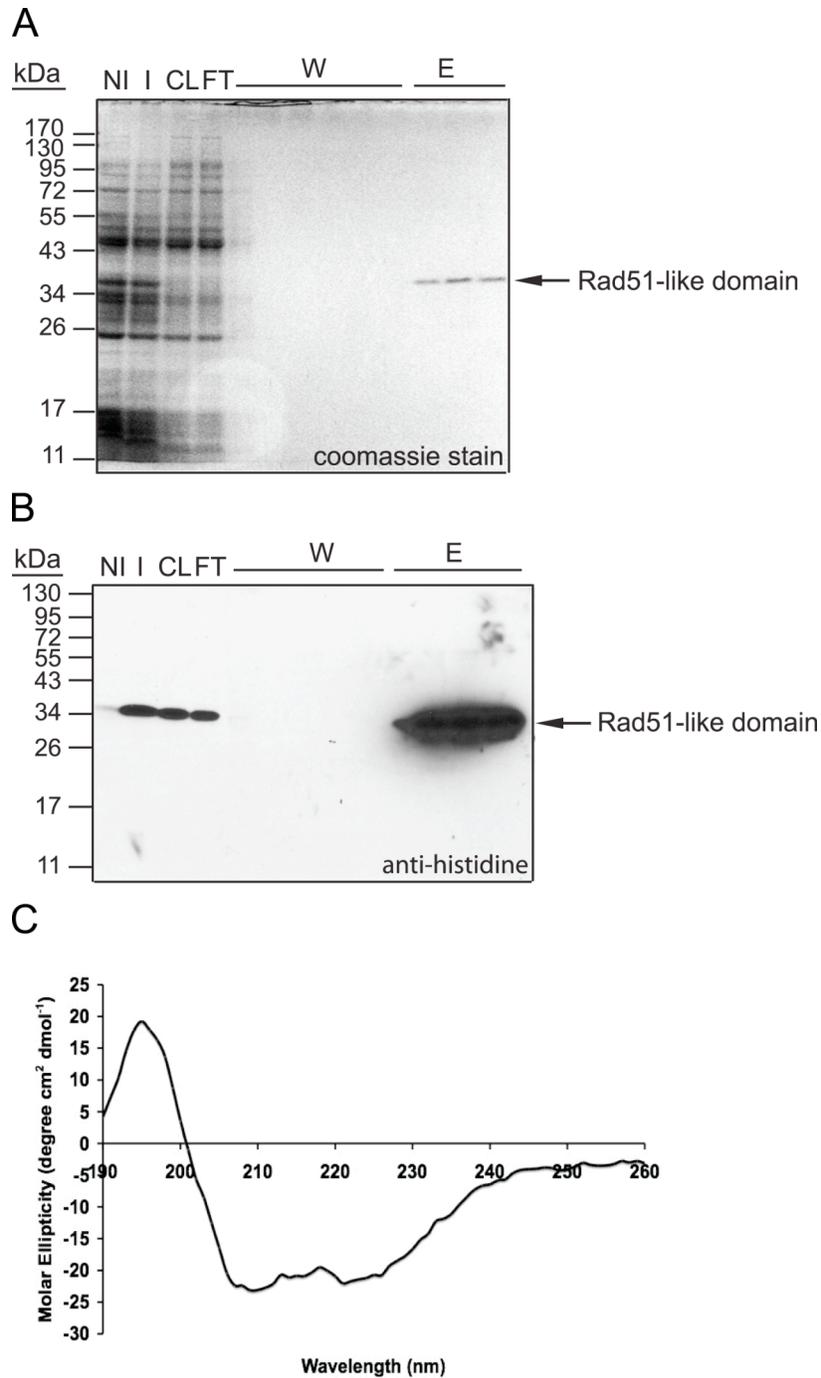


Figure 2.9. Purification and circular dichroism analysis of Kansl2 Rad51 N-terminal like domain.
 A. Coomassie-stained gel showing the purification of Kansl2 Rad51-like domain under native conditions. NI, not induced; I, induced; CL, cell lysate; FT, flow through; W, wash; E, eluate. B. Western blot analysis of the purification using an antibody against the 6X-histidine tag. C. Circular dichroism analysis of the purified Kansl2 Rad51-like domain. Data is expressed as molar ellipticity (degree cm² d mol⁻¹) and the x-axis is the wavelength from 190 to 260 nm.

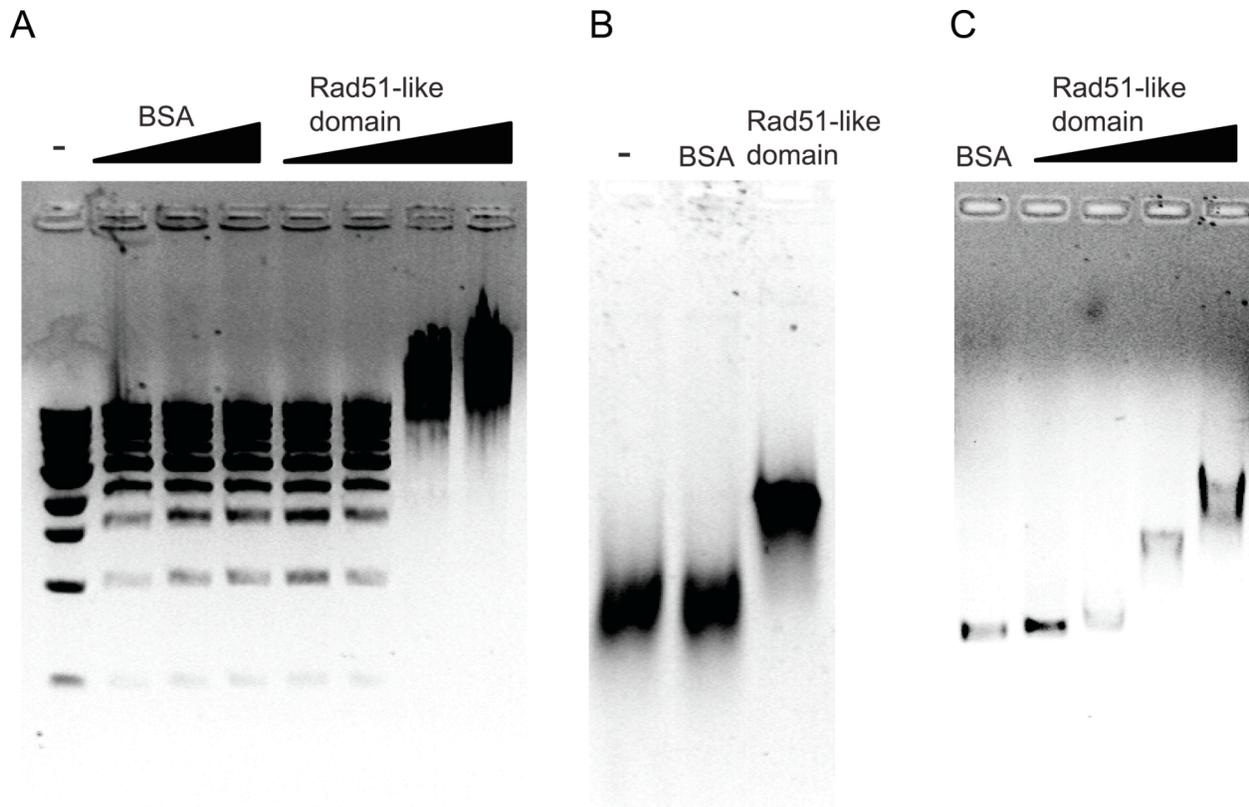


Figure 2.10. The Kansl2 Rad51 N-terminal like domain binds to DNA. A. DNA mobility shift assay with 0.5 μ g of a 1 kb DNA ladder and a 200 ng to 2 μ g Rad51 N-terminal like domain. B. As in 'A' with isolated WT zebrafish mtDNA and 2 μ g Rad51 N-terminal like domain. BSA was included as a negative control. C. As in 'A', but the template is the PCR-amplified D-loop region (536 bp) from zebrafish.

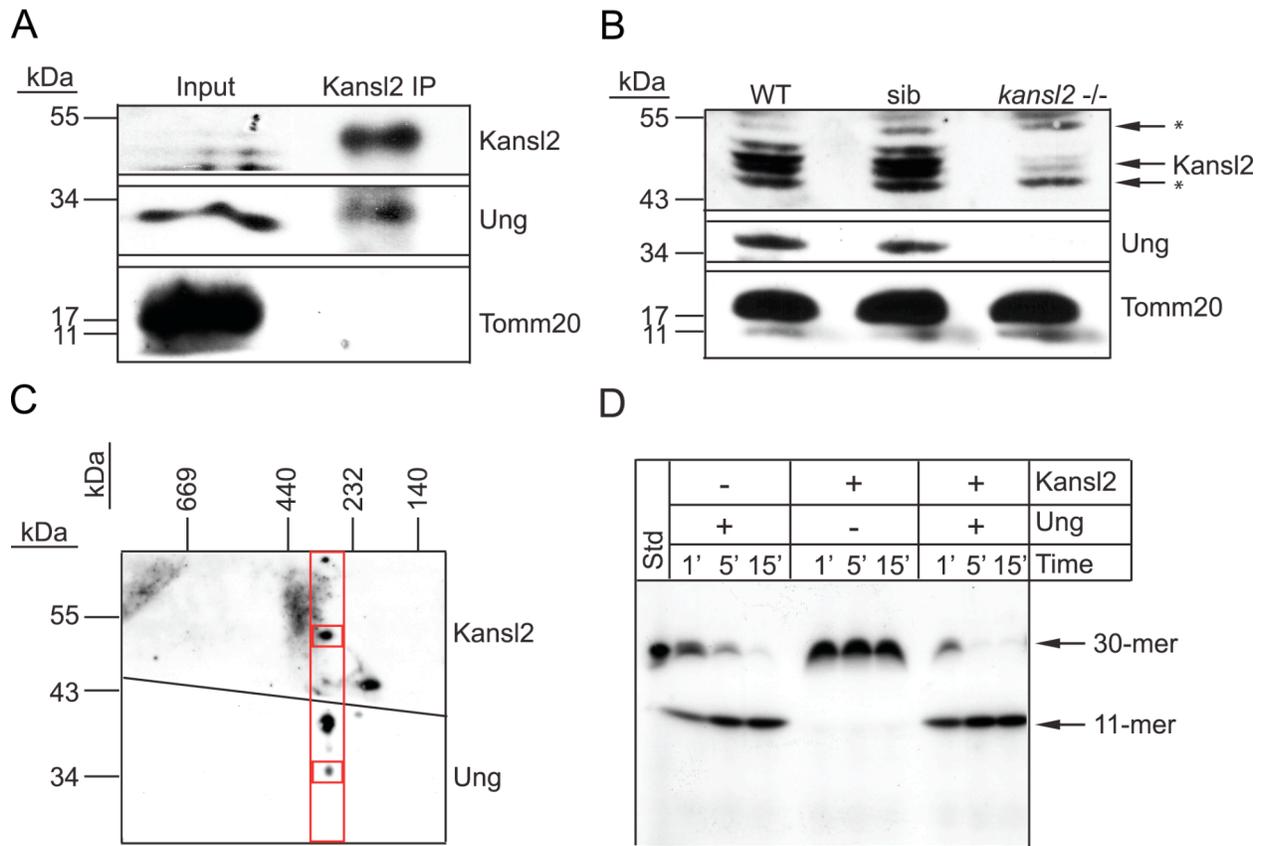


Figure 2.11. KANSL2 and UNG are partner proteins. A. Analysis of co-immunoprecipitation from zebrafish mitochondrial lysates with polyclonal anti-KANSL2, followed by immunoblot detection with antibodies against KANSL2, Ung and Tomm20 (control). Total (T) represents 20% of the input. B. Immunoblot analysis of mitochondrial extracts from WT, sib and *kansl2* ^{-/-} embryos with antibodies against KANSL2, Ung and Tomm20. The asterisks denote non-specific bands that cross-reacts with the KANSL2 antibody. C. Two-dimensional CNPAGE/ SDS-PAGE analysis of the KANSL2 complex using antibodies against Ung and KANSL2. A 300 kDa complex of Ung and KANSL2 is indicated by the red boxes. D. The 5'-³²P-labeled uracil-containing oligonucleotide duplex (30 nucleotides) was incubated with recombinant Ung, KANSL2 or both at indicated time. The cleaved 11-nucleotides fragment is indicated. 'Std' represents labeled oligonucleotide duplex input.

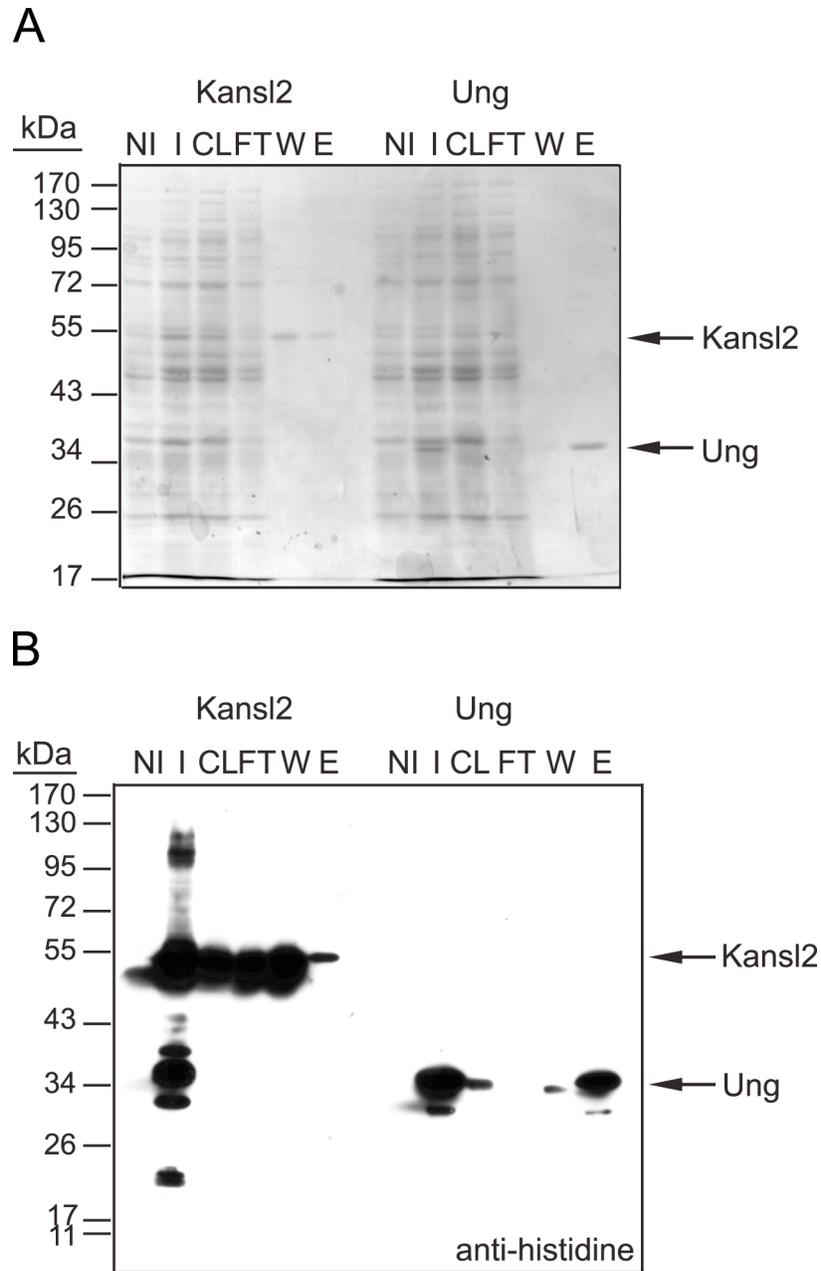


Figure 2.12. Purification of Kansl2 and Ung. A. Coomassie-stained gel showing the purification of Kansl2 without MTS and full-length Ung under native conditions. NI, not induced; I, induced; CL, cell lysate; FT, flow through; W, wash; E, eluate. B. Western blot analysis of the purification using an antibody against the 6X-histidine tag.

2.5 REFERENCES

- 1 **Aihara, H., Ito, Y., Kurumizaka, H., Yokoyama, S. and Shibata, T.** (1999). The N-terminal domain of the human Rad51 protein binds DNA: structure and a DNA binding surface as revealed by NMR. *J Mol Biol* **290**, 495-504.
- 2 **Akbari, M., Otterlei, M., Pena-Diaz, J. and Krokan, H. E.** (2007). Different organization of base excision repair of uracil in DNA in nuclei and mitochondria and selective upregulation of mitochondrial uracil-DNA glycosylase after oxidative stress. *Neuroscience* **145**, 1201-12.
- 3 **Asakawa, K., Suster, M. L., Mizusawa, K., Nagayoshi, S., Kotani, T., Urasaki, A., Kishimoto, Y., Hibi, M. and Kawakami, K.** (2008). Genetic dissection of neural circuits by Tol2 transposon-mediated Gal4 gene and enhancer trapping in zebrafish. *Proc Natl Acad Sci U S A* **105**, 1255-60.
- 4 **Boesch, P., Ibrahim, N., Paulus, F., Cosset, A., Tarasenko, V. and Dietrich, A.** (2009). Plant mitochondria possess a short-patch base excision DNA repair pathway. *Nucleic Acids Res* **37**, 5690-700.
- 5 **Boesch, P., Weber-Lotfi, F., Ibrahim, N., Tarasenko, V., Cosset, A., Paulus, F., Lightowlers, R. N. and Dietrich, A.** (2010). DNA repair in organelles: Pathways, organization, regulation, relevance in disease and aging. *Biochim Biophys Acta* **1813**, 186-200.
- 6 **Bogenhagen, D. and Clayton, D. A.** (1974). The number of mitochondrial deoxyribonucleic acid genomes in mouse L and human HeLa cells. Quantitative isolation of mitochondrial deoxyribonucleic acid. *J Biol Chem* **249**, 7991-5.
- 7 **Bogenhagen, D. F.** (2011). Mitochondrial DNA nucleoid structure. *Biochim Biophys Acta*.
- 8 **Brown, T. A., Tkachuk, A. N., Shtengel, G., Kopek, B. G., Bogenhagen, D. F., Hess, H. F. and Clayton, D. A.** (2011). Superresolution fluorescence imaging of mitochondrial nucleoids reveals their spatial range, limits, and membrane interaction. *Mol Cell Biol* **31**, 4994-5010.
- 9 **Cai, Y., Jin, J., Swanson, S. K., Cole, M. D., Choi, S. H., Florens, L., Washburn, M. P., Conaway, J. W. and Conaway, R. C.** (2010). Subunit composition and substrate specificity of a MOF-containing histone acetyltransferase distinct from the male-specific lethal (MSL) complex. *J Biol Chem* **285**, 4268-72.
- 10 **Chen, H. W., Rainey, R. N., Balatoni, C. E., Dawson, D. W., Troke, J. J., Wasiak, S., Hong, J. S., McBride, H. M., Koehler, C. M., Teitell, M. A. et al.** (2006). Mammalian

- polynucleotide phosphorylase is an intermembrane space RNase that maintains mitochondrial homeostasis. *Mol Cell Biol* **26**, 8475-87.
- 11 **Claros, M. G. and Vincens, P.** (1996). Computational method to predict mitochondrially imported proteins and their targeting sequences. *Eur J Biochem* **241**, 779-86.
 - 12 **Claypool, S. M., Oktay, Y., Boonthung, P., Loo, J. A. and Koehler, C. M.** (2008). Cardiolipin defines the interactome of the major ADP/ATP carrier protein of the mitochondrial inner membrane. *J Cell Biol* **182**, 937-50.
 - 13 **Copeland, W. C. and Longley, M. J.** (2008). DNA2 resolves expanding flap in mitochondrial base excision repair. *Mol Cell* **32**, 457-8.
 - 14 **de Souza-Pinto, N. C., Eide, L., Hogue, B. A., Thybo, T., Stevnsner, T., Seeberg, E., Klungland, A. and Bohr, V. A.** (2001). Repair of 8-oxodeoxyguanosine lesions in mitochondrial dna depends on the oxoguanine dna glycosylase (OGG1) gene and 8-oxoguanine accumulates in the mitochondrial dna of OGG1-defective mice. *Cancer Res* **61**, 5378-81.
 - 15 **Dizdaroglu, M., Jaruga, P., Birincioglu, M. and Rodriguez, H.** (2002). Free radical-induced damage to DNA: mechanisms and measurement. *Free Radic Biol Med* **32**, 1102-15.
 - 16 **Draper, B. W., Morcos, P. A. and Kimmel, C. B.** (2001). Inhibition of zebrafish fgf8 pre-mRNA splicing with morpholino oligos: a quantifiable method for gene knockdown. *Genesis* **30**, 154-6.
 - 17 **Evans, M. D., Dizdaroglu, M. and Cooke, M. S.** (2004). Oxidative DNA damage and disease: induction, repair and significance. *Mutat Res* **567**, 1-61.
 - 18 **Fisher, R. P. and Clayton, D. A.** (1988). Purification and characterization of human mitochondrial transcription factor 1. *Mol Cell Biol* **8**, 3496-509.
 - 19 **Fujiki, Y., Hubbard, A. L., Fowler, S. and Lazarow, P. B.** (1982). Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J Cell Biol* **93**, 97-102.
 - 20 **Glick, B. S. and Pon, L. A.** (1995). Isolation of highly purified mitochondria from *Saccharomyces cerevisiae*. *Methods Enzymol* **260**, 213-23.
 - 21 **Gough, J., Karplus, K., Hughey, R. and Chothia, C.** (2001). Assignment of homology to genome sequences using a library of hidden Markov models that represent all proteins of known structure. *J Mol Biol* **313**, 903-19.

- 22 **Gredilla, R., Bohr, V. A. and Stevnsner, T.** (2010). Mitochondrial DNA repair and association with aging--an update. *Exp Gerontol* **45**, 478-88.
- 23 **Griparic, L., Kanazawa, T. and van der Bliek, A. M.** (2007). Regulation of the mitochondrial dynamin-like protein Opa1 by proteolytic cleavage. *J Cell Biol* **178**, 757-64.
- 24 **Habib, S. J., Neupert, W. and Rapaport, D.** (2007). Analysis and prediction of mitochondrial targeting signals. *Methods in Cell Biology* **80**, 761-81.
- 25 **Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., van Eeden, F. J., Jiang, Y. J., Heisenberg, C. P. et al.** (1996). The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development* **123**, 1-36.
- 26 **Hamilton, M. L., Van Remmen, H., Drake, J. A., Yang, H., Guo, Z. M., Kewitt, K., Walter, C. A. and Richardson, A.** (2001). Does oxidative damage to DNA increase with age? *Proc Natl Acad Sci U S A* **98**, 10469-74.
- 27 **Harris, M. P., Rohner, N., Schwarz, H., Perathoner, S., Konstantinidis, P. and Nusslein-Volhard, C.** (2008). Zebrafish *eda* and *edar* mutants reveal conserved and ancestral roles of ectodysplasin signaling in vertebrates. *PLoS Genet* **4**, e1000206.
- 28 **Haug, T., Skorpen, F., Aas, P. A., Malm, V., Skjelbred, C. and Krokan, H. E.** (1998). Regulation of expression of nuclear and mitochondrial forms of human uracil-DNA glycosylase. *Nucleic Acids Res* **26**, 1449-57.
- 29 **Higashijima, S., Okamoto, H., Ueno, N., Hotta, Y. and Eguchi, G.** (1997). High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. *Dev Biol* **192**, 289-99.
- 30 **Hoogenraad, N. J., Ward, L. A. and Ryan, M. T.** (2002). Import and assembly of proteins into mitochondria of mammalian cells. *Biochim Biophys Acta* **1592**, 97-105.
- 31 **Ishikawa, K., Takenaga, K., Akimoto, M., Koshikawa, N., Yamaguchi, A., Imanishi, H., Nakada, K., Honma, Y. and Hayashi, J.** (2008). ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. *Science* **320**, 661-4.
- 32 **Karahalil, B., de Souza-Pinto, N. C., Parsons, J. L., Elder, R. H. and Bohr, V. A.** (2003). Compromised incision of oxidized pyrimidines in liver mitochondria of mice deficient in NTH1 and OGG1 glycosylases. *J Biol Chem* **278**, 33701-7.
- 33 **Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F.** (1995). Stages of embryonic development of the zebrafish. *Dev Dyn* **203**, 253-310.

- 34 **Klungland, A., Rosewell, I., Hollenbach, S., Larsen, E., Daly, G., Epe, B., Seeberg, E., Lindahl, T. and Barnes, D. E.** (1999). Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage. *Proc Natl Acad Sci U S A* **96**, 13300-5.
- 35 **Koehler, C. M., Jarosch, E., Tokatlidis, K., Schmid, K., Schweyen, R. J. and Schatz, G.** (1998a). Import of mitochondrial carriers mediated by essential proteins of the intermembrane space. *Science* **279**, 369-73.
- 36 **Koehler, C. M., Merchant, S., Oppliger, W., Schmid, K., Jarosch, E., Dolfini, L., Junne, T., Schatz, G. and Tokatlidis, K.** (1998b). Tim9p, an essential partner subunit of Tim10p for the import of mitochondrial carrier proteins. *Embo J* **17**, 6477-86.
- 37 **Lakshminpathy, U. and Campbell, C.** (2001). Antisense-mediated decrease in DNA ligase III expression results in reduced mitochondrial DNA integrity. *Nucleic Acids Res* **29**, 668-76.
- 38 **Levesque, M. P., Krauss, J., Koehler, C. M., Boden, C. and Harris, M. P.** (2012). *Zebrafish*.
- 39 **Liu, P., Qian, L., Sung, J. S., de Souza-Pinto, N. C., Zheng, L., Bogenhagen, D. F., Bohr, V. A., Wilson, D. M., 3rd, Shen, B. and Demple, B.** (2008). Removal of oxidative DNA damage via FEN1-dependent long-patch base excision repair in human cell mitochondria. *Mol Cell Biol* **28**, 4975-87.
- 40 **Mably, J. D., Mohideen, M. A., Burns, C. G., Chen, J. N. and Fishman, M. C.** (2003). Heart of glass regulates the concentric growth of the heart in zebrafish. *Curr Biol* **13**, 2138-47.
- 41 **Maynard, S., de Souza-Pinto, N. C., Scheibye-Knudsen, M. and Bohr, V. A.** (2010). Mitochondrial base excision repair assays. *Methods* **51**, 416-25.
- 42 **McCaffery, J. M. and Farquhar, M. G.** (1995). Localization of GTPases by indirect immunofluorescence and immunoelectron microscopy. *Methods Enzymol* **257**, 259-79.
- 43 **Michikawa, Y., Mazzucchelli, F., Bresolin, N., Scarlato, G. and Attardi, G.** (1999). Aging-dependent large accumulation of point mutations in the human mtDNA control region for replication. *Science* **286**, 774-9.
- 44 **Mitra, S., Izumi, T., Boldogh, I., Bhakat, K. K., Chattopadhyay, R. and Szczesny, B.** (2007). Intracellular trafficking and regulation of mammalian AP-endonuclease 1 (APE1), an essential DNA repair protein. *DNA Repair (Amst)* **6**, 461-9.

- 45 **Nilsen, H., Rosewell, I., Robins, P., Skjelbred, C. F., Andersen, S., Slupphaug, G., Daly, G., Krokan, H. E., Lindahl, T. and Barnes, D. E.** (2000). Uracil-DNA glycosylase (UNG)-deficient mice reveal a primary role of the enzyme during DNA replication. *Mol Cell* **5**, 1059-65.
- 46 **Parisi, M. A. and Clayton, D. A.** (1991). Similarity of human mitochondrial transcription factor 1 to high mobility group proteins. *Science* **252**, 965-9.
- 47 **Pinz, K. G. and Bogenhagen, D. F.** (2000). Characterization of a catalytically slow AP lyase activity in DNA polymerase gamma and other family A DNA polymerases. *J Biol Chem* **275**, 12509-14.
- 48 **Raja, S. J., Charapitsa, I., Conrad, T., Vaquerizas, J. M., Gebhardt, P., Holz, H., Kadlec, J., Fraterman, S., Luscombe, N. M. and Akhtar, A.** (2010). The nonspecific lethal complex is a transcriptional regulator in *Drosophila*. *Mol Cell* **38**, 827-41.
- 49 **Robin, E. D. and Wong, R.** (1988). Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. *Journal of Cellular Physiology* **136**, 507-13.
- 50 **Schagger, H., Cramer, W. A. and von Jagow, G.** (1994). Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal Biochem* **217**, 220-30.
- 51 **Stuart, J. A., Mayard, S., Hashiguchi, K., Souza-Pinto, N. C. and Bohr, V. A.** (2005). Localization of mitochondrial DNA base excision repair to an inner membrane-associated particulate fraction. *Nucleic Acids Res* **33**, 3722-32.
- 52 **Swerdlow, R. H. and Khan, S. M.** (2009). The Alzheimer's disease mitochondrial cascade hypothesis: an update. *Exp Neurol* **218**, 308-15.
- 53 **Tanji, K., Kaufmann, P., Naini, A. B., Lu, J., Parsons, T. C., Wang, D., Willey, J. Z., Shanske, S., Hirano, M., Bonilla, E. et al.** (2008). A novel tRNA(Val) mitochondrial DNA mutation causing MELAS. *J Neurol Sci* **270**, 23-7.
- 54 **Thisse, C., Thisse, B., Schilling, T. F. and Postlethwait, J. H.** (1993). Structure of the zebrafish *snail1* gene and its expression in wild-type, spadetail and no tail mutant embryos. *Development* **119**, 1203-15.
- 55 **Tienson, H. L., Dabir, D. V., Neal, S. E., Loo, R., Hasson, S. A., Boontheung, P., Kim, S. K., Loo, J. A. and Koehler, C. M.** (2009). Reconstitution of the mia40-erv1 oxidative folding pathway for the small tim proteins. *Mol Biol Cell* **20**, 3481-90.

- 56 **Tuppen, H. A., Blakely, E. L., Turnbull, D. M. and Taylor, R. W.** (2010). Mitochondrial DNA mutations and human disease. *Biochim Biophys Acta* **1797**, 113-28.
- 57 **Van Tuyle, G. C. and Pavco, P. A.** (1985). The rat liver mitochondrial DNA-protein complex: displaced single strands of replicative intermediates are protein coated. *J Cell Biol* **100**, 251-7.
- 58 **Wallace, D. C.** (1999). Mitochondrial diseases in man and mouse. *Science* **283**, 1482-8.
- 59 **Wang, G., Chen, H. W., Oktay, Y., Zhang, J., Allen, E. L., Smith, G. M., Fan, K. C., Hong, J. S., French, S. W., McCaffery, J. M. et al.** (2010). PNPASE regulates RNA import into mitochondria. *Cell* **142**, 456-67.

CHAPTER 3

SUMMARY

The work described in this dissertation can best be described by Santiago Ramón y Cajal's famous quote, "There are no small problems. Problems that appear small are large problems that are not understood". The road to scientific discovery is often long and painstaking, yet full of fascination and excitement. The moment of triumph was excited when I discovered that zebrafish Kansl2 co-purifies with Ung. A detail survey of the literature has led to the establishment of the hypothesis postulating the existence of a stable Ung protein complex within mitochondria and the function of Kansl2 as a scaffold for Ung.

This study has identified that Ung is a partner protein of Kansl2 in mitochondria, and has also characterized the nature of this interaction in some detail. Kansl2 is determined as a subunit of the acetyl transferase complex in the nucleus. It is conserved across eukaryotic kingdom, and expressed ubiquitously. Phenotypic analysis also shows that Kansl2 is essential for tissue and organ development in vertebrates and humans. Sequence analysis and biochemical assay show that Kansl2 localizes to mitochondria. Kansl2 binds to the mtDNA via its Rad51-like domain and assembles in a complex with Ung. This interaction enhances Ung activity. Nevertheless, Ung activity is independent of Kansl2.

Based on these observations, a model for the function of Kansl2 is proposed. Kansl2 acts as a scaffold and forms a stable complex with Ung and the mtDNA at the mitochondrial inner membrane. Once the Kansl2:Ung complex is formed, the substrate (mtDNA) can be channeled more efficiently through the complex, facilitating incision of uracil from the substrate. The exact

biochemical interactions between Kansl2 and Ung are not fully understood and will have to await mutational analysis and co-crystallization of the Kansl2:Ung complex. The roles of Kansl2 in the nucleus and the composition of Kansl2:Ung complex will require further study.

In summary, this work has established a new paradigm in mtDNA biology and opened up new venue, which will further our understanding of the maintenance and repair of the mtDNA, its replication and expression, and the flux and transport of repair proteins in general. Much work remains to be done before a complete understanding of this novel interaction and its implications will emerge.