MODELLING THE EVOLUTIONARILY CONSERVED MANF/CDNF PROTEIN FAMILY IN DROSOPHILA MELANOGASTER

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ACADEMIC DISSERTATION

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Helsinki 2014
“Always watch where you are going. Otherwise, you may step on a piece of the Forest that was left out by mistake.”
— Winnie the Pooh
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I  

II  

III  

The publications are referred to in the text by their roman numerals. The articles are reprinted with kind permission of their copyright holders.

Cover image

Targeted ectopic expression of UAS-DmManf-C129S (middle) or knockdown of *DmManf* expression by UAS-*DmManf*-RNAi (right) with MS1096-GAL4 wing driver results in curved wing phenotype.
Mesencephalic astrocyte-derived neurotrophic factor (MANF) and cerebral dopamine neurotrophic factor (CDNF) proteins form a family of neurotrophic factors. Neurotrophic factors have been intensively studied as a putative therapeutic approach to treat neuronal injuries and neurodegenerative diseases. Mammalian MANF and CDNF have been shown to have protective and restorative effects on the nigrostriatal dopaminergic system. In addition, several studies have reported a role for MANF in the endoplasmic reticulum (ER) stress response. A recently established MANF knockout mouse model revealed that MANF functions in the pancreatic insulin-producing β-cells and might be involved in the pathogenesis of diabetes mellitus. Beyond their neurotrophic properties, MANF and CDNF appear to play a more general role in the maintenance of cellular homeostasis.

In this study, Drosophila melanogaster was used as a model organism to explore the function and interaction of the MANF/CDNF protein family in vivo. The sole member of the MANF/CDNF family in Drosophila, DmAManf, was discovered to be crucial for fly development. The human orthologues, HsMANF and HsCDNF, were found to be able to substitute the endogenous DmAManf. Likewise, DmAManf had the cytoprotective properties of mammalian MANF in cultured murine neurons. These results support that the findings from the Drosophila model can be adapted for research in mammalian systems.

MANF/CDNF proteins consist of amino (N) - and carboxy (C) -terminal domains. In this work, several functional features identified in mammalian MANF structure were explored in the Drosophila model. Separate N- or C-terminal domain constructs, even when co-expressed together, failed to complement for the loss of endogenous DmAManf. The ER retention of DmAManf, mediated by the C-terminal signal sequence, and the positive charge of the N-terminal surface amino acid residues were found to be important for appropriate DmAManf function. Furthermore, entering the secretory pathway via ER was essential for the stability of DmAManf protein.

A CXXC motif characteristic for oxidoreductases is located in the C-terminal domain of MANF. In this study, effects of a point mutation (C129S) in CXXC motif of DmAManf were analysed in vivo. Intact CXXC motif was discovered to be vital for DmAManf function. Furthermore, the expression of DmAManf-C129S in wild type background was harmful for fly viability suggesting that this specific mutation represents either a dominant negative or a gain-of-function allele of DmAManf.

Utilising the unique potential of Drosophila model for in vivo screening, interactions of DmAManf were studied in this work. Consistent with a previous in vitro study, a genetic interaction was found between DmAManf and the fly homologue of the major ER chaperone GRP78. Moreover, DmAManf interacted with other genes that encode components of ER function and the unfolded protein response. Finally, novel interactions with DmAManf and genes involved in the ubiquinone synthesis pathway and mitochondria were discovered.

Taken together, this study demonstrates the functional conservation of mammalian and fly proteins and provides meaningful information on structural and functional features of the MANF/CDNF protein family in vivo. The genetic interaction studies confirmed and expanded the previous knowledge on the ER-associated functions of MANF. Furthermore, novel interactions with mitochondria-related genes and DmAManf were discovered.
## SELECTED ABBREVIATIONS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>ARP</td>
<td>Arginine-rich protein</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL-2 associated X protein</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>CDNF</td>
<td>Cerebral dopamine neurotrophic factor</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<tr>
<td>CHOP</td>
<td>C/EBP (CCAAT/enhancer binding protein) homologous protein</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COQ</td>
<td>Coenzyme Q</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP responsive element binding protein</td>
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<tr>
<td>Debcl</td>
<td>Death executioner Bcl-2 homologue</td>
</tr>
<tr>
<td>Dm</td>
<td><em>Drosophila melanogaster</em></td>
</tr>
<tr>
<td>eIF</td>
<td>Eukaryotic initiation factor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER-associated degradation</td>
</tr>
<tr>
<td>ERSE</td>
<td>ER stress response element</td>
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<tr>
<td>GADD34</td>
<td>Growth arrest and DNA damage protein 34</td>
</tr>
<tr>
<td>GRP78</td>
<td>Glucose-regulated protein 78</td>
</tr>
<tr>
<td>Hs</td>
<td><em>Homo sapiens</em></td>
</tr>
<tr>
<td>Hsc70-3</td>
<td>Heat shock 70-kDa protein cognate 3</td>
</tr>
<tr>
<td>IRE1</td>
<td>Inositol-requiring enzyme 1</td>
</tr>
<tr>
<td>Ku70</td>
<td>Ku autoantigen p70 subunit</td>
</tr>
<tr>
<td>MANF</td>
<td>Mesencephalic astrocyte-derived neurotrophic factor</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>NMG</td>
<td>N-terminal mutation group</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein disulphide isomerase</td>
</tr>
<tr>
<td>PEK</td>
<td>Pancreatic eIF2α kinase</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR (RNA-dependent protein kinase) -like ER kinase</td>
</tr>
<tr>
<td>PP1</td>
<td>Protein phosphatase 1</td>
</tr>
<tr>
<td>SAP</td>
<td>SAF-A/B, Acinus and PIAS domain</td>
</tr>
<tr>
<td>SAPLIP</td>
<td>Saposin-like protein</td>
</tr>
<tr>
<td>SCG</td>
<td>Superior cervical ganglion</td>
</tr>
<tr>
<td>ss</td>
<td>Secretion signal</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
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<tr>
<td>UAS</td>
<td>Upstream activation sequence</td>
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<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
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<tr>
<td>UPRE</td>
<td>Unfolded protein response element</td>
</tr>
<tr>
<td>XBP1</td>
<td>X-box binding protein 1</td>
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1 REVIEW OF THE LITERATURE

1.1 Overview of neurotrophic factors

Neurotrophic factors, a subclass of growth factors, are small, secreted proteins which signal through their specific receptors. They regulate different neuronal processes, including migration, differentiation, synaptic plasticity, survival and regeneration. Additionally, several other growth factors have been found to have neurotrophic function and, conversely, functions in non-neuronal tissues have been revealed for neurotrophic factors. Due to their cell survival promoting activity, neurotrophic factors are considered as promising agents for treatments of neuronal degeneration (reviewed in Ramaswamy and Kordower 2009).

Currently, neurotrophic factors are classified into four main families according to homology on the amino acid sequence level and structural similarity. Neurotrophin family (reviewed in Huang and Reichardt 2001) includes the first neurotrophic factor found, nerve growth factor (NGF) (Levi-Montalcini and Hamburger 1951; Levi-Montalcini 1987). Neurotrophins signal via two receptors, tropomyosin-related kinase (TRK) or p75 neurotrophin receptor. The other families are glial-derived neurotrophic factor (GDNF) family ligands (GFLs) (reviewed in Airaksinen and Saarma 2002), neurokine family (reviewed in Stolp 2013) and MANF/CDNF (mesencephalic astrocyte-derived neurotrophic factor/cerebral dopamine neurotrophic factor) family (reviewed in Lindholm and Saarma, 2010). GFLs signal through RET (ret proto-oncogene) receptor tyrosine kinase that is activated by co-receptor GFRα (GDNF family receptor alpha) and the corresponding GFL complex. Additionally, GDNF can signal through NCAM (neural cell adhesion molecule) receptor that binds both GFRα and GDNF (Paratcha et al. 2003) and via Syndecan-3 (Bespalov et al. 2011).

For a long time, non-cell-autonomous neurotrophic support was thought to exist only in vertebrates and the development of invertebrate nervous system was considered to be cell-autonomously programmed. However, homologues for neurotrophic factors – although partial – and their receptors have been discovered by analyzing the invertebrate genomes and protein structures (Bothwell 2006). In Drosophila, Neurotrophin-1 and -2, members of Spätzle family, show partial structural homology to mammalian neurotrophins and support the survival of peripheral neurons (Zhu et al. 2008). However, no homologues for the TRK receptors have been found in Drosophila. Homologues to GDNF receptor RET (Sugaya et al. 1994) and Gfr-like (Gfrl) co-receptor (Hätinen et al. 2007) have been found in Drosophila while GFL homologues have not been identified (Airaksinen et al. 2006). GFLs have been suggested to evolve from existing TGF-β (transforming growth factor beta) -like proteins (Hätinen et al. 2007). Drosophila Gfrl, expressed in distinct cells from Drosophila Ret, failed to phosphorylate both Drosophila and mammalian RET (Kallijärvi et al. 2012). However, Gfrl genetically interacted with Drosophila NCAM, the other known binding partner of mammalian GFRα.
In contrast to other neurotrophic factor families, the most recently found MANF/CDNF family has a functionally conserved homologue in invertebrates (I, II).

1.2 The MANF/CDNF protein family

1.2.1 Discovery of MANF and CDNF

MANF was originally discovered from screening a cultured media of rat ventral mesencephalic cell line 1 to identify proteins that support embryonic midbrain dopaminergic neurons (Petrova et al. 2003). In vertebrates, a homologous protein, CDNF, was identified (Lindholm et al. 2007). Human MANF and CDNF share 53% identical amino acid sequences (Lindholm et al. 2007). MANF and CDNF form a sole family of neurotrophic factors that includes invertebrate homologues with conserved amino acid sequence (Petrova et al. 2003; Lindholm et al. 2007). In invertebrates only one homologue exists and is more similar to mammalian MANF than CDNF (Lindholm et al. 2007). The Drosophila homologue, DmManf, shows 53% and 49% identity to human MANF (HsMANF) and CDNF (HsCDNF), respectively (Lindholm et al. 2007). The amino acid sequences of HsMANF, HsCDNF and DmManf are presented in Figure 1.

![Figure 1. Amino acid sequence alignment of human MANF (HsMANF), human CDNF (HsCDNF) and Drosophila Manf (DmManf).](image-url)

The numbering above amino acid sequences corresponds to DmManf without signal sequence. The signal sequence, N-terminal domain and C-terminal domain are coloured in orange, blue and green, respectively. The conserved eight cysteine residues are indicated by asterisks (*). Alpha-helices (α) are marked under amino acid sequence and coloured according to ribbon diagram presented in Figure 2. Purple square indicates C-terminal CXXC motif, red square C-terminal endoplasmic reticulum retention signal. Amino acid residues coloured in white and yellow indicate the point mutations in transgenic constructs DmManf-NMG1 and DmManf-NMG2, respectively (see Figure 9 and Chapter 4.2.1 in Results and discussion). Reprinted from II.
Human MANF has also been referred as ARP (arginine-rich protein; Shridhar et al. 1996b) or ARMET (arginine-rich, mutated in early stages of tumor). In line, CDNF has been referred as ARMET-L1 (ARMET-like 1). This originates from studies on human ARP, which has an alternative ORF (open reading frame) and was predicted to hold an arginine-rich amino (N) -terminal region. However, ARP with the arginine-rich tract has never been detected to be expressed at protein level and it seems that this ORF is not synthesized in vivo (Petrova et al. 2003). Mutations in the arginine-rich part of ARP were linked to various forms of cancer (Shridhar et al. 1996a; Shridhar et al. 1996b; Shridhar et al. 1997) but further studies revealed that the identified mutations appear as normal polymorphisms (Evron et al. 1997; Tanaka et al. 2000; Piepoli et al. 2006). Drosophila Manf was originally identified as D-ARP (later also known as ARP-like) from yeast-based signal sequence trap screen to isolate genes encoding secreted and membrane proteins (Goo et al. 1999). A putative signal sequence typical for secreted proteins was identified in the N-terminus of D-ARP lacking the arginine rich domain of human ARP (Goo et al. 1999).

1.2.2 Molecular characteristics of mammalian MANF and CDNF

Both MANF and CDNF are approximately 18 kDa, highly soluble proteins found as monomers in neutral pH (Lindholm et al. 2007; Mizobuchi et al. 2007). They are secreted from transiently transfected cells (Lindholm et al. 2007; Lindholm et al. 2008). Endogenous MANF has also been shown to be secreted in vitro (Apostolou et al. 2008). However, the majority of MANF protein seems to remain intracellular (Glembotski et al. 2012) and MANF is partially retained in endoplasmic reticulum (ER) (Mizobuchi et al. 2007; Apostolou et al. 2008). Mouse MANF and CDNF were found to be relatively widely expressed (Lindholm et al. 2007; Mizobuchi et al. 2007; Lindholm et al. 2008). Particularly high MANF levels have been detected in the secretory cells and tissues.

The premature human MANF consists of 179 amino acids. The first 21 amino acids represent a signal sequence peptide that directs the translation of the polypeptide to the secretory pathway via ER (Petrova et al. 2003). Originally, MANF was reported to be glycosylated (Petrova et al. 2003) but in later studies, post-translational modifications were not detected in secreted MANF in vitro (Lindholm et al. 2008). Human and mouse MANF do not contain putative glycosylation sites (Lindholm and Saarma 2010).

Human CDNF includes a signal sequence of 26 amino acids and the mature protein consists of 161 amino acids (Lindholm et al. 2007). Mouse CDNF lacks putative glycosylation sites but in human CDNF, one putative N-glycosylation and one O-glycosylation sites are found (Lindholm et al. 2007; Sun et al. 2011). Both glycosylated (Apostolou et al. 2008) and unglycosylated (Lindholm et al. 2007) forms have been shown to be secreted from CDNF overexpressing cells. Glycosylation of CDNF is not necessary for its secretion or biological activity (Lindholm et al. 2007; Sun et al. 2011).
1.2.3 Structure of MANF and CDNF

Disulphide bridges are formed between cysteine residues to stabilize the structure of proteins (reviewed in Oka and Bulleid 2013). One notable feature of the MANF/CDNF family is the strict conservation of the spacing between the eight cysteine residues found also in *Caenorhabditis elegans* (*C. elegans*) and *Drosophila* Manf, the invertebrate family members (Petrova et al. 2003). Thus, the three-dimensional structure of MANF/CDNF has been predicted to be conserved during evolution. All eight cysteine residues of MANF form intramolecular disulphide bridges (Parkash et al. 2009; Hellman et al. 2011). The structural studies by crystallography and NMR (nuclear magnetic resonance) spectroscopy showed that MANF and CDNF have two domains, N- and carboxy (C) -terminal, connected with a flexible linker region (Parkash et al. 2009; Hellman et al. 2010; Hoseki et al. 2010; Hellman et al. 2011; Latgé et al. 2013). A ribbon diagram of human MANF is presented in Figure 2.

![Ribbon diagram of human MANF structure](image)

*Figure 2: Ribbon diagram of human MANF structure.* Alpha-helixes (α) are numbered. The C-terminal CXXC motif and non-canonical ER retention signal RTDL are indicated. Reprinted from II.

1.2.3.1 The saposin-like N-terminal domain of MANF and CDNF

The globular N-terminal domain of MANF and CDNF consists of five alpha-helixes and one 3_10 helix (Parkash et al. 2009). The N-terminal domain is stabilized by three disulphide bridges formed by cysteine pairs C6–C93, C9–C82 and C40–C51 (numbering according to human MANF without signal sequence, Figures 1 and 2) (Parkash et al. 2009; Hoseki et al. 2010; Hellman et al. 2010). A structural similarity was found to SAPLIP (saposin-like protein) domain and the closest similarity was found to two membrane-lytic proteins of SAPLIP family, granulysin and NK-lysin (Parkash et al. 2009; Hellman et al. 2011). SAPLIPs form a functionally diverse protein family interacting with lipids and membranes (reviewed in Bruhn 2005). According to the structural similarity to SAPLIPs, the positively charged surface amino acid residues of MANF and CDNF are predicted to interact with negatively charged lipids (Parkash et al. 2009). This putative lipid interaction has not yet been demonstrated.
1.2.3.2 C-terminal domain of MANF is structurally similar to SAP-domain of Ku70

There are two cysteine residues in C-terminal domain of MANF (C-MANF), C127 and C130, which form a disulphide bond (Parkash et al. 2009; Hoseki et al. 2010; Hellman et al. 2011). This CXXC motif is characteristic for the superfamily of thiol-disulphide oxidoreductases (reviewed in Wouters et al. 2010). The CXXC motif of MANF does not have oxidoreductase activity or bind metal ions (Mizobuchi et al. 2007; Hartley et al. 2013). Another feature of the C-terminal domain of MANF/CDNF is the RTDL/KTEL amino acid sequence that resembles the canonical ER retention signal (see Chapter 1.3.4). Although MANF and CDNF have been predicted to possess high structural similarity (Parkash et al. 2009), the structure of C-terminal domain of CDNF has not yet been reported.

The NMR studies revealed that the human C-MANF is structurally similar to the SAP (SAF-A/B, Acinus and PIAS) protein superfamily (Hellman et al. 2011). The highest homology was found to the SAP domain of Ku70 [Ku autoantigen p70 subunit, also known as XRCC6 (X-ray repair complementing defective repair in Chinese hamster cells 6)]. Ku70, via the SAP-domain, binds and inactivates pro-apoptotic BAX [BCL-2 (B cell lymphoma 2) associated X protein] in cytoplasm (Sawada et al. 2003; Cohen et al. 2004). When dissociated from Ku70, BAX activates the mitochondrial cell death pathway and is the main pro-apoptotic effector in neurons (Sawada et al. 2003; Amsel et al. 2008, see also Chapter 1.3.3). Intracellular full-length MANF, injected either as an encoding plasmid into the nucleus or as a purified recombinant protein in the cytoplasm, was found to be cytoprotective towards etoposide-induced BAX-mediated cell death in vitro (Hellman et al. 2011). C-MANF alone also blocked etoposide-induced cell death (Hellman et al. 2011). When added to the medium as a recombinant protein, MANF did not protect cultured neurons from cell death and did not either bind or enter into the neurons (Hellman et al. 2011).

1.2.3.3 Disruption of alpha-helixes alters intracellular trafficking and secretion of CDNF

The relationship of alpha-helical structure of CDNF and function was assessed by proline insertions in individual alpha-helixes and by alanine substitutions in an in vitro study (Sun et al. 2011). While insertion of a proline residue might distract the protein folding, alanines are compatible with native folding (Cunningham and Wells 1989). In the N-terminal domain, the disruption of first alpha-helix (α1) by a proline insertion completely blocked secretion of CDNF via both constitutive and regulatory pathways (Sun et al. 2011). Additionally, transport from the ER to trans-Golgi network was defected and the mutated protein was accumulated in the ER. The secretion of mutated CDNF in which amino acids 15-18 (KEFL) or 19-22 (NRFY) (numbering according to the mature CDNF without signal sequence) of α1 were changed to AAAA was reduced via both constitutive and regulated pathways. For the C-terminal domain, a proline insertion in α7 decreased secretion of CDNF via regulatory pathway while secretion via constitutive pathway was unaltered. Mutations in α7 and α8 increased the O-linked glycosylation of T155 residue.
1.3 Endoplasmic reticulum and the maintenance of its homeostasis

MANF and CDNF have been found to partially localize to the ER. Additionally, a function for MANF in ER stress has been demonstrated (see Chapter 1.3.4). ER is a cell organelle responsible for initiation of protein folding in the secretory pathway. Additionally, it serves as the primary intracellular calcium store and plays an important role in lipid synthesis. ER is a complex membrane network and takes up about one third of the cell volume. Upon an increased need for folding capacity, the ER membrane can be expanded (van Anken et al. 2003; Schuck et al. 2009). Secreted and membrane proteins are folded and matured in the ER lumen and then transported to the trans-Golgi network. The quality of newly synthesized proteins is strictly monitored and misfolded proteins are either retained in the ER by molecular chaperones or directed towards ER-associated degradation (ERAD).

Translation of mRNA into polypeptide is processed by ribosomes. Secretory proteins include hydrophobic N-terminal signal sequence bound by signal recognition particle (SRP) upon initiation of translation (reviewed in Guerriero and Brodsky, 2012). SRP binds to SRP receptor located in the ER membrane and the newly synthesized polypeptide enters into the ER lumen through a translocation complex. Following translocation, signal sequence is cleaved by signal sequence peptidase complex and oligosaccharides can be added to the polypeptide chains by a process known as N-linked glycosylation. Polypeptides then interact with chaperones to prevent their misfolding and aggregation. In the ER lumen, high levels of calcium are needed for both protein folding and protein-chaperone interaction. High energy levels are required for maintaining Ca$^{2+}$ levels and redox homeostasis as well as for chaperone function. Proteins are folded and dimerized through the oxidation of pairs of free thiols on cysteine residues, a process catalyzed by protein disulphide isomerases (PDI) (reviewed in Oka and Bulleid 2013). PDIs belong to a superfamily of thiol-disulphide oxidoreductases for which a characteristic feature is a CXXC motif (reviewed in Ellgaard and Ruddock 2005; Sevier and Kaiser 2006; Wouters et al. 2010). CXXC motif functions in the disulphide bond formation and may also take part in metal ion binding (Narindrasorasak et al. 2003; Horibe et al. 2004).

1.3.1 Activation of unfolded protein response

When the protein folding capacity in the ER is exceeded and unfolded or misfolded proteins are accumulated, ER homeostasis is disturbed. This state is referred as ER stress and induces unfolded protein response (UPR) (reviewed in Ron and Walter 2007; Hetz 2012; Arensdorf et al. 2013). The main purpose of UPR is to retain ER homeostasis by decreasing the load of unfolded proteins. Novel protein synthesis is inhibited, folding capacity is increased by activation of chaperone expression, including the major ER chaperone GRP78 (glucose-regulated protein 78), and ER-directed mRNAs are degraded. Transcription of the genes encoding components of ERAD is induced to remove misfolded proteins from the ER by ubiquitin-proteasome degradation. If UPR fails to relieve ER stress and the accumulation of unfolded proteins persists, cell death pathways
The adaptive signalling cascades to restore ER homeostasis are activated through UPR transducer proteins IRE1 (inositol-requiring enzyme 1), PERK [PKR (RNA-dependent protein kinase) -like ER kinase] and ATF6 (activating transcription factor 6). Overview of UPR is presented in Figure 3.

![Figure 3. Overview of the three signalling branches of UPR. See main text for detailed description of the pathways. Reprinted with minor modifications by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery (Hetz et al. 2013), copyright (2013).](image)

1.3.1.1 The conserved function of IRE1 and signalling through X-box binding protein-1 (XBP1)

The studies in yeast (Saccharomyces cerevisiae) have promoted the understanding how cells respond to ER stress. In yeast, ER stress signalling is mediated solely by IRE1, the most ancient branch of UPR conserved from yeast to mammals (Cox et al. 1993; Mori et al. 1993). IRE1 is an ER transmembrane sensor protein consisting of a luminal dimerization domain and a cytoplasmic part (Tirasophon et al. 1998). The cytoplasmic domain includes a protein kinase domain necessary for IRE1 activation in response to ER stress (Mori et al. 1993). IRE1 is oligomerized and activated by trans-autophosphorylation but whether other phosphorylation targets exist, is not currently known (Shamu and Walter 1996; Papa et al. 2003).

The luminal domain of IRE1 binds GRP78 and dissociates from it upon ER stress (Bertolotti et al. 2000, Okamura et al. 2000). Oligomerization is repressed by binding to GRP78 (Oikawa et al. 2007) or prevented if the stress-sensing regions are deleted (Oikawa et al. 2005; Zhou et al. 2006). However, the GRP78 binding domain is dispensable for IRE1 activation upon ER stress suggesting that another alteration in the luminal domain of IRE1 is necessary for its activation upon ER stress (Kimata et al. 2004; Oikawa et al. 2007). Structural studies indicate that unfolded proteins bind to and are able to activate IRE1 directly (Credle et al. 2005; Oikawa et al. 2007). IRE1 was found to bind a
terminally unfolded protein and oligomerize providing further evidence to support this hypothesis (Gardner and Walter 2011). Thus, the association with GRP78 has been suggested to have a regulatory role rather than being the primary switch in IRE1-mediated induction of UPR (Kimata 2004; Credle 2005; Pincus et al. 2010; Gardner and Walter 2011).

In addition to the kinase activity, the cytoplasmic part of IRE1 exhibits endoribonuclease domain with dual function. First, it is involved in a conserved process termed regulated IRE1-dependent decay (RIDD) in order to degrade mRNAs in response to ER stress (Hollien and Weissman 2006; Hollien et al. 2009). RIDD involves mRNAs that traffic through ER and thus alleviates the burden for protein folding in ER (Hollien and Weissman 2006). However, whether a loose specificity of IRE1 endoribonuclease activity is directly involved or does the activation of IRE1 lead to recruitment of other nucleases, is currently unknown. The RNase activity of IRE1 has also been found to be necessary for autoregulation of mammalian IRE1 mRNA (Tirasophon et al. 2000).

Second consequence of IRE1 RNase activity is the cleavage of its sole known substrate, mRNA encoding a transcription factor Hac1 [ATF/CREB1 (cAMP responsive element binding protein 1) homologue] in yeast or XBP1 in metazoans (Cox and Walter 1996; Yoshida et al. 2001; Calfon et al. 2002; Niwa et al. 2005). Hac1/XBP1 induces expression of ER chaperones and components of ERAD by binding to UPRE (unfolded protein response element) or ERSE (ER stress-response element) (Cox and Walter 1996; Travers et al. 2000; Yamamoto et al. 2007; see Chapter 1.3.1.4). While in yeast the Ire1-Hac1 pathway is the sole inducer of UPR-targeted genes, in metazoans their expression is induced even if IRE1 or XBP1 is abolished (Calfon et al. 2002). In yeast, the site-specific cleavage of Hac1 mRNA excises a 252 nucleotide intron causing a frame shift as an unconventional splicing event (Cox and Walter 1996; Sidrauski and Walter 1997). Both unspliced (Hac1\textsuperscript{u}) and spliced (Hac1\textsuperscript{s}) are translated and able to activate UPRE, but Hac1\textsuperscript{u} is rapidly degraded due to ubiquitin-targeting PEST-sequence in its C-terminal part (Cox and Walter 1996; Pal et al. 2007). Additionally, the presence of unspliced intron represses translation of Hac1\textsuperscript{s}. In metazoans, a 23 (C. elegans and Drosophila) or 26 (mammals) nucleotide intron is cleaved (Yoshida et al. 2001; Calfon et al. 2002). The splicing and thus the translation of XBP1 into protein is the key event in UPR (Calfon et al. 2002). Unspliced XBP1 (XBP1\textsuperscript{u}) is suggested to have a role in negative feedback regulating IRE1 signalling by suppressing the translation of spliced XBP1 (XBP1\textsuperscript{s}) (Yoshida et al. 2006).

In vertebrates, two IRE1 homologues have been described, IRE1\alpha (also known as ER to nucleus signalling 1 (ERN1), hereafter referred as IRE1) (Tirasophon et al. 1998) and IRE1\beta (also known as ERN2) (Wang et al. 1998; Iwawaki et al. 2001). While IRE1 is ubiquitously expressed (Tirasophon et al. 1998), IRE1\beta is detected in the gastrointestinal system only (Bertolotti et al. 2001) and functions in the attenuation of protein synthesis by promoting cleavage of 26S rRNA (Iwawaki et al. 2001). RNase activity of IRE1\beta is necessary for the inhibition of protein synthesis and directed specifically towards secretory proteins (Nakamura et al. 2011; Tsuru et al. 2013).

In Drosophila, the unconventional splicing of Xbp1 by Ire1 is conserved (Ryoo et al. 2007; Souid et al. 2007). Additionally, RIDD was first identified in cultured Drosophila...
Schnieder-2 cells (Hollien and Weissman 2006). Similar to mammals, the expression of *Drosophila* homologue to GRP78, Hsc70-3 (Heat shock 70-kDa protein cognate 3), is upregulated in response to ER stress in Xbp1-dependent manner (Plongthongkum et al. 2007; Loewen and Feany 2010; Malzer et al. 2013; Sekine et al. 2013).

Under normal conditions, in both mouse and *Drosophila*, spliced form of XBP1 has been detected specifically in tissues with high secretory load (Iwawaki et al. 2004; Souid et al. 2007; Sone et al. 2013; Ryoo et al. 2013). This indicates that the cells and tissues producing high levels of secretory proteins are susceptible to constant ER stress (reviewed in Hetz and Glimcher 2008).

### 1.3.1.2 Attenuation of protein synthesis through PERK

One of the mechanisms by which cell tries to overcome ER stress is the attenuation of overall protein synthesis. It is mediated through PERK phosphorylating, and thus inhibiting, $\alpha$-subunit of eIF2 (eukaryotic translation initiation factor 2) (reviewed in de Haro et al. 1996; Donnelly et al. 2013). PERK resides on the ER membrane and has a stress-sensing luminal domain, transmembrane domain and cytosolic part (Shi et al. 1998; Harding et al. 1999). The kinase domain is located in the cytosolic part and is activated by dimerization and trans-autophosphorylation (Harding et al. 1999; Bertolotti et al. 2000). PERK is maintained inactive by GRP78 binding to the luminal domain (Bertolotti et al. 2000). In response to ER stress, GRP78 is dissociated from PERK allowing its activation. After restoring ER homeostasis, PERK is reassociated with GRP78 and dephosphorylated (Bertolotti et al. 2000).

Although the phosphorylation of eIF2$\alpha$ inhibits overall protein synthesis, it was also found to selectively upregulate the translation of specific target mRNAs including ATF4 (activating transcription factor 4) (Hinnebusch 1993; Harding et al. 2000). These mRNAs contain multiple inhibitory upstream ORFs and inactivation of eIF2$\alpha$ allows ribosomes to reach the ORF with a start codon resulting in translation of a functional protein (Hinnebusch 1993). ATF4 activates transcription of genes that function in cell death, amino acid metabolism and resistance to oxidative stress.

The attenuation of protein synthesis and selective translational activation are regulated to certain extent by dephosphorylation of eIF2$\alpha$. GADD34/PPP1R15A (growth arrest and DNA damage protein 34/protein phosphatase 1, regulatory subunit 15A) and CReP/PPP1R15B (constitutive repressor or eIF2$\alpha$ phosphorylation/protein phosphatase 1, regulatory subunit 15B) are subunits of PP1 (protein phosphatase 1) that specifically counteract the inactivation of eIF2$\alpha$ by dephosphorylation (Connor et al. 2001; Novoa et al. 2001; Jousse et al. 2003). While CReP is constitutively expressed (Jousse et al. 2003), GADD34 expression is induced through ATF4 upon ER stress-mediated phosphorylation of eIF2$\alpha$ forming a negative feedback loop (Ma and Hendershot 2003; reviewed in Baird and Wek 2012).

The *Drosophila* homologue of PERK, PEK (pancreatic eIF-2$\alpha$ kinase), phosphorylates eIF2$\alpha$ *in vitro* and localizes to ER membrane when transfected in mammalian cells (Pomar et al. 2003). Additionally, PEK-mediated phosphorylation of eIF2$\alpha$ was increased
in cells with induced ER stress indicating that the UPR branch mediated through PERK is conserved in *Drosophila* (Pomar et al. 2003).

Invertebrate homologues of eIF2α phosphatases were only recently discovered (Malzer et al. 2013). The sole homologue to PP1 subunits in *Drosophila*, DmGadd34 (also known as CG3825), dephosphorylates eIF2α (Carra et al. 2010; Malzer et al. 2013). DmGadd34 is functionally more related to CREP due to the lack of transcriptional regulation upon ER stress. However, the translation of DmGadd34 was induced in response to ER stress. Overexpression of DmGadd34 induced the unconventional splicing of Xbp1 and thus ER stress similarly to the loss of endogenous PEK, suggesting that function of GADD34 and CREP is conserved in *Drosophila* (Malzer et al. 2013).

The *Drosophila* homologue of ATF4, cryptocephal (crc), functions in ecdysone-regulated events during larval molts and pupal metamorphosis (Hewes et al. 2000). During molts, larvae build a new cuticle and shed the old one. Synthesis of the new cuticle involves large amounts of secreted proteins and thus cause developmental ER stress. Similar to mammals, the protein level of crc/ATF4 is increased in response to ER stress (Kang et al. 2012). Interestingly, both mice and flies lacking ATF4 exhibited signs that indicate diabetes-like alterations (Seo et al. 2009). There is increasing evidence linking ER stress with the loss of β-cells in diabetes (reviewed in Lee and Ozcan 2014).

In contrast to mammals, both *Drosophila* and *C. elegans* genomes lack apparent homologues of CHOP [C/EBP (CCAAT/enhancer binding protein) homologous protein], the main transcriptional target of ATF4 (Zinszner et al. 1998) (see Chapter 1.3.3).

### 1.3.1.3 Upgrading the UPR by ATF6

The third signalling branch of UPR is mediated by ATF6. In unstressed cells, ATF6 resides on ER membrane and its ER luminal domain is bound by GRP78 (Haze et al. 1999; Shen et al. 2002). Upon ER stress, ATF6 dissociates from GRP78 and is transported to Golgi. There, ATF6 is cleaved by Site-1 and Site-2 proteases (S1P and S2P) to release the cytosolic part of ATF6 (N-ATF6, ATF6f in Figure 3) (Haze et al. 1999; Ye et al. 2000). N-ATF6 has transcriptional activity and contains a DNA binding domain. After cleavage N-ATF6 is localized to nucleus where it binds to ERSE (Yoshida et al. 1998) in presence of NF-Y [nuclear transcription factor Y, also known as CBF (CCAAT-binding transcription factor)] and activates expression of target genes, *e.g.* encoding for XBP1, GRP78 and CHOP (Haze et al. 1999; Yoshida et al. 2000). N-ATF6 induces expression of ER chaperones and ERAD components (Wu et al. 2007). ATF6 branch has been hypothesized to be responsible for maintaining mRNA level of XBP1 in such a way that a rapid response following activation of IRE1 is possible (Yoshida et al. 2003).

In mammals, a structural homologue to ATF6 was identified and named ATF6β (Haze et al. 2001). While N-ATF6β has transcriptional activity and binds to ERSE, it seems to inhibit the transcriptional activity of N-ATF6, probably to fine-tune the transcriptional regulation during UPR (Thuerauf et al. 2004; Thuerauf et al. 2007).

Invertebrate genomes include only one homologue to ATF6. The *Drosophila* homologue, CG3136, has high homology at amino acid level and contains the DNA binding, transmembrane and ER-luminal domains. However, its putative involvement in
Drosophila UPR has not yet been studied (Ryoo and Steller, 2007; Rasheva and Domingos, 2009). The C. elegans homologue, atf-6, is dispensable for worm development and functional UPR, but is partially redundant with Ire1 pathway (Shen et al. 2005).

1.3.1.4 Activation of chaperone expression through specific cis-acting elements

In response to ER stress, the expression of chaperones is induced through three different cis-acting elements found in their promoter regions, UPRE (Wang et al. 2000; Yoshida et al. 2003), ERSE (Roy and Lee 1995; Roy et al. 1996; Yoshida et al. 1998) and ERSE-II (Kokame et al. 2001; Yamamoto et al. 2004). UPRE contains consensus sequence TGACGTGG/A and is the preferential binding site for XBP1s (Yoshida et al. 2001). The consensus of ERSE element is CCAAT-N(9)-CCACG and N-ATF6 was found to activate the expression of ER stress-inducible genes by binding to the CCACG sequence (Yoshida et al. 2000). Binding of N-ATF6 to ERSE requires NF-Y bound to CCAAT (Yoshida et al. 2000). In the presence of NF-Y, XBP1s is also able to bind to ERSE in similar manner to N-ATF6, but with a lot lower affinity (Yamamoto et al. 2004). The ERSE-II element with consensus ATTGG-N-CCACG has been shown to be activated by both N-ATF6 and XBP1s (Yamamoto et al. 2004). In addition, Luman/CREB3 (cAMP responsive element binding protein 3), another ER stress-inducible factor, was found to associate with ERSE-II element (Liang et al. 2006).

1.3.2 Clearing the misfolded proteins by ERAD

When unfolded and misfolded proteins begin to accumulate in the ER, they are degraded by a cytosolic complex called ubiquitin-proteasome system. Misfolded proteins are bound by chaperones that direct their transport from ER to cytoplasm. The mechanism by which misfolded proteins are translocated back to the cytoplasm is known as ERAD (reviewed in Meusser et al. 2005; Stolz and Wolf 2010). ERAD is also used to regulate certain enzyme levels.

The first step in ERAD is to recognize misfolded region and it is mediated by chaperones. The quality of glycosylated proteins is monitored by chaperone-like lectins. After recognized, the misfolded protein is polyubiquitinated, usually in lysine residues by an ubiquitin ligase (E3). Ubiquitin activating enzyme (E1) and ubiquitin conjugating enzyme (E2) activate ubiquitins prior binding to a misfolded protein. The ubiquitinated protein is removed from the ER either together with the surrounding ER-membrane or through a retrotranslocon and escorted to 26S proteasome. ERAD is divided into three subpathways depending on the subcellular location of the misfolded region. Protein misfolding in the ER-luminal part is degraded by ERAD-L, transmembrane part by ERAD-M and cytoplasmic side by ERAD-C. The interacting chaperone, ubiquitin ligase and retrotranslocation components are selected accordingly.
1.3.3 ER stress and cell death

Cells that encounter ER stress activate signalling cascades through the three branches of UPR in order to return the homeostasis in ER. If cell fails to overcome ER stress, it enters the apoptotic pathway and is destined to die. All three branches of UPR regulate signalling pathways leading to cell death. Although signalling pathways involved in ER stress-related cell death have been identified, the molecular mechanisms behind it are still poorly understood.

BCL-2 protein family plays a critical role in apoptosis and consists of several pro- and anti-apoptotic proteins (reviewed in Tait and Green 2010). Characteristic for these proteins is a BCL-2 homology (BH) domain. Apoptosis is initiated upon activation of pro-apoptotic multidomain BAX and BAK (BH antagonist or killer). Multimerized BAX and BAK promote the permeabilization of the outer mitochondrial membrane allowing the release of cytochrome c into cytoplasm and activation of the caspase cascade. BAX and BAK play a crucial role in the ER stress-induced cell death which is blocked in mouse embryonic fibroblasts (MEFs) derived from mice lacking both endogenous BAX and BAK (Wei et al. 2001). BH3-only proteins are upstream regulators of BAX and BAK and their expression is upregulated by UPR. BH3-only proteins also promote apoptosis by inhibiting anti-apoptotic BCL-2 proteins.

Two BCL-2 family proteins have been identified in Drosophila genome, pro-apoptotic Debcl (death executioner Bcl-2 homologue) and anti-apoptotic Buffy (Chen and Abrams, 2000). Buffy is localized to the ER while Debcl is found in the mitochondria (Doumanis et al. 2007). In contrast to mammalian BAX and BAK, the Drosophila Debcl has only a limited role in apoptosis (Sevrioukov et al. 2007; Galindo et al. 2009). Yet, a role for Debcl or Buffy has not been reported in Drosophila UPR. Homologues to BH3-only proteins have not been identified in the Drosophila genome (Quinn et al. 2003).

Phosphorylation of eIF2α by PERK leads to translational upregulation of ATF4. One of the main targets of ATF4 is the apoptosis-promoting CHOP (also known as GADD153). The mechanism by which CHOP promotes apoptosis is still unclear. CHOP is known to downregulate the expression of anti-apoptotic BCL-2 (McCullough et al. 2001) and overexpression of BCL-2 was found to block CHOP-induced apoptosis (Matsumoto et al. 1996). CHOP also upregulates the expression of eIF2α phosphatase GADD34 as well as pro-apoptotic BH3-only proteins BIM (BCL-2-interacting mediator of cell death) and PUMA (p53 upregulated modulator of apoptosis) (Marciniak et al. 2004; Puthalakath et al. 2007; Galehdar et al. 2010).

1.3.3.1 IRE1-mediated regulation of apoptosis

The activation of IRE1 regulates apoptosis by its downstream targets JNK (c-JUN N-terminal kinase), ERK (extracellular signal-regulated kinase), p38 and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells). The recruitment of TRAF2 [TNFR (tumor necrosis factor receptor)-associated factor 2] by cytosolic domain of IRE1 results in activation of ASK1 (apoptosis signal-regulating kinase 1), a member of MAPK (mitogen-activated protein kinase) family. JNK is a downstream target of ASK1 and its
activation is an important event in induction of apoptosis (Urano et al. 2000; Nishitoh et al. 2002). Phosphorylation by JNK inhibits anti-apoptotic BCL-2 (Pattingre et al. 2009) and activates pro-apoptotic BIM (Lei and Davis 2003).

NF-κB is a regulator of both mitochondrial and death receptor apoptotic pathways. NF-κB transcriptional complex, consisting of NF-κB/Rel (Relish) transcription family members, regulates transcription of genes involved in innate immunity, inflammation and apoptosis (reviewed in Hoesel and Schmid 2013). NF-κB is maintained inactive by IκB (inhibitor of NF-κB) and activated by phosphorylation of IκB by IKK (inhibitor κB kinase). Inhibition of NF-κB was found to suppress ER stress-induced cell death in vitro (Hu et al. 2006) and activation of NF-κB requires phosphorylation of eIF2α (Jiang et al. 2003). IRE1 is also essential for the ER stress-induced activation of NF-κB by forming a complex with IKK through TRAF2 (Hu et al. 2006).

1.3.4 Role of MANF in ER stress

The mammalian and Drosophila MANF has been found to localize to ER (Mizobuchi et al. 2007; Apostolou et al. 2008; Tadimalla et al. 2008; Palgi et al. 2012). The C-terminus of MANF and CDNF resembles the canonical ER retention signal sequence, KDEL. This putative ER retention signal of MANF/CDNF is conserved: RTDL in human and mouse MANF, KTEL in human CDNF and RSEL in Drosophila Manf. RTDL motif has been shown to be responsible for retaining MANF in the ER and evidence suggests an interaction with KDEL-R (KDEL receptor) (Glembotski et al. 2012; Henderson et al. 2013). Three KDEL-R genes are found in human genome (reviewed in Capitani and Sallese 2009). These receptors bind C-terminal KDEL-containing proteins in Golgi and facilitate their trafficking back to ER. In vitro study revealed that the RTDL sequence binds KDEL-R with lower affinity in comparison to canonical KDEL sequence (Raykhel et al. 2007). The different affinity between KDEL and RTDL sequences has been suggested to have crucial role in MANF secretion (Glembotski 2011). This hypothesis is supported by in vitro studies showing that mutated MANF-KDEL, in which the last four C-terminal amino acids (RTDL) were replaced with KDEL sequence, was retained intracellularly more efficiently than the wild type MANF (Glembotski et al. 2012). Additionally, deleting the RTDL sequence has been shown to increase MANF secretion (Glembotski et al. 2012; Oh-Hashi et al. 2012; Henderson et al. 2013).

Silencing of MANF made cells more susceptible to and, vice versa, overexpression of MANF protected cells against tunicamycin-induced ER stress-related cell death (Apostolou et al. 2008). Murine MANF showed high expression level in secretory cells that are prone to ER stress due to high traffic of proteins through the ER (Mizobuchi et al. 2007; Lindholm et al. 2008). The protective role against ER stress-related cell death together with the expression studies support the suggested chaperone-like functions for MANF (Lindholm et al. 2008). Additionally, MANF has been found to bind misfolded proteins in vitro (Hartley et al. 2013).
1.3.4.1 ER stress induces expression of MANF

Microarray analyses have demonstrated that the expression of MANF is altered in response to ER stress. Tunicamycin, thapsigargin and dithiothreitol, agents that induce ER stress, were found to upregulate MANF expression in MEF, HeLa, U2OS (human osteosarcoma) and HEK293 (human embryonic kidney 293) cells in vitro (Lee et al. 2003; Apostolou et al. 2008) as well as in Drosophila adult flies in vivo (Girardot et al. 2004). In MEFs, the upregulation of MANF by tunicamycin treatment was lost in XBP1 knockout cells in which ATF6 was silenced suggesting that the induction of MANF expression is mediated through XBP1 or ATF6 (Lee et al. 2003). Later studies revealed that overexpression of activated ATF6 or XBP1 in cardiomyocytes induced MANF expression in vitro (Tadimalla et al. 2008) and that ATF6 expression led to upregulation of MANF expression in the heart of transgenic mouse in vivo (Belmont et al. 2008). Additionally, both N-ATF6 and XBP1s increased MANF promoter activity in response to thapsigargin-induced ER stress in Neuro2a cells (Oh-Hashi et al. 2013).

Expression of MANF has also been found to be upregulated in response to expression of unfolded proteins (Mizobuchi et al. 2007; Nundlall et al. 2010). Pancreatic β-cells isolated from Insulin-Akita mouse showed increased MANF level (Mizobuchi et al. 2007). This diabetes mellitus mouse strain carries C96Y mutation in Ins2 gene (Ins2\textsuperscript{C96Y}) that prevents the folding of proinsulin (Yoshioka et al. 1997; Wang et al. 1999; reviewed in Ron 2002). Pancreatic β-cells expressing high levels of insulin, a secretory protein, are already susceptible to ER stress (Iwawaki et al. 2004). The expression of Ins2\textsuperscript{C96Y} leads to accumulation of unfolded proinsulin causing continuous ER stress including activation of ATF6 and XBP1 (Nozaki et al. 2004). Additionally, chondrocytes isolated from mouse model of multiple epiphyseal dysplasia (MED) showed increased MANF expression (Nundlall et al. 2010). This transgenic mouse strain carries a V194D mutation in Matrilin 3 (Matn3) encoding gene causing folding defect in the A-domain of Matn3 (Cotterill et al. 2005; Leighton et al. 2007). Matn3\textsuperscript{V194D} mutant form is retained in the ER and activates UPR (Cotterill et al. 2005; Leighton et al. 2007). Matn3\textsuperscript{V194D} mutant has been shown to reduce proliferation and dysregulate apoptosis in chondrocytes (Leighton et al. 2007). Noting that the silencing of MANF increased cell proliferation, protected glucose-deprived cells from apoptosis and thus enhanced the survival of cultured HeLa cells (Apostolou et al. 2008), Hartley et al. (2013) suggested that MANF could have a role either in initiation or progress of MED.

Another indication of a link between ATF6/XBP1 and MANF was found in a study of in vitro maturation (IVM) oocytes (Wang et al. 2011). IVM is associated with lower rates of blastulation and pregnancy in comparison to routine in vitro fertilization (Lee et al. 2008). In comparison to in vivo matured oocytes, MANF mRNA level was increased in IVM oocytes and two-cell embryos as well as in the brain tissue of fetal and newborn mice (Wang et al. 2011). MANF protein levels together with mRNA levels of ATF6 and XBP1 were increased in the brain tissues in corresponding time points suggesting an induction of ER stress response and subsequent upregulation of MANF expression. However, brain morphology or cognition was not significantly altered.
Murine MANF has been found to be upregulated through ERSE-II located in its promoter region at positions $-134$ to $-124$ (Mizobuchi et al. 2007; Tadimalla et al. 2008). Both XBP1/ATF6 and NF-Y binding sites are required for induction of MANF expression upon ER stress (Mizobuchi et al. 2007). MANF promoter also includes the canonical ERSE at position $-492$ to $-473$ but was found to be far less efficient for inducing MANF expression in response to ER stress (Tadimalla et al. 2008). Interestingly, the MANF ERSE-II completely matches the first ERSE-II element discovered in promoter region of HERP [also known as HERPUD1 (homocysteine-inducible, ER stress-inducible, ubiquitin-like domain member 1)] (Kokame et al. 2001). HERP encodes an ubiquitin-domain ER membrane protein and is predicted to function in the ERAD pathway (Kokame et al. 2001, Hori et al. 2004; Schulze et al. 2005). Luman/CREB3 was found to contribute to the ER stress-induced expression of HERP (Liang et al. 2006) but not MANF (Oh-Hashi et al. 2013). Expression of another component of ERAD complex, VIMP (VCP-interacting membrane protein, also known as selenoprotein S), is suggested to be induced upon ERSE-II-like element located in its promoter region (Schulze et al. 2005). Taken together, ERSE-II might regulate the protein quality control and degradation of misfolded proteins during ER stress suggesting that MANF has also a role in these functions (Mizobuchi et al. 2007).

1.3.4.2 MANF expression is essential for pancreatic $\beta$-cells

Recently, a knockout MANF mouse model was reported (Lindahl et al. 2014). Both global and pancreatic-specific knockout of MANF resulted in severe diabetic phenotype with insulin deficiency and progressive loss of $\beta$-cells. MANF knockout mice were postnatal viable but their health was declined drastically soon after reaching adulthood. In accordance with the previous diabetes models (reviewed in Lee and Ozcan 2014), the loss of $\beta$-cells in MANF knockout mice was mediated by decreased proliferation and increased apoptosis (Lindahl et al. 2014). Overexpression of MANF enhanced proliferation and inhibited cell death of $\beta$-cells. Moreover, the loss of MANF resulted in progressive UPR in vivo. These findings are in line with the in vitro studies showing that overexpression protected cells against ER stress-induced cell death (Apostolou et al. 2008). Additionally, silencing of MANF made cells more vulnerable to ER stress-induced cell death in vitro (Apostolou et al. 2008). This could explain why $\beta$-cells, known to be susceptible to ER stress because of the high levels of secretory proteins (Iwawaki et al. 2004), are lost if MANF expression is abolished (Lindahl et al. 2014).

1.3.4.3 MANF interacts with the major ER chaperone GRP78

GRP78 [also known as BiP (binding immunoglobulin protein) and HSPA5 (heat shock 70 kDa protein 5); Haas and Wabl 1983; Munro and Pelhan 1986; reviewed in Lee 1987; Weng et al. 2011; Pfaffenbach and Lee 2011] is a member of the heat shock protein 70 (HSP70) family and associates with other ER-resident proteins. GRP78 is the major ER chaperone assisting the folding of newly synthesized proteins and consists of three domains: ATPase, peptide-binding and C-terminal domains. Overexpression of GRP78
was found to increase Ca\textsuperscript{2+} storage capacity independent of the peptide-binding domain and thus its chaperone activity towards other proteins (Lievrémont et al. 1997). In addition, GRP78 associates with ER stress response transducer proteins, IRE1, PERK and ATF6, and maintains them inactive. GRP78 has also been shown to have cytoprotective and anti-apoptotic properties. ER stress, induced by a presence of unfolded protein, hypoxia or ischemia (see Chapter 1.3.4.5), has been shown to increase expression of GRP78. Transcriptional activity of XBP1s activates GRP78 expression through ERSE element found in the promoter region of GRP78.

Mammalian MANF was found to co-localize with GRP78 in the ER in cardiomyocytes (Mizobuchi et al. 2007; Tadimalla et al. 2008). MANF was also found to interact directly with GRP78 in Ca\textsuperscript{2+}-dependent manner (Glembotski et al. 2012). MANF binding to GRP78 was released upon a treatment with thapsigargin, an inhibitor of SERCA (sarcoplasmic reticulum calcium transporting ATPase) used to deplete ER calcium and induce ER stress, leading to increased secretion of MANF. Overexpression of GRP78 in cardiomyocytes decreased the secreted portion of MANF independent of ER retention signal (Glembotski et al. 2012).

The loss of RTDL sequence did not affect the thapsigargin-induced secretion of MANF in cultured cardiomyocytes (Glembotski et al. 2012). However, an in vitro study in SH-SY5Y neurons revealed that RTDL sequence was essential for the thapsigargin-induced secretion of MANF suggesting a cell type-dependent role of MANF (Henderson et al. 2013). The RTDL sequence was found to mediate MANF binding to cell surface, modulated by KDEL-R (Henderson et al. 2013). The cell type-dependent requirement for ER retention signal in thapsigargin-induced secretion of MANF might be due to cell-specific expression of different KDEL-Rs (Henderson et al. 2013). KDEL-R and GRP78 probably maintain the ER residence and regulate the secretion of MANF together (Glembotski et al. 2012).

1.3.4.4 Atypical secretion of MANF upon ER stress

The increased secretion of MANF in response to ER stress is atypical for ER stress-induced molecules that are usually further retained in the ER (Glembotski 2011). A competence model was suggested by Glembotski (2011). MANF containing an unique RTDL sequence as ER retention signal has weaker affinity to KDEL-R than the canonical KDEL sequence found in the majority of ER-resident proteins (Raykhel et al. 2007). Under normal conditions, MANF mainly binds KDEL-Rs and is retained in the ER. Upon ER stress, the expression of ER-resident proteins is upregulated and more KDEL-containing proteins enter the Golgi. However, the level of KDEL-Rs is not affected by ER stress (Llewellyn et al. 1997). MANF, with lower affinity to KDEL-R, loses its binding to the receptors and is more secreted. Thus, MANF could act as an extracellular signalling molecule and signal to neighbouring cells what is the state of the secreting cell. Additionally, MANF could bind to the cell membrane and activate cell survival promoting pathways cis.
1.3.4.5 MANF and ischemia

Ischemia is a pathological state in which blood supply to tissues is disturbed and subsequently leads to hypoxia i.e. deprivation of oxygen. ER stress is induced by an ischemic injury (reviewed in DeGarcia and Montie, 2004) and the protein folding in the ER lumen requires molecular oxygen (Tu and Weissman, 2002). Drosophila embryos homozygous for a null allele of DmManf showed tolerance to a hypoxic environment (Azad et al. 2012; P. Azad, personal communication; M. Palgi, personal communication). Hypoxia increased murine MANF mRNA level in vitro (Romero-Ramirez et al. 2004). Expression of MANF mRNA was transiently increased after ischemic insult and status epilepticus in rat brains (Lindholm et al. 2008) and MANF protein expression was increased upon ischemic brain injury in rats (Apostolou et al. 2008; Yu et al. 2010). Ischemia-induced MANF expression was found in activated microglial cells and astrocytes which lack endogenous MANF expression under normal physiological conditions (Shen et al. 2012). Increased MANF expression preceded the upregulation of CHOP expression in response to ischemia-induced ER stress (Yu et al. 2010).

1.3.4.6 Mammalian MANF and CDNF are neuroprotective in vivo

Both mammalian MANF and CDNF, when injected as recombinant proteins into striatum, have been shown to protect and repair midbrain dopaminergic neurons in the 6-OHDA (6-hydroxydopamine) rat model of Parkinson’s disease (PD) in vivo (Lindholm et al. 2007; Voutilainen et al. 2009). Injected recombinant CDNF was also protective and neurorestorative for the tyrosine hydroxylase (TH)-positive cells in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model of PD (Airavaara et al. 2012). TH is the rate-limiting enzyme in dopamine synthesis and is commonly used as a marker for dopaminergic cells (reviewed in Daubner et al. 2011). CDNF has been found to be protective in the 6-OHDA rat model also when gene therapy with a recombinant adeno-associated viral (AAV) serotype 2 vector encoding CDNF was used (Bäck et al. 2013). Additionally, chronic infusion of CDNF but not MANF after the 6-OHDA treatment was protective towards degeneration of dopaminergic neurons in vivo (Voutilainen et al. 2011).

Prelesional administration of either a recombinant MANF protein or AAV vector encoding MANF was found to protect cerebral cortex neurons against ischemia in rats and prevent cell death in ischemic cortex (Airavaara et al. 2009; Airavaara et al. 2010). Extracellularly added recombinant MANF was found to protect cultured cardiomyocytes from simulated ischemia (Tadimalla et al. 2008) and protected from tissue damage in an in vivo ischemia mouse model (Glembotski et al. 2012).

A protective role for MANF was also demonstrated in a spinocerebellar ataxia 17 (SCA17) knockin mouse model (Yang et al. 2014). In SCA17, an expanded polyglutamine repeat in TATA box binding protein (TBP) causes age-dependent neurodegeneration (reviewed in Orr and Zoghbi, 2007). In SCA17 knockin mice, chaperone activity together with MANF expression was decreased (Yang et al. 2014). The reduced MANF level was found to be mediated by decreased transcriprional activity through Xbp1s. Overexpression of MANF protected from mutated TBP toxicity. Additionally, MANF was found to
increase the phosphorylation of protein kinase C (PKC) in PC12 cells and in vivo suggesting that MANF is involved in the signalling pathways activated by PKC.

1.3.4.7 MANF and CDNF repress inflammatory response

In response to ER stress caused by an ischemic injury in the nervous system, astrocytes secrete pro-inflammatory and anti-inflammatory cytokines together with trophic factors to modify the surrounding microenvironment. All three branches of mammalian UPR contribute to the inflammatory process by regulating the activity of NF-κB, the main mediator of pro-inflammatory response. Astrocyte damage leads to excessive cytokine release which may have a destructive effect on the survival of neurons. The roles of MANF and CDNF for ER stress-induced inflammatory cytokine expression in cultured rat astrocytes were studied (Cheng et al. 2013; Zhao et al. 2013b). In the first study (Cheng et al. 2013) tunicamycin was used to induce ER stress and subsequent expression and secretion of pro-inflammatory cytokines. Overexpression of CDNF in rat primary astrocytes suppressed both mRNA expression and secreted protein levels of pro-inflammatory cytokines IL1-β (interleukin-1 beta), IL-6 (interleukin-6) and TNF-α (tumor necrosis factor alpha) (Cheng et al. 2013). In further studies, the overexpression of CDNF showed cytoprotective effect against lipopolysaccharide-induced inflammation by suppressing phosphorylation of JNK but not p38 or ERK pathways (Zhao et al. 2013a). In the second study (Zhao et al. 2013b), oxygen-glucose deprivation (OGD) was used to induce inflammatory response monitored by the increased expression and secretion of pro-inflammatory cytokines. In addition, OGD induces expression of GRP78, indicating activation of ER stress response, and p65 subunit of NF-κB. When cells were incubated with extracellular recombinant MANF prior to OGD, the inflammatory response was significantly reduced and cell viability increased. Additionally, preincubation of the OGD cells with MANF-containing medium repressed the induction of GRP78 and p65 protein expression (Zhao et al. 2013b).

1.3.4.8 Valproic acid (VPA) induces expression of MANF and CDNF

VPA is used in treatment of epilepsy, mood disorders and migraine and has been reported to attenuate effects of oxidative and ER stresses (Lee et al. 2014). VPA treatment resulted in upregulation of CDNF and MANF expression in vivo and in vitro (Niles et al. 2012; Almutawaa et al. 2014). VPA acts through MAPK/ERK, phosphatidylinositol 3-kinase/Akt and Wnt/β-catenin pathways and it was suggested that these signalling pathways could be involved in the induction of MANF/CDNF expression (Monti et al. 2009; Almutawaa et al. 2014). Additionally, upregulation of MANF expression together with its protective role towards ER stress-related cell death (Apostolou et al. 2008) could contribute to the VPA-mediated relieve of ER stress (Almutawaa et al. 2014).
1.4 Selected highlights of Drosophila as a model organism

*Drosophila* has served as a model organism for over a century (reviewed in Bier 2005; Rincon-Limas et al. 2012). One of its key features is the modest genome size consisting of approximately 14,000 protein coding genes (http://flybase.org, FB2014_01 release; Adams et al. 2000; McQuilton et al. 2012). The easy and relatively inexpensive husbandry together with the sophisticated genetic tools makes *Drosophila* an attractive *in vivo* model to study not only the biological processes but also human diseases. Although diverging early during evolution of metazoans, the *Drosophila* genome includes homologues for 75% of identified human disease genes (Reiter et al. 2001).

1.4.1 Overview of the Drosophila life cycle

*Drosophila melanogaster* is a holometabolous species which undergoes complete metamorphosis with a four-stage development (described e.g. in Ashburner et al. 2005). The embryos hatch approximately one day after fertilization. During the larval stage growth is rapid and the larvae molt twice in around 24 h intervals dividing the larval stage into three instars. Two days after reaching the 3\(^{rd}\) instar, larvae wander away from the food and form an immobile pupa. The metamorphosis of larvae into adults occurs during the pupal stage and after 4–5 days, the adults eclose. Altogether, the development of *Drosophila* lasts 9–10 days.

1.4.2 Activation or silencing of target gene by binary expression system

One of the most important genetic tools of *Drosophila* model is the binary UAS (upstream activation sequence)/GAL4 expression system adopted from yeast (Brand and Perrimon 1993). The principle of UAS/GAL4 system is illustrated in Figure 4. UAS contains binding sites for GAL4, a yeast transcription factor. UAS fly lines are created by generating transgenic flies using a construct in which the target gene (i.e. marker or the gene of interest) is introduced downstream of UAS. Target gene expression is activated by crossing UAS-[target gene] flies to enhancer-GAL4 flies. In the absence of GAL4, target gene remains inactive although the flanking regions of the transgene insertion site may activate transcription independent of UAS (Potter et al. 2010). GAL4 lines can be created either by placing the GAL4 gene under specific promoter (e.g. heat shock promoter) or by inserting a promoterless GAL4 gene into the fly genome. The latter will use the genomic enhancers of the flanking region and has enabled establishment of thousands of GAL4 lines by mobilizing the GAL4 transgene (Brand and Perrimon 1993; Hayashi et al. 2002). These insertions are referred as “enhancer trap” lines.

Further developments of UAS/GAL4 system have been established for extremely refined genetic manipulations, e.g. MARCM (mosaic analysis with a repressible cell marker) system by Lee and Luo (1999) and Q system by Potter et al. (2010) (reviewed in Duffy 2002). The UAS/GAL4 system is also adopted for gene knockdown studies by
RNAi (RNA interference) process (Piccin et al. 2001; Kalidas and Smith 2002). In RNAi, the mRNA of target gene is degraded by double-stranded RNA (dsRNA) (Fire et al. 1998). In UAS-RNAi flies, dsRNA is introduced under UAS and can be expressed by GAL4 driver for tissue-specific inactivation. Transgenic genome-wide Drosophila RNAi libraries have been established and maintained in stock centers (Dietzl et al. 2007; http://www.shigen.nig.ac.jp/fly/nigfly/).

Figure 4. *Schematic presentation of the binary UAS/GAL4 in vivo expression system.* See text for details. P stands for 5’- and 3’- inverted repeats of P-element between which the transgene is located. Drawings based on Brand and Perrimon (1993), Duffy (2002), and Dietzl et al. (2007).

### 1.4.3 Examples of ER stress models in Drosophila

#### 1.4.3.1 Autosomal dominant retinitis pigmentosa (ADRP) and rhodopsin-1 (Rh-1)

The first *Drosophila* model focusing to study UPR in human disease was established in a study of Rh1 [also known as ninaE (neither inactivation nor afterpotential E)] (Ryoo et al. 2007). Mutated Rh1 (Rh1\(^{G69D}\)) was previously reported to cause retinal degeneration and disturb ER-Golgi transport displaying similar molecular and phenotypic characteristics to human ADRP (Colley et al. 1995; Kurada and O’Tousa 1995). Ryoo et al. (2007) cloned Rh1\(^{G96D}\) allele into the UAS construct for tissue-specific expression. UPR was discovered
to contribute to the retinal degeneration caused by ectopic expression of Rh1<sup>G96D</sup>, detected by the unconventional splicing of Xbp1 and upregulation of Hsc70-3, the Drosophila homologue of GRP78.

1.4.3.2 Diabetes mellitus and Ins2<sup>C96Y</sup>

A fly model corresponding to Insulin-Akita mouse was recently developed (Park et al. 2014; see Chapter 1.3.4.1). Both wild type and mutated (Ins<sup>C96Y</sup>) human proinsulin encoding constructs were introduced to Drosophila for conditional expression by UAS/GAL4 system. Ectopic expression of Ins<sup>C96Y</sup> in the Drosophila eye resulted in retinal degeneration similar to ADRP model (Ryoo et al. 2007, see also previous chapter). The unconventional splicing of Xbp1 was detected and mRNA levels of PEK, Hsc70-3 and Xbp1 were upregulated indicating the presence of ER stress. Similar to Insulin-Akita mouse model (see Chapter 1.3.4.1), a microarray analysis revealed that DmManf expression was upregulated in response to expression of Ins<sup>C96Y</sup> in the eye imaginal disc.

1.4.3.3 Alzheimer’s disease - Amyloid-β toxicity

Indirect links between pathogenesis of Alzheimer’s disease and alterations in ER function have been found, e.g. increased phosphorylation of eIF2α as well as elevated mRNA levels of GRP78 and XBP1s (Katayama et al. 1999; Chang et al. 2002; Kakimura et al. 2002; Hoozemans et al. 2005; Lee et al. 2010). In a recently established, improved Drosophila model of Alzheimer’s disease, GAL4-directed expression of a construct encoding two copies of human amyloid-beta peptide (Aβ) under UAS sequence caused neurotoxicity (Casas-Tinto et al. 2011). The splicing of Xbp1 and expression of Hsc70-3 were induced upon Aβ expression indicating ER stress. In this model, overexpression of either endogenous Drosophila Xbp1 or the spliced form of murine XBP1 rescued the Aβ-related neurotoxicity. Additionally, murine Xbp1s was found to induce expression of Hsc70-3 in Drosophila.

1.4.4 Genetic screens in the Drosophila model

A unique advantage of Drosophila as a model system is the possibility to perform large-scale genetic screens in vivo (reviewed in St. Johnston 2002). A screen can be carried out to identify genes involved in a particular cellular or developmental process. Additionally, they can be used to discover interacting partners of certain proteins or novel components of already established signalling pathways. The beauty of Drosophila genetic screens was revealed by the Nobel-prize winning mutagenesis screen to identify genes modulating the patterning of the Drosophila embryo (Nüsslein-Volhard and Wieschaus 1980). In this screen, mutations and thus genes affecting the normal patterning of the embryo were identified by altered embryonic morphology – one of the main reasons for its success was the simplicity of the phenotype detection.
In addition to mutations, modifier screens performed in *Drosophila* model have been fruitful. One advantageous strategy is to use flies heterozygous for a recessive mutation of the gene of interest and screen for enhancers and suppressors that cause a phenotype in the heterozygous – *i.e.* sensitised – background. This approach also allows identification of lethal mutations. After the establishment of UAS/GAL4 system (Brand and Perrimon 1993) and its derivatives, *e.g.* libraries of EP lines for gain-of-function (Rørth 1996) and UAS-RNAi lines for loss-of-function (Dietzl et al. 2007), more delicate tools exist to identify genetic modifiers. Moreover, genomic deletions of different sizes (also known as deficiencies, Merrill et al. 1988) can be assessed to roughly identify the chromosomal location of modifier genes and then narrowed down to found the actual interacting genes. Combining the available genetic tools enables identification of almost any genetic interaction.
2 AIMS OF THE STUDY

The MANF/CDNF family of neurotrophic factors has been characterized by their protective and neurorestorative functions. Based on animal model and in vitro studies, MANF and CDNF may have a potential as a treatment for neuronal degeneration. Currently, the drugs used to treat neurodegenerative diseases only alleviate the symptoms and are not able to halt or reverse the degeneration. However, development of an efficient and safe treatment requires that the molecular characteristics and mode of action of the drug candidates, i.e. MANF and CDNF, are thoroughly studied and understood. In this work, Drosophila melanogaster was used as a model to study the MANF/CDNF protein family function within a multicellular organism in vivo.

The specific aims of this study were:

I Explore the functional conservation between Drosophila Manf and its human homologues

II Characterize the structural and functional components of Drosophila Manf protein

III Identify genetic interaction partners of Drosophila Manf
3 MATERIALS AND METHODS

Materials and methods used in the individual studies (I, II and III) are presented in the original articles. In this chapter, I summarize the methods I have applied myself in the work presented in this thesis. These and the methods used by the other authors of studies I, II and III are listed in Table 1.

Table 1. Methods used in this study. The methods numbered in “Chapter” column I have used myself in at least one of the articles indicated. U, unpublished data.

<table>
<thead>
<tr>
<th>Method</th>
<th>Article</th>
<th>Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fly work</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fly husbandry and strains</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Generation of transgenic flies</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Rescue experiments</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td><strong>Molecular methods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmid constructs</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>RNA extraction</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>RT-PCR analysis</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Quantitative RT-PCR (qPCR)</td>
<td></td>
<td>III</td>
</tr>
<tr>
<td>Northern blot analysis</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Protein extraction</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Western blot analysis</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Measurement of dopamine concentration</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Transmission electron microscopy</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td><strong>In vitro cell culture and transfection</strong></td>
<td></td>
<td></td>
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<tr>
<td>Schneider-2 cell culture</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Chinese hamster ovary (CHO) cell culture</td>
<td></td>
<td>II</td>
</tr>
<tr>
<td>Injection of superior cervical ganglion (SCG) neurons</td>
<td></td>
<td>II</td>
</tr>
<tr>
<td><strong>Data acquisition and analysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microscopes, imaging and image analysis</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>I</td>
<td>II</td>
</tr>
</tbody>
</table>

3.1 Fly husbandry, strains and transgenesis

Flies were maintained at 25 °C if not mentioned otherwise. The growing medium consisted of malt, agar, semolina and yeast. Propionic acid and methyl 4-hydroxybenzoate were used as antifungal and antimicrobial compounds, respectively.

As a wild type, w strain maintained in our laboratory was used. Mutations and balancer chromosomes essential for the work presented in this thesis are listed in Table 2.
In the work presented in this thesis, \textit{DmManf} mutant stands for \textit{DmManf}^{\text{D96}} allele if not stated otherwise. The GAL4 lines used and their expression patterns are described in Table 3. UAS-lines and the purpose they were used for are presented in Table 4. The UAS-lines generated in II are comprehensively described in the original article, and the main characteristics of these constructs are illustrated in Figure 9 in Results and discussion section. UAS-RNAi lines used in III are listed in the original manuscript.

\textbf{Table 2. Mutations and balancer chromosomes.} \textit{Cy} (Curly) results in dominant curly wing phenotype. BDSC, Bloomington Drosophila Stock Center.

<table>
<thead>
<tr>
<th>Mutation/ Balancer</th>
<th>Description</th>
<th>Source and citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{DmManf}^{\text{D96}}</td>
<td>Null allele of \textit{DmManf}</td>
<td>1</td>
</tr>
<tr>
<td>debcl^{\text{W105}}</td>
<td>Putative dominant negative allele of \textit{debcl} (fly BAX homologue)</td>
<td>BDSC; Sevrioukov et al. 2007</td>
</tr>
<tr>
<td>debcl^{\text{E26}}</td>
<td>Amorphic allele of \textit{debcl}</td>
<td>BDSC; Sevrioukov et al. 2007</td>
</tr>
<tr>
<td>TM6B P{Dfd-EYFP}3 Sb Tb</td>
<td>Third chromosome balancer with \textit{Tb} and \textit{Sb} markers (see Chapter 3.3)</td>
<td>BDSC</td>
</tr>
<tr>
<td>T(2;3)SM6a-TM6B Tb</td>
<td>Translocation balancer against second and third chromosomes with \textit{Cy} and \textit{Tb} markers</td>
<td>BDSC</td>
</tr>
<tr>
<td>sqh-EYFP-ER</td>
<td>Ubiquitous ER-directed expression of EYFP used as an ER marker</td>
<td>BDSC; LaJeunesse et al. 2004</td>
</tr>
</tbody>
</table>

\textbf{Table 3. GAL4 lines with description of the expression patterns.} Most of the GAL4 lines are expressed in the larval salivary glands (Brand and Perrimon 1993). BDSC, Bloomington Drosophila Stock Center; \textit{da}, daughterless; GMR, Glass Multimer Reporter; repo, reversed polarity; salm, spalt major; tub, tubulin.

<table>
<thead>
<tr>
<th>GAL4 line</th>
<th>Expression pattern</th>
<th>Source and citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>69B-GAL4</td>
<td>Central nervous system (CNS), epidermis, gut, imaginal discs, proventriculus, ring gland, salivary glands, trachea</td>
<td>J. Mattila; Brand and Perrimon 1993</td>
</tr>
<tr>
<td>C135-GAL4</td>
<td>Proventriculus, salivary glands</td>
<td>L. Hrdlicka; Hrdlicka et al. 2002</td>
</tr>
<tr>
<td>\textit{da}-GAL4</td>
<td>Ubiquitous excluding proventriculus</td>
<td>BDSC; Wodarz et al. 1995</td>
</tr>
<tr>
<td>GMR-GAL4</td>
<td>Eye disc</td>
<td>BDSC; Freeman 1996</td>
</tr>
<tr>
<td>MS1096-GAL4</td>
<td>Wing pouch, partially in CNS</td>
<td>O. Shimmi; Capdevila and Guerrero, 1994</td>
</tr>
<tr>
<td>Repo-GAL4</td>
<td>Glial cells excluding midline glia</td>
<td>I. Salecker; Sepp and Auld 1999</td>
</tr>
<tr>
<td>salm-GAL4</td>
<td>Imaginal discs, ring gland, salivary gland, trachea</td>
<td>BDSC; Hinz et al. 1994</td>
</tr>
<tr>
<td>tub-GAL4</td>
<td>Ubiquitous</td>
<td>BDSC; Lee and Luo 1999</td>
</tr>
</tbody>
</table>
Table 4. UAS lines and the purposes for which they were used in this study. BDSC, Bloomington Drosophila Stock Center, VDRC, Vienna Drosophila RNAi Center.

<table>
<thead>
<tr>
<th>UAS line</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAS-DmManf</td>
<td>Wild type allele of DmManf, transformant lines 133/L3 and 135/L5</td>
<td>I</td>
</tr>
<tr>
<td>UAS-HsMANF</td>
<td>Wild type allele of human MANF</td>
<td>II</td>
</tr>
<tr>
<td>UAS-HsCDNF</td>
<td>Wild type allele of human CDNF</td>
<td>II</td>
</tr>
<tr>
<td>UAS-DmManf-RNAi</td>
<td>RNAi construct against DmManf mRNA, transformant line 12835</td>
<td>VDRC; Dietzl et al. 2007</td>
</tr>
<tr>
<td>UAS-lacZ</td>
<td>lacZ reporter; a dose control for UAS/GAL4 binary expression system</td>
<td>J. Larsson; Brand and Perrimon 1993</td>
</tr>
<tr>
<td>UAS-mCD8-GFP</td>
<td>GFP reporter with membrane directing tag (murine CD8); a dose control for UAS/GAL4 binary expression system</td>
<td>BDSC, Lee and Luo 1999</td>
</tr>
<tr>
<td>UAS-GFP.nls</td>
<td>GFP reporter with nuclear localization sequence (nls)</td>
<td>BDSC, Shiga et al. 1996</td>
</tr>
<tr>
<td>UAS-Hsc70-3^{WT}</td>
<td>Wild type allele of Hsc70-3 (fly GRP78 homologue)</td>
<td>BDSC, Elefant and Palter 1999</td>
</tr>
<tr>
<td>UAS-Hsc70-3^{K97S}</td>
<td>Dominant negative allele of Hsc70-3</td>
<td>BDSC, Elefant and Palter 1999</td>
</tr>
<tr>
<td>UAS-Hsc70-3^{D231S}</td>
<td>Dominant negative allele of Hsc70-3</td>
<td>BDSC, Elefant and Palter 1999</td>
</tr>
<tr>
<td>UAS-PEK</td>
<td>Wild type allele of PEK (fly PERK homologue)</td>
<td>S.J. Marciniak; Malzer et al. 2010</td>
</tr>
</tbody>
</table>

The constructs used to generate transgenic fly lines were cloned according to the descriptions in the original articles (I, II, see also Chapter 3.3). The injections to generate the transgenic lines were done by us (I) or Genetic Services, Inc. (II). Independent insertions were isolated and made homozygous, when possible. Insertion chromosome of each transformant line was analysed by in situ hybridization on larval giant polytene chromosomes (I) or by genetic crosses (II).

### 3.2 Plasmid constructs

A schematic design of the DmManf constructs is presented in Figure 5. The constructs were cloned according to standard molecular methods and the details are presented in the original articles (I, II). The mutations and tags to DmManf, HsMANF and HsCDNF cDNA (II) were introduced by inverse PCR mutagenesis using Phusion® Hot Start High-Fidelity DNA polymerase (Finnzymes). pBlueScript SK was used as an intermediate vector. In human MANF and CDNF, honeybee mellitin was used as a secretion signal peptide. pUAST vector was used in the generation of transgenic lines (I, II), pMT vector in the expression studies on transfected Drosophila Schneider-2 cells (I, II) and pCR3.1
vector in transfection (CHO cells) or injection (SCG neurons) of the cultured mammalian cells (II).

Figure 5. Schematic presentation of DmManf construct design. The number of amino acids in each part, secretion signal sequence (ss), N-terminal domain and C-terminal domain, are indicated. In tagged constructs, the V5-tag was inserted between ss and N-terminal domain. Start, start codon; ss, secretion signal sequence; Stop, stop codon; aa, amino acid.

3.3 Rescue experiments

The lethal phenotype of larvae homozygous for a loss of function allele of DmManf (I) was used in the rescue experiments in which a UAS-transgene was expressed by a GAL4 line in homozygous DmManf mutant background (Figure 6). The rescue of larval lethality was evaluated by appearance of pupae and pupal lethality by presence of adults representing the lethal genotype. A third chromosome balancer with Tb (Tubby, short and rounded body phenotype) and Sb (Stubble, short and blunt-ended bristle phenotype) dominant markers was used and emerging of the rescued individuals was monitored by appearance of Tb⁺ pupae and Sb⁺ adults. Parents were allowed to lay eggs for 2–3 days. The quantification of the rescue efficiency was evaluated by counting the amount of rescued pupae from 5 or 15 vials 9–10 days after the beginning of egg laying (I, II). The amount of rescued pupae was normalized by dividing it by 0.33 (homozygous UAS lines) or 0.17 (heterozygous UAS lines) according to Mendelian inheritance. Emerged rescued adults were counted 10–17 days after the beginning of egg laying. The amount of emerged adults was divided by the number of Tb⁺ pupae.

Figure 6. Schematic example of rescue crosses. The genotypes, pupal phenotypes (caused by Tb marker) and expected proportions (based on Mendelian inheritance) of progeny are presented in the diagram below the cross. Homzygous DmManf mutants are lethal at early larval stage Grey box indicates the rescued genotype. For description of the genetic components, see Tables 2 and 3.

3.4 RNA extraction and RT-PCR

Total RNA was extracted from larvae or adults (II, III). In II, RNeasy Mini Kit (Qiagene) was used in RNA extraction and RQ1 RNase-Free DNase (Promega) in DNase treatment. In III, NucleoSpin® RNA II (Macherey-Nagel) was used to extract RNA with on-column
DNase treatment by rDNase included in the kit. RNA was further purified using NucleoSpin® RNA II and the concentration and quality of purified RNA were analysed by NanoDrop.

M-MLV Reverse Transcriptase (Promega) was used to synthesize cDNA and GoTaq® Hot Start Polymerase in PCR reaction (II). PCR products were analysed on 1% agarose gel by electrophoresis.

### 3.5 Protein extraction and Western blot analysis

Protein extraction was made by homogenizing embryos (I, II), larvae (I, II, III) or adults (I) in lysis buffer supplemented with Complete proteinase inhibitor tablets (Roche). Cultured cell media was collected and stored, and the cells were lysed with lysis buffer. Buffers used are described in II. Western blotting was done according to the instructions provided by the manufacturer. The following rabbit polyclonal antibodies were used: anti-DmManf (I, II, III; generated in I), anti-HsMANF (II; Lindholm et al. 2008), anti-HsCDNF (I, II; Lindholm et al. 2007) and anti-V5 (II; Invitrogen). Anti-Twinfilin (I; Wahlström et al. 2001) or anti-Tubulin (II, III; Sigma) was used as a loading control.

### 3.6 Immunohistochemistry, imaging and image analysis

Larvae were dissected and fixed in 4% paraformaldehyde in PEM (100 mM PIPES pH 7.0, 2 mM EGTA, 1 mM MgSO₄) for 30 min. Tissues were incubated with primary antibody at 4 ºC overnight and with secondary antibody at room temperature for 1 h. Anti-DmManf (I) was used to detect DmManf protein expression and anti-pHis3 [phospho-Histone H3 (Ser10), 06-570, Upstate] to identify mitotic cells (Hendzel et al. 1997). Tissues were mounted with VECTASHIELD® Mounting Medium with or without DAPI (Vector Laboratories) and imaged with TCS SP5 laser scanning microscope (Leica Microsystems) equipped with HCX PL APO 20x/0.7 Imm Corr or HCX APO 63x/1.30 Corr CS 21 glycerol immersion objective. Imaris (Bitplane Inc.) and ImageJ (Abràmoff et al. 2004) softwares were used for image analysis. For the quantification of pHis3 positive cells, automatic “Spots” algorithm in Imaris 7.6.0 was used.

### 3.7 Statistical analysis

The means were compared by Student’s t-test and the null hypothesis was rejected at p<0.05. The statistical analysis was performed by using Microsoft® Excel Analysis ToolPak. The statistical significance of the survival of SCG neurons was analysed by one-way ANOVA and post hoc Dunnett’s test (II).
4 RESULTS AND DISCUSSION

4.1 DmManf is vital for normal Drosophila development (I)

To understand the function of DmManf, its expression was examined during embryogenesis (I) and larval stage (Palgi 2012) by immunohistochemical and in situ hybridization analyses. Similar to mammalian MANF, DmManf showed relatively wide expression pattern with preference for the secretory tissues. Studies using germline mosaic clones in vivo and transiently transfected Schneider-2 cells in vitro indicated that DmManf is a secreted protein (I). Later, a detailed intracellular analysis has revealed that DmManf is also partially localized to ER and mitochondria (Palgi et al. 2012; Palgi 2012).

For further functional analysis, deletions to DmManf gene were generated by P-element excision (I). A loss of function allele of DmManf gene, DmManf^D96, was used in studies presented in this thesis. Flies homozygous for DmManf^D96 allele (hereafter referred as DmManf mutant) failed to complete the 1st larval molt and died as early 2nd instar larvae. Heterozygous DmManf mutants showed no obvious phenotype.

According to Northern blot analysis, DmManf mRNA was expressed throughout the development and in adult flies (I). Strong mRNA and protein expressions were detected in the early stages of embryogenesis indicating that DmManf was maternally deposited. The lethality of DmManf mutant larvae occurred after maternal DmManf load had worn out. In order to study the effects of the complete loss of DmManf, the maternal DmManf was abolished by germline mosaic technique. The loss of both zygotic and maternal DmManf lead to lethality during the final stage of embryogenesis and the embryos failed to hatch. In contrast to zygotic mutant larvae, the maternal and zygotic DmManf mutant embryos showed dramatic disorganization of the central and peripheral nervous systems. Additionally, the cuticle of these mutant embryos was severely defective.

4.1.1 The loss of DmManf alters dopaminergic system (I, II)

Mammalian MANF was originally identified as a survival-promoting factor for dopaminergic neurons (Petrova et al. 2003). In later studies, MANF and CDNF exhibited neuroprotective and neurorestorative effects on dopamine neurons in rodent models of PD (Lindholm et al. 2007; Voutilainen et al. 2009). In CNS, DmManf was expressed in glial cells excluding midline glia (I). The strongest expression was found in the cell body glia surrounding neurons immunoreactive for TH, the rate-limiting enzyme in dopamine synthesis commonly used as a marker for dopaminergic neurons (reviewed in Daubner et al. 2011). In DmManf mutants, a selective loss of dopaminergic neurites was detected just prior to lethality of the larvae. In the mutant embryos lacking both maternal and zygotic DmManf, the dopamine level was drastically lower in comparison to wild type. In these mutant embryos, the layering of the cuticle was severely disorganized probably due to the loss of dopamine. The cuticular layers are crosslinked by dopamine-derived quinones.
during hardening of the cuticle (Wright et al. 1996; reviewed in Jane et al. 2005). The components used to build the cuticle are secreted by hypodermal cells. In zygotic DmManf mutant larvae, the expression of the hypoderm-specific transcript of *pale*, the *Drosophila* gene encoding for TH, was upregulated (Birman et al. 1994; Palgi et al. 2012). Accordingly, the dopamine levels were elevated in zygotic DmManf mutants probably reflecting the increased *pale* expression in the hypodermal cells (II).

4.1.2 *The loss of zygotic DmManf is rescued by ectopic DmManf expression (I, unpublished)*

To study whether the lethality observed in homozygous DmManf mutants was exclusively due to the loss of endogenous DmManf expression, transgenic fly lines with UAS-construct encoding DmManf were generated (I; Figure 7). When UAS-DmManf was ubiquitously expressed by *da*-GAL4 or *tub*-GAL4 driver in homozygous DmManf mutant background, the lethality of DmManf mutants was rescued to adulthood. The rescued adults were fertile and showed no obvious phenotype. This demonstrated that DmManf mutant lethality was solely due to the loss of endogenous DmManf.

To explore in which tissues DmManf expression is required for development, UAS-DmManf construct was expressed in homozygous DmManf mutant background in a tissue-specific manner. DmManf was found to be a secreted protein and function as an extracellular trophic factor (I). Thus, expression in exactly same cells in which endogenous DmManf is expressed might not be necessary but – instead – achieved via secreted DmManf pool from neighbouring cells. Proventriculus-specific expression of UAS-DmManf by C135-GAL4 was not able to rescue DmManf mutant lethality suggesting that a single, concerted source of DmManf was not enough for viability (I). To summarize the rescue experiments with various GAL4 drivers, the ectopic DmManf expression either in or in close proximity to hypodermal cells was sufficient to rescue the larval lethality of homozygous DmManf mutants (data not shown). These data indicate that the essential quantity of DmManf for viability can be achieved as a secreted protein. This also suggested that the primary reason for the lethality of DmManf mutants was disturbances in the development of cuticle (I and data not shown). In contrast, glial-specific expression of UAS-DmManf by repo-GAL4 failed to rescue DmManf mutants indicating that glial-derived DmManf is not sufficient for proper development of the cuticle. However, the role of DmManf in cuticle formation should be addressed in more detailed studies at the molecular level.

One of the GAL4 lines with such an expression pattern in which the ectopic DmManf expression was sufficient to complement for the loss of endogenous DmManf was 69B-GAL4 (I). The expression of transgenic DmManf by 69B-GAL4 alone was sufficient to maintain the viability and fertility of DmManf mutants. Actually, a fly line with such a genotype (DmManf$^{696}$/ DmManf$^{696}$/69B-GAL4/69B-GAL4/DmManf$^{696}$/UAS-DmManf$^{L3}$ on the 3rd chromosome) has been successfully maintained in our laboratory for several years. The absence of wild type DmManf allele in this stock was verified by PCR (data not shown). The 69B-GAL4 driver has been commonly employed as an epidermal driver. However, further analysis
revealed that GAL4 is expressed almost ubiquitously excluding fat body and gastric caeca. Additionally, a patterned expression in imaginal discs, cuticle and CNS was detected. Thus, it is termed as a semi-ubiquitous driver in this thesis.

<table>
<thead>
<tr>
<th>Construct</th>
<th>da</th>
<th>69B</th>
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<tbody>
<tr>
<td>DmManf</td>
<td>22 aa</td>
<td>94 aa</td>
</tr>
<tr>
<td>HsMANF</td>
<td>20 aa</td>
<td>95 aa</td>
</tr>
<tr>
<td>HsMANF-HsCDNF</td>
<td>20 aa</td>
<td>HsMANF - 95 aa</td>
</tr>
<tr>
<td>HsCDNF</td>
<td>20 aa</td>
<td>100 aa</td>
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Figure 7. Rescue of *DmManf* mutant lethality by ectopic expression of DmManf, HsMANF and HsCDNF. Schematic presentation of DmManf, HsMANF and HsCDNF transgenes as well as recombinant construct with N-terminal domain of HsMANF and C-terminal domain of HsCDNF (HsMANF-HsCDNF, red corresponds to the HsCDNF sequence inserted into HsMANF transgene and green to a fragment of C-terminal domain of HsMANF). Results of rescue experiments with ubiquitous (da) and semi-ubiquitous (69B) GAL4 drivers are presented. Markings used: ++, full rescue; (+), subtle (only few pupae observed) rescue; --, no rescue of *DmManf* mutant lethality. In constructs with human MANF and CDNF, honeybee mellitin (20 aa) was used as a secretion signal peptide. aa, amino acid. Modified from II.

4.1.3 *HsMANF and HsCDNF are able to substitute for the endogenous DmManf (I, II)*

To study the functional conservation, transgenic fly lines carrying an UAS-construct encoding either human MANF or CDNF were generated (I, II, Figure 7). Similar to UAS-DmManf construct, the ubiquitous expression of either human MANF or CDNF were able to rescue *DmManf* mutant lethality to adulthood (II). The emerged adults were fertile and showed no obvious phenotype. These results confirm that MANF is functionally conserved during evolution and both of the human paralogues, HsMANF and HsCDNF, are able to complement for the lack of endogenous fly protein.

To test whether restricted expression of human transgenes affects their ability to complement for the loss of endogenous DmManf expression, 69B-GAL4 was used in the rescue experiments (II). This driver was selected because ectopic DmManf in its non-ubiquitous expression pattern is able to rescue *DmManf* mutant lethality to adulthood (II). The emerged adults were fertile and showed no obvious phenotype. These results confirm that MANF is functionally conserved during evolution and both of the human paralogues, HsMANF and HsCDNF, are able to complement for the lack of endogenous fly protein.

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However, when HsCDNF was used, the amount of rescued pupae was drastically decreased. To dissect the differences between the human paralogues, a recombinant HsMANF-HsCDNF construct was built. In this construct, C-terminal domain of HsMANF (amino acid residues 105-158) was replaced with that of HsCDNF (amino acid residues 101-164). This recombinant construct rescued *DmManf* mutant lethality when ubiquitously expressed but failed to do so with 69B-GAL4 driver. This result indicated that the functional differences between the two human paralogues reside in the C-terminal domain. To confirm this hypothesis, a construct with N-terminal domain of HsCDNF and C-terminal domain of HsMANF (HsCDNF-HsMANF) was generated. Unfortunately, no transformant lines of this construct were obtained and the hypothesis could not be tested.
4.1.4 **Knockdown of DmManf is efficient and specific in vivo (III)**

In order to study tissue-specific requirements for DmManf, a transgenic UAS-\(DmManf\)-RNAi line was used to knock down \(DmManf\) expression (III). First, the efficiency and specificity of the knockdown by UAS-\(DmManf\)-RNAi construct was verified. When DmManf was ubiquitously knocked down with \(tub\)-GAL4, qPCR and Western blot analyses showed that both mRNA and protein levels of DmManf were reduced. Ubiquitous knockdown of \(DmManf\) with \(tub\)-GAL4 and \(da\)-GAL4 drivers resulted in lethality at larval stage with few escapers (approximately 5%) which died during pupal stage. To further confirm the specificity, heterozygous \(DmManf\) mutant background was used to reduce the endogenous \(DmManf\) mRNA expression. When \(DmManf\) was ubiquitously knocked down with \(tub\)-GAL4 in heterozygous \(DmManf\) mutant background, no pupae or wandering 3\(^{rd}\) instar larvae were detected closely resembling the zygotic \(DmManf\) mutant phenotype. The larval lethality caused by ubiquitous knockdown of \(DmManf\) was rescued to adulthood by simultaneous overexpression of UAS-DmManf, UAS-HsMANF or UAS-HsCDNF. These data indicate that UAS-\(DmManf\)-RNAi specifically and efficiently knocked down endogenous \(DmManf\) expression.

4.1.5 **DmManf is involved in wing disc cell proliferation (III)**

DmManf is ubiquitously expressed in the 3\(^{rd}\) instar larval wing disc (II). The knockdown of \(DmManf\) by GAL4 drivers with expression in the wing, e.g. MS1096-GAL4, resulted in altered adult wing phenotype. In MS1096-GAL4 driver line GAL4 is expressed in the wing pouch area and also partially in CNS (Capdevila and Guerrero 1994; Neumann and Cohen 1996; our unpublished observations). Additionally, the GAL4 insertion is located on the X chromosome. In \(Drosophila\) males, the X chromosomal dosage compensation is achieved by hyperactivation of the X-linked genes (reviewed in Laverty et al. 2010; Georgiev et al. 2011). Thus, the GAL4 expression in MS1096-GAL4 driver is expected to be stronger in the males. Silencing of \(DmManf\) by MS1096-GAL4 resulted in a bent-up wing phenotype and this phenotype was stronger in males consistent with the X-chromosomal GAL4 insertion (III, Figure 8A). When heterozygous \(DmManf\) mutant background was used, phenotype was stronger, even leading to a complete loss of the wing in males. This also provided additional evidence for the specificity of UAS-\(DmManf\)-RNAi construct.

To confirm the specificity of \(DmManf\) knockdown by MS1096-GAL4, UAS-\(DmManf\)-RNAi line was co-expressed together with overexpression constructs UAS-DmManf, UAS-HsMANF and UAS-HsCDNF. The wing phenotype in both females and males was reverted back to wild type (III, Figure 8A). In wild type background, no phenotype was detected when these constructs were expressed by MS1096-GAL4 (III, data not shown).

The silencing of human MANF has been shown to stimulate cell proliferation in vitro (Apostolou et al. 2008). In contrast, the loss of MANF suppressed and overexpression enhanced proliferation of mouse pancreatic \(\beta\)-cells in vivo (Lindahl et al. 2014). To investigate whether DmManf is involved in regulation of cell proliferation in \(Drosophila\), the larval wing discs in which \(DmManf\) was knocked down by MS1096-GAL4 were
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studied (III). Proliferating cells were identified by a mitotic marker pHis3 (Hendzel et al. 1997) within the GFP-expressing wing disc area of 3rd instar male larvae. The number of pHis3 positive cells was significantly increased when DmManf was knocked down in heterozygous DmManf mutant background in comparison to heterozygous DmManf mutant alone (Figure 8B). However, this result is controversial to the phenotype observed in adult males in which the wing blade was small or even completely lost. In a developing wing disc, few apoptotic cells are found and they can be detected e.g. by acridine orange staining (Milán et al. 1997). However, the larval wing discs from males in which DmManf was knocked down with MS1096-GAL4 showed no difference in acridine orange staining in comparison to wild type (data not shown). Thus, the molecular mechanisms resulting in the drastic wing phenotype subsequent to the loss of DmManf remain unsolved.

Figure 8. Knockdown of DmManf in the wing results in altered adult wing phenotype. A) The observed wing phenotype in adult males, see text for details. B) Quantification of pHis positive cells within GFP-expressing wing disc area of 3rd instar male larvae. UAS-mCD8-GFP was used to detect the GAL4 expressing region. *, P < 0.05, Student’s t-test. Modified from III.
4.2 Studies on the structural and functional features of MANF/CDNF in vivo (II)

Several features identified in the amino acid sequence (Petrova et al. 2003; Lindholm et al. 2007; Apostolou et al. 2008; Lindholm et al. 2008) and three-dimensional structure (Parkash et al. 2009; Hellman et al. 2011) are predicted to be involved in the function and interaction of MANF/CDNF proteins. To dissect the structural and functional components of the MANF/CDNF family, a series of mutations in DmManf were designed (II, Figure 9). Mutated DmManf cDNAs were cloned into pUAST constructs and transgenic flies were generated. The rescue model (see Figure 6 and Chapter 3.3) was used to test whether the transgenes could complement for the loss of endogenous DmManf despite the mutations. First, transgenes were expressed ubiquitously by da-GAL4 driver and their ability to rescue DmManf mutant lethality was evaluated. However, if the mutation affects intracellular trafficking or extracellular distribution of DmManf protein, abundant expression of the mutated protein by da-GAL4 might hide these effects. Thus, 69B-GAL4 was used to express transgenes in homozygous DmManf mutant background in a non-ubiquitous manner (see Chapter 4.1.2). Western blot analysis revealed that the overall GAL4 expression level in 69B-GAL4 was mildly decreased when compared to da-GAL4 (II). In comparison to the endogenous DmManf expression, the ectopic expression of DmManf by 69B-GAL4 produced clearly higher DmManf protein level. Apparently, the possible differences in the rescue experiments with da-GAL4 and 69B-GAL4 drivers do not result from deficient DmManf expression level but from incomplete expression pattern of 69B-GAL4. In comparison to ubiquitous da-GAL4, 69B-GAL4 lacks expression in muscles, gastric caeca and fat body. Additionally, the expression in CNS and epidermis is decreased in comparison to da-GAL4. To address the issues with expression pattern and tissue-specific expression level, targeted mutagenesis to endogenous DmManf gene should be performed to express the mutated gene under endogenous DmManf promoter (reviewed in Venkel and Bellen 2005). However, this approach was beyond the scope of current work.

To study the effects of mutations on secretion of DmManf, Schneider-2 cells were transfected with V5-tagged DmManf constructs in pMT vector. The V5-DmManf protein expressed by transfected plasmids was analysed in cell lysates and media with an antibody against V5-tag by Western blotting (II). To avoid disturbing the secretion signal peptide or the putative interaction with KDEL-R, V5-tag was inserted after the secretion signal sequence and prior to the N-terminal domain of mature DmManf protein (Figure 5).
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Figure 9. Mutations in DmManf transgenes and their ability to rescue DmManf mutant lethality. A schematic presentation of designed DmManf constructs and results of the rescue experiments with ubiquitous da-GAL4 and semi-ubiquitous 69B-GAL4 drivers. Red bars indicate point mutations, broken line a deletion (for detailed explanations, see Chapters 4.2.1–4.2.5). Colouring is according to amino acid sequence alignment presented in Figure 1. Markings used: ++, full rescue; (+), subtle (only few pupae observed) rescue; --, no rescue of DmManf mutant lethality. Below, an example of a combination of N- and C-terminal domain constructs is presented (see Chapter 4.2.5). Modified from II.

4.2.1 Positively charged surface amino acids affect DmManf functionality (II)

The N-terminal domain of MANF (N-MANF) shows high similarity to SAPLIPs, a protein family with lipid-interacting functions (Parkash et al. 2009). N-MANF has two patches of positively charged surface amino acids that are predicted to interact with the negatively charged membrane lipids (Parkash et al. 2009). The first patch consists of amino acid residues R15, K80, K84, K86, and K87 and the second patch of R44, K46, R49 and K96 (numbering according to HsMANF amino acid sequence without secretion signal). Two mutated DmManf constructs were created in which selected amino acid residues from each of the patches were replaced with neutral alanine (II, Figure 9). In the first construct, DmManf-NMG1 (N-terminal mutant group 1), mutations K79A, K83A and K86A were created corresponding to amino acid residues K80, K84 and K87 in HsMANF. In the second construct, DmManf-NMG2, mutations K43A, K45A and R95A were introduced corresponding to amino acid residues R44, K46 and K96 in HsMANF. When ubiquitously expressed, both of these constructs were able to rescue the DmManf mutant lethality and thus replace the endogenous DmManf (II, Figure 9).

When semi-ubiquitous 69B-GAL4 driver was used, DmManf-NMG2 construct was able to rescue DmManf mutant lethality (II). However, DmManf-NMG1 failed to rescue mutant lethality indicating that the positive surface potential in the corresponding patch is crucial for proper DmManf functionality. Either mutation group (NMG1 or NMG2) did not notably affect the levels of secreted or cellular amounts of V5-tagged DmManf in comparison to wild type construct (II). Thus, the surface potential affects other molecular characteristics of DmManf than secretion. In further studies, the intracellular trafficking
and putative lipid interaction of both constructs should be studied in detail. Additionally, a construct in which all nine positively charged surface amino acid residues are neutralised should be studied.

4.2.2 ER retention is necessary when the availability of DmManf is restricted (II)

Similar to mammalian MANF, DmManf has been shown to localize to ER (Palgi et al. 2012). A putative ER retention signal sequence, RSEL, is located at the very C-terminal end of DmManf. To test whether this sequence is responsible for localizing DmManf to the ER, a DmManf construct with deleted RSEL sequence was created (DmManf-ΔRSEL) (II, Figure 9). UAS-DmManf (wild type construct) and UAS-DmManf-ΔRSEL were expressed by da-GAL4 driver and DmManf co-localization with an ER marker (transgenic sqh-EYFP-ER construct) was studied in homozygous DmManf mutant background in order to avoid detecting the endogenous DmManf. The ER localization of DmManf-ΔRSEL was significantly decreased in comparison to wild type DmManf in vivo (II, Figure 10). In vitro studies showed that the deletion of ER retention signal clearly increased the level of secreted V5-DmManf-ΔRSEL in the medium and decreased the amount of V5-DmManf-ΔRSEL in cell lysates in comparison to wild type construct. This demonstrated that the suggested role of RSEL sequence as an ER retention signal is functional and conserved (II).

Figure 10. Deletion of ER retention signal affects intracellular localization of DmManf.

UAS-DmManf and UAS-DmManf-ΔRSEL were expressed by ubiquitous da-GAL4 driver in homozygous DmManf mutant background. In 3rd instar larval Garland cells, the expression from UAS constructs was detected by immunostaining with anti-DmManf. The co-localization of DmManf (red) and EYFP (green) is shown as yellow. Reprinted from II.

In rescue experiments, the ubiquitous expression of DmManf-ΔRSEL construct by da-GAL4 resulted in full rescue of the larval and pupal lethality of homozygous DmManf mutants (II, Figure 9). However, when semi-ubiquitous 69B-GAL4 was used, the ability of DmManf-ΔRSEL to rescue DmManf mutant larval lethality was notably decreased. This indicated that the ER-retained DmManf has a crucial role for fly viability when the availability of DmManf is restricted.

The ER retention of mammalian MANF has been shown to be mediated by its C-terminal RTDL sequence (Glembotski et al. 2012; Oh-Hashi et al. 2012; Henderson et al. 2013). To study the conserved role of ER-retained MANF, HsMANF transgene with a deletion of the ER retention signal (HsMANF-ΔRTDL) was used (II). Similar to
corresponding DmManf construct (DmManf-ΔRSEL), HsMANF-ΔRTDL was able to rescue DmManf mutant lethality when ubiquitous da-GAL4 driver was used but failed to do so when expressed by semi-ubiquitous 69B-GAL4 (II). This demonstrated that the functional importance of the ER-retained MANF is conserved. It also opens an intriguing opportunity to study the functional difference between the C-terminal domain of human MANF and CDNF (see Chapter 4.1.3). In future studies, the ER retention signal of HsCDNF, KTEL, could be replaced with the unique ER retention signal of HsMANF, RTDL. The rescue experiments with restricted expression pattern of 69B-GAL4 driver could give a hint whether the different ER retention signals contribute for the functional difference between the human paralogues. Additionally, the replacement of the ER retention signal of DmManf and HsMANF with the canonical KDEL sequence could further enlighten the role of ER retained MANF protein in this in vivo model.

4.2.3 Disrupting the C-terminal CXXC motif by C129S mutation (II)

The C-terminal domain of MANF has a CXXC motif characteristic for thiol-disulphide oxidoreductases. A disulphide bridge between the cysteines in CXXC motif stabilizes the folding of C-terminal domain of MANF and CDNF (Parkash et al. 2009; Hoseki et al. 2010; Hellman et al. 2011). To study the importance of this CXXC motif in DmManf, one of the cysteine residues was mutated by point mutation C129S (II, Figure 9). Interestingly, ubiquitous expression of DmManf-C129S failed to rescue DmManf mutant lethality. The transfected Schneider-2 cells showed similar levels of cellular and secreted V5-DmManf-C129S in comparison to wild type V5-DmManf suggesting that interrupting the C-terminal CXXC motif does not affect the secretion of DmManf. These data demonstrated that the intact CXXC motif has a crucial role in DmManf function in vivo but affects other cellular functions than secretion. The effects of C129S mutation are further discussed in Chapter 4.3.1.

4.2.4 Entering the secretory pathway via ER is crucial for DmManf functionality (II)

Both mammalian and Drosophila MANF are secreted proteins and their role as extracellular trophic factors have been demonstrated in vivo (I; Lindholm et al. 2008; Voutilainen et al. 2009). To study the importance of signal sequence-directed entrance of DmManf to the secretory pathway via ER, a DmManf construct with a deletion of the N-terminal secretion signal (ss) sequence was created (DmManf-Δss) (II, Figure 9). Interestingly, ubiquitous expression of this construct failed to rescue DmManf mutant lethality. DmManf-Δss or V5-DmManf-Δss was not detected in the medium of transfected CHO or Schneider-2 cells, respectively, indicating that the deletion of the secretion signal peptide actually abolished the secretion of DmManf. The Western blot analysis from 3rd instar larval lysates demonstrated that ubiquitously expressed DmManf-Δss, even when UAS/GAL4 expression system was enhanced by doubling both UAS- and GAL4 insertions, showed clearly defective expression level in vivo. In transfected Schneider-2 cells, the expression level of V5-DmManf-Δss was also lower in comparison to wild type
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V5-DmManf. This suggests that either the translation of DmManf-Δss in cytosol is defected or the cytosolic DmManf protein is degraded. The RT-PCR analysis revealed that the mRNA was expressed from UAS-DmManf-Δss transgene in vivo when ubiquitous da-GAL4 driver was used. DmManf-Δss mRNA expression level did not differ from the mRNA expressed by the wild type construct supporting the hypothesis that the expression defect occurs post-transcriptionally.

The molecular events affecting the expression level of DmManf-Δss protein are intriguing. MANF and CDNF are folded proteins and, putatively, the loss of ER-mediated folding of DmManf might lead to its ubiquitination and degradation via proteasome system. In future studies, the putative ubiquitination of cytosolic DmManf should be addressed. Additionally, the loss of secretion signal in DmManf-Δss has two consequences: it does not enter the ER and it is not secreted. While the loss of ER retained DmManf (DmManf-ΔRSEL, see Chapter 4.2.2) affected functionality only when its expression was restricted, it would be important to address whether the loss of secreted or ER-retained DmManf results in the deficient functionality. In order to reduce the secretion of DmManf, a construct in which the ER retention signal RSEL is replaced with the canonical KDEL sequence – and expected to result in higher affinity to KDEL-R and decreased secretion – could reveal whether secreted DmManf has a crucial role during development.

4.2.5 Both N- and C-terminal domains as an intact full-length protein are needed for functional DmManf protein (II)

Human MANF and CDNF consist of two domains, N-terminal and C-terminal (Parkash et al. 2009). Amino acid sequence of MANF is evolutionarily conserved and because the spacing between the eight cysteine residues is also conserved, DmManf is predicted to be structurally very similar to human MANF (I). To explore whether either N- or C-terminal domain alone includes the functional characteristics of DmManf important for fly viability, constructs of independent N- and C-terminal domains, with and without secretion signal sequence, were generated (II, Figure 9). Each of these transgenes was expressed individually by ubiquitous da-GAL4 driver in homozygous DmManf mutant background but none of them could complement for the lack of endogenous DmManf. Additionally, none of these constructs were able to rescue the wing phenotype observed in flies in which DmManf was knocked down by MS1096-GAL4 (data not shown, see Chapter 4.1.5). This indicates that both domains of DmManf have important functions during development.

Simultaneous expression of N- and C-terminal domain constructs could retain the functional properties of DmManf essential for viability. Thus, combinations of N- and C-terminal domain construct, with or without secretion signal sequence (see an example in Figure 9) were expressed by ubiquitous da-GAL4 driver in homozygous DmManf mutant background (II). However, co-expression of N- and C-terminal domain constructs, in any of the combinations, failed to rescue DmManf mutant lethality. These data indicate that in
addition to the requirement of both N- and C-terminal domains for the DmManf functionality, they also need to be expressed as an intact full-length protein.

Western blot analysis from larval lysates revealed that N- and C-terminal domain constructs with a secretion signal peptide (DmManf-N and DmManf-C) were abundantly expressed at the protein level. In contrast, the protein expressions produced by N- and C-terminal domain constructs without the secretion signal peptide (DmManf-N-Dss and DmManf-C-Dss) were under the detection limit (II). Similar to DmManf-Dss construct, the mRNA expression of these constructs was not affected (see Chapter 4.2.4). Taken together, these data suggest that the ER-directed translation of DmManf is necessary for producing a stable DmManf protein.

Although the data presented above strongly indicates that the N- and C-terminal domains need to be expressed as an intact full-length protein, the absolute requirement was not proven in this study. The peptide produced by C-terminal domain construct might be either degraded or misdirected. Based on the expression studies with the constructs encoding for independent domains, the C-terminal domain without the secretion signal sequence (DmManf-C-Dss) was most likely degraded even when co-expressed with the N-terminal domain. Furthermore, the artificial DmManf C-terminal domain with the secretion signal peptide (DmManf-C) has both secretion signal (directing the translated peptide to ER) and ER retention signal (retaining the protein in ER). This might lead to incorrect intracellular localization for DmManf-C and result in its impaired function.

The importance of N-terminal domain also remains uncertain. While secretion signal sequence directs the translation of DmManf-N to the ER, it then continues to the trans-Golgi network and is secreted instead of retained to the ER due to the lack of ER retention signal. However, if DmManf is cleaved in the ER after its translation, this would be the outcome of endogenous expression as well. In Western blot analysis of larval lysates in which wild type DmManf was ubiquitously overexpressed, a faint band corresponding to the size of C-terminal domain was detected (II). This might result from a cleavage of DmManf protein, but such has never been found to occur endogenously. It is more probable that the appearance of a smaller protein band results from degradation of excess DmManf protein in the ER. However, no experiments were performed during this study to explore whether endogenous DmManf protein is cleaved into separate N- and C-terminal domains after its translation in the ER.

To further test whether both N- and C-terminal domains of DmManf, as an intact full-length protein, are necessary for fly viability in vivo, the N- and C-terminal domain constructs with connecting linkers should be generated and studied in the rescue model. This would reveal whether the loss of physical connection or other implications mentioned above are responsible for the inability of individual domain constructs to rescue DmManf mutant lethality. Furthermore, a DmManf-N construct with the DmManf ER retention signal (RSEL) added to the C-terminus would reveal whether retaining the DmManf-N in the ER would provide the important function of DmManf.
4.3 Effects of DmManf overexpression (III, unpublished)

Previously, overexpression of DmManf was reported not to cause evident phenotypes in adult flies or embryonic nervous system (Palgi et al. 2012). Accordingly, the ubiquitous overexpression of DmManf by da-GAL4 or tub-GAL4 did not show any obvious phenotype or affect the larval or pupal viability quantified in current study (III). Similarly, overexpression of DmManf with either wing-specific MS1096-GAL4 or semi-ubiquitous 69B-GAL4 driver did not cause any obvious phenotype (III).

The alterations designed in the DmManf protein (II, Figure 9) could have dominant negative effects when expressed in wild type background. Additionally, they could interfere with the endogenous DmManf expression. The ubiquitous expression by da-GAL4 of mutated DmManf constructs without the secretion signal sequence (DmManf-Δss, DmManf-N-Δss and DmManf-C-Δss) did not affect the overall viability (data not shown). This indicates that cytoplasmic DmManf, either as a full-length protein or as an independent N- or C-terminal domain is not harmful for cells. However, because the protein expression of these constructs was defected (II), the lack of damaging effects might be merely due to the low expression levels.

Except for the DmManf-C129S construct (see Chapter 4.3.1 below), ubiquitous overexpression of the mutated DmManf constructs with the secretion signal sequence, DmManf-ΔRSEL, DmManf-NMG1, DmManf-NMG2, DmManf-N and DmManf-C did not affect the viability or show any obvious phenotype under normal conditions (data not shown). This indicates that none of the modifications listed above exhibit negative effects on fly viability.

Despite the normal phenotype of flies in which wild type DmManf is overexpressed with 69B-GAL4, a previous microarray analysis revealed altered expression of genes involved in various functions and biological processes in these flies (Palgi et al. 2012). Expression of genes annotated to function in regulation of cell cycle and related processes as well as cell death were upregulated. Evaluation of downregulated genes in response to DmManf overexpression indicated that several ER-related processes were deteriorated. Under normal conditions, these processes are probably in balance and do not affect the overall viability of DmManf-overexpressing flies. In future studies, flies overexpressing DmManf could be challenged with various stress conditions, e.g. ER stress or the fly model of PD.

4.3.1 Overexpression of DmManf-C129S impairs normal development (unpublished)

In contrast to any other DmManf constructs tested, normal fly development was disturbed by ectopic expression of the transgene carrying C-terminal cysteine mutation, DmManf-C129S. Overexpression with ubiquitous tub-GAL4 (Table 5) and da-GAL4 (data not shown) led to lethality before pupal stage. The rescue studies suggested that DmManf-C129S failed to complement for the loss of endogenous DmManf (II, Chapter 4.2.3). In fact, the overexpression studies indicated that C129S mutation does not only compromise the functionality of DmManf protein but also causes damaging effects. Additionally, the
overexpression of DmManf-C129S with wing driver MS1096-GAL4 resulted in defected adult wing similar to DmManf knockdown flies (Figure 11). These data indicate that DmManf-C129S represents either a dominant negative or a gain-of-function allele of DmManf. Co-expression of transgenic wild type DmManf and DmManf-C129S with ubiquitous tub-GAL4 did not rescue the lethality caused by DmManf-C129S expression (Table 5) suggesting that increasing the expression level of wild type DmManf does not alter the harmful effects of DmManf-C129S expression. Accordingly, the wing phenotype observed in flies overexpressing DmManf-C129S with wing-driver MS1096-GAL4 was not either rescued or worsened by simultaneous overexpression of transgenic wild type DmManf (Figure 11).

The similarity between the wing phenotype in DmManf knockdown and DmManf-C129S expressing flies suggests that DmManf-C129S actually impairs the function of wild type DmManf. Additionally, ubiquitous overexpression of DmManf-C129S resulting in lethality prior to the pupal stage remotely resembles the DmManf mutant phenotype. However, these phenotypic analyses were too coarse and more sophisticated readout for the loss of DmManf function should be found to address this hypothesis.

The mutated DmManf-C129S protein contains an unpaired cysteine residue (C132) which prevents folding of the C-terminal domain. Recently, a Drosophila model for conditional expression of an unfolded protein (human proinsulin with mutation Ins2C96Y) was reported (Park et al. 2014, see Chapter 1.4.3.2). In 3–5 days old adult males, the expression of Ins2C96Y by eye-driver GMR-GAL4 resulted in retinal degeneration indicated by uneven surface of the compound eye. It was also shown that ER stress was induced by ectopic expression of Ins2C96Y. In order to study whether the disturbed C-terminal CXXC motif in DmManf-C129S and the presence of unpaired cysteine residue (C132) has similar impact on eye development, GMR-GAL4 driver was used to express DmManf-C129S in the wild type background. However, no signs of retinal degeneration were detected and the eyes of this genotype showed no phenotype in 5 days old adult flies (Figure 11). Although the time point was same than used in the previous study (Park et al. 2014), it does not exclude the possibility of age-dependent degeneration that was not assessed in this work. As a control for effective expression by GMR-GAL4, overexpression constructs of PEK (UAS-PEK) and a dominant negative allele of Hsc70-3 (UAS-Hsc70-3D231S) were used. Overexpression of either of these constructs has been found to cause ER stress (Elefant and Palter 1999; Malzer et al. 2010). The overexpression of PEK showed similar degenerated small eye phenotype to previously reported data (Malzer et al. 2010) and the overexpression of Hsc70-3D231S resulted in severe retinal degeneration phenotype (Figure 11B). Based on these results, the presence of unpaired cysteine (C132) in the C-terminal domain of DmManf does not induce – at least severe – UPR in the eye. This is further supported by the co-expression studies of DmManf-C129S and wild type form of Hsc70-3 (UAS-Hsc70-3WT). Hypothetically, the elevated amount of Hsc70-3 should increase the binding of unfolded DmManf-C129S and relieve its negative effects. However, the pupal lethality of DmManf-C129S overexpression by ubiquitous tub-GAL4 driver was not rescued by simultaneous overexpression of Hsc70-3 (Table 5). Thus, the implications of DmManf-C129S at developmental, cellular and molecular level remain unsolved and should be thoroughly studied.
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Table 5. Overexpression of DmManf, DmManf-C129S and Hsc70-3WT by tub-GAL4. Tb+ and Tb−, number of observed pupae of indicated phenotype. Adults, observed percentage of emerged adults out of Tb+ pupae. For each genotype, pupae and adults from six vials were counted.

<table>
<thead>
<tr>
<th>Constructs overexpressed</th>
<th>Tb+</th>
<th>Tb−</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (wild type)</td>
<td>489</td>
<td>423</td>
<td>100%</td>
</tr>
<tr>
<td>UAS-DmManfL5</td>
<td>444</td>
<td>359</td>
<td>93%</td>
</tr>
<tr>
<td>UAS-Hsc70-3WT</td>
<td>409</td>
<td>369</td>
<td>100%</td>
</tr>
<tr>
<td>UAS-DmManf-C129S L4</td>
<td>0</td>
<td>613</td>
<td>–</td>
</tr>
<tr>
<td>UAS-DmManfL5 + UAS-DmManf-C129S L4</td>
<td>0</td>
<td>538</td>
<td>–</td>
</tr>
<tr>
<td>UAS-Hsc70-3WT + UAS-DmManf-C129S L4</td>
<td>0</td>
<td>572</td>
<td>–</td>
</tr>
</tbody>
</table>

Figure 11. Effects of ectopic DmManf-C129S mutant expression on Drosophila wing (A) and eye (B). A) Simultaneous overexpression of wild type DmManf (UAS-DmManf) did not affect the wing phenotype caused by DmManf-C129S overexpression. In comparison, the phenotype in DmManf-knockdown by UAS-RNAi construct was rescued by overexpression of wild type DmManf. B) In 5 days old adults, ectopic DmManf-C129S in the eye showed no retinal degeneration. UAS-PEK was driven by GMR-GAL4 at 18 °C as its overexpression resulted in lethality at 25 °C, consistent with the original study (Malzer et al. 2010).
SXXC motif has been shown to be involved in enzymatic redox functions in prokaryotes (Fomenko and Gladyshev 2002). SXXC motif has also been identified in a mammalian ER-resident protein disulphide isomerase-like in the testis (PDILT) (van Lith et al. 2005). Although lacking the CXXC motif, PDILT was able to interact with oxidoreductase Ero1α, and is involved in intermolecular disulphide-dependent complex formation. Additionally, a SXXC motif has been identified to reside next to the cysteine-rich vitelline membrane domain of a Drosophila protein VM26Ab (Vitelline membrane 26Ab, also known as sV23) (Wu et al. 2010). This protein is involved in the composition of oocyte vitelline membrane and in formation of intermolecular disulphide linked complexes. Thus, it is possible that the C129S mutation in DmManf, forming a CXXS motif, could gain disulphide-dependent functions and hamper disulphidation pathway. This would also explain why simultaneous overexpression of wild type DmManf did not rescue the phenotypes caused by expressing the DmManf-C129S mutant. Functions for DmManf related to disulphide bond formation are disputed by the finding that both N- and C-terminal domain CXXC motifs in mammalian MANF lack PDI activity (Hartley et al. 2013). However, this does not completely exclude the possibility that PDI-like activities would exist in the CXXS motifs of DmManf.

In conclusion, the C129S mutation could have two consequences. First, it results in an unpaired cysteine residue (C132) and prevents folding of the C-terminal domain which might have a toxic effect, e.g. by causing constant ER stress and activating UPR. Second, the loss of CXXC motif could alter DmManf function and gain unknown detrimental effects. To explore whether the unpaired cysteine residue (C132) is responsible for the negative effects of DmManf-C129S expression, a construct in which both of the cysteine residues within the CXXC motif are mutated should be studied. This construct could give further answers whether the harmful effects are due to the deficient functionality of DmManf protein or the unpaired cysteine residue. Additional characterization of the C-terminal CXXC motif in DmManf would be of great importance for understanding the function and conservation of MANF/CDNF family.

### 4.4 Identification of genetic interaction partners of DmManf (III)

Despite the studies during the last decade, very little is known on how MANF/CDNF impact biological processes and which proteins they interact with. To address this issue, genetic interacting partners of DmManf were studied by utilizing the transgenic UAS-RNAi method from the genetic toolbox of Drosophila (III). The knockdown of DmManf in the wing resulted in a drastic wing phenotype and strongly indicated a role for DmManf in wing development (see Chapter 4.1.5). Thus, the UAS-RNAi lines of target genes were driven in the wing disc and the adult wings were monitored for phenotypes. Both candidate and unbiased screen approaches were used. For the candidate genes, two sets of UAS-RNAi lines were tested: a) a selection of genes encoding for proteins known to have a function related to ER or ER stress response (hereafter referred as “ER genes”) and b) genes encoding for transcription factors that are predicted to bind DmManf promoter
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region. Additionally, a partial unbiased screen of genome-wide UAS-RNAi libraries was performed. In both approaches MS1096-GAL4 was used as a wing driver (see Chapter 4.1.5 for more detailed description of the expression pattern) and 69B-GAL4 as a semi-ubiquitous driver (see Chapter 4.1.2) to knock down the target gene expression in wild type background. The phenotypes were then compared to knockdown of target gene in heterozygous *DmManf* mutant (to reduce DmManf expression level) or DmManf-overexpressing (to increase amount of DmManf) backgrounds. These backgrounds alone did not show any obvious phenotypes. The genes showing altered phenotype in either heterozygous *DmManf* mutant or DmManf-overexpressing backgrounds in comparison to wild type background were considered as genetic interacting partners of *DmManf*. A schematic presentation of the screen approach is presented in Figure 12A.

4.4.1 Overall evaluation of the approach to identify genetic interactions

Although the screen approach used in this study enabled identification of genetic interacting partners of *DmManf*, it was not further evaluated whether the observed association represented a direct, physical interaction or did it demonstrate a biochemical interaction. The interactors might act in the same signalling pathway but the detected genetic interaction might only result from an additive effect on general cellular function, e.g. cell proliferation or apoptosis. Further studies are necessary to explore on what level the identified genetic interactions are executed.

For several genes assessed in the unbiased screen, knockdown with wing-driver MS1096-GAL4 resulted in more severe, but similar, phenotypes in both heterozygous *DmManf* mutant and DmManf-overexpressing backgrounds (III). This is surprising because reducing and increasing the DmManf level are expected to have opposite effects. MS1096-GAL4 driver has a patterned expression in the developing wing disc. When simultaneous DmManf overexpression is used, both knockdown of the target gene and overexpression of DmManf occurs in the GAL4-expressing cells while in the surrounding cells, endogenous expression levels are expected to remain (illustrated in Figure 12B). This creates a concentration gradient of DmManf expression. The overexpression level is not affected by the knockdown of target gene as it is not regulated by endogenous *DmManf* promoter. In the case of heterozygous *DmManf* mutant background, the target gene is silenced within GAL4 expression pattern while all cells have heterozygous DmManf expression level. If the knockdown of a target gene alters DmManf expression via the endogenous *DmManf* promoter, there might actually be either lower (downregulation) or higher (upregulation) DmManf levels within the GAL4 expression pattern in comparison to surrounding cells. This in turn could create a gradient of DmManf expression resembling the overexpression situation. In other words, the two genetically different *DmManf* backgrounds studied, heterozygous mutant and overexpression, were not comparable to each other. However, the actual cause for these observations was not studied further and, thus, remained speculative.
Figure 12. Schematic presentation of the screen for interacting partners of *DmManf*. A) To identify genetic interacting partners of DmManf, a 4-stage approach was assessed. For candidate gene approach, stages 2-4 were used. B) Schematic presentation of the difference between knockdown of target genes by UAS-RNAi with MS1096-GAL4 in DmManf overexpression (OE, above) and heterozygous mutant (below) backgrounds (see text for details).
4.4.2 DmManf interaction with ER genes

4.4.2.1 Interaction between GRP78 and MANF is conserved in Drosophila (III, unpublished)

Mammalian MANF has been shown to interact directly with the major ER chaperone GRP78 (Glembotski et al. 2012). In the screen of candidate genes with ER-related function, a genetic interaction between the *Drosophila* gene encoding the homologue to GRP78, *Hsc70-3*, and *DmManf* was found (III). The knockdown of *Hsc70-3* with wing driver MS1096-GAL4 in the wild type background resulted in severely bent-up wing phenotype in adult females. When simultaneous DmManf overexpression was used, the wing phenotype was stronger resulting in almost complete loss of the wing.

In embryos in which *Hsc70-3* was knocked down by semi-ubiquitous 69B-GAL4 driver, the mRNA level of *DmManf* was elevated in comparison to wild type (data not shown). Additionally, both total and spliced Xbp1 mRNA levels were increased (data not shown) suggesting a transcriptional activation of Xbp1. These data also imply that ER stress response is induced and are further discussed in Chapter 4.4.2.5.

The knockdown of *Hsc70-3* with ubiquitous tub-GAL4 driver was lethal prior to pupal stage (III). Additionally, overexpression of dominant negative alleles of *Hsc70-3*, UAS-*Hsc70-3*<sup>D231S</sup> and UAS-*Hsc70-3*<sup>K97S</sup> was used (Elefant and Palter 1999). Similar to the knockdown of *Hsc70-3* by UAS-RNAi construct, the ubiquitous overexpression of UAS-*Hsc70-3*<sup>D231S</sup> and UAS-*Hsc70-3*<sup>K97S</sup> resulted in lethality prior to pupal stage (data not shown). Simultaneous overexpression of DmManf did not rescue any of these lethal phenotypes demonstrating that DmManf cannot complement for the functional loss of major ER chaperone Hsc70-3 (III, unpublished).

In order to test whether Hsc70-3 could complement for the loss of endogenous DmManf, an overexpression construct for wild type allele of *Hsc70-3* (UAS-*Hsc70-3<sup>WT</sup>*) was used (III). This construct was expressed ubiquitously by da-GAL4 driver in homozygous *DmManf* mutant background similar to the rescue experiments described above (see Chapter 3.3). However, overexpression of *Hsc70-3* did not rescue *DmManf* mutant lethality suggesting that DmManf and Hsc70-3 have distinct functions during development. The simultaneous overexpression of both DmManf and Hsc70-3 in wild type background did not affect overall viability or show any obvious phenotypes in emerged flies. This demonstrated that a concurrent, abundant expression of both DmManf and Hsc70-3 does not have deleterious effects.

Although mammalian MANF has been shown to bind unfolded proteins *in vitro* (Hartley et al. 2013), the increased DmManf protein expression could not complement for the loss of major ER chaperone Hsc70-3 *in vivo* (III). In contrast, overexpression of DmManf actually disturbed even further the wing phenotype caused by decreased levels of Hsc70-3. This suggests that the presence of large amounts of DmManf in the ER acquires Hsc70-3, perhaps by regulating the folding of DmManf or maintaining an appropriate DmManf protein level inside the ER. Alternatively, the loss of interaction between mammalian MANF and GRP78 was associated with increased secretion of MANF (Glembotski et al. 2012). The knockdown of *Hsc70-3* could lead to increased secretion of
DmManf and, similar to rescue experiment with DmManf-ΔRSEL (see Chapter 4.2.2), could compromise viability. On the other hand, the excess amounts of DmManf could bind the residual Hsc70-3 and retain it from its important functions. In any case, the interaction between *Drosophila Manf* and *Hsc70-3* should definitely be studied in depth.

### 4.4.2.2 DmManf interacts with PEK, a transducer of UPR (III)

Expression of MANF has been shown to be induced in response to ER stress (Lee et al. 2003; Girardot et al. 2004; Mizobuchi et al. 2007; Apostolou et al. 2008; Lindholm et al. 2008; Nundlall et al. 2010). Studies on mammalian systems indicate that regulation of MANF expression upon ER stress is mediated by transcription factors ATF6 and XBP1s (Lee et al. 2003; Mizobuchi et al. 2007; Belmont et al. 2008; Wang et al. 2011; Oh-Hashi et al. 2013). Of the genes encoding for the UPR transducer proteins (*Ire1, PEK* and *Atf6*), a genetic interaction with *DmManf* was identified for *PEK* in this study (III). The knockdown of *Ire1* or *Atf6* with MS1096-GAL4 or 69B-GAL4 drivers did not cause any obvious phenotype in wild type, heterozygous *DmManf* mutant or DmManf-overexpressing backgrounds. However, the approach used here does not exclude an interaction between *DmManf* and *Ire1* or *Atf6*.

With all tested drivers, MS1096-GAL4, 69B-GAL4 and *tub*-GAL4, the knockdown of *PEK* showed more severe phenotype when DmManf was overexpressed in comparison to wild type background. Heterozygous *DmManf* mutant background did not alter the phenotypes caused by knocking down *PEK*. The qPCR analysis revealed that *DmManf* mRNA level in 2nd instar larvae was increased when *PEK* was knocked down with ubiquitous *tub*-GAL4 in wild type background. Interestingly, these larvae showed no altered mRNA levels of either total or spliced *Xbp1* (data not shown). This is in contrast with the previous study demonstrating that the loss of endogenous PEK resulted in the unconventional splicing of *Xbp1* (Malzer et al. 2010). This suggested that the knockdown of *PEK* with *tub*-GAL4 in wild type background alone was not efficient enough to induce UPR.

In a previous study, the loss of zygotic *DmManf* was shown to upregulate *PEK* mRNA level and to increase the phosphorylation of eIF2α, a target of PEK (Pomar et al. 2003; Palgi et al. 2012). Putatively, overexpression of DmManf could either downregulate *PEK* expression or disturb the proper activation of PEK. Thus, it would impair the inhibition of overall protein synthesis. However, this hypothesized direct interaction between DmManf and PEK has not been explored, yet.

### 4.4.2.3 Interaction between DmManf and Xbp1 (III)

A genetic interaction was detected between *DmManf* and *Xbp1*, the gene encoding for the transcription factor activated by Ire1-mediated unconventional splicing which has a central role in the activation and regulation of UPR. Knockdown of *Xbp1* with MS1096-GAL4 and 69B-GAL4 showed no phenotype in wild type background. When simultaneous overexpression of DmManf was used, knockdown of *Xbp1* resulted in severe phenotypes (III). The UAS-*Xbp1*-RNAi line used in this study targets both the unspliced and the
unconventionally spliced mRNA of \textit{Xbp1} and the efficient knockdown of \textit{Xbp1} was verified by qPCR (data not shown).

Knockdown of \textit{Xbp1} with ubiquitous \textit{tub-GAL4} did not alter \textit{DmManf} mRNA levels analysed by qPCR in 2\textsuperscript{nd} instar larvae (III). During normal development, ER stress is detected especially in secretory cells and silencing of XBP1 has been found to disturb the maintenance of this developmental ER stress (Iwawaki et al. 2004; Souid et al. 2007; Hess et al. 2011; Sone et al. 2013; Ryoo et al. 2013). Previous studies of mammalian MANF \textit{in vitro} indicate that XBP1s mediates the UPR-induced upregulation of MANF (Lee et al. 2003; Oh-Hashi et al. 2013). As knockdown of \textit{Xbp1} did not enable the upregulation of \textit{DmManf} in response to presumed ER stress (III), altogether these data suggest that XBP1 is essential for regulation of MANF expression.

The genetic interaction between \textit{Xbp1} and \textit{DmManf} indicates that \textit{Ire1} could also – at least genetically – interact with \textit{DmManf}. Further studies would reveal whether this putative interaction actually exists.

\textbf{4.4.2.4 Overexpression of DmManf induces Xbp1 expression (unpublished)}

To study the effect of DmManf overexpression on UPR, UAS-DmManf was driven by ubiquitous \textit{tub-GAL4} and the \textit{Xbp1} mRNA levels were analysed by qPCR in 2\textsuperscript{nd} instar larvae. Intriguingly, the overexpression of DmManf not only induced the mRNA expression of \textit{Xbp1} but also increased the level of the spliced from, \textit{Xbp1s}. Consequent of the increased level of total \textit{Xbp1} the ratio of spliced/total \textit{Xbp1} was not affected. This strongly indicated that DmManf overexpression caused ER stress and activated the transcriptional upregulation of \textit{Xbp1}. Nonetheless, expression of secretory proteins in general causes ER stress (reviewed in Hetz and Glimcher 2008) – thus the observed activation of \textit{Xbp1} expression might not specifically result from DmManf overexpression.

\textbf{4.4.2.5 Summary and implications of DmManf interaction with ER genes (III)}

A genetic interaction was detected between \textit{DmManf} and ER genes \textit{Hsc70-3}, \textit{PEK} and \textit{Xbp1}. Additionally, similar interaction was found between \textit{DmManf} and \textit{sip3} (septin interacting protein 3, \textit{Drosophila} homologue of synoviolin/HRD1), a protein involved in ERAD (III). Non-ubiquitous knockdown of each of these genes (excluding \textit{Hsc70-3}) had only minor implications on overall fly viability or morphology in wild type background. However, simultaneous overexpression of DmManf (which alone does not cause any obvious phenotype or affect fly viability, see Chapter 4.3) together with knockdown of these genes resulted in drastic phenotypes. Reducing the DmManf expression level by heterozygous \textit{DmManf} mutant background did not affect the knockdown of ER genes.

It is puzzling why DmManf overexpression has negative effect on the knockdown of genes with ER-related functions. MANF expression has been shown to be induced upon ER stress and it has been hypothesized to have a protective role against ER stress (Apostolou et al. 2008; Airavaara et al. 2009; Voutilainen et al. 2009). However, the overexpression levels obtained by UAS/GAL4 system are probably a lot higher than the upregulated levels of mammalian MANF detected \textit{in vitro} studies in which ER stress
upregulated MANF expression approximately 2-fold (Glembotski et al. 2012; Hartley et al. 2013). Although not analysed in detail, overexpression of DmManf by ubiquitous da-GAL4 and semi-ubiquitous 69B-GAL4 drivers resulted in notably higher DmManf protein levels in comparison to endogenous expression level (II). The consequences of DmManf overexpression could mimic the effects of increased overall protein expression on ER homeostasis rather than indicate specific ER-related functions of DmManf. However, this issue was not studied in detail during this work. It is still possible that at certain concentrations DmManf could have a protective role and only excess amounts have negative effects. The suggested protective role of MANF against ER stress should be studied with more delicate methods to regulate the DmManf expression levels, e.g. by increasing the copy number of DmManf genomic regions by transgenic constructs.

A previous microarray analysis revealed that the transcription of several UPR-related genes, including PEK and sip3, was downregulated in DmManf-overexpressing larvae (Palgi et al. 2012). The decreased sip3 expression was also verified by qPCR analysis (Palgi et al. 2012). Knockdown by UAS-RNAi construct results in silencing of the target gene and the efficiency differs between different UAS-RNAi lines. If overexpression of DmManf downregulates the expression of important UPR components, there would be less mRNA to be degraded. Thus, the efficiency of knockdown would be improved and enhance the silencing of target gene. This would give a simple explanation why DmManf overexpression has negative effects on the knockdown of ER-related genes. In future, the mRNA levels of UPR-related genes should be analysed in DmManf-overexpressing flies to examine this hypothesis.

Knocking down certain genes involved in ER-related functions might disturb UPR process. For example, the loss of transcription factor Xbp1s could interrupt the transcriptional regulation of UPR-induced gene expression, and decrease in Hsc70-3 expression levels could severely compromise the protein folding capacity in the ER. Indeed, the knockdown of Hsc70-3 resulted in increased levels of both total and spliced Xbp1 mRNAs indicating induction of UPR. Under normal conditions in wild type background, cells still manage to overcome these alterations. However, when DmManf is simultaneously overexpressed, cellular homeostasis is disrupted and cells are probably directed towards apoptosis. The translation of DmManf is targeted to the ER by N-terminal secretion signal peptide and it could simply act as a substrate for UPR in non-specific manner. In this scenario, overexpression of DmManf would cause a massive protein load in the ER. Under normal conditions, the ER homeostasis is retained by increasing the folding capacity through UPR. The putative degradation of overexpressed DmManf supports this hypothesis (II, see also Chapter 4.2.5). When an important piece of normal ER function or UPR is missing, e.g. Hsc70-3 or PEK, cells fail to overcome ER stress and the cell death pathways are activated. This is supported by the increased Xbp1 mRNA levels in response to overexpression of DmManf. However, whether overexpression of DmManf is involved in ER stress-related apoptosis is yet unsolved.

Although the approaches used in this work provide evidence for the contribution of DmManf in Drosophila UPR, it needs to be more comprehensively studied in the future.
### 4.4.3 Screening the candidate genes encoding for TFs (unpublished)

*Drosophila* Interactions Database (DroID, www.droidb.org) lists computationally and experimentally determined interactions between transcription factors (TFs) and genes that they may regulate based on REDFly database (Halfon et al. 2008) and modENCODE project (Roy et al. 2010). For DmManf, 20 candidate TFs are listed (Data version 2013_07). By using the UAS-RNAi approach, *Centrosomal protein 190kD* (*Cp190*) and Chromator (*Chro*) were identified to genetically interact with *DmManf* (Figure 13). The knockdown of *Cp190* by 69B-GAL4 showed a wrinkled wing phenotype with incomplete penetrance (Figure 13). In contrast, adult males with wrinkled wing phenotype were not found when DmManf was overexpressed. Thus, overexpression of DmManf rescued the loss of *Cp190* indicating that Cp190 could act as a positive regulator of DmManf expression. The knockdown of *Chro* by MS1096-GAL4 showed a mildly wrinkled wing phenotype in wild type background. In heterozygous *DmManf* mutant background, knockdown of *Chro* by MS1096-GAL4 led to strongly wrinkled wings (data not shown). These data indicate that *Cp190* and *Chro* might act in transcriptional regulation of DmManf expression. However, more detailed analyses, e.g. by luciferase assay with DmManf promoter or by qPCR analysis of DmManf mRNA levels in Cp190 and Chro knockdown flies, should be performed to confirm these interactions.

### 4.4.4 The genetic interaction of DmManf and genes with mitochondrial function (III)

The screen of approximately 2800 *Drosophila* genes resulted in identification of 21 genes as interacting partners of *DmManf* (III, Figure 12A). Four of these genes were unannotated. A Gene Ontology (GO) term analysis of the remaining 17 genes strongly indicated that *DmManf* interacts with genes that function in mitochondria. The most enriched GO terms were ubiquinone related processes (2 genes), mitochondrial cellular compartment (6 genes) and cellular metabolic process (13 genes). Additionally, three genes with unknown cellular compartment had human homologues annotated to mitochondria. Mitochondria are often referred as the power plants of cells and they have an important role in oxidative phosphorylation and calcium signalling. Previously, DmManf has been shown to partially co-localize with a mitochondrial marker in salivary glands (Palgi 2012). However, no co-localization of mammalian MANF and mitochondrial marker was detected in SH-SY5Y in vitro (Henderson et al. 2013). This might indicate distinct roles for MANF in different cell types or during evolution.

#### 4.4.4.1 DmManf and ubiquinone synthesis pathway (III)

Ubiquinone (Q), also known as coenzyme Q, is an electron carrier involved in the electron transport chain in mitochondria (reviewed in Hatefi 1985; Aguilaniu et al. 2005; Crane 2007). Q also participates in other cellular processes, e.g. protects phospholipids and lipoproteins against lipid peroxidation, and is found in membranes of different organelles in all cells. In *Drosophila*, only six genes are annotated to be involved in the
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Figure 13. Screening the candidate TFs predicted to bind DmManf promoter region. Results from UAS-RNAi fly experiments (this study) and previous microarray analysis (MAA, Palgi et al. 2012) are shown. UAS-RNAi lines were crossed to MS1096-GAL4 and 69B-GAL4 drivers in wild type, heterozygous DmManf mutant and DmManf-overexpressing backgrounds. Observed phenotypes of knockdown flies in heterozygous DmManf mutant background (het vs. wt) or DmManf-overexpressing background (OE vs. wt) were compared to phenotypes of knockdown flies in wild type background. In MAA results, mutant larvae stands for zygotic DmManf mutant larvae, OE larvae for 69B-GAL4>UAS-DmManf L3 larvae, and mutant embryos for maternal and zygotic DmManf mutant embryos.

Q synthesis pathway or closely related processes (Figure 14). The partial but unbiased screen for DmManf interacting partners contained two of these genes, encoding for enzymes COQ7 (coenzyme Q7 homologue) and CG9249 [homologue of coenzyme Q3 (COQ3)]. Both of these genes were identified as genetic interacting partners of DmManf (III). With all three drivers used, MS1096-GAL4, 69B-GAL4 and tub-GAL4, knockdown of COQ7 showed more severe phenotype in DmManf-overexpressing background.
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compared to wild type. In wild type background, knockdown of \textit{COQ3}/\textit{CG9249} with ubiquitous \textit{tub}-GAL4 and semi-ubiquitous 69B-GAL4 drivers resulted in lethality. With MS1096-GAL4, knockdown of \textit{COQ3}/\textit{CG9249} showed a bent-up wing phenotype. This phenotype was more severe leading to wrinkled wings when either heterozygous \textit{DmManf} mutant or \textit{DmManf}-overexpressing backgrounds were used (see Chapter 4.4.1).

Another four genes annotated to be involved in the Q synthesis were analysed in further studies (III). Knockdown of \textit{COQ2}/\textit{CG9613} by ubiquitous \textit{tub}-GAL4 resulted in decreased pupal viability when \textit{DmManf} was simultaneously overexpressed in comparison to wild type background. Taken together, three genes from the Q synthesis pathway were identified to genetically interact with \textit{DmManf} (Figure 14). The SAPLIP-like N-terminal domain of \textit{MANF} and CDNF might interact with lipids (Parkash et al. 2009) and the protective role of Q against lipid peroxidation could give further answers for the link between \textit{DmManf} and ubiquinone synthesis pathway.

In addition to Q synthesis, other indications of \textit{DmManf} involvement in the electron transport chain were found in the unbiased screen. Two genes identified as genetic interacting partners of \textit{DmManf}, encoding for ND75 (NADH:ubiquinone reductase 75 kD subunit precursor) and lethal(2)37Bb, have human homologues that have been found to be mutated in patients diagnosed with mitochondrial complex I deficiency (Online Mendelian Inheritance in Man, phenotype number 252010). The data obtained from the unbiased screen (III) together with the previously reported subcellular localisation studies (Palgi 2012) suggests that \textit{DmManf} functions in mitochondria. These findings deserve a further evaluation in future studies.

\textbf{Figure 14. Simplified schematic presentation of ubiquinone synthesis pathway.} Three components were found to interact with \textit{DmManf} (marked in grey). \textit{COQ3} is involved in earlier steps but also acts in the final stage of ubiquinone synthesis. Adapted from III, drawn according to Kyoto Encyclopedia of Genes and Genomes.

4.4.4.2 Role of ubiquinone in mitochondrial dysfunction and human disease

Dysfunctional mitochondrial biogenesis and metabolism are related to variety of human disorders (reviewed in Scharfe et al. 1999; Balaban et al. 2001). The loss of Q, a state referred as Q deficiency, is associated mainly with neuronal and muscular defects (Quinzii et al. 2008). One of the causes for Q deficiency is the loss of \textit{COQ7},
demethoxyubiquinone (DMQ) hydroxylase which also leads to impaired ATP synthesis and accumulation of Q precursor, DMQ (Marbois and Clarke 1996; Jonassen et al. 1998; Jonassen et al. 2001; Stenmark et al. 2001; Takahashi et al. 2012). Mitochondrial function has also been suggested to play a major role in pathology of PD (reviewed in Schon and Przedborski 2011) and the studies on neuroprotective properties of Q in PD models have been promising (reviewed in Jin et al. 2013). Q deficiency leads to destabilization of mitochondrial complex I (García-Corzo et al. 2013). Mutations in the subunits of complex I have been linked to familial forms of PD (reviewed in Schon and Przedborski 2011). MPTP, paraquat and rotenone, toxins used to induce PD-like symptoms, are also known to interfere with complex I function (Langston et al. 1983; Betarbet et al. 2000).

4.4.5 Interaction between DmManf and Drosophila homologue of Ku70 (III)

C-terminal domain of MANF is structurally similar to SAP-domain of Ku70 (Hellman et al. 2011). In the unbiased genetic screen, an interaction between DmManf and Irbp (Inverted repeat-binding protein), the Drosophila gene encoding for the homologue of Ku70 was identified (III). In wild type background, knockdown of Irbp by wing-driver MS1096-GAL4 resulted in wrinkled wing phenotype. In heterozygous DmManf mutant background, adult flies showed clearly milder wing phenotype than in wild type background. In a previous microarray analysis, heterozygous DmManf mutant embryos (with abolished maternal DmManf) showed upregulation of Irbp mRNA (Palgi et al. 2012). In homozygous mutant embryos lacking both zygotic and maternal DmManf, Irbp mRNA expression was even more upregulated (Palgi et al. 2012) indicating that the decrease of DmManf levels results in transcriptional upregulation of Irbp. The microarray results are in accordance with the knockdown phenotypes – the partial rescue of wing phenotype observed in DmManf heterozygous mutant background could be due to elevated Irbp expression. Taken together, these data suggest that decreased DmManf expression induces Irbp expression (III; Palgi et al. 2012).

4.4.5.1 Intracellular neuroprotectivity of C-MANF is conserved in Drosophila (II)

C-MANF shows a remarkable structural similarity to SAP-domain of Ku70 via which Ku70 binds BAX and inhibits BAX-mediated apoptosis (Sawada et al. 2003). In addition, mammalian MANF and C-MANF were found to be cytoprotective against BAX-mediated cell death in cultured mouse SCG neurons (Hellman et al. 2011). To test the functional conservation, plasmids encoding for full-length DmManf, with its endogenous secretion signal, and C-terminal domain of DmManf without secretion signal (DmManf-C-Dss) were microinjected into mouse SCG neurons (II). Both DmManf and DmManf-C-Dss, the corresponding constructs for the human counterparts used in the original study (Hellman et al. 2011), significantly inhibited the etoposide-induced cell death. This indicated that while human MANF and CDNF are able to function in Drosophila in vivo, DmManf possesses the cytoprotective functions of MANF/CDNF protein family conserved during evolution.
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4.4.5.2 Partial redundancy of Irbp and DmManf functions? (III)

Ubiquitous knockdown of Irbp by tub-GAL4 in wild type background resulted in lethality at pupal stage and no adults emerged. When DmManf was simultaneously overexpressed, this lethality was partially rescued to adulthood. The eclosed adults showed no obvious phenotype. Similar to mammalian Ku70, both mammalian and Drosophila MANF were capable of supporting mammalian SCG neurons from induced cell death 

in vitro (II; Hellman et al. 2011). These data together indicate that DmManf and Irbp could have partially redundant functions. However, this hypothesis remains to be verified in future studies.

4.4.5.3 Loss of the pro-apoptotic member of Drosophila BCL-2 family does not rescue DmManf mutant lethality

The structural and functional similarity between C-MANF and SAP-domain of Ku70 (II; Hellman et al. 2011) together with the genetic interaction between DmManf and Irbp (III) suggest that MANF could interact with BAX. Hypothetically, the loss of endogenous DmManf could lead to decreased inhibition of Debcl, the BAX homologue in Drosophila, and subsequent increased cell death. This could account for the molecular cause of DmManf mutant lethality. In order to test this hypothesis, two alleles of debcl, amorphic debcl^{E26} and a putative dominant negative debcl^{W105}, as well as UAS-debcl-RNAi line were used to abolish debcl expression (III). Alleles of debcl were combined with DmManf mutant and UAS-debcl-RNAi was driven by ubiquitous da-GAL4 driver in homozygous DmManf mutant background. However, the larval lethality of homozygous DmManf mutant was not rescued by abolishing the Debcl expression. This indicates that during development, the molecular functions of DmManf other than related to Debcl are more important for fly viability. Accordingly, the number of apoptotic cells in CNS of DmManf mutant embryos was not increased compared to wild type (I). Putatively, the increased expression of Irbp upon loss of DmManf could take over the Debcl-related functions of DmManf. In addition, the role of BCL-2 family members in Drosophila apoptosis is less important in comparison to mammals (Sevrioukov et al. 2007; Galindo et al. 2009).

Another indirect link between DmManf and Debcl was identified in the unbiased genetic screen. One of the genes identified as interacting partners of DmManf was Tom20 (Translocase of outer membrane 20) that encodes a receptor component of translocase of outer membrane (TOM) complex (III). TOM is responsible for directing proteins encoded by nuclear genes into the mitochondria. In Drosophila, the TOM complex is built by seven subunits (Hwa et al. 2004). Another component of TOM, Maggie/TOM22 is involved in the localization of Debcl to mitochondria and modulates the permeabilization of the mitochondrial outer membrane during apoptosis (Colin et al. 2009). The genetic interaction identified between DmManf and Tom20 might be a secondary effect of the proposed interaction between DmManf and Debcl, or vice versa. In future studies it would be necessary to study whether DmManf co-localizes with TOM proteins and if either Irbp or Debcl are involved in this putative interaction.
5 CONCLUSIONS

In this study, *Drosophila melanogaster* was used as a model to explore the function and genetic interactions of the MANF/CDNF protein family *in vivo*. These proteins have neurotrophic properties and there is increasing evidence suggesting that they are involved in the ER stress response and, more generally, in cellular homeostasis beyond that of neuronal function. Here, the main findings of this work are summarized.

DmManf is essential during *Drosophila* development. The endogenous expression of DmManf can be replaced by ectopic expression of either DmManf or its human orthologues, HsMANF and HsCDNF. The functional conservation between fly and human proteins opened a window for studies of the MANF/CDNF protein family in the *Drosophila* *in vivo* model. In addition to the conserved role of human orthologues in *Drosophila* *in vivo*, the *Drosophila* Manf protein possesses the cytoprotective properties of mammalian MANF *in vitro*. This indicates that the functional properties of the MANF/CDNF family proteins have evolved early during evolution of metazoans and further justifies the use of the powerful *Drosophila* model for analysing the MANF/CDNF protein family. While the *in vitro* research has revealed important aspects of the molecular mechanism of MANF/CDNF proteins, *in vivo* analyses are essential to study their function and interaction pathways in multicellular environment. The recently established MANF knockout mouse model will surely benefit in untangling several important issues in the future. However, the diverse genetic tools of the *Drosophila* model will substantially enhance this research.

The ability of the transgenic DmManf construct to rescue *DmManf* mutant lethality was applied to explore how designed mutations affect DmManf functionality. The ER conserved retention mediated by a C-terminal RSEL sequence of DmManf was discovered to be important for DmManf functionality. Similarly, neutralizing the positive charge of the surface amino acid residues K79, K83 and K86 of DmManf had a negative impact on fly viability when availability of DmManf was restricted. Deletion of the secretion signal leads to instability of DmManf at the protein level and this mutated DmManf cannot complement the loss of endogenous DmManf. Furthermore, both N- and C-terminal domains as an intact full-length protein are necessary for proper function of DmManf. This characterization of the structural and functional determinants revealed several important aspects of DmManf function and forms an essential basis for further studies.

The modification of the C-terminal CXXC motif of DmManf by a C129S point mutation does not only disturb the functionality of DmManf but also results in deleterious consequences during development in wild type background. These presumably dominant negative impacts are not alleviated by overexpression of wild type DmManf. For example, the presence of unpaired cysteine residue (C132) may cause UPR. However, the eye-specific expression of DmManf-C129S does not cause the retinal degeneration phenotype observed in previous studies on *Drosophila* UPR models. Furthermore, overexpression of the *Drosophila* homologue of the major ER chaperone GRP78 does not protect from the harmful influence of DmManf-C129S expression. The molecular basis behind the effects of DmManf-C129S on cellular homeostasis remains to be studied in the future.
An interaction between mammalian MANF and GRP78 has previously been shown *in vitro*. In this work, a genetic interaction between the fly homologue of GRP78 and *DmManf* was demonstrated *in vivo*. Furthermore, *DmManf* was found to interact with other genes that encode proteins known to be involved in ER function and UPR. These results offer further evidence for MANF functioning in ER-related processes and support the studies of MANF/CDNF protein in *Drosophila* UPR. Additionally, the genetic interaction studies presented in this thesis revealed novel functions for DmManf related to ubiquinone synthesis and mitochondria.
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