



The longevity gene INDY (*I'm Not Dead Yet*) in metabolic control: Potential as pharmacological target[☆]

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A B S T R A C T

The regulation of metabolic processes by the Indy (*I'm Not Dead Yet*) (SLC13A5/NaCT) gene was revealed through studies in *Drosophila melanogaster* and *Caenorhabditis elegans*. Reducing the expression of Indy in these species extended their life span by a mechanism resembling caloric restriction, without reducing food intake. In *D. melanogaster*, mutating the Indy gene reduced body fat content, insulin-like proteins and reactive oxygen species production. Subsequent studies indicated that Indy encodes a citrate transporter located on the cell plasma membrane. The transporter is highly expressed in the mammalian liver. We generated a mammalian knock out model deleting the mammalian homolog mIndy (SLC13A5). The knock out animals were protected from HFD induced obesity, fatty liver and insulin resistance. Moreover, we have shown that inducible and liver selective knock down of mIndy protects against the development of fatty liver and insulin resistance and that obese humans with type 2 diabetes and non-alcoholic fatty liver disease have increased levels of mIndy. Therefore, the transporter mINDY (NaCT) has been proposed to be an 'ideal target for the treatment of metabolic disease'. A small molecule inhibitor of the mINDY transporter has been generated, normalizing glucose levels and reducing fatty liver in a model of diet induced obese mice. Taken together, studies from lower organisms, mammals and humans suggest that mINDY (NaCT) is an attractive target for the treatment of metabolic disease.

1. Introduction

The non-electrogenic solute transporter INDY – an acronym for *I'm*

Not Dead Yet – carries di- and tricarboxylates across the plasma membrane in *D. melanogaster* (Inoue, Zhuang, & Ganapathy, 2002). In *D. melanogaster* and *C. elegans*, reduced expression of Indy and its

Abbreviations: Aak2, 5'AMP-activated protein kinase catalytic subunit alpha-2; ACC, acetyl CoA carboxylase; Acly, ATP citrate lyase; Adipo-IR, adipose tissue insulin resistance index; ADME, absorption, distribution, metabolism, excretion; AGA, appropriate-for-gestational-age; AhR, arylhydrocarbon receptor; AMPK, 5'AMP-activated protein kinase; ASO, 2'-O-methoxyethyl chimeric anti-sense oligonucleotide; ATP, adenosine triphosphate; BMI, body mass index; *C. elegans*, *Caenorhabditis elegans*; cAMP, cyclic adenosine monophosphate; cDNA, complementary DNA; CeNac2, *Caenorhabditis elegans* sodium cotransporter 2; CIC, citrate carrier; CoA, coenzyme A; CREB, cAMP responsive element-binding protein; *D. melanogaster*, *Drosophila melanogaster*; DAG, diacylglyceride; EEG, electroencephalography; EST database, expressed sequence tags database; F1,6BPase, fructose 1,6 biphosphatase; FATP, fatty acid transport protein; FFA, free fatty acids; HEK-293, human embryonic kidney 293; HepG2, hepatoma G2; HFD, high-fat diet; HP, hairpin; IGF-1, insulin-like growth factor 1; IL-6, interleukin 6; INDY/Indy, *I'm Not Dead Yet*; INS, insulin; KO, knockout; mINDY/mIndy, mammalian INDY; mRNA, messenger ribonucleic acid; Na, sodium; NaCT, sodium citrate transporter; NaDC, sodium- di- and tri-carboxylate cotransporters; NADH, nicotinamide adenine dinucleotide; NAFLD, non-alcoholic fatty liver disease; NaS, sodium-sulfate cotransporter; NASH, non-alcoholic steato-hepatitis; NEFA, non-esterified fatty acids; OAA, oxaloacetic acid; oGTT, oral glucose tolerance test; PGC-1 α , peroxisome-proliferator-activated receptor-gamma coactivator 1 alpha; pH, potentia hydrogenii; PXR, NR1I2, pregnane X receptor; SGA, small-for-gestational-age; siRNA, small interfering RNA; SLC13A5, solute carrier 13A5; STAT, signal transducer and activator of transcription; T2DM, type 2 diabetes; TAG, triacylglyceride; TCA, tricarboxylic acid cycle; TG, triglyceride; Vc, *Vibrio cholerae*; VLDL, very low density lipoprotein

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homolog CeNac2 promoted longevity in a manner akin to caloric restriction, one of the most reliable interventions prolonging healthy life span over a wide range of species (Anderson & Weindruch, 2012; Rogina, Reenan, Nilsen, & Helfand, 2000). In mammals, mIndy encodes the sodium-coupled citrate transporter NaCT, which shares the highest sequence and functional similarity with *D. melanogaster* Indy. mINDY knockout mice are protected from obesity and insulin resistance associated with a high fat diet or with normal aging (Birkenfeld et al., 2011). The effect is mediated by a switch in mitochondrial metabolism and reduced hepatic lipid generation. These promising findings led to the development of new pharmacological approaches targeting mINDY as a candidate in the context of metabolic diseases like insulin resistance and type 2 diabetes.

In this review, we summarize the role of mIndy in metabolic control in different cells and tissues and describe the most recent advances on structure, expression and function of the dimer. We present a previously unpublished three dimensional structure of mINDY/NaCT. In addition, we discuss new pharmacological approaches using mINDY/NaCT as a target structure.

2. INDY — molecular structure and function

In *D. melanogaster*, Indy is a cation independent, electroneutral transmembrane transporter carrying di- and tricarboxylates across the plasma membrane, whereas the mammalian INDY transporter is cation dependent (Inoue, Zhuang, & Ganapathy, 2002; Rogina, 2017). mINDY belongs to the SLC13 protein family, consisting of sodium-coupled di- and tri-carboxylate/sulfate transporters (Markovich & Murer, 2004; Willmes & Birkenfeld, 2013). The SLC13 family comprises five genes Slc13a1–Slc13a5; encoding multi-spanning transporters with 8–13 transmembrane α -helices flanked by an intracellular N-terminus and an extracellular C-terminus, containing putative consensus glycosylation sites (Markovich, 2008; Markovich & Murer, 2004; Willmes & Birkenfeld, 2013). Slc13a1–5 orthologues are present in pro- and eukaryotes. The SLC13A family members contain numerous predicted consensus phosphorylation and N-myristoylation sites with unknown functional significance. A highly conserved consensus sequence motif is present in each of the five family members (Markovich, 2001). Mammalian SLC13A sodium-coupled cotransporters are located in the plasma membrane of epithelial cells with ubiquitous expression, but primarily in liver, kidney, small intestine, placenta and the central nervous system. All the mammalian transporters are electrogenic with a general sodium:substrate ratio of 3:1 or 4:1. The SLC13A family

members are functionally divided into two groups: SLC13A1 and SLC13A4 belong to the sodium-sulfate cotransporters (NaS) mainly transporting sulfate, selenate and thiosulfate whereas SLC13A2, SLC13A3 and SLC13A5 are sodium- di and tri-carboxylate cotransporters (NaDC) carrying Krebs-cycle intermediates such as citrate, succinate, and α -ketoglutarate (Willmes & Birkenfeld, 2013) (Table 1).

The bacterial INDY homolog protein binds one molecule of citrate and one molecule of sodium. Conserved amino acids, serving as the structural basis for the transporter specificity, are found over a wide range of species (Mancusso, Gregorio, Liu, & Wang, 2012). The sodium: citrate stoichiometry for mINDY is 4:1, which is in contrast to the other SLC13 family members. In humans, the mIndy gene is located on chromosome 17p13 with a size of approximately 30 kb containing 12 exons (Inoue, Zhuang, & Ganapathy, 2002). A number of common splice variants have been documented in the human EST database, especially in the liver. A splice variant lacking 43 N-terminal residues has previously been reported, but function and specific tissue distribution are still unknown (Pajor, 2006). Another common splice variant for mIndy, that is found at > 30% abundance in the mRNA of liver, leaves out one of the exons, resulting in a shorter mINDY protein, lacking the entire 10th membrane domain, and is predicted to be functionally inactive (NCBI database). Given mINDY's dimeric nature, a non-functional protein could conceivably dimerize with the normal protein. A dominant negative effect on mINDY cellular activity may ensue. mINDY has high substrate specificity for the tricarboxylate citrate, and exhibits the inward electrogenic sodium-coupled substrate cotransport. Moreover, mINDY mediated citrate transport appears to be pH-sensitive. Maximal activity is exhibited at pH 7.0–7.5 and the transporter is inhibited at acidic and alkaline pH (Inoue et al., 2004). The transporter shows lower affinities to other Krebs-cycle intermediates such as succinate, malate or fumarate (Inoue, Zhuang, & Ganapathy, 2002; Inoue, Zhuang, Maddox, et al., 2002; Inoue et al., 2004). mINDY is less selective to other organic anions. For example, mINDY does not mediate the uptake of glutarate derivatives into neurons (Brauburger, Burckhardt, & Burckhardt, 2011). Interestingly, mINDY is lithium-sensitive with substantial quantitative and qualitative species differences. Human mINDY is stimulated by lithium although INDY is inhibited or unaffected in most other species (Gopal et al., 2015; Inoue, Zhuang, Maddox, Smith, & Ganapathy, 2003).

Several homology models of human SLC13 family members, NaDC1, NaDC3, and mINDY/NaCT, were recently reported in the literature (Colas, Pajor, & Schlessinger, 2015; Colas, Schlessinger, & Pajor, 2017; Mancusso et al., 2012; Mulligan et al., 2016; Nie, Stark, Symersky,

Table 1
Overview of the human, mouse and rat SLC13A family members.

Name	Synonym	Expression	Substrate	Reference
Slc13a1	NaS1, NaSi-1	Human: kidney Mouse: kidney, ileum, duodenum/jejunum and colon, caecum, testis, adrenal, adipose tissue Rat: kidney, small intestine	Sulfate, thiosulfate, selenate	Markovich (2000), Busch et al. (1994), Markovich and Murer (2004) and Markovich, Forgo, Stange, Biber, and Murer (1993)
Slc13a2	NaDC1, NaC1, SDCT1	Human: kidney, intestine Mouse: kidney, intestine Rat: small intestine, large intestine, kidney, liver, lung, epididymis	Succinate, α -ketoglutarate citrate	Chen, Shayakul, Berger, Tian, and Hediger (1998), Pajor, Sun, and Valmonte (1998) and Pajor and Sun (2000)
Slc13a3	NaDC3, NaC3, SDCT2	Human: brain, kidney, placenta, liver, pancreas, eye Mouse: kidney, brain Rat: kidney, liver, brain, placenta	Succinate, α -ketoglutarate, citrate	Chen, Tsukaguchi, Chen, Berger, and Hediger (1999), Kekuda et al. (1999), Pajor, Gangula, and Yao (2001) and Wang et al. (2000)
Slc13a4	NaS2, SUT-1	Mouse: placenta, brain, lung, eye, heart, testis, thymus, liver Human: placenta, brain, heart, testis, thymus, liver Rat: placenta, brain, liver	Sulfate	Dawson et al. (2005), Dawson et al., 2006 and Markovich, Regeer, Kunzelmann, and Dawson (2005)
Slc13a5	NACT, INDY	Human: liver, testis, brain, (kidney, heart) Mouse: liver, brain, testis Rat: liver, testis, brain	Citrate, succinate, malate fumarate	Inoue, Zhuang, and Ganapathy (2002), Inoue, Zhuang, Maddox, Smith, and Ganapathy (2002) and (Gopal et al., 2007)

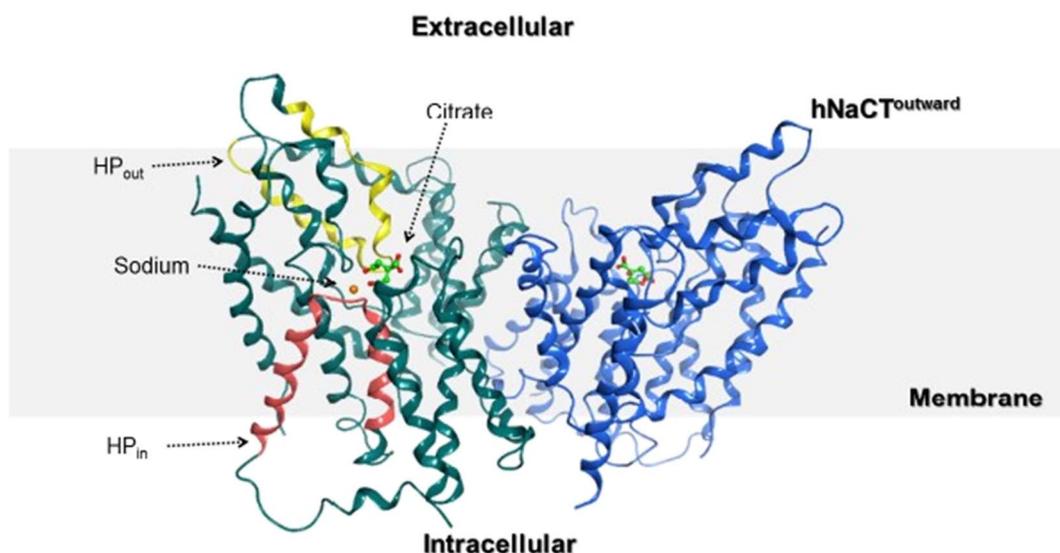


Fig. 1. Homology model of human mINDY/NaCT^{outward} (modelled with the homology modeling tool as implemented in the MOE software package (Chemical Computing Group, version 2016.08) — side-view). The backbone of NaCT is represented as a ribbon when the subunit A is colored in dark green and subunit B in blue, HP_{out} and HP_{in} in monomer A are yellow and pink respectively. The carbon and oxygen atoms of citrate are colored green and red respectively when the atom of sodium is shown as an orange sphere. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Kaplan, & Lu, 2017; Pajor et al., 2016; Schlessinger, Sun, Colas, & Pajor, 2014). These models were constructed based on the crystal structure of bacterial INDY (VcINDY, PDB code 4F35) used as a template (Mancusso et al., 2012).

To understand human mINDY/NaCT's transport mechanism we propose a new human mINDY/NaCT homology model (Fig. 1). Published homology model and crystal structure of the VcINDY were used to create *in-silico* models of inward and outward conformations of human NaCT, referred to as NaCT^{inward} and NaCT^{outward} respectively. The NaCT^{inward} model was based on the crystal structure of VcINDY's inward conformation (PDB code 4F35). The NaCT^{outward} model was based on VcINDY's homology model in outward conformation as reported by Schlessinger et al. (2014).

Structural comparison between NaCT^{inward} and NaCT^{outward} suggests significant secondary structure transformation between both conformations during the transport process, as was recently observed for VcINDY (Pajor, 2014). In the proposed mechanism, two central helical hairpins called HP_{out} and HP_{in} (Fig. 1) act as inner and outer gates covering the substrate-binding site while regulating substrate binding and release. In NaCT these hairpins contain conserved residues that are essential for citrate binding, including motives SNT in HP_{in} (residues: S140, N141 and T142) and SNV in HP_{out} (residues: S464, N465 and V466). These observations are consistent with the SDM data performed for VcINDY on similar conserved residues (Pajor, 2014). The conformational change of these hairpins between mINDY/NaCT^{inward} and mINDY/NaCT^{outward} has a direct effect on the NaCT-citrate affinity and therefore on substrate binding and release from the transporter. For this reason, restricting the protein flexibility in this region can have significant effects on the rate of substrate transport.

Furthermore, residues G228, V231, V232 and G409, affect both, citrate transport and inhibition (Pajor et al., 2016). Intriguingly, residues Q77 and T86, located outside of the putative citrate binding site, were also involved in mINDY/NaCT inhibition. These findings may suggest alternative ligand binding sites. Further exploration of mINDY/NaCT^{inward} and mINDY/NaCT^{outward} surface topology offers that these alternative binding sites could be used for the discovery of allosteric mINDY/NaCT inhibitors. These data provide important insights into the mechanism of transport and inhibition in mINDY/NaCT and can be applied to guide structure-based drug design of new generation of mINDY/NaCT inhibitors.

3. Metabolic actions of citrate

Citrate, as the substrate with highest affinity to mINDY/NaCT, is a key metabolite involved in intracellular signaling as it coordinates glycolysis and lipid synthesis pathways. Citrate inhibits phosphofructokinase (PFK) by allosteric modulation, thereby reducing glycolytic flux (Ros & Schulze, 2013). Furthermore, citrate promotes polymerization and, thus, activation of acetyl CoA carboxylase (ACC), which catalyzes the rate limiting step in *de novo* lipogenesis (Gregolin, Ryder, Kleinschmidt, Warner, & Lane, 1966; Huard et al., 2015). Additionally, citrate has been reported to stimulate gluconeogenesis through fructose 1,6 bisphosphatase (F1,6BPase) activation (Fu & Kemp, 1973; Nielsen, 1983). Citrate, as a tricarboxylic acid (TCA) intermediate is involved in the generation of biochemical energy in the form of adenosine triphosphate (ATP) from acetyl-CoAs derived from carbohydrates and fatty acids. In addition, the TCA cycle provides precursors of certain amino acids as well as the reducing agent NADH that is required for numerous other biochemical reactions.

Cytosolic citrate is known as the prime carbon source for synthesis of fatty acids, triacylglycerols, cholesterol and low-density lipoproteins. Moreover, citrate activates fatty acid synthesis and affects gluconeogenesis and β -oxidation (Bloch & Vance, 1977; Ruderman, Saha, Vavvas, & Witters, 1999; Spencer & Lowenstein, 1962). Major organs involved in lipogenesis are the liver and white adipose tissue. Lipogenesis directly correlates with cytosolic citrate concentrations, in part through direct import across the plasma membrane by mINDY/NaCT (Gopal et al., 2007; Inoue, Zhuang, & Ganapathy, 2002). Fig. 2 shows the cellular fate of citrate in hepatocytes.

Serum citrate concentration is relatively constant, ranging from 50 to 200 μ M (Nielsen, 1983; Nielsen & Sorensen, 1981; Nielsen & Thomsen, 1979). While the K_t value of rat mINDY is around $18 \pm 4 \mu$ M, the K_t value of $604 \pm 73 \mu$ M is much higher for human mINDY/NaCT (Inoue, Zhuang, & Ganapathy, 2002; Inoue, Zhuang, Maddox, et al., 2002). Since physiological citrate concentrations in human circulation range around 135 μ M, we and others showed that the human transporter is hardly saturated under physiological conditions, such that human mINDY/NaCT is a transporter with a low affinity but high capacity ($K_m = 2254 \pm 207 \mu$ M, $V_{max} = 25,117 \pm 1051$ [pmol/mg-min⁻¹]) whereas mouse mINDY has high affinity but lower capacity for citrate ($K_m = 49 \pm 9$ mM, $V_{max} = 3760 \pm 160$

mechanisms. Profiles of whole genome integrative methylation and gene expression analysis in glioblastoma patients demonstrated an inverse correlation between promoter methylation and mIndy expression level in glioblastomas. In this setting, mIndy was downregulated with hypermethylation of its promoter (Etcheverry et al., 2010). In renal cell cancer samples, hypermethylation of a gene cluster including mIndy was associated with poor clinical outcomes (Tian et al., 2014). A recent study analyzed placentas and cord blood of term infants born small for gestational age. The authors observed mIndy hypermethylation together with downregulation of the mINDY/NaCT protein. Children with reduced mIndy expression had reduced circulating IGF-1 levels as well as decreased total and abdominal fat stores at an age of two weeks (Diaz et al., 2016).

4. Proposed metabolic and life span extending function

Indy first came to the attention of the general scientific community when it was demonstrated that mutations in the Indy gene are associated with lower body fat content, insulin-like proteins and reactive oxygen species leading to lifespan extension by mechanisms resembling caloric restriction in most (Neretti et al., 2009; Rogina et al., 2000; Wang et al., 2009) but not all studies (Toivonen et al., 2007). In *D. melanogaster*, INDY preferentially transports citrate and succinate across the plasma membrane. The transporter also shows affinity for alpha-ketoglutarate and fumarate. Fly Indy is mainly expressed in tissues involved in energy homeostasis, is found at lower levels in the nervous system, and encodes an electroneutral tricarboxylate carrier. Interestingly, Indy mRNA is down-regulated in dietary restricted healthy *D. melanogaster*. Moreover, Indy mutated long-lived *D. melanogaster* share several phenotypes with long-lived caloric restricted flies. Food intake was not reduced in Indy mutant long-lived flies and fertility was normal (Inoue, Zhuang, & Ganapathy, 2002; Mancusso et al., 2012; Neretti et al., 2009; Rogers & Rogina, 2014; Rogina et al., 2000; Rogina & Helfand, 2013; Wang et al., 2009; Willmes & Birkenfeld, 2013; Willmes, Helfand, & Birkenfeld, 2016).

Likewise, knockdown of the Indy homolog CeNac2 in *C. elegans* extended life span, an effect mediated partly via AMPK/aak2 (Fei et al., 2004; Schwarz et al., 2015). *C. elegans* with reduced Indy expression exhibited lower lipid content, as shown by two independent groups, while one group failed to observe this phenotype (Fei et al., 2004; Schwarz et al., 2015; Toivonen et al., 2007). The *C. elegans* Indy homolog CeNac2 is expressed mainly in tissues functioning as sites of nutrient absorption and fat storage (Fei, Inoue, & Ganapathy, 2003; Knauf, Rogina, Jiang, Aronson, & Helfand, 2002; Wang et al., 2009). CeNac2 is also located on the plasma membrane and transports TCA cycle intermediates, which are used to generate biochemical energy in the form of ATP.

The cloning of mIndy demonstrated that substrate transport via mINDY is, in contrast to flies, sodium dependent (Inoue, Zhuang, & Ganapathy, 2002). mINDY mediates the cotransport of citrate, succinate and other dicarboxylates together with sodium across the plasma membrane in an electrogenic manner, coupling three to four sodium ions to the transport of each divalent anion substrate (Birkenfeld et al., 2011; Gopal et al., 2007; Inoue, Zhuang, Maddox, et al., 2002; Pajor, 1996, 2006). The transport process for cellular citrate uptake is several times more selective than for other TCA cycle intermediates (Inoue, Zhuang, Maddox, et al., 2002). mINDY shows a highly conserved amino acid sequence of the N-terminal sodium and carboxy-binding motif between many species and is highly expressed in the liver and moderately in brain, testis and kidney (Inoue, Zhuang, & Ganapathy, 2002; Inoue, Zhuang, Maddox, et al., 2002; Mancusso et al., 2012; von Loeffelholz, Docke, et al., 2017).

Genetic whole-body mIndy deletion in mice produced a metabolic phenotype with striking similarities to caloric restriction. Moreover, the gene deletion protected mice from high fat diet (HFD) induced obesity, hepatic steatosis, and insulin resistance. mINDY knockout mice showed

increased energy expenditure, improved hepatic mitochondrial biogenesis, enhanced hepatic fatty acid oxidation and reduced hepatic lipid synthesis (Birkenfeld et al., 2011). mINDY knockout mice displayed reduced uptake of citrate from the circulation in the liver, but not kidney and adipose tissue, concomitant with elevated circulating citrate levels (Birkenfeld et al., 2011; Shulman & Helfand, 2011). Body weight gain with age was also reduced with mINDY deletion. Unexpectedly, oxygen consumption, carbon dioxide production and energy expenditure were increased. In parallel, an elevated hepatic mitochondrial density and increased gene expression of the peroxisome-proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 α) were observed in the livers of mINDY knockout mice. The liver of mINDY knockout mice share approximately 80% similar transcriptional changes, including the expression of electron transport chain components, with caloric restricted mice, as shown by microarray studies. Hepatic triglyceride level and liver lipid deposits were lower in mIndy deleted mice. Lipid oxidation was increased and citrate incorporation into fatty acids and sterols was reduced in livers of diet-induced mINDY knockout mice. The findings are consistent with elevated lipid oxidation and reduced lipid synthesis upon mIndy deletion in obese mice (Birkenfeld et al., 2011; Willmes & Birkenfeld, 2013).

In rat primary hepatocytes, mIndy expression and [¹⁴C]-citrate uptake was induced by physiological concentrations of glucagon via a cAMP-dependent mechanism through a confirmed cAMP responsive element binding protein (CREB) binding site within the mIndy promoter (Neuschafer-Rube et al., 2014). The rat mIndy promoter sequence located upstream of the most frequent transcription start site was determined by 5'-rapid amplification of cDNA ends. *In silico* analysis identified a CREB-binding site within the promoter fragment of mIndy. Functional relevance for the CREB-binding site was demonstrated by reporter gene constructs that were induced by CREB activation when under the control of a fragment of a wildtype promoter, whereas promoter activity was lost after site-directed mutagenesis of the CREB-binding site. Moreover, CREB binding to this promoter element was confirmed by chromatin immunoprecipitation in rat liver. *In vivo* studies revealed that mIndy was induced in livers of fasted as well as in high fat-diet streptozotocin diabetic rats, in which CREB is constitutively activated. mIndy induction was completely prevented when CREB was depleted by antisense oligonucleotides. These data suggest that mIndy is a CREB-dependent glucagon target gene that is induced in fasting and in type 2 diabetes (Neuschafer-Rube et al., 2014). In line with these results, mIndy siRNA-mediated knockdown reduced overall lipid content in the stable human hepatocarcinoma cell line HepG2 (Li et al., 2015).

Diet-induced hepatic steatosis is enhanced by arylhydrocarbon receptor (AhR) activation, both, in humans and animal models (Neuschafer-Rube et al., 2015). Benzo[a]pyrene induced mIndy expression in primary rat hepatocytes in an AhR-dependent manner. The induction resulted in an increased citrate uptake and citrate incorporation into lipids which were probably enhanced by the benzo[a]pyrene-dependent induction of key enzymes of fatty acid synthesis. A potential AhR binding site in the rat mIndy promoter appears to be conserved in the human promoter. Elimination or mutation of this site largely abolished the activation of the mIndy promoter by benzo[a]pyrene. This study thus identified mIndy as a AhR target gene (Neuschafer-Rube et al., 2015).

Very recently, interleukin-6 was shown to regulate mIndy by binding to its cognate receptor (von Loeffelholz, Docke, et al., 2017). In obese, insulin-resistant patients with NAFLD, hepatic mIndy expression was increased and mIndy gene expression was independently associated with hepatic steatosis. In non-human primates, a two-year westernized diet also increased hepatic mIndy expression. Liver microarray studies in non-human primates showed an association of high mIndy gene expression levels and pathways involved in hepatic lipid metabolism and immunological processes. Studies in human primary hepatocytes confirmed that IL-6 markedly induced mIndy transcription via the IL-6-

receptor and activation of the transcription factor Stat3 and a putative start site of the human mIndy promoter was determined. Activation of the IL-6/Stat3 pathway stimulated mIndy expression, enhanced cytoplasmic citrate influx and augmented hepatic lipogenesis *in vivo* whereas deletion of mIndy completely prevented the stimulating effect of IL-6 (von Loeffelholz, Docke, et al., 2017).

mINDY mediates cellular citrate uptake, which plays important roles in the *de novo* fatty acid and cholesterol synthesis. The pregnane X receptor (PXR, NR1I2), which was initially characterized as a xenobiotic sensor, has been functionally linked to the regulation of various physiologic processes associated with lipid metabolism and energy homeostasis. The mIndy gene is also a transcriptional PXR target (Li et al., 2015). mIndy mRNA and protein expression were markedly induced by the prototypical PXR activator rifampicin in human primary hepatocytes. Two enhancer modules located upstream of the mIndy gene transcription start site, that are associated with regulation of PXR-mediated mIndy induction were characterized by cell-based luciferase reporter assays, electrophoretic mobility shift assays, and chromatin immunoprecipitation assays. Further functional analysis revealed that rifampicin can enhance lipid accumulation in human primary hepatocytes, and knockdown of mIndy expression alone leads to a significant decrease of the lipid content in HepG2 cells. Overall, these results revealed mIndy as a novel target gene of PXR and may contribute to drug-induced steatosis and metabolic disease in humans.

Low weight at birth is associated with subsequent susceptibility to diabetes. A genome-wide DNA methylation analysis in placentas of term infants born appropriate-for-gestational-age (AGA) or small-for-gestational-age (SGA) was performed and analyzed in cord blood to identify new genes related to fetal growth and neonatal body composition (Diaz et al., 2016). mIndy was downregulated and hypermethylated in both, SGA placenta and cord blood. SGA infants have less adipose and are more insulin sensitive than AGA infants (de Zegher et al., 2013). These data suggest that reduced mIndy expression may be a programming mechanism attempting to protect those fetuses from excessive fat accumulation and impaired insulin action (Diaz et al., 2016).

5. Role of INDY in metabolic disease and related disorders

5.1. Interaction between NAFLD, insulin resistance and obesity

Non-alcoholic fatty liver disease (NAFLD), central obesity and insulin resistance are closely linked to each other, which is highlighted by the fact that obese subjects and patients with type 2 diabetes are often affected by NAFLD (Birkenfeld & Shulman, 2014; Gaggini et al., 2013; Gastaldelli et al., 2007; Yang, Hung, et al., 2016). The condition is defined as the presence of cytoplasmic lipid droplets in > 5% of hepatocytes or triglyceride levels exceeding the 95th percentile for lean, healthy individuals in the absence of significant alcohol consumption or viral and autoimmune liver disease. Moreover, patients with NAFLD are at increased risk for cardio-metabolic complications such as type 2 diabetes (T2DM) and cardiovascular disease in addition to hepatic complications (Amaro et al., 2010; Bhatia, Curzen, Calder, & Byrne, 2012; Chalasani et al., 2012; Gastaldelli et al., 2007; Kantartzis, Machicao, et al., 2009; Kantartzis, Peter, et al., 2009; Monetti et al., 2007; Sevastianova et al., 2011; Targher et al., 2007; Visser et al., 2011). NAFLD is a chronic condition ranging from relatively benign steatosis to more significant liver injury including cirrhosis and hepatocellular carcinoma. Histology may show lobular inflammation, hepatocyte swelling, fibrosis and overt cirrhosis like for example in non-alcoholic steatohepatitis (NASH) (Browning et al., 2004; Day, 2002; Nati et al., 2016; Szczepaniak et al., 1999). Insulin resistance is a characteristic feature of NAFLD, even when subjects are not obese (Bugianesi et al., 2005; Sanyal et al., 2001; Stefan, Schick, & Haring, 2017; Yki-Jarvinen, 2010).

Increased lipid species, such as diacylglycerols and ceramides

interact directly with the insulin signaling cascade in the liver. While DAGs activate novel protein kinase ϵ , phosphorylating Thr1160 on the insulin receptor, ceramides inhibit AKT (Petersen et al., 2016; Turpin et al., 2014). Together, these mechanisms promote hepatic insulin resistance. However, insulin resistant subjects with NAFLD also show reduced insulin sensitivity in skeletal muscle and adipose tissue (Bugianesi et al., 2005; Gastaldelli et al., 2007; Lomonaco et al., 2012). Indeed, adipose tissue becomes resistant to insulin's antilipolytic actions. Therefore, free fatty acid (FFA) release is increased, despite an increase in, both, hepatic and systemic lipid oxidation. Increased FFA availability promotes VLDL-TG secretion (Arner, 2002; Miles & Nelson, 2007; von Loeffelholz, Lieske, et al., 2017). FFA are taken up by tissues for oxidation and accumulate as ectopic fat (Bugianesi et al., 2005; Hwang et al., 2007; Machado et al., 2012). Possibly, ectopic fat could serve as a defense mechanism against lipotoxicity and individuals with NAFLD could develop NASH and cirrhosis as a consequence of a second hit elicited through inflammation and excess reactive oxygen species (Choi & Diehl, 2008; Day, 2002; Neuschwander-Tetri, 2010). Moreover, adaptation of mitochondrial energetics, gene expression, morphology, and content appear to play a key role in the transition from steatosis to full blown NASH (Patterson et al., 2016; Satapati et al., 2012; Sunny, Parks, Browning, & Burgess, 2011; Sunny, Bril, & Cusi, 2017; Yang, He, et al., 2016). Finally, the fibrosis associated with NASH may progress to hepatic cirrhosis predisposing patients to hepatocellular carcinoma. Therefore, preventing NAFLD, NASH and hepatic cirrhosis also protects against malignant liver disease (De Minicis, Marzoni, Benedetti, & Svegliati-Baroni, 2013; Li et al., 2017).

5.2. Indy in metabolic disease

In the last few years, genetic mINDY deletion and pharmacological mINDY inhibition was shown to ameliorate NAFLD, obesity and insulin resistance in model organisms (Birkenfeld et al., 2011; Neuschafer-Rube et al., 2014; von Loeffelholz, Docke, et al., 2017; Willmes et al., 2016; Willmes & Birkenfeld, 2013). Therefore, mINDY has been proposed to be an 'ideal target for the treatment of metabolic diseases' (Birkenfeld et al., 2011; Brachs et al., 2016; Neretti et al., 2009; Neuschafer-Rube et al., 2014; Pesta et al., 2015; Rogina, 2017; Schwarz et al., 2015; Shulman & Helfand, 2011; von Loeffelholz, Docke, et al., 2017; Willmes et al., 2016; Willmes & Birkenfeld, 2013).

Whole body deletion of mINDY in mice protected them from high fat diet and age-related insulin resistance, NAFLD and obesity. The phenotype was mediated by reduced hepatic *de novo* lipogenesis along with increased hepatic lipid oxidation, improved mitochondrial lipid oxidation and AMPK activation. However, this approach did not mimic a therapeutic setting.

The impact of a selective inducible hepatic knockdown of mIndy on whole body lipid and glucose metabolism using 2'-O-methoxyethyl chimeric anti-sense oligonucleotides (ASOs) in high fat fed rats was examined in a preventive setting at the onset of HFD feeding. A 4-week ASO treatment reduced mIndy mRNA expression by 91%. The ASO treated and control rats had similar body weights. Yet, the ASO-induced mIndy reduction led to a 74% reduction in fasting plasma insulin concentrations and a 35% reduction of plasma triglycerides. Additionally, hepatic triglyceride content was reduced by the ASO mIndy knockdown, likely mediating a trend to decreased basal rates of endogenous glucose production and an increased suppression of hepatic glucose production during a hyperinsulinemic-euglycemic clamp (Pesta et al., 2015). Together, these data corroborate the mIndy KO phenotype of improvements in hepatic glucose production, insulin responsiveness and protection from NAFLD.

Independently, another study confirmed that knockdown of hepatic mIndy expression by a liver-selective siRNA resulted in > 60% mIndy knockdown, improved insulin sensitivity in a hyperinsulinemic-euglycemic clamp and prevented triglyceride accumulation in the liver, independent of changes in body weight (Brachs et al., 2016).

Protection from NAFLD and insulin resistance might also prevent NASH, cirrhosis and hepatocellular carcinoma. Along these lines, RNAi-mediated silencing of INDY expression in the human hepatoma cell lines HepG2 and Huh7 suppressed cell proliferation, colony formation and induced cell cycle arrest. The mechanism seems to be mediated via metabolic actions: mIndy knockdown in HepG2 and Huh7 cells led to reductions in intracellular citrate concentrations, the ATP/ADP ratio, phospholipid content, and ATP citrate lyase expression (Li et al., 2017). These findings confirm observations in mINDY knockout mice (Birkenfeld et al., 2011). Moreover, *in vitro* and *in vivo* assays demonstrated that mIndy depletion promoted AMPK activation. The response was accompanied by deactivation of the oncogenic mechanistic target of rapamycin signaling mTOR. Together, these data show, that mIndy by regulating hepatic energy homeostasis, also affects fibrotic and oncogenic pathways (Li et al., 2017). Indeed, obese patients with NAFLD exhibited increased mIndy expression compared with lean individuals with normal hepatic lipid content. However, the above mentioned studies do not definitively demonstrate the concept that mINDY/NaCT inhibition is effective in treating type 2 diabetes and NAFLD (von Loeffelholz, Lieske, et al., 2017).

Proof of concept could be provided by using mINDY/NaCT inhibitors. Small mINDY inhibitor molecules have been identified via virtual docking using a homology model of the plasma membrane transporter and a proteoliposome-based assay to measure their inhibitory activity on citrate transport (< 73% inhibition at 1 mM) (Sun et al., 2010). Inhibitors of NaDC1 and NaDC3 weakly inhibited mINDY in transfected CUBS cells (Pajor & Randolph, 2007). However, these compounds exhibited cytotoxicity in HEK-293-derived cell-based assays thereby confounding the interpretation of citrate uptake data (Huard et al., 2015). Moreover, previously reported mINDY inhibitors displayed poor ADME (absorption, distribution, metabolism, excretion) properties precluding their use in *in vivo* experiments (Sun et al., 2010).

Better suited for a proof of concept study was the following compound: this novel small dicarboxylate molecule (compound 2 or PF-06649298) selectively and potently inhibited citrate transport through mINDY/NaCT, both *in vitro* and *in vivo* (Huard et al., 2015). Binding and transport experiments indicated that compound 2, which was discovered using a substrate-based design strategy, specifically binds to mINDY in a competitive and stereosensitive manner, and is, itself, a substrate for mINDY transport. The favorable pharmacokinetic properties of compound 2 permitted *in vivo* experiments to evaluate the effect of inhibiting hepatic citrate uptake on metabolic endpoints. Mice were fed a high fat diet for 12 weeks and subsequently treated with either 250 mg/kg of compound 2 or vehicle twice a day for three weeks. Glucose intolerance on high fat diet was completely reversed in mice treated with compound 2 during an oral glucose tolerance test. Further, livers of treated mice tended to exhibit lower hepatic di- and triglycerides, with higher levels of acylcarnitine. The finding may indicate greater flux through β -oxidation pathways and was corroborated by a trend for increased β -hydroxybutyrate concentrations. Subsequently, molecular modeling and site-directed mutagenesis of human mINDY, transport characterization and cell-surface biotinylation were applied to examine the residues involved in inhibitor binding and transport (Pajor et al., 2016). The results indicate that residues located near the putative citrate binding site (G228, V231, V232, and G409) affect both citrate transport and inhibition of citrate uptake by compounds 2 and 4. V231 appears to distinguish between compounds 2 and 4 as inhibitors. Furthermore, residues located outside the putative citrate binding site (Q77 and T86) may also play a role in mINDY/NaCT inhibition by compounds 2 and 4. These results provide new insight into the mechanism of transport and inhibition of mINDY/NaCT and supply a basis for future drug design of SLC13 inhibitors (Pajor et al., 2016). Another compound 4a, which is less selective, was published recently (Huard et al., 2016). Treatment with 4a reduced citrate uptake in liver, kidney and testis of rodents resulting in modest improvement of glucose metabolism. However, compound 4a shows only partial selectivity for

mINDY, over NaDC1 and NaDC3 and additionally increased citrate concentrations in plasma and urine (Huard et al., 2016).

Recently, the pharmacology of these newly identified mINDY/NaCT inhibitors compounds 2 and 4a was elucidated using a combination of ^{14}C -citrate uptake, membrane potential assays, and electrophysiology. In contrast to their previously proposed mechanism of action, both compounds 2 and 4a elicited allosteric, state-dependent mINDY/NaCT inhibition with low-affinity substrate activity in the absence of citrate. As allosteric state-dependent modulators, the inhibitory potency of both compounds appears to be highly dependent on the ambient citrate concentration. Therefore, the detailed mechanism of action studies in this publication may be of value in interpreting the *in vivo* effects of these compounds (Rives, Shaw, Zhu, Hinke, & Wickenden, 2016).

In pharmacokinetic studies, the unbound partition coefficient (K_{puu}) was determined for a set of compounds from the SLC13A family that are inhibitors and substrates of transporters in hepatocytes and in transporter-transfected cell lines (Riccardi et al., 2016). K_{puu} describes the asymmetric free drug distribution of a compound between cells and medium *in vitro* and plasma and tissue *in vivo*. Enantioselectivity was observed, with (R)-enantiomers achieving much higher K_{puu} than the (S)-enantiomers in mIndy-transfected human embryonic 293 cells. The intracellular free drug concentration correlated directly with *in vitro* pharmacological activity rather than the nominal concentration in the assay because of the high K_{puu} mediated by mINDY/NaCT transporter uptake.

Together, these data provide strong evidence for a role of mIndy in the control of glucose and lipid metabolism in the liver and other tissues. Through these mechanisms, mIndy may also affect fibrosis and carcinogenesis. These findings were the basis for pharmacological investigations testing the therapeutic potential of mINDY/NaCT. In proof of concept studies, small molecule inhibitors of mINDY/NaCT improved glucose tolerance and fatty liver, confirming genetic studies and further strengthening the rationale for developing medications targeting mINDY/NaCT in patients with insulin resistance and NAFLD. It will be important to determine whether or not other conditions, such as NASH and HCC can also be prevented or treated using this approach (Table 2).

6. Emerging relevance of Indy in neuronal metabolism

mIndy is expressed in the human brain, in neurons and possibly glia of the mouse cerebral cortex, hippocampal formation, cerebellum and olfactory bulb, and in neurons of the rat brain (Inoue et al., 2002; Pajor et al., 2001; Wada, Shimada, & Fujita, 2006). In human glioblastoma cells, mIndy was down-regulated suggesting that it may normally be expressed in human glial cells (Bergeron, Clemencon, Hediger, & Markovich, 2013; Etcheverry et al., 2010). In the cerebral cortex of rats, mIndy expression is known to increase during postnatal development and is much higher in the cerebral cortex of adult rats (Yodoya et al., 2006).

The interest in the activity of mIndy and other plasma membrane transporters of TCA cycle intermediates in the brain is driven in part by knowledge that neurons do not express pyruvate carboxylase. Therefore, neurons may not have the usual mechanisms for synthesizing TCA intermediates and could require plasma membrane transporters of TCA cycle intermediates to maintain normal metabolism (Cesar & Hamprecht, 1995; Shank, Bennett, Freytag, & Campbell, 1985; Yu, Drejer, Hertz, & Schousboe, 1983). Surprisingly normal neuronal pyruvate carboxylation rates have been described that resemble those seen in glial cells (Hassel, 2001; Hassel & Brathe, 2000a, 2000b). The pathway by which neurons synthesize TCA cycle intermediates, through carboxylation of pyruvate without pyruvate carboxylase, is not yet understood. However, the fact that neurons can synthesize TCA cycle intermediates *de novo* nevertheless indicates that the brain may not critically depend upon transporters such as mINDY to maintain normal metabolism.

Recently discovered mutations in the human mIndy/SLC13A5 gene

Table 2
Studies using INDY as a target to prevent or treat metabolic disease.

Publication	Species	Method/treatment	Metabolic intervention	Results
Li et al., JBC, 2017 (Li et al., 2017)	Human	RNAi-mediated silencing of SLC13A5 expression in human hepatoma cell lines, HepG2 and Huh7	Subcutaneous injection of SLC13A5-shRNA transfected HepG2 cells into nude mice	Suppression of cell proliferation and colony formation and induction of cell cycle arrest accompanied by increased expression of cyclin-dependent kinase inhibitor p21 and decreased expression of cyclin B1 Reduction of tumor growth and weight in nude mice treated with SLC13A5-shRNA transfected HepG2 cells Reduction of intracellular citrate levels, ATP/ADP ratio, phospholipid content and ATP citrate lyase expression Activation of the AMPK, accompanied by deactivation of mTOR
Birkenfeld et al., Cell Metab, 2011 (Birkenfeld et al., 2011)	Mouse	Whole body Indy knockout	One cohort normal chow diet, one cohort high fat diet (60% kcal from fat) <i>ad libitum</i>	Reduction in hepatocellular ATP/ADP ratio Activation of hepatic AMPK and induction of PGC1 α Inhibition of ACC-2, reduction of SCREBP-1c levels Promotion of hepatic mitochondrial biogenesis, lipid oxidation and energy expenditure Attenuation of hepatic <i>de novo</i> lipogenesis 33% reduction in hepatic citrate uptake 90% reduction incorporation of citrate into fatty acids was in isolated hepatocytes Reduction in hepatic lipid production and in plasma glucose levels (oGTT) Trend for lower hepatic TAG and DAG
Huard et al., Sci Rep, 2015 (Huard et al., 2015)	Mouse	Small dicarboxylate molecule (compound 2 or PF-06649298), ubiquitous effectivity, 3 week treatment	Before treatment, 13 weeks of high fat diet (60% kcal from fat) <i>ad libitum</i>	91% reduction of hepatic mINDY mRNA expression Amelioration of diet-induced hepatic steatosis Reduction of plasma insulin, lipid and amino acid levels Improvement of hepatic insulin sensitivity (hyperinsulinemic euglycemic clamp) No effect on body weight Reduction of total cholesterol, stearic acid and palmitic acid levels
Pesta et al., Aging, 2015 (Pesta et al., 2015)	Rat	2'-O-methoxyethyl chimeric anti-sense oligonucleotides (ASOs), liver specific, 4 week treatment	During 4 weeks of treatment, high fat diet (60% kcal from fat), <i>ad libitum</i>	> 60% reduction of hepatic mINDY mRNA expression Improvement of hepatic insulin sensitivity (hyperinsulinemic-euglycemic clamp) Prevention of hepatic neutral lipid storage and triglyceride accumulation No effect on body weight
Brachs et al., Molecular Metabolism, 2016 (Brachs et al., 2016)	Mouse	mINDY specific chemically modified siRNA, liver-selective, weekly injection for 8 weeks	During 8 weeks of treatment, high fat diet (60% kcal from fat), <i>ad libitum</i>	> 60% reduction of hepatic mINDY mRNA expression Improvement of hepatic insulin sensitivity (hyperinsulinemic-euglycemic clamp) Prevention of hepatic neutral lipid storage and triglyceride accumulation No effect on body weight

have been linked to the development of early-onset epileptic encephalopathy (Hardies et al., 2015; Thevenon et al., 2014). Affected individuals have subclinical seizures as early as the first days of life (Berg et al., 2010; Covanis, 2012). The mechanisms by which mutations in SLC13A5 lead to severe seizure activity and the other accompanying clinical signs are unknown (Bhutia, Kopel, Lawrence, Neugebauer, & Ganapathy, 2017). Functional transport studies *in vitro* for over a dozen different mutations in SLC13A5 show little to no transport activity (Hardies et al., 2015; Klotz, Porter, Colas, Schlessinger, & Pajor, 2016). Interestingly, in a recent report from four affected individuals, analysis of patients' CSF samples revealed changes in multiple metabolites associated with carbohydrate metabolism, lipid synthesis and amino acid pathways, such as beta-hydroxyisovaleroylcarnitine, an intermediate of leucine metabolism and 2-methylcitrate and 3-hydroxybutyrate (Bainbridge et al., 2017).

It is too early to draw a definitive conclusion as to whether and how mIndy is involved in neuronal metabolism and whether modifications in citrate-transporting function contribute to epileptic encephalopathy. So far, mice with complete loss of mIndy activity do not exhibit a deleterious neurological phenotype, and as noted in earlier sections of this review show various metabolic benefits (Birkenfeld et al., 2011). Clearly, more mechanistic studies are warranted to shed light on this emerging aspect of mIndy.

7. Summary

An increasing wealth of data confirm that findings gleaned from lower organisms, such as *D. melanogaster* and *C. elegans*, can be translated into mammals as well as humans. Moreover, genetic data from flies, mice and rats show that deletion or reduction of mIndy gene

expression attenuates body fat, liver fat and/or insulin and glucose levels. Most exciting is that recently identified chemical compounds that inhibit mINDY/NaCT transport function have been shown to ameliorate insulin resistance and NAFLD in mice. Since INDY expression is increased in livers of obese humans with insulin resistance, it is tempting to speculate that patients with type 2 diabetes and the metabolic syndrome could be treated with these or similar compounds in the future. Apart from thiazolidinediones, which are rarely used in the treatment of type 2 diabetes due to their side effects, there are no other anti-diabetic agents currently available that can simultaneously treat insulin resistance and NAFLD. It will be of great interest in the future to see whether mINDY inhibiting compounds will be amendable to therapeutic interventions in patients. The ultimate question then will be whether such a compound will also promote healthy aging and longevity.

Conflict of interest

Grit Zahn is employed at Eternygen, and owns shares of Eternygen. Jens Jordan and Andreas Birkenfeld own shares of Eternygen.

All other authors declare that there are no conflicts of interest concerning the review titled "The longevity gene INDY (*Tm Not Dead Yet*) in metabolic control: Potential as pharmacological target".

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