JEM Journal of Experimental Medicine

INSIGHTS

NNT in NSCLC: No need to worry?

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In this study, Ward et al. (https://doi.org/10.1084/jem.20191689) provide exciting evidence that nucleotide nicotinamide transhydrogenase (NNT), a mitochondrial matrix–located enzyme harnessing the proton gradient to generate NADPH using NADH, markedly contributes to non-small cell lung carcinoma (NSCLC), which is abrogated in the murine C57BL/6J background, a strain known to be deficient in NNT.

In the present work in this issue of *JEM*, Ward et al. set out to interrogate whether mitochondrial nucleotide nicotinamide transhydrogenase (NNT) and consequently NADPH/NADH ratios, which are key to sustain proper mitochondrial redox homeostasis, contribute to lung tumorigenesis and aggressiveness using a conditional $Kras^{G12D}$-driven lung tumor model (LSL-$Kras^{G12D/+}$). Ward et al. (2020) focused on the lung, as this tissue is naturally exposed to high oxygen tensions and, as such, shows robust mitochondrial respiratory activity. The authors took advantage of the Jackson Laboratory mouse strain C57BL/6J, which has been long known to be inherently resistant to tumor development. By the beginning of this millennium, it was recognized that this particular mouse strain harbors a naturally occurring missense mutation in both the mitochondrial leader sequence encoded by exon 1 and an in-frame five-exon deletion in the *Nnt* gene causing deletion of exons 7–11 and thus no detectable NNT protein (Toye et al., 2005). NNT is a mitochondrial matrix–located enzyme anchored in the mitochondrial inner membrane that generates up to 50% of the mitochondrial NADPH pool by catalyzing the transfer of hydride from NADH to $NADP^+$ using the proton motive force built up across the inner mitochondrial membrane by the electron transport chain (ETC; Klingenberg and Slenczka, 1959). Other sources of mitochondrial NADPH include isocitrate dehydrogenase of the tricarboxylic acid cycle (TCA), methylenetetrahydrofolate dehydrogenase of the one-carbon metabolism of serine, and malic enzyme converting malate into pyruvate. NADPH, in turn, is used for fatty acid and iron-sulfur (Fe-S) cluster biosynthesis and for maintaining mitochondrial redox balance by supplying electrons for proper glutathione recycling via glutathione reductase and for mitochondrial thioredoxin reductase (TXNRD2). The latter, along with thioredoxin-2, peroxiredoxin 3, and peroxiredoxin 5, presents the prime defense system in the mitochondrial matrix against potentially deleterious H_2O_2 and peroxynitrite generated by the decomposition of superoxide anion ($O_2^{\cdot-}$). Null mutations of NNT in man have been described, causing familial glucocorticoid deficiency syndrome in affected patients (Meimaridou et al., 2012). Remarkably, while knockout of *Txnrd2* in mice was shown to cause embryonic lethality during midgestation due to severe anemia and defects in cardiodevelopment (Conrad et al., 2004), human patients carrying a homozygous null mutation are born but also suffer from familial glucocorticoid deficiency (Prasad et al., 2014), again highlighting a functional linkage of both redox systems.

Ward et al. (2020) now demonstrate that on a p53 wild-type background, NNT expression significantly contributes to increased tumor burden, while on a p53-deficient background, tumor initiation was similar between groups regardless of NNT expression, albeit tumor aggressiveness (i.e., grade 3 adenocarcinoma) was still clearly increased in NNT-proficient mice. In search of the underlying mechanisms of NNT in tumor initiation and aggressiveness, the authors compared lung tumor cell lines of non-small cell lung carcinoma (NSCLC) origin with or without NNT expression. Although cells with suppressed expression showed reduced proliferation, surprisingly, NNT loss had only a mild effect on H_2O_2 levels in mitochondria and did not cause a major weakening of the mitochondrial antioxidant machinery. Rather, a profound impairment of mitochondrial oxidative capacity and a shift from oxidative toward glycolytic metabolism was

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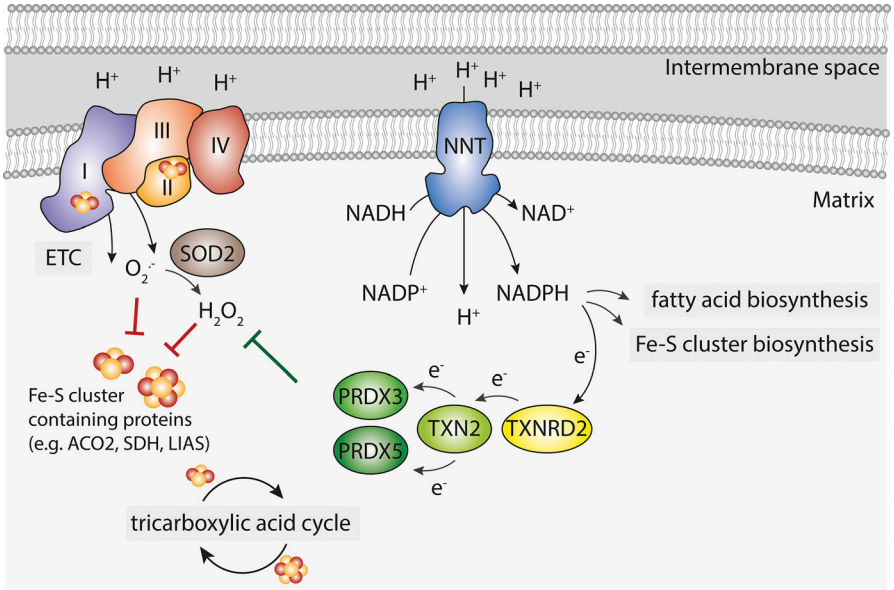

NNT increases tumorigenesis and aggressiveness of NSCLC by maintaining Fe-S cluster stability. NNT is a mitochondrial inner membrane protein that generates substantial amounts of NADPH by harnessing the proton gradient. NADPH is used for various biosynthetic processes and sustains the prime antioxidant system in mitochondrial matrix comprised of TXNRD2/TXN2/PRDX3 and PRDX5 to keep peroxide levels in check. Superoxide anion ($O_2^{\cdot-}$), generated at ETC complexes I and III, and hydrogen peroxide (H_2O_2) cause the oxidative destruction of Fe-S clusters (symbolized by red and orange circles), which are essential prosthetic groups of multiple enzymes of the ETC and the TCA. In the absence of NNT, lower NADPH/NADH ratios cause impaired mitochondrial respiration and TCA cycle activity due to the oxidative disruption of Fe-S clusters, which are present on many related enzymes, leading to an overall decreased tumor burden and aggressiveness of lung cancer. e^-, electrons; H^+, protons; LIAS, lipoic acid synthetase; PRDX3, peroxiredoxin-3; PRDX5, peroxiredoxin-5; SOD2, superoxide dismutase-2; SDH, succinate dehydrogenase; TXN2, thioredoxin-2; TXNRD2, thioredoxin reductase-2.

unmasked on the *Nnt* deficient background, likely contributing to reduced tumorigenesis and aggressiveness of lung adenocarcinoma. To further explore the impact of the skewed NADPH/NADH ratios in tumor cell growth, the authors discovered that the activity of certain Fe-S cluster–containing proteins, including the TCA enzymes aconitase 2 (ACO2) and succinate dehydrogenase, and electron transport across mitochondrial respiratory chain complexes I-III and II-III were strongly compromised. Remarkably, restoring the NADPH/NADH ratios in NNT-deficient cells by expressing the yeast mitochondrial NADH kinase pos5p, previously reported to be required for efficient Fe-S cluster biogenesis (Pain et al., 2010), blunted the defects in mitochondrial respiration and ACO2 activity. Comparing biogenesis and stability of Fe-S clusters by knocking down key enzymes of the core Fe–S biosynthetic enzymatic complex, such as NFS1 cysteine desulfurase (NFS1) and Fe-S cluster assembly enzyme, revealed that deletion of these enzymes impaired the activities of respiratory chain complexes and ACO2, in a manner similar to that induced by NNT deletion. This was accompanied by alterations in the signature of metabolites of the TCA cycle such as pyruvate, malate, fumarate, and succinate. By contrast, turnover of lipoic acid synthetase, an enzyme known to be inherently sensitive to aberrations in Fe-S biogenesis, was differently affected and strongly compromised by NFS1 and Fe-S cluster assembly enzyme knockdown as expected, but remained unaffected by NNT deficiency. This indicates that factors other than Fe-S cluster biosynthesis must be impaired by NNT loss. In addition to the changes in metabolites of the Krebs cycle, suppressed NNT expression caused accumulation of fatty acyl-carnitines as a result of decreased β-oxidation and, interestingly, increased uptake, rendering cells highly sensitive to fatty acid–induced cell death. This complies with the defects seen in enzymes of the TCA cycle along with an impaired ETC and the fact that fatty acid biosynthesis necessitates high levels of NADPH.

Fe-S cluster proteins have been long known to be inherently sensitive to oxidative stress (Gardner and Fridovich, 1991). Moreover, NFS1 was recently found to be positively selected in adenocarcinoma, showing the highest expression in well-differentiated adenocarcinomas to prevent an iron-starvation response, and consequently iron accumulation and ferroptosis (Alvarez et al., 2017), a recently described form of necrotic cell death marked by iron-dependent lipid peroxidation (Conrad and Pratt, 2019). Despite the lack of massive accumulation of H_2O_2 in NNT–deprived cells, Ward et al. (2020) nonetheless asked whether antioxidants might subvert some of the defects induced by NNT loss. In fact, expression of mitochondrial matrix–targeted catalase (but not its cytosolic counterpart) and treatment of NSCLC cells with suppressed NNT expression with the cysteine donor N-acetyl cysteine and the superoxide anion scavenger MitoTempo partially rescued the defects in electron transport chain and ACO2 activity. Hence, these findings strongly suggest that a locally restricted accumulation of partially reduced forms of oxygen in mitochondrial matrix causes oxidation and inactivation of Fe-S cluster–containing enzymes.

The work presented by Ward et al. (2020) is not only intriguing in terms of the tumor-promoting role of NNT in adenocarcinoma, but also has far-fetched implications, particularly in the field of oxidative stress response. In this context, it was repeatedly shown that backcrossing of mice with targeted deficiencies of key redox enzymes, such as cytosolic thioredoxin reductase (Bondareva et al., 2007; Jakupoglu et al., 2005), TXNRD2 (Conrad et al., 2004), and glutathione peroxidase 4 (GPX4; Ingold et al., 2018), on the C57BL/6J background worsens the phenotype by shifting embryonic lethality to earlier developmental stages or even causing mouse embryonic death. This may also likely explain why mice with homozygous null mutations of these key redox enzymes cause lethal phenotypes, while humans with null mutations are born yet display specific pathological phenotypes, such as familial glucocorticoid deficiency (TXNRD2) or Sedaghatian-type spondylometaphyseal dysplasia (GPX4; Smith et al., 2014). As such, a careful interpretation of results obtained with mice on different genetic backgrounds—as convincingly shown here—remains an absolute requirement.

In light of the tumor-promoting activity of NNT, this and other studies advocate for the development of novel anticancer strategies based on NNT inhibition, not only for

the treatment of highly aggressive lung cancer as proposed here, but also against adrenocortical carcinoma and likely against many other tumors with high NNT expression (Chortis et al., 2018). Besides the pressing need to develop clinically suitable NNT-specific inhibitors for the treatment of aggressive tumors, the study presented here should also spark further investigations in systematically evaluating the general importance of NNT in other tumor entities including therapy-resistant and dedifferentiating tumors, which are known to undergo substantial metabolic alterations.

Acknowledgments

M. Conrad received funding from the Deutsche Forschungsgemeinschaft (CO 291/5-2 and CO 291/7-1), the German Federal Ministry of Education and Research through the Joint Project Modelling ALS Disease In Vitro (01EK1611B), the VIP+ program NEUROPROTEKT (03VP04260), the Government of the Russian Federation (2019-220-07-7053), and the m4 Award provided by the Bavarian Ministry of Economic Affairs, Regional Development, and Energy.

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