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Molecular characteristics of the multi-functional FAO enzyme ACAD9 illustrate the importance of FADH2/NADH ratios for mitochondrial ROS formation

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Abstract
A decade ago I postulated that ROS formation in mitochondria was influenced by different FADH2/NADH (F/N) ratios of catabolic substrates. Thus, fatty acid oxidation (FAO) would give higher ROS formation than glucose oxidation. Both the emergence of peroxisomes and neurons not using FAO, could be explained thus. ROS formation in NADH:ubiquinone oxidoreductase (Complex I) comes about by reverse electron transport (RET) due to high QH2 levels, and scarcity of its electron-acceptor (Q) during FAO. The then new, unexpected, finding of an FAO enzyme, ACAD9, being involved in complex I biogenesis, hinted at connections in line with the hypothesis. Recent findings about ACAD9's role in regulation of respiration fit with predictions the model makes: cementing connections between ROS production and F/N ratios. I describe how ACAD9 might be central to reversing the oxidative damage in complex I resulting from FAO. This seems to involve two distinct, but intimately connected, ACAD9 characteristics: (i) its upregulation of complex I biogenesis, and (ii) releasing FADH2, with possible conversion into FMN, the crucial prosthetic group of complex I. Also see the video abstract here: https://youtu.be/N7AT_HBNumg

KEYWORDS
ACAD9, Beta-oxidation, ECSIT, FADH2/NADH ratio, MCIA complex, peroxisomes, reverse electron transport (RET)

INTRODUCTION: ILLUMINATING THE NEW ACAD9 FINDINGS FROM AN OLD PERSPECTIVE

When I first proposed a hypothesis linking overall mitochondrial ROS formation to FADH2/NADH (F/N) ratios in this journal, I predicted links between the oxidation of high F/N substrates, such as fatty acids (FAs) during fatty acid oxidation (FAO), and damage to the ETC, especially at complex I.[1] After acceptance, an article appeared which demonstrated the unexpected involvement of an Acyl-CoA-Dehydrogenase, ACAD9, an FAO enzyme normally forming double bonds between carbon-2 and carbon-3 of FAs, in complex I biogenesis.[2] I referred to that publication by a note added in proof.[1] In this first paper the authors implied that ACAD9 was not a "real dehydrogenase" anymore, but in later research they demonstrated clear in vivo activity in human fibroblasts, though they still referred to it as "residual", despite the fact that loss of enzymatic function would probably quickly evolve in the absence of evolutionary constraints.[3] And indeed, further research shows ACAD9 to be a real, authentic, FAO enzyme.[4] These results
also fit with older experimental work, in which purified mature ACAD9 demonstrated activity with various long-chain unsaturated acyl-CoAs as substrates. In the meantime, a lot of follow-up research has culminated in recent, impressive, research papers, which delineate many of the molecular characteristics of this multi-functional enzyme, allowing it to play the roles in mitochondrial metabolic regulation it does. However, the interpretation of these latest findings leaves something to be desired. Giachin et al., show that losing its FAD-cofactor (‘deflavination’) induces the FAO enzyme ACAD9 to switch from a role in FAO to becoming a crucial part of the mitochondrial complex I assembly (MCIA) machinery. The authors only state that their findings “suggest a unique molecular mechanism for coordinating the regulation of the FAO and OXPHOS pathways to ensure an efficient energy production” and do not go beyond that. But just simply describing the complete oxidation of FAs in mitochondria already shows this to be a highly superficial way of interpreting their results. Acyl-CoA-Dehydrogenases, such as ACAD9, catalyse the first step of a recurrent, cyclical, pathway in which every chain of FA’s is shortened by 2 carbons at the time, resulting in one FADH$_2$ (formed during this first step), an NADH, and an acetyl-CoA. The further oxidation of acetyl-CoA in the 8-step TCA-cycle will, in turn, generate another FADH$_2$, coming from succinate oxidation by complex II, and 3(!) additional NADH molecules. Thus, high complex I activity is also essential during FAO, and from that perspective, no switch is needed.

A more enlightening way of looking at things is that upon extensive oxidative damage to complex I during FAO, a programme can be activated to repair complex I, while at the same time shutting down further FAO. This is exactly what follows the repurposing of ACAD9. In that light, the fact that the major site of ROS-induced oxidative damage is located in the FMN containing, NADH binding, “N-module” of complex I, is also telling. Possibly, FMN could be derived from the “ejected” FAD and repurposed as well. Further details and experimental results supporting ideas along these lines are discussed below. An overview of the two repurposing pathways in the context of FAO-related oxidative damage is given in Figure 1. But first, some extra background.

**BACKGROUND I: ROS, THE ENEMY INSIDE**

The respiratory ETC allows redox reactions in which a large part of the energy present in “high energy” electrons from different food sources is stored in a proton motive force (PMF; $\Delta p$) across mitochondrial inner membranes. Complex electron transfer reactions along the chain are directly coupled to proton “pumping” across the inner membrane. An important part of the explanation for the extraordinary levels of ATP that can be generated in this way lies in the extreme subdivision of the reaction from start to finish and the use of molecular oxygen (O$_2$) as the final electron acceptor. This last redox reaction is catalysed by the ultimate complex of the chain, Cytochrome c oxidase, forming water, see and references therein. However, this process allows reverse reactions to occur. On top of that, O$_2$ can act as a doubly edged sword by occasional premature reactions with some of the reduced centres of the ETC, giving rise to internal superoxide anions and other reactive oxygen species (ROS) which are all highly damaging to the eukaryotic cell as they can initiate detrimental reaction cascades involving almost every major group of biological molecules.

Eukaryotic evolution has been heavily influenced by internal, mitochondrial, ROS formation, which has given rise to a host of mechanisms to both suppress ROS formation and repair the damage done. This constant co-evolution with internal ROS formation is sometimes reflected in surprising ways. As an example, low amounts of ROS will induce efficient antioxidant responses. They would thus have beneficial effects.

![Figure 1](https://example.com/figure1.png)

**FIGURE 1** From ROS to restoration (highly schematized). After ROS damage in Complex I, due to high QH$_2$ levels and reverse electron transport (RET) during beta oxidation, repair is needed. (A) High F/N ratios with insufficient electron-acceptor (Q) for Complex I; ROS formation at Complex I via RET (*). RET depends on high QH$_2$ levels and a high delta p (indicated). (B) ROS reduction by lessening FA oxidation (because release of FAD from ACAD9 inhibits initiation of $\beta$-oxidation and destabilizes its dimerization with possible implications for the multifunctional FAO complex and OXPHOS supercomplexes; see main text). Possible restoration of Complex I activity by two separate routes: (1) ACAD9 (minus FAD) involvement in mitochondrial complex I assembly (MCIA) complex formation, (2) Hydrolysing the released FAD-cofactor to FMN for use in Complex I. For details see the main text. IMS = intermembrane space; Complex I light green, Complex II light green, ACAD9 (shown as a monomer) dark green, ECSIT pink, NDUFAF1 grey (together forming the core subunits of MCIA). Ubiquinone/ubiquinol (Q) red, electron flow black arrows. ROS-generating site of complex I upon RET: The FMN containing site (IF). Q binding site at IQ, F = FADH$_2$ oxidising complex, I = NADH dehydrogenase complex. For details see text. Extended and adapted from. Chemical structures of the co-factors (oxidised forms, see absence of hydrogens at N1 and N5) incorporated under open source licence. Complexes not to scale. ROS generation in complex III is not indicated.
The observation of health improving, low-level, ROS induction is thus explained by the so-called “mitohormesis” concept. An extensive overview of the different ROS forms and their oxidative activities can be found in so only a brief outline is given here. The most reactive species is the hydroxyl radical (OH), which does not seem to be formed directly in the ETC much. Instead, amongst others, complex I generates superoxide anions (O$_2^-$). Such superoxide anions are, for the most part, rapidly converted to a relatively stable ROS form: hydrogen peroxide (H$_2$O$_2$), in a reaction catalysed by superoxide dismutase. However, this specific type of ROS easily passes membrane barriers, allowing it to function as a long-range signalling molecule. Of course, its long range effects can also be highly detrimental. Although differences of opinion still exist, nowadays most researchers in the field think that some of the largest contributions to mitochondrial ROS formation come from Complex I; on the matrix side in the NADH binding, FMN containing N-module and ubiquinol cytochrome c oxidoreductase (Complex III); in this case on the intermembrane side. It should be stated that many of the findings with regard to pinpointing the elusive sources of mitochondrial ROS formation come from studies that have to manipulate the experimental set-up to such extents that their results have been questioned when considering real life. As the $\Delta p$ contains a lot of chemical potential energy, this can be converted to large amounts of ATP, by ATP-synthase (complex V). The potential energy of ATP is released upon hydrolysis, and its highly efficient synthesis in mitochondria enables the many costly eukaryotic cellular processes. As we will see below, internal ROS formation is always intimately associated with such highly efficient ATP generation, but it is especially a problem for metazoans geared for maximal output at high $\Delta p$ (e.g., compare human and yeast mitochondria). States in which both the QH$_2$/Q ratio and $\Delta p$ are high, can easily give ROS formation in complex I. This is one of the reasons why $\Delta p$ can also be dissipated as heat by uncoupling agents/proteins, thus influencing ROS formation. Of note, lowering $\Delta p$ generally means less ROS formation, but a low $\Delta p$ does not simply equal a low amount of ROS production.

**BACKGROUND II: FADH$_2$/NADH (F/N) RATIOS AND ROS FORMATION**

The redox state of the central electron carrier in the first part of the ETC, coenzyme Q, thus seems to be a crucial determinant for ROS formation. A high QH$_2$/Q ratio can of course most easily occur upon oxidation of abundant energy-rich substrates. When comparing oxidation of glucose and FAO (via beta-oxidation) we can calculate to which extent the electron carrier pairs FAD/FADH$_2$ and NAD$^+$/NADH are involved. The electrons entering the respiratory chain are either derived from soluble NADH using Complex I, or use a prosthetic FADH$_2$ group, which can be attached to different ETC, complexes (see Figure 1A). Complete breakdown of a glucose molecule (using glycolysis with the aspartate/malate shuttle to import NADH, mitochondrial breakdown of pyruvate by oxidative decarboxylation and the TCA cycle) will generate 2 FADH$_2$ and 10 NADH molecules, resulting in an FADH$_2$/NADH (F/N) ratio of 0.2. With mitochondrial oxidation of (almost completely) saturated FAs, involving an ACAD – ETF/ETF:QO complex (see Figure 1A), much higher F/N ratios (approaching 0.5 as the FAs become longer) will be generated. Such high F/N ratios (especially when confronted with a large number of reducing equivalents in the form of NADH) would translate into acceptor problems for Complex I. On top of this, reverse electron transport (RET) from a combination of raised membrane potential ($\Delta p$) and high QH$_2$/Q ratios, might ensue. By also taking the Q-cycle of complex III into account, high F/N ratios might be expected to lead to ROS formation by that complex as well. The relevant models are described in.

**BACKGROUND III: OBSERVATIONS LINKING F/N RATIOS AND ROS FORMATION**

Quite a lot of observations (in)directly link F/N ratios and ROS formation (especially in Complex I), at cellular, but also at higher order levels. Severe oxidative stress inside eukaryotes could explain why FAO started to occur in a new cellular organelle, the peroxisomes. Generating NADH without FADH$_2$ for the ETC (the electrons instead ending up at H$_2$O$_2$, with catalase returning that compound to water and molecular oxygen) is likely the oldest role of peroxisomes. Thus, they could have evolved to lessen the total amount of FAO in mitochondria, lowering overall F/N ratios, together with another eukaryotic innovation, carnitine, controlling the overall rate of mitochondrial FAO, and oxidative damage. Peroxisomal FAO is almost completely copied from the endosymbiont, except, as expected, for the step involving FAD/FADH$_2$. Also, peroxisomes can be formed from ER-derived and mitochondrial pre-peroxisomes. Interestingly, in the trade-off between efficient ATP generation and ROS formation, our mitochondria only allow partial peroxisomal breakdown of very-long chain FAs (the ones with the highest F/N ratios). Indirect evidence is also found in studies of supercomplex formation, mitochondrial uncoupling proteins (UCPs), and the strictly carnitine dependent mitochondrial import of FAs. Catabolism of substrates characterised by high F/N ratios (e.g., FAs and succinate) is used by animal cells to differentiate and/or respond to a changing metabolic environment: in such cases ROS formation plays an indispensable signalling role. Often UCPs are involved. FAO upregulates UCPs both in activity and in number. By allowing protons to return to the matrix, they lower $\Delta p$, and thus lessen RET and ROS formation. UCP2 is upregulated in response to FAs (by PPAR transcription factors), as well as by high QH$_2$/Q and ROS. UCP2 and UCP3 transcription is co-activated with Glycerol-3-Phosphate Dehydrogenase expression (which leads to high F/N and QH$_2$/Q ratios). The catabolism of succinate induces UCP1 in brown (mitochondria rich) adipose tissue. Specific aspects of neuronal metabolism also make sense in light of the hypothesis. For instance: surprisingly, neurons, though consuming huge amounts of ATP do not use FAs as a catabolic substrate, and strictly prefer glucose/lactate (F/N of 0.2). This can be understood, invoking the model, because of the extreme sensitivity of neurons to oxidative damage, especially in complex I (and references therein).
It is also reflected in the preponderance of complex I containing supercomplexes, mostly absent in astrocytes.[39] Only upon prolonged starvation can maximally half of the total neuronal energy consumption be supplied by ketone bodies (with F/N ratios only going up slightly). Of note, acetoacetate (lower in energy content, and with a higher F/N ratio) constitutes only 20% of the ketone body supply, beta-hydroxybutyrate making up the rest.[29,40]

HOW DOES ACAD9 FIT IN: FILLING IN THE DETAILS

We are now equipped to interpret the recent findings surrounding ACAD9 in much more detail. New publications shed light on several unexpected aspects of its regulation.[6,7,10] As mentioned, the central player involved in complex I biogenesis is the so-called mitochondrial complex I assembly (MCIA) complex. In MCIA, ECSIT (see below) fulfills an important function.[41] The careful recent studies by Giachin and co-workers and Xia et al., delineated how the (different domains of) central players at its core (ACAD9, ECSIT and NDUFAF1) interact and how these interactions allow them to integrate mitochondrial metabolic signals into an appropriate response. First of all, they demonstrate the two functions of ACAD9 to be mutually exclusive, as binding of the C-terminal domain of ECSIT to the so-called vestigial dehydrogenase domain of ACAD9, not only induces the loss of FAD (deflavination), but also actively counters re-uptake. This deflavination, and the accompanying conformational change, allows ACAD9 to switch from a role in FAO to being an MCIA factor. In all this, ECSIT also fulfills a bridging function, because its N-terminal domain binds to NDUFAF1 making up the complete core of MCIA. This core allows the MCIA holocomplex (with membrane components COA1, TIMMDC1, TMEM126B, and TMEM186) to form and complex I biogenesis to start. A few remarks are in order here: the authors do not discuss what happens to the FAD molecule: it is either a (strongly rate-limiting?) dehydrogenase function in FAO or a necessary component of MCIA involved in the biogenesis of complex I. The main switch is best understood when we consider, for example, hepatocytes, cells that highly express ACAD9.[45] These highly ROS-susceptible cells have to forego FAO, but need a high capability of repairing oxidatively damaged complex I, implying a constant need for MCIA.[6,7,18,38] But what happens with that prosthetic group?

COULD FAD INDEED BE REPURPOSED AS WELL?

Above I indicated that the best way to interpret all the experimental data is by stressing the mutually exclusive nature of the ACAD9 molecule: it is either a (strongly rate-limiting?) dehydrogenase functioning in FAO or a necessary component of MCIA involved in the biogenesis of complex I. The main switch is best understood when we consider, for example, hepatocytes, cells that highly express ACAD9.[45] and also can use FAO to cover their normally high-energy needs. An imbalance between ATP production and its use might lead to a high Δp, combined with high F/N and QH2/Q ratios, resulting in RET and oxidative damage around the FMN containing, NADH binding, "N-module" of complex I (see Figure 1A). Repurposing of ACAD9 allows the simultaneous shutdown of FAO and activation of complex I restoration. When we look at the latest insights in the dynamics of the N-module and its flavin binding site, as well as the mitochondrial import and further processing of flavins in the organelle (nicely reviewed in,[10]) an exciting possibility arises. FMN could be derived from the "ejected" FAD and repurposed as well.[8–10] There are promising candidates for the role of a hydrolysing FAD – FMN converter inside mitochondria.[46,47] This would constitute the most literal form of lowering the F/N ratio: converting the FAD of ACAD9 into the NADH recognising FMN of complex I (see Figure 1B).

HOW DOES ECSIT FIT IN: FILLING IN THE DETAILS

It is somewhat surprising that the researchers studying the versatile role of ACAD9 in both FAO and the MCIA complex stress "metabolic efficiency", but are silent on its function as a ROS suppressor and restoration enzyme, given another core MCIA constituent described above: ECSIT. ECSIT-ROS connections have been found in
the context of immunology. ECSIT got its name (Evolutionarily Con-
served Signalling intermediate in Toll pathway), from the fact that
bacterial activation of Toll receptors allows downstream binding of
TRAF6 to ECSIT. This binding then increases mitochondrial ROS pro-
duction to kill bacterial pathogens.\cite{48,49} Interestingly, ECSIT-deleted
macrophages display high mitochondrial ROS production preventing
further induction by Toll receptors.\cite{50} The discovery that this cytosol-
lic signalling protein could also localise to mitochondria and interact,
amongst others, with chaperone NDUFAF1 to function in complex I
biogenesis constituted a major breakthrough, shedding light on other
observations and making links with variations in ROS production logi-
cal (as complex I is an important ROS producer). Thus, the availability,
molecular conformation, and location of the three core subunits of
MCIA are key determinants of mitochondrial ROS formation.

**DISCUSSION AND FUTURE RESEARCH**

Since I proposed that mitochondrial F/N ratios and internal ROS
formation could be considered major determinants in eukaryotic evolution,\cite{11} several new findings turned out to be supportive of the
model. For instance, in the case of the evolution of peroxisomes: peroxi-
osomal FA oxidation is mostly derived from the mitochondrial
ancestor,\cite{31} and peroxisomal biogenesis can be physically linked to
complex I biogenesis.\cite{32} The arrival of peroxisomes can thus be conceiv-
ed of as an instance of symbiogenesis, the position that tries to under-
east eukaryogenesis as a series of mutual adaptations of archael “host” and bacterial endosymbiont.\cite{23,31,32,51} As mentioned above,
high F/N ratios can also come about in other conditions, for example,
when succinate has accumulated during ischaemia in mammalian
tissue and indeed becomes responsible for RET induced mitochon-
drial ROS production during reperfusion\cite{24,52} or upon use of the
Glycerol-3-Phosphate Dehydrogenase shuttle.\cite{53} Just as in the case of
FAO, UCP action is enhanced.\cite{18} Upregulation of UCP2 and UCP3
activation of the Glycerol-3-Phosphate Dehydrogenase by T3 go hand in hand.\cite{36,37} Though overall all these findings are consistent
with the hypothesis, layers of adaptations to internal ROS forma-
tion make the interpretation of experimental results complicated. Let
me illustrate possible pitfalls with one final example, by discussing
the effects of the Glycerol-3-Phosphate shuttle in neutrophils, as very
recently described.\cite{44} The experiments show that neutrophils use the
glycerol 3-phosphate pathway upon glycolysis, to maintain polarised
mitochondria (under hypoxia!) and produce ROS, which, in turn, sta-
bilises HIF-1α. Using HIF-1α as a readout for ROS formation, they show
(in Figure 1 of their publication) that ROS seems to be coming from
both complex I and III (using rotenone and antimycin A as inhibitors of
each, respectively). This seems to fit perfectly with the predictions of
the F/N ratio hypothesis.\cite{25} But, when using exolactate as a compet-
titive inhibitor of complex II, a stark increase in HIF-1α stabilisation is
observed. As one of the sources of “FADH2-linked” electrons, complex II inhibition seems to be behaving unexpectedly here. However, the “layers of adaptation” might be contributing to the observed effects.
In a recent broad-ranging, highly worthwhile, review, the multiple-
layered effects of succinate (e.g., increased upon inhibition of complex II) are listed. Succinate is eloquently described as a mitochondrial coen-
zyme Q (and thus, F/N ratio) redox sentinel.\cite{53} and in the review we
can find that succinate inhibits cytosolic HIF-alpha prolyl hydroxylases,
thus stabilising HIF-1α via a non-ROS pathway.\cite{54} This example makes
it abundantly clear that direct, fully convincing, evidence for the F/N
hypothesis will be hard to obtain. Maybe studying ROS production by
proteobacteria upon shifts between FAO and aerobic glycolysis would
be illuminating. Is there significant ROS formation? Is it bigger when
going from glycolysis to FAO than vice versa? What complexes are
involved?

In conclusion, all these experimental results, though highly sugges-
tive, only support the importance of the F/N concept indirectly. The
latest insights regarding the dynamic nature of ACAD9 function, how-
ever, seem to me a rather strong illustration of the lowering of F/N
ratios as a way of both suppressing and repairing ROS-related damage.
Thus, the concept may indeed turn out to be a highly enlightening one
when it comes to understanding the mechanics of mitochondrial ROS
formation, and its possible role during eukaryotic evolution.

Though the switch in the role of ACAD9 itself is now very well
documented, the repurposing of its prosthetic group is still hypothet-
ical. Labelling experiments could quickly give us the answer as regards
to a possible physiological relevance. Dynamic mitochondrial super-
complex formation seems clearly linked with respiratory efficiency
and ROS formation\cite{39,57,58} but insight with regard to the involve-
ment of the FAO machinery is still somewhat lacking.\cite{46} And how
about the possible role of ACAD9 in such higher order structures? The
complicated, multi-layered, and subtle nature of mitochondrial ROS
formation keeps on posing daunting experimental challenges. Probably
for years to come.

**CONFLICT OF INTEREST**
The author declares no conflict of interest.

**DATA AVAILABILITY STATEMENT**
Data sharing is not applicable to this article as no new data were
created or analyzed in this study.

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