BBA Review: Cell Biology of Metals III

The balancing act of NEET proteins: Iron, ROS, calcium and metabolism

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Abstract

NEET proteins belong to a highly conserved group of [2Fe–2S] proteins found across all kingdoms of life. Due to their unique 2Fe-2S cluster structure, they play a key role in the regulation of many different redox and oxidation processes. In eukaryotes, NEET proteins are localized to the mitochondria, ER and the membranes connecting these organelles (MAM), and are involved in the control of multiple processes, ranging from autophagy and apoptosis to ferroptosis, oxidative stress, cell proliferation, redox control and iron and iron-sulfur homeostasis. Through their different functions and interactions with key proteins such as VDAC and Bcl-2, NEET proteins coordinate different mitochondrial, MAM, ER and cytosolic processes and functions and regulate major signaling molecules such as calcium and reactive oxygen species. Owing to their central role in cells, NEET proteins are associated with numerous human maladies including cancer, metabolic diseases, diabetes, obesity, and neurodegenerative diseases. In recent years, a new and exciting role for NEET proteins was uncovered, *i.e.*, the regulation of mitochondrial dynamics and morphology. This new role places NEET proteins at the forefront of studies into cancer and different metabolic diseases, both associated with the regulation of mitochondrial dynamics. Here we review recent studies focused on the evolution, biological role, and structure of NEET proteins, as well as discuss different studies conducted on NEET protein function using transgenic organisms. We further discuss the different strategies used in the development of drugs that target NEET proteins, and link these with the different roles of NEET proteins in cells.

I. Introduction

Iron-sulfur (Fe–S) clusters are among the oldest cofactors known to drive electron transfer reactions [1]. Because of the relatively small energy shifts of Fe–S clusters, when they transit from their oxidized to reduced forms, Fe–S clusters allow for fast and ideal electron-transfer conditions [2]. This made Fe-S clusters ubiquitous electron carriers across the three kingdoms of life. Indeed, Fe-S proteins are involved in many vital and diverse biochemical reactions, such as photosynthesis, respiration, nitrogen fixation, iron homeostasis and gene expression [1].

The primary forms of iron-sulfur clusters include [2Fe–2S], [3Fe–4S], and [4Fe–4S] (Fig. 1A). The reduction potential of Fe–S clusters, is dependent on the properties and structure of their coordinating residues in each specific protein, which in most cases are thiol side groups of cysteines, with the classical example of ferredoxins [1]. More rarely, His, Asp, Arg and Thr may coordinate the iron atoms [3]. In particular, histidine ligands constitute the most common evolutionary choice after cysteines [3], and most of the Fe–S His-ligand structures are conserved in their respective protein families [3]. In contrast to cysteine ligands, histidine-coordinated metal ions can have a neutral and deprotonated state at physiological pH. The former features a higher reduction potential, whereas the latter exhibits a significantly decreased reduction potential [2, 3]. The proton-coupled electron transfer (PCET) capabilities of His-ligated Fe–S are pervasive in redox reactions in complicated biochemical processes [4]. Because the His-ligation of Fe–S clusters impacts their lability (Fig. 1B), proteins containing His-ligated Fe–S clusters have been tied to different redox, iron-sulfur cluster biogenesis and cluster-delivery and/or chaperone functions, as well as iron sensing. These proteins include among others the ISC machinery component IscU, glutaredoxins and NEET proteins [2, 5].

The first report of a NEET protein, mitoNEET (mNT), was published by Colca *et al.* [6]. The protein was predicted to be a zinc-finger protein because of the presence of the *zf*-CDGSH zinc-finger domain. However, subsequent biophysical, biochemical and X-ray structural analyses revealed that mNT contains [2Fe–2S] clusters [5]. The clusters' ligands are 3Cys:1His, part of the CDGSH domain (**C**-X-**C**-X2-(S/T)-X3-P-X-**C**-D-G-(S/A/T)-**H**) [5]. The presence of the histidine ligand leads to a pH-dependent lability of the clusters [5]. Molecular simulation suggested that the protonation states of the His-ligand can affect the coordination bond polarity and the H-bond with the N_{ε} of the His-ligand and thereby control the break of the Fe–His bond [7] (Fig. 1B, red arrow). This lability allows NEET proteins to transfer their [2Fe–2S] cluster to an apo-acceptor protein [2]. As a result, NEET proteins display unique biochemical properties associated with their labile [2Fe–2S] clusters [2, 5], which are exploited in iron, reactive oxygen species (ROS) and calcium homeostasis [5].

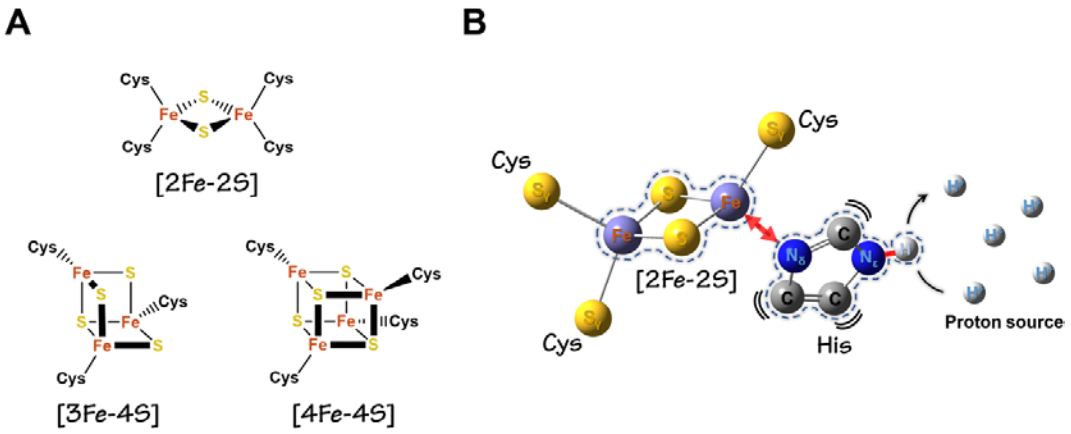

Fig. 1. Structural motifs of common Fe–S clusters and lability of the His-ligated NEET [2Fe–2S] cluster. (**A**) The most common coordination structures of Fe–S clusters originated during evolution. (**B**) An [2Fe–2S] cluster coordinated by three Cys and one His. Protonation of the His-ligand residue decreases dramatically its affinity for the [2Fe–2S] cluster [5]. The lability of the cluster under acidic conditions is used in nature to induce its transfer to apo-receptor proteins [2].

NEET proteins are present in all kingdoms of life from archaea to eukaryote, and only few organisms from the fungi phyla do not contain NEET-encoding genes in their specialized genomes [8]. The CDGSH domain of NEET proteins most likely appeared close to the emergence of life around 4 billion years ago (BYA) [8]. Phylogenetic analysis of the CDGSH domain of NEET proteins revealed that their evolution is linked with that of the *Fer4_19* domain that binds [4Fe–4S] and is a part of many Fe–S proteins found to be present in the genome of the putative last universal common ancestor (LUCA) [8]. The fact that no *Fer4_19* domain was found in association with the CDGSH domain in eukaryotes may suggests that the function of this domain could be different from that existing in prokaryotic organisms (Fig. 2A). NEET proteins can be divided into two classes: I) NEET proteins with one CDGSH domain per monomer, and II) NEET proteins with two CDGSH domains per monomer. Phylogenetic studies suggested that the separation of these two classes of NEET proteins most likely coincided with the appearance of eukaryotes on earth (~2.3–2.6 BYA) [9].

In vertebrates, the NEET family is composed of three distinct proteins (Fig. 2B): the membrane-bound mitoNEET (mNT) and nutrient-deprivation autophagy factor-1 (NAF-1) proteins, encoded respectively by the *CISD1* and *CISD2* genes that belong to class I NEET proteins; and the soluble mitochondrial inner NEET (MiNT) protein, encoded by *CISD3* that belongs to class II NEET proteins. While mNT and NAF-1 are homodimeric proteins anchored to the outer membrane of the mitochondria (OMM) [5, 9, 10]. NAF-1 is also localizes to the endoplasmic reticulum (ER) and the membrane structures connecting the ER to the mitochondria (MAM). In the model plant *Arabidopsis thaliana*, only one type of class I NEET proteins was found to be localized to the outer membranes of the chloroplast, and probably the mitochondria, and was termed AtNEET (Fig. 2B) [11].

The separation of plant and animal NEET proteins is thought to have occurred about 1.5 BYA and CISD1 (mNT) and CISD2 (NAF-1) diverged during the emergence

of vertebrates around 622–768 million years ago (MYA) [8]. The basic structure, coordination, and labile nature of the [2Fe–2S] clusters of NEET proteins have been conserved since their initial appearance on Earth [8, 9]. This degree of conservation highlights the importance of the unique cluster properties of NEET proteins to the role they play in different organisms, as well as support their involvement in numerous fundamental cellular processes [2, 5, 12].

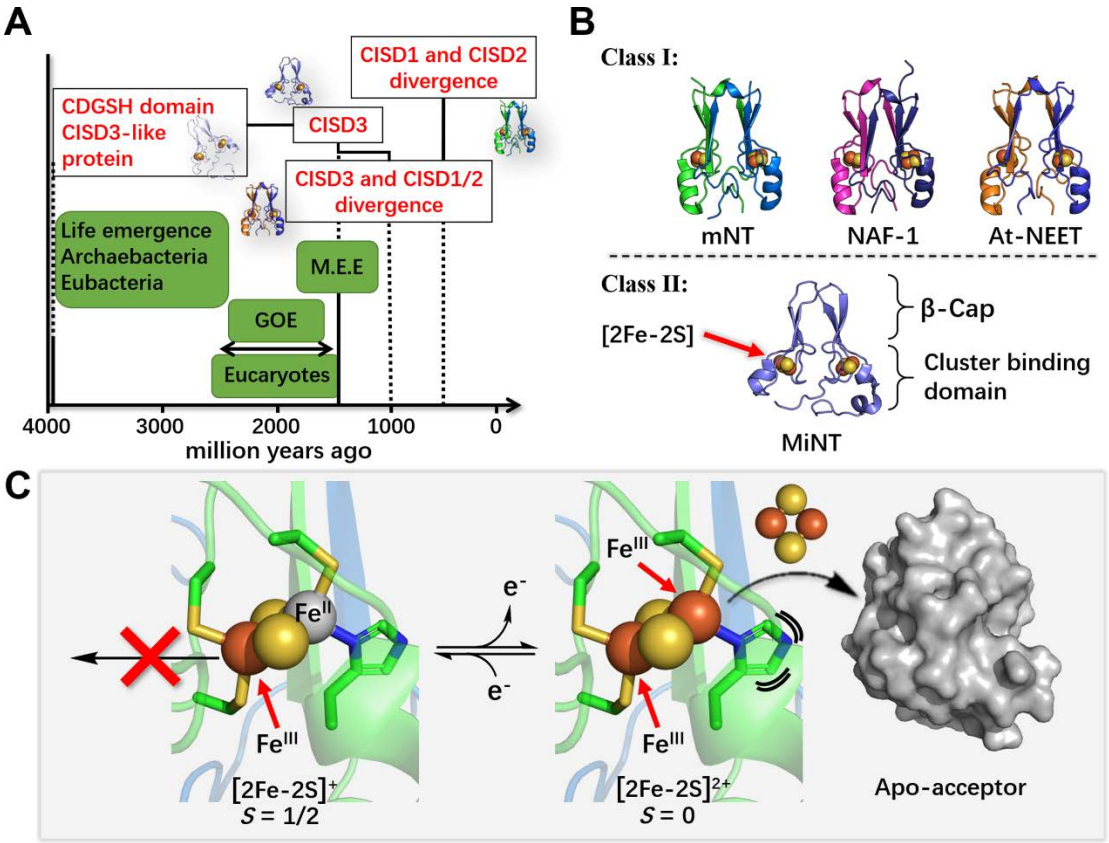

Fig. 2. Phylogenetic structural relationships and cluster transfer functions of NEET proteins. (**A**) Hypothetical evolutionary time-frame of NEET proteins from the appearance of the CDGSH domain to the divergence of the latest forms of human NEET proteins; M.E.E. - mitochondrial endo-symbiotic event, GOE - Great oxygenation event. (**B**) The two different classes of NEET proteins. Top: The soluble parts of the membrane-bound human mNT (PDB ID: 2QH7), NAF-1 (4OO7), and the plant AtNEET (3S2Q). mNT, NAF-1 and AtNEET are homodimeric proteins with one CDGSH domain per monomer and are representatives of the NEET Class I family. Bottom: The monomeric and soluble human MiNT protein (6AVJ) that possess two CDGSH domain. (**C**) The lability of the [2Fe–2S] clusters of NEET proteins depends on the oxidation and protonation state of their His-ligand residue. When the [2Fe–2S] cluster is reduced (left) the cluster remains stable even under acidic pH. Oxidation of the Fe and protonation of the His residue increases the lability of the cluster and allows its transfer to an apo-acceptor-protein (right).

In this review, we will focus on the role of NEET proteins in the regulating iron, ROS, and calcium metabolism and homeostasis in eukaryotic organisms, as well as on their involvement in regulating mitochondrial dynamism and different human diseases.

II. The role of NEET proteins in protecting the mitochondria from over accumulation of iron and ROS

Studies across different organisms ranging from mammalian cells and mice to plants and *C. elegans* demonstrated that NEET protein dysfunction leads to disruptions in the structure and function of specific subcellular organelles such as the mitochondria and chloroplast [5, 11, 13, 14], accompanied by alterations in subcellular levels of iron and ROS in these organelles. These studies highlighted the key role NEET proteins play in regulating iron and ROS levels [2, 5, 12] in mammalian cells (discussed below), plants and *C. elegans* (section VII).

In eukaryotes, mitochondria provide a key source of ATP, as well as serve as the main site for iron-sulfur [2Fe–2S] cluster biogenesis, heme synthesis, and fatty acids metabolism [1, 15]. In an effort to understand the mechanism of action of thiazolidinediones (TZDs), Colca's group investigated new mitochondrial binding targets of the anti-diabetes type II compound pioglitazone - PGZ [6]. Their study identified mNT, the first NEET protein that was found to bind PGZ. A successive study identified a total of three NEET proteins, mNT, NAF-1 and MiNT encoded by *CISD1*, *CISD2* and *CISD3* genes, respectively in mammalian cells [5]. mNT was found to be exclusively localized on the OMM, where each of its monomers is anchored by a single α-helix trans-membrane domain with the main part of the protein facing the cytosol [2, 5]. NAF-1 was found to be localized to the OMM, the ER membranes and the membranes that connect the mitochondria to the ER (MAM) [5]. The monomeric globular MiNT protein was shown to be localized inside the mitochondria [5] (Fig. 3).

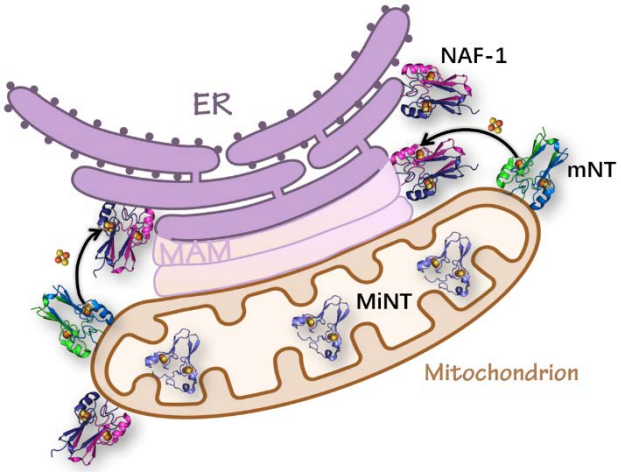

Fig. 3. NEET proteins localization in mammalian cells. mNT is localized to the outer mitochondrial membrane (OMM) [5], NAF-1 is localized to the OMM as well as to the ER membranes and the mitochondrial associated membranes (MAM) [5], whereas MiNT is localized inside of the mitochondria [5].

A recent study revealed that mNT and NAF-1 interact and that mNT can transfer its [2Fe–2S] to NAF-1 (but not *vice-versa*) [16]. In addition to interacting with NAF-1, mNT was also recently shown to have an interaction with the outer-mitochondrial

membrane protein voltage-dependent anion channel (VDAC1) [17, 18].

IIa. NEET proteins role in ROS hemostasis, respiration (via Complex 1), ATP production, redox regulation, and nitric oxide and fatty acids metabolism

Two hypotheses regarding the functional role of NEET proteins were proposed based on NEET proteins' unique properties of having redox active and labile [2Fe–2S] clusters [5]: 1. A role for NEET proteins in electron transfer reactions, based on the interactions of mNT with mitochondrial proteins such as the mitochondrial outer membrane import complex protein 1 (MTX1), glutathione reductase, glutamate dehydrogenase 1 [19], and reduced flavin mononucleotide (FMNH2) [20]; and 2. A role for NEET proteins in iron-sulfur cluster transfer based on the *in vitro* ability of all three NEET proteins to transfer their clusters to apo-acceptor proteins such as apo-ferredoxin [2, 5]. In addition, mNT was proposed to play a novel role in a pathway that repairs the cluster of the cytosolic iron regulatory protein1 (IRP1) in mammalian cells [21]. mNT and/or NAF-1 were also shown to transfer their clusters to anamorsin, a protein required for iron sulfur cluster assembly in the cytosol [22]. NEET proteins were further suggested to have a sensing function and to balance and protect cells from environmental changes such as nutrient deprivation, oxidative stress, or iron overload, triggering different cellular mechanisms such as autophagy, apoptosis and ferroptosis [12]. The sensing mechanism, which controls the activity of NEET proteins in response to redox signals via changes in the redox state of NEET proteins' clusters, can also impact their role as electron or [2Fe–2S] cluster transfer proteins.

Recent studies, using a wide array of genetic and biochemical tools, revealed that NEET proteins are involved in many other essential biological processes, such as energy metabolism via OXPHOS for ATP production [5] and β-oxidation of lipids [5, 23]. NEET proteins also play a major role in iron-ROS homeostasis [2, 5, 10] and Fe–S biogenesis [10]. The latter implicates them in the regulation of inflammation [5], autophagy and apoptosis [5, 11, 24], neuronal development [5], and longevity [25, 26]. In particular, mNT is involved in energy metabolism in mitochondria affecting OXPHOS and lipid metabolism via binding to components in complex I or other partners such as GDH1 [5, 23], in the regulation of iron and ROS homeostasis via transferring clusters to possible acceptor proteins such as ferredoxin, iron regulatory protein 1 and anamorsin [12, 21, 22], in β-cell insulin secretion [27], in cell proliferation of human breast cancer [12] and in lipid accumulation in adipocytes [23]. Suppression of NEET protein expression may increase ADP/ATP ratio, along with NAD+/NADH ratio [28]. When mNT expression is suppressed, disruptions in mitochondrial respiration occur and this is associated with decreased mitochondrial volume and function [29]. In contrast to mNT, NAF-1 was shown to be primarily involved in regulating intracellular calcium homeostasis [30], in the maintenance of mitochondrial integrity and in controlling ER functions and lifespan [25] as well as potentially playing a role in the inflammatory response [31]. MiNT is the least characterized among NEET proteins. It resides inside the mitochondrial matrix and was shown to have a role in regulating mitochondrial iron and reactive oxygen homeostasis [10]. MiNT can also

bind NO (nitric oxide) when its [2Fe–2S] clusters are reduced while the other two human NEET proteins, mNT and NAF-1, fail to bind NO. But interestingly a single amino acid mutation, (D96V in mNT, or D123V in NAF-1) facilitates the binding of NO to the [2Fe–2S] cluster indicating that subtle changes to these proteins may switch their ability to bind NO, and thereby facilitate signaling in cells and modulation of mitochondrial function through NO signaling [32]. Binding of nitric oxide can inhibit the electron transfer activity of MiNT [2Fe–2S] clusters suggesting that mitochondrial NEET proteins may play a novel role in energy metabolism in cells, and that nitric oxide may regulate the electron transfer activity of NEET proteins and modulate energy metabolism in mitochondria [33]. MiNT may also be involved in protein-protein interaction(s) inside the mitochondrial matrix. Potential functional partners of MiNT include multiple components of the respiratory complex I, ribosomal RNA binding proteins, glutathione-S transferase and many other proteins in Fe–S biogenesis [10]. In different cellular models, low expression levels of NEET proteins commonly showed a decrease in mitochondrial membrane potential (MMP) [5, 34, 35], an increase in the accumulation of mitochondrial iron and ROS, and an increase in autophagy and apoptosis [10, 12]. All of these mitochondrial dysfunctions were mitigated by iron chelators such as Deferiprone (DFP) or by re-expression of the suppressed NEET proteins to normal levels (personal communication). Similar to mNT and NAF-1, MiNT could also have a key role, albeit functioning from within the mitochondria, in regulating iron and ROS homeostasis.

An accumulating lines of evidence suggests that NEET proteins have a balancing and/or protecting role from over-accumulation of iron and ROS in intra-cellular organelles such as mitochondria. Recent studies demonstrated that NEET proteins have a protecting role in human melanoma cells by alleviating mitochondrial dysfunctions and apoptosis [36]; in liver cells they can antagonize mitochondrial lipid peroxidation and inhibit ferroptosis [37]; and in an Alzheimer's disease (AD) mouse model they could be involved in attenuating amyloid β-mediated mitochondrial damage and loss of neurons [38]. Recently, mNT and NAF-1 were shown to cooperate in the control of iron and ROS homeostasis in mitochondria [12], and the link between these proteins, as balancing factors for iron, Fe–S, and ROS homeostasis, was confirmed by genetically manipulating their levels. Furthermore, it appears that MiNT is similarly involved in this balancing act (personal communication). Through their role in regulating iron and ROS, NEET proteins could therefore regulate cellular proliferation, apoptosis and autophagy activation, as well as many other essential processes (Fig. 4) [16].

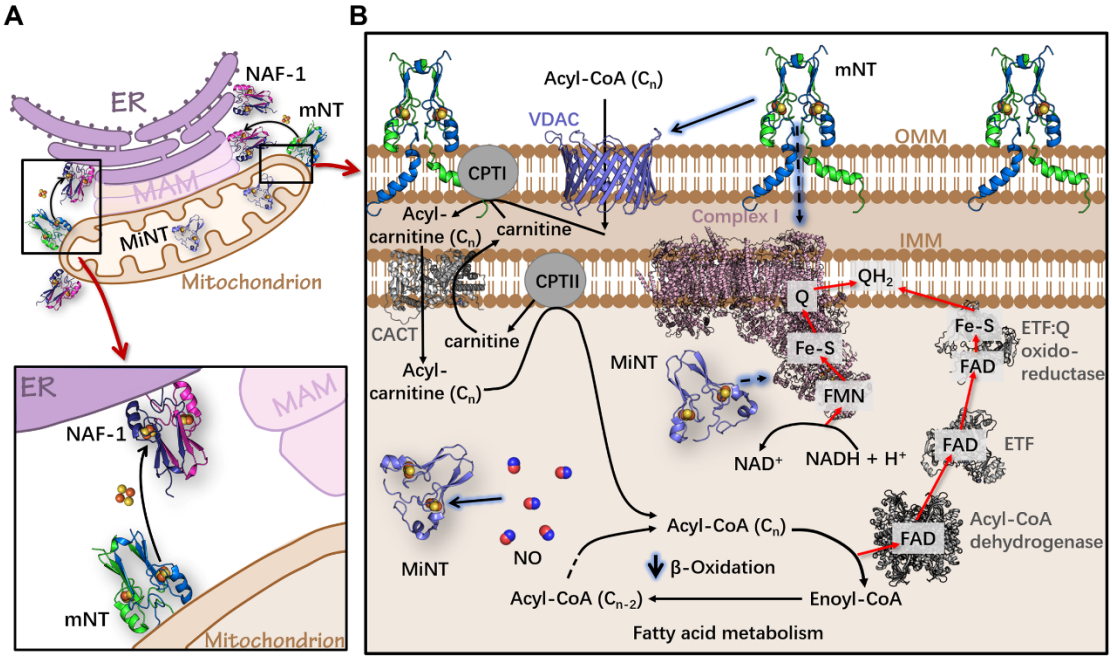

Fig. 4. NEET proteins participation in different mitochondrial functions. mNT participates in several mitochondrial metabolic functions, as well as in energy transduction through interactions with respiratory complex I, glutathione-S transferase, fatty acids metabolism and Fe–S biogenesis. MiNT is known to regulate mitochondrial iron and reactive oxygen homeostasis, electron transfer and NO hemostasis.

IIb. Mitochondrial dynamics and NEET proteins

Control of mitochondrial dynamics through processes such as fusion, fission and degradation, highlights the high plasticity needed for the regulation of mitochondrial hemostasis, metabolism and function [39]. Mitochondrial adaptations through morphological changes - fragmentation or elongation, depend on nutrient availability, as well as ATP production capacity and demand in response to changes in environmental, metabolic or developmental conditions [40]. Mitochondrial dynamics is a highly controlled process. Fusion requires three large GTP-hydrolyzing enzymes. The first two, mitofusin 1 and mitofusin 2 (MFN1/2) are located on the outer mitochondrial membrane and are responsible for the fusion of this membrane. The third enzyme, called Optic Atrophy 1 (OPA1), is localized to the inner mitochondrial membrane. It is responsible for the fusion of the inner mitochondrial membrane [39, 41]. Mitochondrial fission uses the so-called GTP-hydrolyzing enzyme dynamin-related protein 1 (DRP1). This protein is being recruited from the cytosol onto the mitochondrial surface to induce fission [39, 41].

ER/mitochondria cross talk regulates ER stress signaling, the unfolded protein response (UPR) and iron homeostasis. The mitochondrial associated membranes (MAM) interface plays a key role for pathological abnormalities such as diabetes and neurodegenerative diseases [28, 41]. It was suggested that mitochondrial dynamic

abnormalities impact the entire inter-organelle communication network of cells, in turn impacting overall cellular survival and bioenergetics [40]. Mitochondria play a major role in iron metabolism through iron-sulfur Fe–S cluster and heme biosynthesis processes, controlled by several regulatory proteins, among which are NEET proteins [2, 5, 12, 15]. Mitochondrial iron hemostasis is considered a regulator of mitochondrial morphology [42]. Disrupting the expression of any of the three human NEET proteins *i.e.*, mNT, NAF-1 or MiNT, was shown to result in the over accumulation of mitochondrial labile iron levels [2, 5, 10, 12]. Increased mitochondrial iron, or cellular iron in general, causes disturbances in mitochondrial dynamic, and will interfere with the balance between mitochondrial fission and fusion [40], through increased production of ROS and other radicals [43]. Decrease in the expression of mNT or NAF-1 causes abnormalities in mitochondrial membrane potential (MMP) [5, 12, 35], and loss of mNT was shown to cause intra-mitochondrial junctions and network contacts abnormalities (Fig. 5) [29].

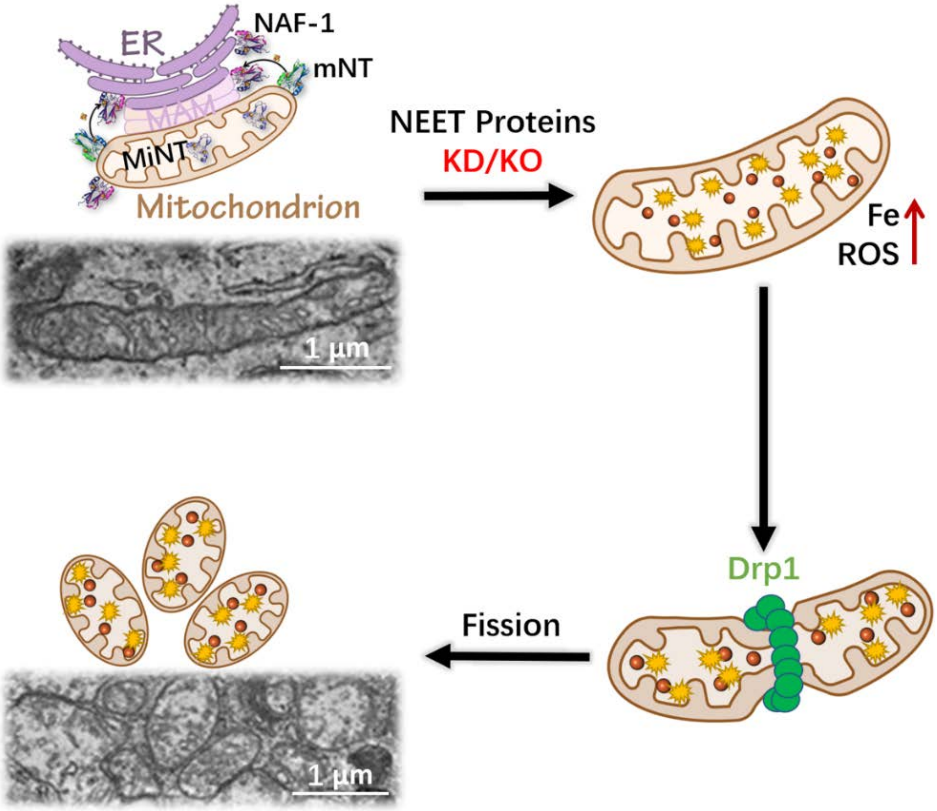

Fig. 5. Lowering the expression of NEET proteins results in mitochondrial fission. NEET proteins are involved in controlling iron and oxidative stress. When the mNT or NAF-1 expression is knocked down (KD) or knocked out (KO), an increase in mLI (represented as red circle) and ROS (represented as yellow stars) is observed. These stimulate fission of the mitochondria probably mediated by the DRP1 protein [43]. Mitochondrial images were adapted from Holt, et. al. 2016 [34].

Similarly, decrease in NAF-1 levels is accompanied by disruptions in mitochondrial morphology, including fission, fusion, inner membrane translocation, outer membrane translocation, mitochondrial protein import and membrane

polarization and potential [35]. In addition, decreased expression of MiNT results in disturbances in MMP [10]. The increase in mitochondrial labile iron (mLI) caused by a deficiency in any of the NEET proteins could therefore disrupt mitochondrial dynamics favoring fission and triggering mitophagy and ferroptosis in cell [40, 43]. This disturbance could also be linked to disruptions in Ca^{2+} signaling caused by NEET abnormalities (detailed in the section below) with the latter causing mitochondrial fragmentation and fission by DRP1 [43]. However, the findings that iron chelators that correct the NEET-derived abnormalities in mLI [12], can lead to mitochondrial elongation by decreasing the expression of mitochondrial fission modulators of DRP1 [40, 44], suggest that the effect of iron on these processes is more pronounced than that of calcium.

Numerous proteins found to impact or control mitochondrial dynamics are present at the MAM region, side-by-side with mNT and NAF-1, and many of these proteins were shown to physically, or functionally, interact with mNT and NAF-1 [12, 17, 28, 45, 46]. The main proteins controlling MAM integrity found to be affected by NEET protein function include inositol 1,4,5-trisphosphate receptor (IP3R) and voltage-dependent anion channel (VDAC), and these two proteins communicate with each other via the HSC70 chaperon family protein GRP75 [41]. In addition to the different roles of these proteins linking Ca^{2+} signaling to the tethering of MAM and the formation of MAM contact sites, their interaction with NEET proteins such as mNT and NAF-1 could expand the control of these processes to include responses to changes in redox, ROS, iron and Fe–S metabolism. NEET proteins could therefore serve as important links between these processes and mitochondrial dynamics through interactions with different proteins found at the MAM [41]. Altogether, NEET proteins may play an important physiological role in controlling the mitochondria, ER and MAM hemostasis and structure [29, 35, 41], linking mitochondrial iron/ Ca^{2+} imbalanced and Fe–S hemostasis with mitochondrial ROS production [37] and mitochondrial dynamic morphological changes.

III. NEET proteins constitute an important link between calcium signaling and iron metabolism

Interactions between the mitochondria and the ER are critical for maintaining normal cellular functions. These interactions primarily occur at the contact sites between the two organelles known as the ER- mitochondrial associated membrane (MAM) network [31]. The MAM network is believed to be the site in which the ER and mitochondria cooperate to exchange signals and regulate proper cellular functions including Ca^{2+} signaling, lipid metabolism, autophagy, cell survival and cell death [41]. Maintaining optimal distance between the mitochondria and the ER through numerous regulatory proteins that reside in the MAM secures proper Ca^{2+} transport and signaling between these two organelles [47]. Mitochondria, ER and MAM integrity is therefore important for preventing abnormalities that may cause sever diseases such as neurodegenerative diseases, diabetes, inflammation, and cancer [31]. Under normal physiological

conditions, iron is known to regulate ROS generation that alters normal Ca^{2+}-dependent signaling pathways. Yet, excessive iron levels which promote oxidative stress lead to disturbances in Ca^{2+} signals, which among other downstream effects induce damage to mitochondrial function [48]. On the other hand, increasing mitochondrial Ca^{2+} to non-physiological levels also causes mitochondrial dysfunction and loss of iron hemostasis. This highly controlled self-sustained cycle of iron and Ca^{2+} is essential for the control of mitochondrial health and the regulation of key cellular functions [48].

While NAF-1 was shown to play a regulatory role in maintaining mLI, Fe–S and mROS hemostasis [2, 5, 12, 49], and could impact Ca^{2+} levels through altering iron and ROS, NAF-1 was also shown to be a more direct regulator of Ca^{2+} hemostasis between the mitochondria and the ER [5, 50, 51]. It is thought that this control is maintained by the interactions of NAF-1 with different Ca^{2+} associated proteins such as IP3R [52], as well as its effect on the unfolded protein response (UPR) related to Ca^{2+} regulation and ER stress [28, 41]. NAF-1 is known to interact with Bcl-2 during autophagy [45, 46], an interaction controlled by the absence or presence of its [2Fe–2S] clusters [5], and the process of autophagy is thought to be linked to Bcl-2-NAF-1 regulated ER-Ca^{2+} stores [2, 5]. Suppression of NAF-1 in knock-out cellular models, disrupts cytosolic and ER-stores of Ca^{2+} suggesting a role for NAF-1 and Bcl-2 in the regulation of autophagy upon Ca^{2+} release from the ER (Fig. 6) [52-54].

NAF-1 KO models, have shown alterations in Ca^{2+} concentration at the ER lumen that lead to increases in ER stress resulting in the activation of UPR [28]. Moreover, NAF-1 was shown to be a part of the IP3R macro-complex that is required for controlling ER Ca^{2+} stores and signaling through the MAM [45, 46, 53, 54]. IP3R is known to mediate ER Ca^{2+} efflux through its redox-sensitive cysteines [28]. KO models of NAF-1 also show cellular dysregulation of Ca^{2+} that results in lower basal levels of cytosolic Ca^{2+}, depletion of the ER Ca^{2+} stores and dramatic increases of mitochondrial Ca^{2+} load [28]. The latter suggested the involvement of proteins mediating ER Ca^{2+} influx activity, such as the ER sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA2) protein, which takes up Ca^{2+} from the cytosol and delivers it to the ER lumen [28, 51, 52]. The KO models of NAF-1 also showed alteration in mitochondrial function and structure that are further affected by increased mitochondrial Ca^{2+} levels [28, 51]. Suppression of NAF-1 was also reported to impair intracellular Ca^{2+} hemostasis, through the SERCA2 protein especially SERCA2b isoform in hepatocytes [51], SERCA 2a in the heart [55], or SERCA1 in skeletal muscle [56]. It was suggested that NAF-1 absence altered the redox status of the cell and increased oxidative modifications and glutathionylation of SERCA proteins, impairing Ca^{2+} pumping activity from the ER, resulting in ER stress and mitochondrial abnormalities [51-53, 55-57]. These abnormalities were shown to be related to aging, in which the activity of SERCA is significantly decreased, especially when the NAF-1 protein is absent (Fig. 6) [55, 56].

NAF-1 was also shown to interact at the MAM area with GTPase of immune-associated protein 5 (Gimap5). This interaction suggested that both proteins are essential for mitochondrial integrity and the buffering capacity of Ca^{2+} levels through the control of mitochondrial Ca^{2+} uptake that was shown to be important in the

maintenance of intracellular Ca^{2+} hemostasis in cells [30, 35, 52, 54]. On the other hand, deficiency in NAF-1 that increases cytosolic Ca^{2+} levels, affecting Ca^{2+}-dependent phosphatase signaling pathways as a secondary response, is known to involve calcineurin and affects different cellular functions including adipogenesis in adipose tissues, as well as the regulation of glucose hemostasis [30, 52]. In another study of Ca^{2+} hemostasis, NAF-1 was found to be involved in the negative regulation of Calpin2, a Ca^{2+}-dependent protease, known to be activated by high cytosolic Ca^{2+}. When NAF-1 is absent from cells this process could cause hyperactivity of Calpin2 to different signals and the activation of cell death (Fig. 6) [58].

An important recent study further identified a redox-sensitive binding interaction between mNT and the voltage-dependent anion channel 1 (VDAC1) protein complex, a crucial crosstalk point between the mitochondria and the cytosol that regulates the transfer of ions, including Ca^{2+}, different metabolites and ROS (Fig. 6) [17, 59]. The ability of the redox sensitive [2Fe–2S] cluster protein mNT to bind and block VDAC in response to changes in cellular redox states highlights a new and exciting way in which NEET proteins could regulate mitochondria-to-cytosol calcium signals in response to changes in redox, iron, ROS and [Fe–S] levels [17]. This regulation is thought to play a hemostatic role in protecting mitochondria, ER and the MAM [17].

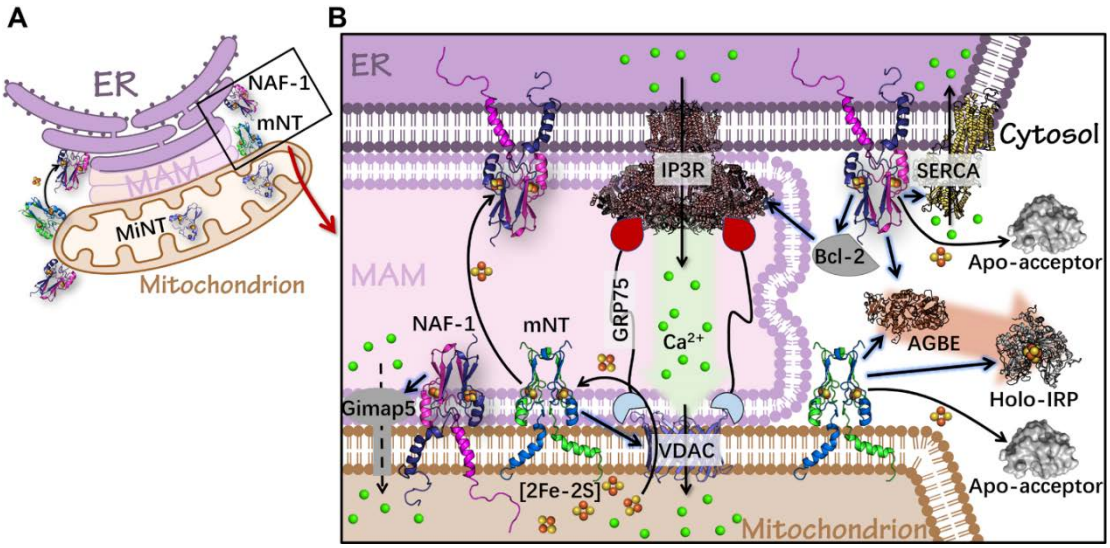

Fig. 6. NEET proteins involvement in calcium signaling at the mitochondrial ER and MAM junction. The role of NEET proteins in the regulation of Ca^{2+} and iron hemostasis at the mitochondria-ER-MAM interface is depicted. The complementary role of mNT and NAF-1 in [2Fe–2S] cluster transfer and interactions with different ER and mitochondrial proteins (SERCA, IP3R, Gimap5 and VDAC) that regulate proper Ca^{2+} signaling through the MAM is shown in the model. mNT could also contribute to [2Fe–2S] translocation from the mitochondria via VDAC. GRP75 regulates the interaction between IP3R and VDAC that plays a role in the integrity of the MAM. mNT and NAF-1 bind with AGBE, an iron regulator that ensures the function of the holo-IRP. mNT also has a role in the reactivation of holo-IRP.

VDAC may have a further role in the transferring of [2Fe–2S] clusters from inside the mitochondria to mNT [17]. In addition to the mNT-VDAC interaction, a C-terminal truncated version of VDAC interacts with NAF-1 to confer resistance to cellular

apoptosis [18]. Moreover, mNT and/or NAF-1 binds specifically with 1,4-Alpha-Glycogen Branching Enzyme (AGBE) that is known as a regulator for iron hemostasis, to ensure that holo-IRP1 remains functional and can enter to the nucleus [60].

IV. Involvement of NEET proteins in metabolic and genetic diseases

IVa. NEET proteins and Diabetes

Diabetes is a worldwide epidemic with significant social and economical burdens [61]. It is an important public health challenge, and considered as a leading cause for morbidity and mortality worldwide [62]. Diabetes is a group of metabolic diseases characterized by hyperglycemia due to a defect in insulin secretion, insulin sensitivity or both [63]. These pathogenic defects range from autoimmune-related destruction of pancreatic β-cells causing insulin deficiency to abnormalities which cause resistance to insulin uptake and action [63]. Several different factors are thought to be associated with the development and progression of diabetes, among them is oxidative stress. Diabetes is usually accompanied by the over accumulation of free radicals and/or impaired antioxidant mechanism [63, 64]. Iron hemostasis was also shown to play a key role in diabetes and other metabolic diseases [65], and it is thought that disturbances in iron homeostasis may affect glucose metabolism, insulin sensitivity and insulin secretion, but the exact mechanism of iron-induced diabetes is yet to be determined [64]. Systemic metabolic disorders such as diabetes and neurodegeneration were further linked to disturbances in mitochondrial function resulting from iron and oxidative stress derived processes [66].

NEET proteins are known to participate in iron, [Fe–S], Ca^{2+} and ROS hemostasis in mitochondria [2, 5, 12], and the first NEET protein identified, mNT was recognized as a novel mitochondria protein that cross-linked to the anti-diabetic drug Thiazolidinedione (TZD), used in the treatment for Type-2-Diabetes (T2D). TZD is known as a direct activator of peroxisome proliferator activated receptor-γ (PPAR-γ) [6]. TZD treatment leads to improvement in insulin action and sensitivity in all tissues [6]. TZD was also shown to stabilize the [2Fe–2S] clusters of NEET proteins, preventing their loss or their transfer of their [2Fe–2S] clusters to apo-acceptor proteins [5, 67]. NEET proteins were confirmed to have a role in the pathology of diseases related to mitochondrial dysfunction, including diabetes [5]. It is thought that overexpression of mNT preserves insulin sensitivity in adipose tissue (see below), whereas decrease in mNT expression leads to increased oxidative stress and glucose intolerance [23]. Mitochondrial dynamics is also affected by oxidative stress causing dysfunctions in insulin secretion or action [68]. Mitochondrial damage and dysfunction are important defects associated with a decrease in the level or function of NEET proteins [5, 10, 12, 34]; the latter were related to decreased insulin production by the pancreas in type 1 diabetes [49]. NEET proteins were shown to be powerful factors in mitochondrial metabolism in pancreatic cells, and to affect glucose hemostasis in type 1 and type 2 diabetes [23, 69]. Increasing the activity of NEET proteins is crucial to the

maintenance of fat reserves and energy hemostasis, along with resistance to diabetes [23]. While reduction in the expression of NEET proteins causes impaired glucose metabolism and diabetes [23, 68]. Decrease in NEET protein expression causes an increase in mitochondrial ROS and oxidative stress, due to the increase of mLI [12], and disruption in mLI and ROS affect mitochondrial dynamics and function [5, 10]. Induction of mNT in β-pancreatic cells, responsible for glucose stimulated insulin secretion, causes hyperglycemia and glucose intolerance due to the activation of Parkin-dependent mitophagy [27]. While mNT induction in pancreatic α-cells, which play an important role controlling hyperglycemia by hyperglucagonemia, leads to hypoglycemia and hyper secretion of glucose induced insulin [27]. However, decreasing the expression of mNT in pancreatic α- and β-cells causes reduced mitochondrial function in α-cells, while protecting β-cells viability and mass [27]. The differential role displayed by mNT in pancreatic α- and β-cells, reveals a critical mechanism by which compromised mitochondrial function alters β-cell insulin secretion and α-cell glucagon production, preserving insulin sensitivity under metabolic challenges [27].

Mutations in NAF-1, the causative agent of the rare genetic disease Wolfram Syndrome Type 2 (WFS-T2) described below, result in the development of diabetic features that mimic type 1 diabetes [70]. Absence of NAF-1 was shown to be responsible for the loss of β-pancreatic cells function [49, 69]. In WFS-T2 the destruction of pancreatic β-cells and their associated insulin secretion was related to the unique localization of NAF-1 to the MAM (detailed above) [5, 41]. Adipose-specific loss of NAF-1 is also associated with a reduction in ER-mitochondrial interactions, mitochondrial dysfunction and altered insulin signaling in adipose tissue [71, 72]. The critical roles NAF-1 plays in mitochondria, MAM and ER hemostasis [25, 28, 41, 49], as well as in the regulation of autophagy [5, 45, 73], apoptosis [24, 34] and ferroptosis [12, 74], makes NAF-1 a crucial player in pancreatic β-cell integrity. NAF-1 absence could therefore cause disruptions and increased program cell death (PCD) of pancreatic β-cells associated with type 1 or 2 diabetes [49, 50, 69, 70, 75, 76].

IVb. NEET proteins and obesity

Obesity is a worldwide disease associated with type 2 diabetes and metabolic disorders [77]. Obesity is primarily characterized by an increase in circulating fatty acids, and accumulation of triacylglycerol, that contributes to mitochondrial alterations, increased lipotoxicity, elevated oxidative stress and impaired energy substrate metabolism and oxidative phosphorylation (OXPOX), which is related to diabetes [78]. Obesity is a multifactorial disease that is influenced by diet and results from mitochondrial disorders [77]. Mitochondria manage the production of energy by controlling the fate of lipids through β-oxidation or storage in adipose tissue. These mechanisms require that mitochondrial energy production will be coordinated with the tri-carboxylic acid cycle and the electron transport chain (ETC). Failure in controlling these highly coordinated functions was shown to be associated with obesity and type 2 diabetes [77]. NEET proteins were shown to be essential for the maintenance of mitochondrial energy

production and fatty acid metabolism. An *ob/ob* mice model, with a genetic background that induces an expansion of adipocyte tissues leading to an obesity phenotype, was shown to have a diminished expression of mNT [23]. In the same model, the overexpression of mNT was found to decrease β-oxidation rates of lipids and to exhibit higher lipid accumulation due to an increase in adiponectin production [23]. Lower rates of β-oxidation were attributed to lower mitochondrial iron content that affected the ETC resulting in a decrease in ROS production [23]. Interestingly, this observed phenotype was accompanied by persistent insulin sensitivity. In contrast, when the expression level of mNT was decreased, opposite phenotypes were observed including increased oxidative stress and diminished glucose tolerance [23]. The observed lower insulin sensitivity may result from increased mitochondrial ROS production when the level of mNT expression is decreased [12]. Treatment of *ob/ob* mice with antioxidants lowered lipid oxidation [23], suggesting that lowering oxidative stress could be a therapeutic approach for these disease, highlighting the potential importance of mNT expression and function in the development of obesity and associate metabolic diseases. A study of the transcriptome signature of mice white adipocyte tissue (WAT), in which mNT was overexpressed, supported the idea that mNT impacts the management of inflammatory mechanisms and mitochondrial iron and ROS homeostasis associated with obesity and metabolic diseases [79].

The expression of mNT is increased during the differentiation of human adipocytes and is maintained in these tissues [80]. Interestingly, similar to the observations made with the *ob/ob* mice model [23], mNT expression was downregulated in the subcutaneous (SAT) and visceral (VAT) adipose tissues of human patients with obesity phenotype [80]. The expression level of mNT was also correlated with an adipogenesis expression pattern in the VAT but not in the SAT [80]. In patients with morbid obesity, mNT expression was positively correlated with insulin sensitivity and the expression of the protein Sirtuin 1 (SIRT1) in both types of adipocyte tissues [80]. SIRT1 deacetylase was shown to be related to mitochondrial biogenesis and the browning of white adipocyte tissues [81]. Expression of SIRT1 was shown to have a beneficial effect on the physiology of adipocyte tissues. In contrast, lower expression of this protein is associated with the development of obesity and associated metabolic diseases [82]. Moreover, the expression of mNT was also positively correlated with transcripts involved in iron homeostasis [80]. Another protein, ISCA2 which is involved in [Fe–S] cluster biogenesis [1], was shown to positively correlate with the expression of mNT. These findings support the idea that NEET proteins are an important factor in this biogenesis mechanisms [5].

Development of obesity, and related metabolic diseases, appear to be linked with the expression of NEET proteins (mainly mNT), and their unique [2Fe–2S] cluster lability properties. Loss of insulin sensitivity (type 2 diabetes) is correlated with the development of obesity [77] and was shown to depend on the expression level of mNT, not only in mice models [23], but also in humans [80]. The function of NEET proteins in mitochondrial morpho-dynamism (see above), could therefore affect mitochondrial dysfunction associated with different metabolic diseases such as obesity and type 2 diabetes. NEET proteins may therefore be novel targets for drug development and

treatment of metabolic abnormalities including, obesity and type 1 and type 2 diabetes [49, 83].

IVc. NAF-1 and Wolfram Syndrome Type 2 (WFS-T2)

The first genetic study of WFS-T2 syndrome was reported in 2007. This study, conducted on three families from Jordan, identified a single missense mutation at nucleotide 109 that converts G→C leading to an amino acid change from Glutamic acid to Glutamine (E87Q) (Fig. 7) [69]. Moreover, haplotype analysis of these patients revealed a common haplotype with similar markers in the linked region of this gene, which indicates a common ancestor founder for this mutation [69]. In WFS-T2, the mutated base (G109C) is located six bases away from the Intron-Exon junction, at the 5' end of Exon 2. Moreover, this missense mutation disrupts mRNA splicing, leading to skipping of Exon 2 in the final transcript [69]. This change is considered to be a disruption of an exonic splice enhancer (ESE) that affects the accurate splicing of the mRNA for this gene [69] leading to a splice-site mutation [69]. In addition, the (G109C) mutation also leads to a frameshift in exon 3, which creates a premature stop codon. The resulting spliced mRNA product encodes for a 34 amino acids peptide (26 encoded from exon 1 and 8 amino acids from Exon 3 [69]. At the protein level, this WFS-T2-mRNA encodes a NAF-1 protein that comprises of only 25% of native NAF-1; *i.e.* an elimination of 75% of the wild type NAF-1 protein occurs [5, 69]. The eliminated protein parts include the NAF-1 transmembrane α-helix domain, which is the leader sequence of the entire NAF-1 which targets the protein to the mitochondria, ER, and MAM membranes [5, 69]. Also, all soluble parts of NAF-1, including the β-cap domain and the cluster-binding domain with its [2Fe–2S] clusters, are missing in WFS-T2 patients [5, 69]. The amino acids that remain comprise mainly of the inner organelle part of NAF-1 that cannot penetrate the organelles due to the absence of a leader sequence in the truncated WFS-T2-NAF-1 protein [2]. Hence, it is reasonable to assume that this short version of NAF-1 is degraded by cytoplasmic proteases (Fig. 7) [84].

In 2014 a first case of European Caucasian WFS-T2 patient was reported. A genetic study for this patient revealed a novel homozygous deletion of Exon 2, with a proximal breakpoint predicted deletion size of about 2,050 base pairs, spanning from Intron 2 to Intron 3 [75]. Exon 2 of the patient was completely absent, while exon 1 and 3 were present [75]. Altogether, the novel homozygous deletion of Exon 2 affecting NAF-1 protein, due to the elimination of amino acids from position 102 to 106 of the polypeptide, that are in the [2Fe–2S] domain region [2, 75].

In 2015 two Italian siblings were also diagnosed with WFS-T2. Their mutation was a homozygous substitution in a conserved site of Guanine nucleotide with adenine residue; at the position 103+1 (G103→A) in the donor splice site of Intron 1 [76]. This mutation impaired mRNA splicing pattern, producing multiple splice variants, resulting in the retaining of a large segment of Intron 1. Consequently producing a skipping effect causing whole or partial absence of Exon 1 in the transcripts of the patients [76, 84, 85]. By investigating the cells with the (G103→A) mutation, it was found that the mRNA levels of *CISD2* were decreased by almost 99-100% in homozygote cells compared to

normal cells. Heterozygotes cells showed 64-65% decrease in NAF-1 mRNA levels compared to control normal cells. This analysis demonstrated the high instability of the mutated mRNA [84, 85]. Using a polyclonal antibody against the C-terminus of NAF-1, NAF-1 was confirmed to be completely absent in patient cells, whereas its expression was approximately decreased by 50% in the heterozygous parents compared to healthy controls [84].

Another case of WFS-T2 was reported in a Moroccan patient in 2017. *CISD2* gene sequencing revealed a novel homozygous variant, in which Adenine is substituted with Guanine at position 215 in Exon 2 (A215→G). This missense variant changed a highly conserved Asparagine amino acid at the position 72 into a Serine (N72S), which is present within a random coil region of the cluster binding domain of the NAF-1 protein [2, 50]. Analyzing mRNA splicing revealed no abnormal expression or mis-splicing effect. In addition, protein expression levels appeared normal [50]. At the cellular level, ER and mitochondria structure and function were impacted due to the dysfunctional protein caused by the mutation, which highlight the important regulatory part of the affected NAF-1 protein domain in these cells. The expression of a non-fully functional NAF-1 variant in Fibroblast cells of these patients, caused an increase contact between the mitochondria and the ER as well as increased elongation of the mitochondria, suggesting that disrupting NAF-1 function can trigger a process of stress-induced mitochondrial hyper-fusion [50]. A full mechanistic explanation for the impact of this mutation on NAF-1 function awaits however further investigation [50].

Two additional unrelated new cases of WFS-T2 were further reported at 2017 [50, 86]. Unfortunately, there was no genetic analysis to confirm these cases [86]. In 2019 a homozygous deletion of two nucleotides TG c.272-273del was reported in a Chinese family. The latter induced a frameshift mutation at codon 91 in exon 2 of the *CISD2* gene (Leu91fs) patient. This mutation was inherited from the parents who were first cousins [87].

Since NAF-1 is localized to organelles involved in numerous metabolic and bioenergetics processes, *i.e.,* mitochondria, ER and MAM, its absence impacts multiple cells, tissues and organs; mainly those with high metabolic and ATP demands [41, 88]. The latter lead to systemic abnormalities and manifestations including juvenile-onset insulin-dependent diabetes mellitus associated with pancreatic β-cell dysfunction which causes diabetes ‘Type 1-like’ pathophysiology [69]. WFS-T2 patients are however negative for anti-Glutamate dehydrogenase (anti-GAD that is the gold standard marker characteristic antibody of the autoimmune disease Type 1 Diabetes Mellitus) [89]. It was also shown that WFS-T2 patients do not suffer from diabetes insipidus, which is one of the WFS-T1 pathophysiological disorders [69]. Additionally, patients of WFS-T2 suffer from a progressive optic-nerve atrophy, sensorineural hearing loss and peptic ulcers disease [69]. Furthermore, WFS-T2 patients suffer from bleeding tendency characterized by abnormal platelet aggregation test against adenosine diphosphate (ADP), while this test was normal against collagen, restocetin and epinephrine [49, 75]. As they age, WFS-T2 patients suffer more often from neurological and psychiatric manifestations including anxiety, severe depression and psychosis [69, 70]. WFS-T2 patient typically die at a relatively early age, with median

age at death less than 30 years old (range, 24-45 years) [41, 70].

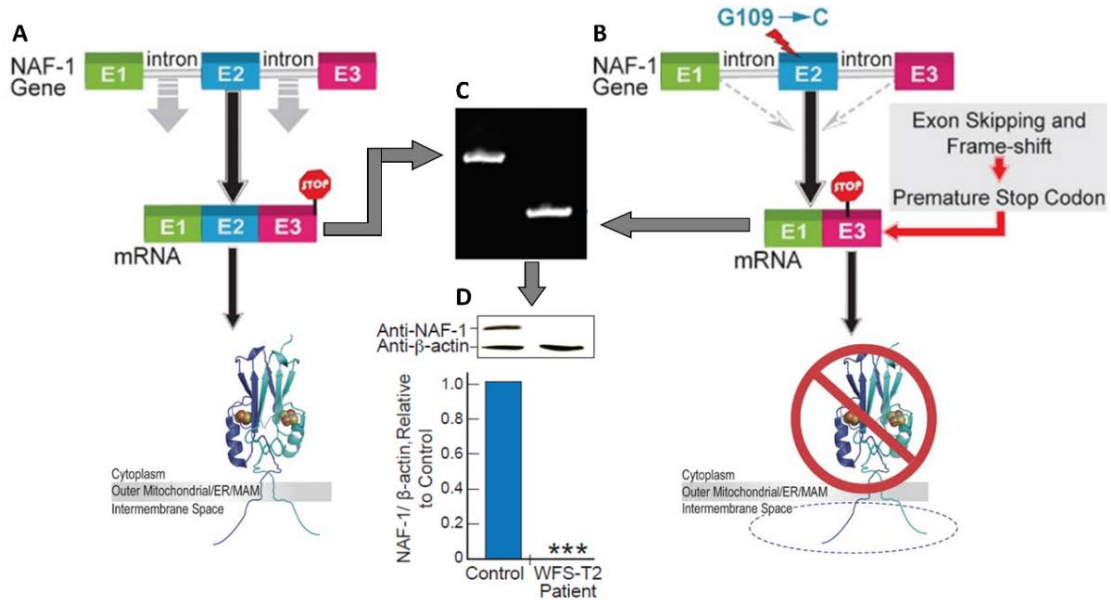

Fig. 7. The original *CISD2* gene mutation (G109C) discovered as the causative agent of WFS-T2. **A.** A normal *CISD2* gene is composed of three exons that transcribe the mature normal mRNA that translates into a normal NAF-1 protein. **B.** The point mutation (G109C) in exon 2 causing exon skipping, frameshift and premature stop codon that transcribe a much shorter mRNA that translates only 25% of NAF-1, the inter organelle domain (circled dashed blue line). **C.** mRNA length that is translated in normal human individual *vs.* the WFS-T2 patient cells. **D.** Western blot form normal fibroblasts *vs.* WFS-T2 fibroblasts showing the complete absence of NAF-1 expression in this disease.

IVd. *In vivo* mice model studies of NEET proteins

Animal models are key to our understanding of the biology of different diseases, as well as for the testing of possible treatments [90]. In the past several years, mice models provided an excellent system for studying NEET proteins and their associated pathophysiological diseases [25]. Due to its association with the rare monogenic disease WFS-T2 and its potential involvement in aging (3), the *CISD2* gene was an interesting target in different mice model systems. In 2009 Chen et al., were the first to report on generating a *CISD2-/-* knock out (KO) mice model system [25]. Their study revealed that *CISD2*-KO mice had a shortened life span compared to control, and that KO mice showed a premature aging phenotype, which correlates with the observation that the expression of NAF-1 is decreased with aging in wild type mice [25]. Other pathophysiological disorders observed in the *CISD2*-KO mice were prominent eyes, optic nerve degeneration resulting in blindness, protruding ears, early depigmentation and gray hair, hair follicle atrophy, and decreased density of hair follicles. In addition, the skin of *CISD2*-KO mice exhibits a hyperplastic epidermis, decrease in subcutaneous fat and muscle, and an increased thickness of the dermis layer. Furthermore, osteopenia resulting from thinner femur trabecular thickness, muscle degeneration and muscle atrophy caused by neuron degeneration were also observed [25, 35]. Diabetic features

of impaired glucose tolerance and decreased insulin levels were further observed in the *CISD2*-KO mice [25]. The phenotype of this mice model system resembled therefore the pathophysiology observed in WFS-T2 patients [25]. The same research group also established a second model system in which the *CISD2* gene was overexpressed and demonstrated that overexpression of NAF-1 extended lifespan of mice, without any apparent side effects [26]. They showed that NAF-1 overexpression protects the mitochondria from age-associated damage and functional decline [26]. In addition to the obvious correction between age-associated reduction in whole-body energy metabolism, NAF-1 protein levels and mitochondrial function, their findings highlighted a new role for *CISD2* in human longevity [26, 52]. In agreement with these phenotypic observations, the transcriptomics pattern of the *CISD2*-KO model resembled that of naturally aged mouse. Furthermore, increasing the expression level of NAF-1 delayed the aging progress of cardiac tissues and attenuated multiple age-related structural defects and functions [55].

Another role for NAF-1, i.e., the maintenance of energy metabolism and glucose homeostasis, was established from an adipocyte-specific *CISD2*-KO mice model [30]. This model system revealed a role for NAF-1 in the development of epidermal white adipose tissue (eWAT) [30]. This study demonstrated that the function of eWAT adipocytes, that perform insulin-stimulated glucose uptake and adiponectin secretion, is impaired in the *CISD2*-KO model. It was hypothesized that adipogenesis is linked to the regulation of Ca^{2+} signaling by NAF-1, that is part of NAF-1 regulatory role as outlined above [30, 52]. To further investigate the role of mNT and NAF-1 in eWAT and skeletal muscle function, normal wild type mice were subjected to chronic exercise revealing that expression of both mNT and NAF-1 increased with exercise [91]. This exercise-induced adaptation was strongly correlated with mitochondrial protein expression and biogenesis, and with the increased expression of mNT and NAF-1, which may have an important role in maintaining the integrity of mitochondria [91]. Proper cardiac function was also shown to depend on NAF-1 expression [26], and the absence of NAF-1 in *CISD2*-KO mice is associated with intercalated disc defects due to mitochondrial degeneration, thereby impairing the electromechanical function of the heart [55]. This is thought to result from the disruption of Ca^{2+} hemostasis as explained above [46, 55]. The main Ca^{2+} regulatory role and the other cellular processes in which NAF-1 participates, *e.g.* different mitochondrial and ER functions, were further studied using embryonic cell lines obtained from a *CISD2*-KO mouse model or the transgenic model that over-expresses NAF-1 [55]. In this system it was shown that NAF-1 absence stimulated ER stress and increased the unfolded protein response process. Moreover, mouse embryonic fibroblasts (MEFs) were derived from the different NAF-1 models showing that Ca^{2+}-regulation disturbances between the ER and the mitochondria (through the MAM) lead to disruptions in the glutathione cycle, and increased NAD^+/NADH and ADP/ ATP ratios [28, 52].

In the Alzheimer's disease (AD) mouse model JAX004462, NAF-1 protein levels were found to modulate the severity of certain AD phenotypes (*e.g.* acceleration of amyloid β-plaque formation) [38]. Overexpressing of NAF-1 promoted the survival and alleviates the pathophysiological defects in this AD model, through protecting against

amyloid β-mediated mitochondrial damage and preventing the loss of neurons and neuronal progenitor cells [38]. Moreover, NAF-1 overexpression reverted the expression AD-dysregulated genes (*e.g.* synapse-related functions, ion hemostasis and cell death) to normal levels [38]. This neuroprotective property of NAF-1 places it as a promising new target for AD treatment [54].

Mouse models for mNT reveal that mNT overexpression enhanced lipid uptake and storage, leading to increased adiponectin levels that caused expansion of WAT mass, causing massive obesity as well as increased insulin sensitivity [23]. Significant reduction in inflammation and oxidative stress were also observed in the mNT overexpression models. In contrast, reduced mNT expression decreased mitochondrial respiratory capacity and decreased weight gain on a high fat diet. Different expression levels of mNT were therefore found to affect the dynamics of cellular and whole-body lipid hemostasis [23]. Expressing mNT in different mice tissues of the pancreas show the variability of mNT role in different tissues [27]. These findings further highlighted the interaction of mNT with Parkin that is proposed to modulate mitophagy [27, 92]. The expression of mNT either in α- or β- pancreatic cells identified a role for mNT in the control of glucose level and metabolism in mice [6, 19, 27].

KO of mNT in *CISD1-/-* mice model was used to study how mNT expression levels affect chronic ethanol-fed mice. These studies showed a protective role for mNT in hepatic cells against alcoholic steatohepatitis [93]. These findings also suggested that mNT could be a therapeutic target [93]. In another *CISD1(-/-)* KO model of C57BL/6 mice, an evaluation of pioglitazone-mediated neuroprotection was studied. It was found that in WT pioglitazone can protect against mitochondrial dysfunction, while in the KO model of mNT pioglitazone loses its neuroprotective effects. These findings highlighted mNT as an important regulator of Ca^{2+}-mediated mitochondrial dysfunction in neurons that can be corrected by pioglitazone or other drugs targeting mNT [94].

Overexpression of mNT in HL1 cardiomyocytes cells isolated from a transgenic mouse, had a protective effect against oxidative stress induced by hydrogen peroxide [95, 96]. KO of *CISD1* in mice, causes decrease in the oxidative phosphorylation capacity of the mitochondria [5]. When mNT was disrupted (KO) in diabetic Zucker rats, cardiac cells showed increased damage and oxidative stress, suggesting that mNT can be used as a pharmacological target for protecting cells after transplanting as they will be transitioning into an oxidative environment [96]. The loss of mNT results in mitochondrial dysfunction and loss of dopamine and tyrosine hydroxylase, with elevated ROS and reduced capacity to synthesize ATP. Reports of decreased performance of mNT KO model mice are consistent with the decrease in the level of dopamine in the striatum and and Parkinson's disease-type motor defects [97].

V. NEET proteins support cellular proliferation and enhance oxidative stress tolerance of cancer cells

Va. Mitochondria dynamics and cancer

As explained above, mitochondria are dynamic organelles able to adapt their structure and shape transitioning between fusion and fission based on the energetic and physiological needs of the cell. Mitochondria produce energy through oxidative phosphorylation (OXPHOS) and ATP production and are a major source of ROS that could cause oxidative damage to proteins, lipids, and DNA resulting in the development of cancer. The biology of mitochondrial support of tumorigenesis is considered at multiple stages. Tumor initiation, growth and survival can result from mutations in mitochondrial enzymes that induce cancer through mitochondrial signaling and oxidative stress. Redox homeostasis of the mitochondria can also regulate cell death through alterations in mitochondrial morphology and mitochondrial-associated signaling pathways. The survival of cancer cells can be promoted by alterations in mitochondrial mass by changes in the regulation of biogenesis and mitophagy. In addition, mitochondrial metabolic reprogramming, biogenesis, and redox homeostasis can contribute to the metastatic potential of cancer cells [98]. Mitochondrial dynamics of cancer cells can be linked to cancer development and progression, and has a strong impact on invasive and metastatic potential of cancer cells. Several publications demonstrated a link between mitochondrial dynamics and cancer [98]. Cancer cells and tumors can undergo a massive metabolic change, reducing the tricarboxylic acid (TCA) cycle and mitochondrial OXPHOS caused by mutations that affect TCA enzymes and the activity of the OXPHOS complexes leading to utilizing glycolysis as the main source for ATP production, also known as the Warburg effect [98]. It has been shown that cancer cells can use nutrients from host cells by inducing catabolic processes such as autophagy, mitophagy, aerobic glycolysis, and lipolysis [98, 99]. In addition to the Warburge effect, changes in nuclear and mitochondrial DNA expression, mutations and changes in the migartion protential of cells associated with mitochondrial dysfunction can induce cancer cell development, growth and metastasis [98]. Changes and imbalances in mitochondrial dynamics, such as changes in regulated degradation processes, can cause an alteration in the homeostasis of cells that can induce tumor inititiation, growth, and mestastsis, suggesting an important role for mitochondrial dymanics, autophagy, and mitophagy in cancer deveolopment. Autophagy can either support cancer cell survival or promote cancer cell death, depending on its cellular context, while mitophagy dysfunction can induce cancer development [98]. Accumulation of damaged mitochondria caused by a decrease in mitophagy can lead to an increase in oxidative damage and to a disrupt redox balance, causing ROS-induced DNA mutations and genetic instability [99]. OMM proteins play a major role in promoting cancer cell progression, VDAC1 is important for the metabolic phenotype of cancer cells; it regulats mitochondrial activity and glucose metabolism. Moreover, VDAC1 can directly bind to hexokinase II (HK II), and induce its activity. HK expression is upregulated in multiple cancer types and this enzyme catalyzes the first reaction of glycolysis that sustains the elevated rates of glucose catabolism leading to increased tumor growth [100]. The interaction between VDAC1 and HK II inhibits mitochondria-induced apoptosis, helping tumor cells to increase survival and growth. VDAC1 is also a mitochondrial target of Parkin, required for its efficient targeting of damaged mitochondria and mitophagy [101], demonstrating that, in addition to fusion

and fission proteins, OMM proteins also have an important role linking mitochondrial dynamics with cancer development.

Vb. The role of NEET protiens in cancer

NEET proteins were shown to have an important role in cancer, promoting proliferation and survival of cancer cells, and inducing cancer cell metastasis and tumor growth [12]. Recent studies indicated that mNT regulates the channel function of VDAC1 and that this interaction is dependent on the redox state of the [2Fe–2S] clusters of mNT [17]. Analysis of mNT expression using the human atlas tool (https://www.proteinatlas.org/ENSG00000122873-CISD1/pathology) strongly suggests that mNT could be used as a prognostic marker for breast, liver and urothelial cancer (unfavorable) all with $p < 0.001$, demonstrating that cancer patients displaying high expression levels of *CISD1* have a lower survival rate. Interestingly, most of the published work in the field of NEET protein family and cancer focused on NAF-1, although recently in our hands (personal communication), we also find a strong role for mNT in cancer.

A study of Pancreatic cancer demonstarted that high levels of NAF-1 are associated with advanced clinical stage, increased tumor size and increased metastasis. In addition, multivariate analysis suggests that NAF-1 is an independent prognostic marker for pancreatic cancer. NAF-1 silencing in pancreatic cell line showed an inhibition of survival and growth of these cells and inactivation of the Wnt, β-catenin pathway. NAF-1 silencing inhibited the epithelial-to-mesenchymal transition (EMT) through the Wnt/β-catenin pathway and suppressed the tumorigenesis of pancreatic cancer cells, suggesting that NAF-1 and the Wnt/β-catenin pathway contribute to the proliferation of pancreatic cancer [102]. A study on Gastric cancer (GC) found that NAF-1 was upregulated in gastric cancer cells and is a prognostic factor for poorer survival of gasrtic cancer patiens. High expression level of NAF-1 was correlated with clinical stage and metastasis of GC. Overexpressing NAF-1 promoted the proliferation of gastric cancer cells, while silencing NAF-1 inhibited tumor growth *in vivo*. Downregulation of cyclin-dependent kinase inhibitor p21Cip1 and p27Kip1, and activation of AKT signaling were found to be associated to NAF-1 effect on induced proliferation of gastric cells [103]. An additional study on GC showed that NAF-1 enhanced sensitivity to 5-fluorouracil (5-FU) through an increase in apoptosis and inhibition of protective autophagy through the activation of the AKT/mTOR pathway [104]. In a more recent study on prostate cancer [105] phloretin treatment increased oxidative stress, as demonstrated through lower antioxidant enzymes. This study also found that increased ROS significantly downregulated multiple components of the Wnt/β-catenin signaling pathway, suggesting that phloretin anticancer activity could occur through generating ROS to influence Wnt/β-catenin signaling that is regulated by NAF-1. A study aimed at investigating the expression pattern and clinicopathological significance of NAF-1 in patients with hepatocellular carcinoma (HCC) found that NAF-1 expression in liver cancer cell lines and tissues was significantly up-regulated at both the RNA and protein levels. NAF-1 was found as an independent marker for

poor prognosis of liver cancer [12, 106]. HCC patients with high NAF-1 expression displayed a shorter survival and a higher recurrence rate than those with low expression. In addition, down regulation of NAF-1 in hepatoma cells suppressed cell proliferation *in vitro* and inhibited tumor size *in vivo*. This study concluded that NAF-1 may serve as a prognostic marker and a novel therapeutic target for HCC, as well as that NAF-1 plays an important role in promoting proliferation and enhanced progression of HCC. A study of patients with early-stage cervical cancer [12, 107] found that *CISD2* expression was significantly upregulated in cervical cancer cells at the mRNA and protein levels. Statistical analysis showed a significant correlation between *CISD2* expression and the expression of squamous cell carcinoma antigen, myometrium invasion, recurrence, lympho-vascular space involvement and especially pelvic lymph node metastasis. Patients with higher NAF-1 expression had shorter overall survival rate than patients with lower NAF-1 expression.

Multivariate analysis also suggested that NAF-1 expression may be a prognostic indicator for the survival of patients with early-stage cervical cancer and suggested that NAF-1 may serve as a novel biomarker for early-stage cervical cancer progression. In laryngeal squamous cell carcinoma (LSCC), NAF-1 was found to be up-regulated in LSCC tissues compared with adjacent noncancerous tissues both at mRNA and protein levels. NAF-1 was significantly correlated with T stage, lymph node metastasis, clinical stage and disease progression [108].

NAF-1 is thought to support mitochondrial iron and ROS metabolism, promoting cancer cell survival via stabilization of HIF1α and suppression of apoptosis. It was shown that increased uptake of iron into cells and mitochondria causes a metabolic shift that enhances oxygenic glycolysis, and the activation of cellular stress pathways associated with HIF1α stabilization and mTOR inactivation have been demonstrated to result from suppression of NAF-1 in breast cancer cells [5, 34]. The role of NAF-1 in promoting cancer cell proliferation studied by Darash-Yahana et al., [73] showed that overexpression of NAF-1 in xenograft tumors resulted in a dramatic increase in tumor size and aggressiveness. Breast cancer cells with enhanced expression of NAF-1 were more tolerant to oxidative stress and undergo less apoptosis and autophagy. The degree of lability of the NAF-1 [2Fe–2S] cluster was found to be critical for NAF-1 function in cancer cells. A mutated form of NAF-1 with a single point mutation (H114C), which stabilizes the NAF-1 cluster, resulted in a dramatic decrease in tumor size, enhanced mitochondrial iron and ROS accumulation, and reduced cellular tolerance to oxidative stress. Breast cancer cells treated with pioglitazone revealed a similar effect on mitochondrial iron and ROS accumulation, demonstrating for the first time that the cluster of NAF-1 is critical for its function in cancer cells. This study suggests that drugs that target the lability or stability of NAF-1 and mNT [2Fe-2S] clusters could be used as a theraputic approach for patients with tumors that display high expression level of NAF-1 [73].

In addition to mNT and NAF-1, knockdown of MiNT also leads to increased accumulation of mitochondrial labile iron, as well as increased mitochondrial reactive oxygen production [10]. The MiNT protein could therefore function in the same pathway as its homodimeric counterparts (mNT and NAF-1), and could be a key player

in this pathway within the mitochondria. As such, it represents an additional NEET target for anticancer or antidiabetic drug development.

Through their effect on mitochondrial function and dynamics, NEET proteins could therefore impact cancer growth, development and metastasis (Fig. 8).

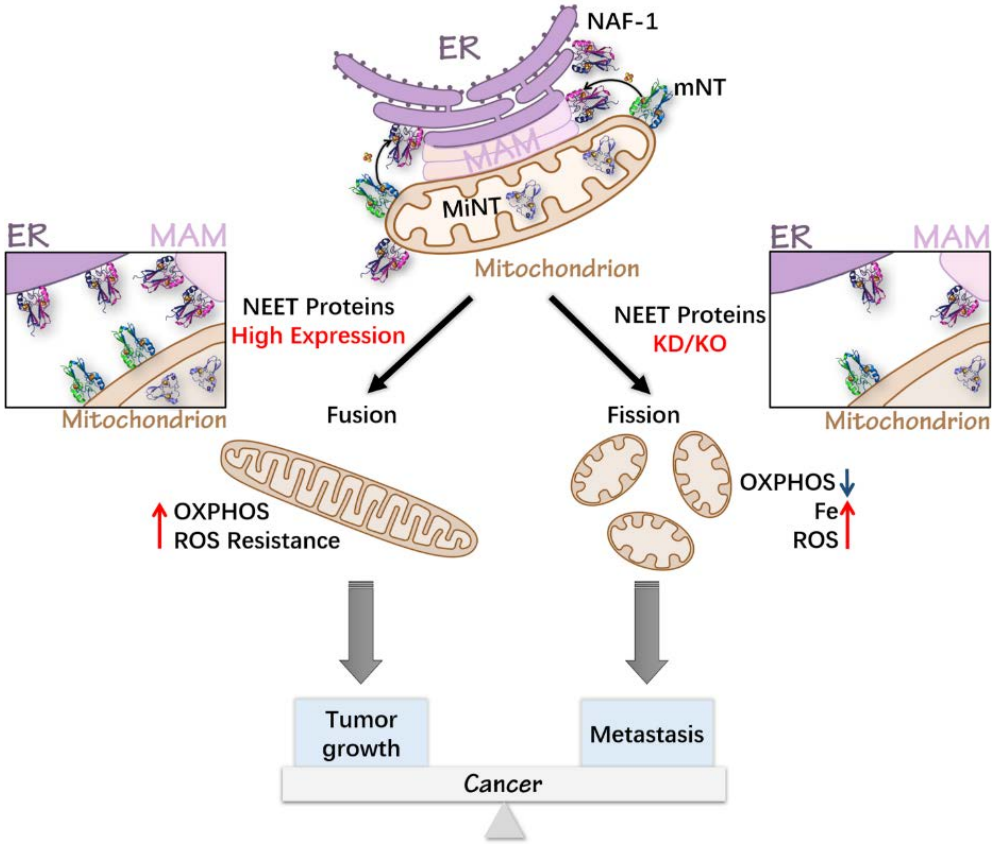

Fig. 8. Mitochondrial dynamics can impact tumor growth and/or metastasis of cancer cells through cluster transfer reactions involving NEET proteins. Mitochondrial fusion promotes oxidative phosphorylation (OXPHOS) at the expense of anaerobic glycolysis, leading to increased ROS tolerance that will inhibit migration of cancer cells but may increase tumor growth. On the other hand, mitochondrial fission inhibits oxidative metabolism by increasing the energetic yield of glycolysis, easing the process of metastasis. Based on our published data [73] we found that high expression levels of NAF-1 increases tumor growth, oxidative phosphorylation and ROS resisitance. We also showed that knockdown of NEET proteins causes decreased OXPHOS while increasing ROS and Fe accumulation in the mitochondria [5, 10, 34]. The fine balance between tumor growth and tumor metastasis, critical for the fate of cancer cells, needs to be further studied in relation to NEET expression and mitochondrial dynamics in cancer cells, and the potential use of different NEET-targeting drugs to treat cancer should be further evaluated.

VI. NEET proteins as drug targets

The expression level of NEET proteins, as detailed above, is correlated with the development of many different diseases (*e.g.*, cancer, neurodegeneration, metabolic diseases and genetic disorder such as WFS-T2). In cancer, the expression of NEET

proteins is significantly increased supporting the proliferation of cancer cells by maintaining mitochondrial homeostasis (described above [5, 12]). In contrast, reduced or lack of expression of NEET proteins leads to early senescence and the development of neurodegeneration and metabolic diseases [25, 27, 97]. Disrupting the lability of NEET clusters was found to play an important role for the development of cancer [73], and can be also involved in many other mitochondria-associated diseases. Hence, NEET proteins represent novel and highly promising targets for drug design. As mentioned above, NEET proteins were discovered through their binding of the thiazolidinedione (TZD) drug, pioglitazone (PGZ, see Fig. 9), used as a treatment for types 2 diabetes [6]. TZD binding to NEET proteins stabilizes their [2Fe–2S] clusters and increases mitochondrial labile iron and ROS levels in cancer cell [73]. Such stabilization in turn inhibits the ability of NEET proteins to transfer their [2Fe–2S] clusters to different apo-acceptor proteins. This leads to the blocking of cluster mobilization from the mitochondria to the cytosol and to impaired mitochondrial iron and ROS homeostasis [5, 73]. Accordingly, a His to Cys mutation of the histidine ligand of NAF-1 [2Fe–2S] cluster (H114C) leads to a 25-fold increase in cluster stability and to the inhibition of H114C cluster transfer abilities to apo-acceptor proteins [5, 73]. The overexpression of this mutated form of NAF-1 in breast cancer cells revealed that in H114C cells, the levels of mitochondrial iron and ROS were significantly increased, and the tolerance to oxidative stress was drastically decreased [73]. The stabilization of the [2Fe–2S] further reduced cancer cell proliferation and tumor growth [73]. These findings are similar to those of PGZ binding to NEET proteins, suggesting that in cancer cells, modulating NEET protein cluster stability by different drugs could suppress cancer cell proliferation [73]. This can be achieved by designing of ligands to the cluster [5, 12, 73]. Several ligands were indeed shown to have an effect on the stability of the NEET cluster. They could present a promising potential for therapeutic use, not only for cancer but also for neurodegenerative and metabolic diseases such as diabetes [109]. While the stabilization of the NEET [2Fe–2S] cluster impacts mitochondrial homeostasis, some compounds were found to destabilize the NEET [2Fe–2S] cluster [12]. Destabilization of the [2Fe–2S] cluster in cancer cells induced alterations in mitochondrial homeostasis (*e.g.* respiration) and decreased cell proliferation similar to what was found with reduced NEET proteins expression [12]. To date, despite the critical role that NEET proteins play in many human diseases, only few efforts were directed at the development of new ligand and drugs that target these proteins [5, 73, 83, 109].

NEET ligands identified so far range from synthetic molecules to natural compounds and their derivatives. The former are mostly TZD derivatives such as the cluster stabilizer PGZ [5]. The binding pose of several of these ligands onto mNT was obtained so far via molecular docking. While docking may be a rather approximate for proteins containing a transition metal cluster [110], very important insights could be achieved already with this simple approach. Indeed, subsequent structure-activity relationship studies [111, 112] showed that the binding affinity of TZD-type ligands to NEET proteins is largely dependent on the aromatic moiety (*e.g.* phenol group) in these molecules. Modifications of the nitrogen atom on the TZD ring with an acidic group is

also beneficial for increasing its binding affinity to mNT. Changing the TZD ring into a TTD (2-thioxothiazolidin-4-one) ring can also cause a relative potency improvement. These findings provided an important reference for enhancing the affinity of TZD-type ligands and reducing side effects. Due to the lack of the PPAR-γ binding region, NL-1 pharmacological effects acts primarily through NEET proteins interaction [94]. Using nanotechnology to encapsulate, NL-1, into poly (lactic-co-glycolic acid) nanoparticles can further overcome low solubility of the molecule and enable it to cross the brain blood barriers (BBB) [113]. These strategies laid the basis for the research and development of drugs targeting NEET proteins [83, 113]. Takahashi *et al*. [109] designed and synthesized a PGZ derivative, named TT01001 that interacts with mNT specifically without interacting with PPAR-γ, originally targeted by PGZ [6]. In addition to other TZD-like ligands, diuretic furosemide (see Fig. 9) was also shown to bind to mNT and stabilize its [2Fe–2S] cluster. The first furosemide-mNT co-crystal structure recently confirmed that furosemide binds to the junction between the NEET protein [2Fe–2S] cluster-binding domain and the β-cap domain [83]. This structure laid a critical molecular structural foundation for further development of rational stabilizer designs targeting NEET protein by the "*cluster stabilizer mechanism*".

NEET protein ligands of natural products were primarily determined by molecular docking and experimental screening, and include chromen-4-one compounds with symmetrical chemical scaffold, such as magnolol, curcumin, cromolyn, enterobactin and others [111]. Although many natural product ligands have similar, or better, theoretical calculation binding affinity compared to PGZ, many of them were not identified as stabilizers or destabilizers of the [2Fe–2S] cluster. Nevertheless, the unique chemical framework of natural products expands the ligand design ideas [12], and include studies of the molecular interactions of the *Garcinia xanthone* derivative, cluvenone (CLV, stabilizer) and its derivatives MAD-28 (destabilizer) and MAD-44 (stabilizer, see Fig. 9) with mNT and NAF-1. Within the limitations of the methodology reported above, the MAD-28 ligand was suggested to form an additional hydrogen bond with Cys83 of mNT, which causes energy penalty and deflects the coordination of the iron-sulfur cluster-ligated His, increasing the coordination bond length, thereby accelerating the release of the iron-sulfur cluster [114].

With very few exceptions, the mechanism by which NEET ligands exerts a stabilizing or destabilizing effect is not yet known. Most reported ligands are stabilizers [12, 83, 109]. Identifying destabilizers is therefore very important from a pharmacological perspective. Indeed, in diseases like cancer a stabilizer may perform well but in other diseases (such as WFS-T2) a destabilizer may provide the solution (personal communication). A more refined understanding and drug control of NEET protein function, as well as better delivery tools, may therefore be required for utilizing NEET proteins as powerful drug targets for many different human diseases.

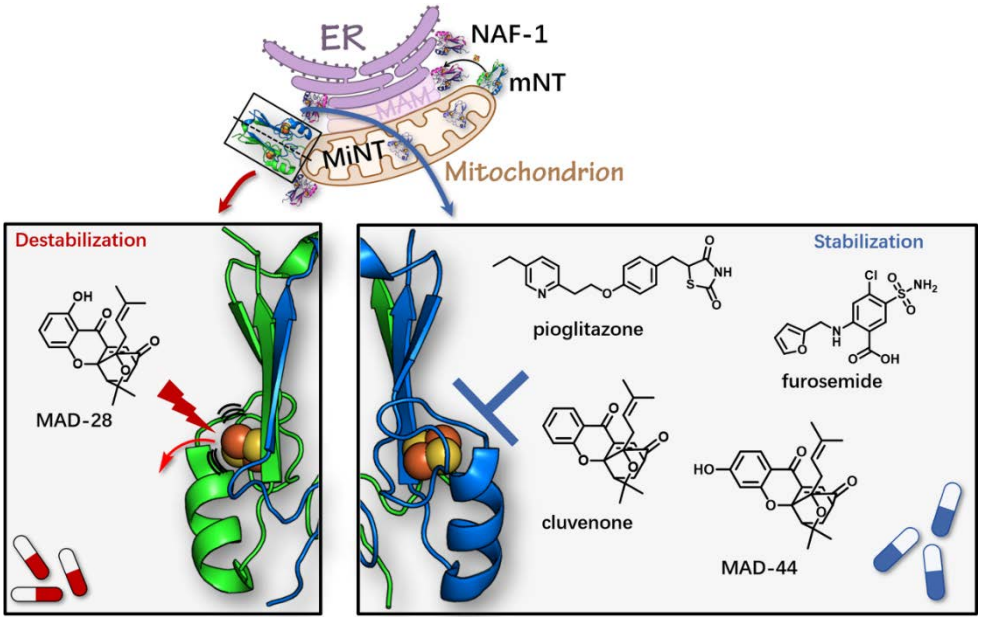

Fig. 9. The stabilization and destabilization effects of different NEET protein ligands. Known destabilizing drugs are shown on left and known stabilizing drugs are shown on right.

VII. Other organisms in which NEET protein structure and function were studied

VIIa. NEET proteins and programmed cell death in *C. elegans*

The programmed cell death (PCD) pathway is a tightly regulated pathway, key to the survival and development of almost all multicellular organisms. The core mammalian PCD pathway is regulated by the pro-survival Bcl-2 protein, as well as by the pro-death APAF-1 and caspase proteins. One pathway of regulation specifically involves interactions between pro-survival Bcl-2 proteins and pro-death Bcl-2 homology region 3 (BH3) domain-containing proteins, highlighting the BH3 domain as a pro- or anti-apoptotic modulating domain of Bcl-2 activity. Several studies have implicated NEET proteins as involved in the regulation of PCD or apoptosis via BH3-associated interactions with Bcl-2. Crosslinking followed by mass spectrometry identified NAF-1 as a protein that binds to the pro-survival Bcl-2 protein at the ER. The displacement of NAF-1 from Bcl-2 binding, thought to occur through the ER localized BH3-only protein Bik, lead to the hypothesis that in mammalian cells, a Bcl-2-NAF-1 complex at the ER has a role in the regulation of apoptosis and autophagy [5]. Further investigations of this interaction via binding interface mapping between Bcl-2 and NAF-1 discovered that NAF-1 binds to Bcl-2 at a BH3-only binding site, providing structural justification for the displacement of Bik by NAF-1 [5]. Additional support for NAF-1 involvement in the regulation of autophagy and apoptosis comes from studies utilizing cancer cell lines and xenograft tumors with altered NAF-1 expression in mice. In these studies, NAF-1 suppression, or altered function, resulted in the activation of apoptosis [5, 73]. These studies further linked NAF-1 with the regulation

of apoptosis, however they did not identify the different proteins and genes involved in this process.

Recent genetic and molecular studies conducted in the model system *Caenorhabditis elegans*, discovered that the *CISD* gene family regulates germline apoptosis. Thus, in the absence of NEET protein function apoptosis of germline cells was enhanced and abnormalities occurred in *C. elegans* reproduction. This process was shown to be dependent on the core apoptotic machinery of *C. elegans* since disruptions in core apoptotic machinery genes such as deletions of *ced-3*/caspase or *ced-4*/APAF-1, or gain-of-function of *ced-9(n1950)*/Bcl-2 reduced the number of germline cell corpses in *CISD1* null animals. Furthermore, disruption of the pro-apoptotic BH3 protein, CED-13, significantly reduced the number of germ cell corpses observed in *cisd* dysfunctional animals. These findings support a model in which CISD proteins function as anti-apoptotic proteins that regulate PCD in the *C. elegans* germline by competing with the pro-apoptotic protein CED-13 for the binding of Bcl-2 [13]. NEET proteins could therefore perform a pro-survival/anti-apoptotic function through inhibiting CED-9 (Bcl-2) at the CED-13 (BH3 domain) interaction site, preventing CED-9(Bcl-2)-CED-13 interaction that induces PCD and promoting germ cell survival [13]. The *in vivo* studies in *C. elegans* described above supported previous computational and *in vitro* experimental analysis of NAF-1-Bcl-2 binding that further dissected the binding interface between these two proteins and revealed that it was mediated through the Bcl-2 BH3-binding domain [114].

The *C. elegans* studies described above, as well as work by others [115] further demonstrated that knocking out NEET proteins in *C. elegans* results in a dramatic effect on metabolic processes, as well as on mitochondrial morphology, and that these processes were found to occur under high levels of ROS and low levels of ATP [115].

VIIb. NEET proteins in Plants

Arabidopsis thaliana contains a single gene encoding a NEET protein (At5g51720, named AtNEET) that shows 50 and 57% similarity to the mammalian NEET proteins mNT and NAF-1, respectively, and displays biochemical, biophysical, and structural characteristics common to those of other NEET proteins [11]. Similar to mNT and NAF-1, the plant protein contains a typical NEET fold with a strand-swapped β-cap domain and a cluster binding domain [11]. Expressing AtNEET-GFP fusion protein in Arabidopsis plants indicated that the AtNEET protein is localized to chloroplasts and mitochondria [11] and its expression is restricted to leaves [116]. The complete absence of AtNEET from any of the mutant collections currently available suggests that this protein could be essential for plants. Knockdown and RNA interference (RNAi) lines with suppressed expression of AtNEET showed a late bolting and early senescence phenotype and accumulated higher levels of Fe and ROS, indicating that AtNEET could play a key role in maintaining Fe and ROS homeostasis in Arabidopsis plants [11].

One of the suggested functions of NEET proteins in different biological systems is that of [2Fe–2S] cluster transfer [2]. Indeed, mimicking the function of the mammalian NEET protein mNT in human cells, AtNEET was shown to transfer its [2Fe–2S]

clusters to apo-Ferredoxin (FD) and mitochondria [11], as well as to the cytosolic [2Fe–2S] protein DRE2 [14], further supporting the involvement of AtNEET in Fe metabolism in plants (Fig. 10). In addition, growth of AtNEET knockdown seedlings was previously shown to be insensitive to high Fe levels, but sensitive to low levels of Fe [11], suggesting a role for AtNEET in Fe transfer, distribution, and/or management in plant cells.

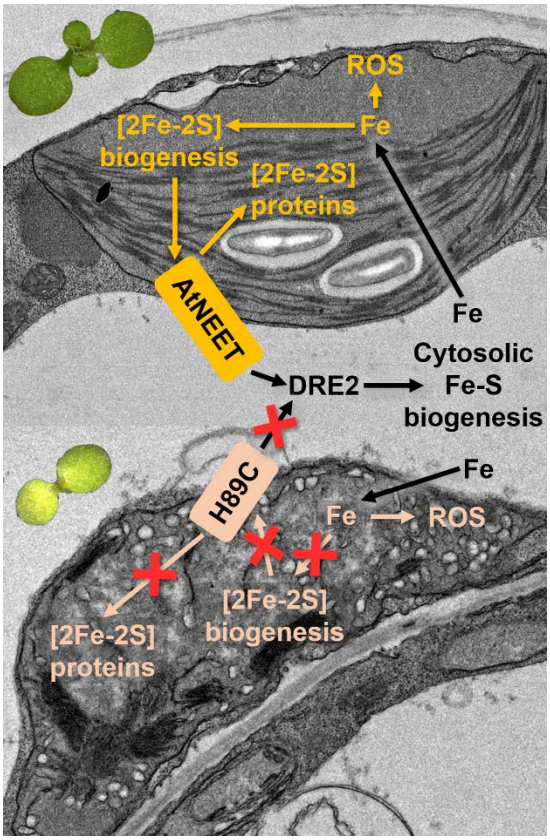

Fig. 10. Model showing the putative role of AtNEET in the mobilization of [2Fe–2S] clusters from the chloroplastic Fe–S biogenesis machinery to chloroplastic and cytosolic Fe–S proteins. A disruption in the function of AtNEET via expression of the dominant-negative AtNEET variant H89C blocks the mobilization of some [2Fe–2S] clusters within and outside of the chloroplast, and triggers over-accumulation of Fe in the chloroplast that enhances ROS accumulation, resulting in chlorosis, structural damage to chloroplasts and a high seedling mortality rate. Representative images of wild type AtNEET (top) and AtNEET-H89C (bottom) seedlings and chloroplasts are shown (After [14]).

Because chloroplasts and mitochondria contain many Fe-S proteins and require a supply of Fe from the cytosol for the biogenesis of Fe–S cluster proteins, the localization of AtNEET to these subcellular compartments [11] could be central to the proposed role of this protein in Fe–S cluster transfer. To shed light on the function of AtNEET in relation to its localization, a dominant-negative strategy [73] in which a mutated form of AtNEET (H89C) that is unable to donate its cluster to an acceptor protein, was recently used (Fig. 10) [14]. Disrupting AtNEET function by using this strategy resulted in transcriptional re-programming of several networks mediating ROS metabolism, Fe–S biogenesis and Fe– deficiency responses, as well as in a decrease in

the level of key Fe–S proteins, such as FD, and an over-accumulation of Fe at the whole plant level, and in particular in chloroplasts [14]. These changes were accompanied by chlorosis, severe structural damage to chloroplasts and a high mortality rate of seedlings. In addition, the disruption in AtNEET function blocked the transfer of Fe–S clusters from the chloroplastic [2Fe–2S] biogenesis machinery to chloroplastic (FD1) and cytosolic (DRE2) [2Fe–2S] proteins, suggesting that AtNEET could function in mobilizing clusters from the chloroplastic [2Fe–2S] biogenesis pathway to target proteins such as FD1 and DRE2 [14]. Similar to animal cells [5, 11, 12], AtNEET could be localized inside the chloroplast, as well as on its outer membrane (and at the mitochondria), implicating AtNEET in mediating [2Fe–2S] cluster transfer between these different subcellular compartments and the cytosol [14]. The conserved role for NEET proteins in Fe–S metabolism/Fe homeostasis between different organisms and kingdoms could therefore highlight an ancient function as well as a central role for these Fe–S proteins in linking organelle and cytosol homeostasis and function.

VIII. Summary

Since the discovery of NEET proteins in 2004, many advances were made in their structural and functional characterization. A key role for NEET proteins was identified in numerous human pathologies including cancer, diabetes, neurodegenerative diseases such as Alzheimer and Parkinson's, obesity and heart disease. At the cellular level, NEET proteins were found to be involved in the regulation of autophagy, apoptosis, ferroptosis, ROS production, tolerance and signaling, calcium signaling, cell proliferation, electron transfer reactions, redox control and iron and iron-sulfur homeostasis. Key interactions between NEET proteins and central regulators such as VDAC and Bcl-2 were also identified.

We believe that the future of NEET protein research will greatly benefit from addressing the following research avenues:

- Determining the mechanisms and outcomes of NEET protein involvement in the regulation of mitochondrial dynamics and morphology.
- Revealing and studying the scope of signaling networks, and protein-protein interactions, impacted by NEET proteins in different cells and tissues.
- Developing drugs with high specificity and efficacy in treating the different diseases NEET proteins are associated with. A special effort should be directed at the development of novel drugs that can alter the lability of NEET [2Fe–2S] clusters, as a particularly exciting venue of research.
- Fostering research on plants, which appear to contain a single member of the NEET protein family that could be used in the production of crops with high tolerance to different stress conditions.

Although these research avenues are highly challenging, their potential benefits could be plentiful, making the future of NEET research highly exciting and promising!

Acknowledgments

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