Genetic modifications of *Mecr* reveal a role for mitochondrial 2-enoyl-CoA/ACP reductase in placental development in mice

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Abstract: Mitochondrial fatty acid synthesis (mtFAS) is an underappreciated but highly conserved metabolic process, indispensable for mitochondrial respiration. It was recently reported that dysfunction of mtFAS causes childhood onset of dystonia and optic atrophy in humans (MEPAN). To study the role of mtFAS in mammals, we generated three different mouse lines with modifications of the *Mecr* gene, encoding mitochondrial enoyl-CoA/ACP reductase. A knock-out-first type mutation, relying on insertion of a strong transcriptional terminator between the first two exons of *Mecr*, displayed embryonic lethality over a broad window of time and due to a variety of causes. Complete removal of exon 2 or replacing endogenous *Mecr* by its functional prokaryotic analogue *fabI* (*Mecr*\textsuperscript{tm}*fabI*) led to more consistent lethality phenotypes and revealed a hypoplastic placenta. Analyses of several mitochondrial parameters indicate that mitochondrial capacity for aerobic metabolism is reduced upon disrupting mtFAS function. Further analysis of the synthetic *Mecr*\textsuperscript{tm}*fabI* models disclosed defects in development of placental trophoblasts consistent with disturbed PPAR signaling. We conclude that disrupted mitochondrial fatty acid synthesis leads to deficiency in mitochondrial respiration, which lies at the root of the observed pantropic effects on embryonic and placental development in these mouse models.
Introduction

Fatty acid synthesis (FAS) in mammalian cells takes place in two compartments; in the cytosol where it follows the canonical integrated pathway (FAS I) and in mitochondria (mtFAS). The mtFAS pathway resembles bacterial type II FAS where each step is carried out by separate enzymes (1). Currently, the only mtFAS product with a confirmed physiological function is octanoic acid, which is used in endogenous lipoic acid (LA) synthesis. LA acts as a cofactor of enzymes of oxidative decarboxylation reactions like pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (KGD) as well as two dehydrogenases in amino acid metabolism, the glycine cleavage system and branched chain dehydrogenase (2). Studies in *Saccharomyces cerevisiae* indicate that inactivation of any enzyme in the mtFAS pathway leads to respiratory defects, lack of cytochromes due to disturbed mitochondrial translation and respiratory complex assembly, rudimentary mitochondria and complete loss of protein lipoylation in yeast (3, 4, 5, 6). In mouse models, adult mice subjected to tamoxifen–induced inactivation of the *Mcat* gene, encoding malonyl-CoA transferase in mtFAS, suffered from loss of lipoylation, premature aging symptoms and disturbed energy metabolism (7).

The 2-enoyl-thioester reductase (ETR) catalyzes the NADPH-dependent reduction of enoyl-ACP (acyl carrier protein) to saturated acyl-ACP in the mtFAS pathway. The disruption of the *ETR1* gene encoding this reductase in yeast leads to the typical mtFAS defect phenotype described above, while overexpression of *ETR1* results in dramatically enlarged mitochondria (3). The mitochondrial enoyl-CoA /ACP reductase (Mecr) is the mammalian orthologue of ETR and is highly conserved in higher eukaryotes (8). The overexpression of Mecr in mouse resulted in cardiac myopathy, as well as mitochondrial enlargement in this organ (9) similar to the phenotype observed in yeast. Recently, the first human individuals suffering from a disorder caused by recessive mutations in the *MECR* gene have been described. These patients display childhood onset dystonia, optic atrophy and basal
ganglia signal abnormalities on MRI together with decreased MECR function and mitochondrial protein lipoylation (10). Due to the defects observed in the nervous system of the patients, the term MEPAN (mitochondrial enoyl-CoA reductase protein-associated neurodegeneration) was proposed as name for the disorder.

Etr1, Mecr/MECR and other eukaryotic homologs belong to the family of medium-chain dehydrogenase/reductase proteins whereas most prokaryotic functional analogues, like fabI, are of the short-chain dehydrogenase/reductase type (1). Expression of MECR or mitochondrially targeted bacterial fabI in the yeast etr1Δ mutant alleviated the respiratory defect of these cells, indicating that it is the enoyl reductase activity which is required for growth (3, 8). A role of Mecr in transcriptional regulation mediated by peroxisome proliferator-activated receptors (PPAR) has recently been proposed independently by two different groups (11,12) although it is controversial whether this effect is due to a product of mtFAS or direct action of Mecr as a transcriptional co-activator.

In the study presented here, we demonstrate that Mecr inactivation in mice leads to embryonic lethality. We report the results of our studies of the time points and causes of embryonic lethality in three different Mecr models: embryos produced by a leaky “knock-out-first” mouse line, a complete knock-out line generated by removal of a critical exon, and synthetic hypomorphic embryos carrying a replacement of mammalian Mecr with the bacterial fabI analogue. This is the first report on the roles of Mecr or mtFAS in mouse embryonic development.

**Results**

**Generation of “knock-out-first” type (Mecr<sup>tm1a</sup>) and Mecr exon 2 deleted (Mecr<sup>tm1d</sup>) mice**

*Mecr<sup>tm1a</sup>* mouse embryos were obtained from the European Mouse Mutant Archive (EMMA) consortium. In this model, *Mecr* expression is abrogated by insertion of a marker gene, followed by
a strong transcriptional terminator, between exons 1 and 2 of Mecr. This construct also introduced FRT and loxP sites allowing for subsequent removal of the marker cassette and exon 2. These tools were used to generate the Mecr<sup>tm1d</sup> model missing exon 2, as described in Materials and Methods (Fig. 1A and B). During this process the second exon of Mecr was deleted by crossing mice carrying a floxed second exon of the Mecr gene with Cre-expressing mice. The lack of this sequence results in removal of a part of Mecr encoding a conserved N-terminal section of the protein. In addition, splicing between the remaining exons 1 and 3 would generate an out-of-frame fusion and yields an inactive protein. The cross breeding of heterozygous Mecr<sup>+/−</sup> did not produce any homozygous Mecr<sup>−/−</sup> pups from either the Mecr<sup>tm1a</sup> or the Mecr<sup>tm1d</sup> line.

**Generation of Mecr<sup>tm(fabI)</sup> mice**

As mitochondrially targeted bacterial fabI complements respiratory growth of etr1 deficient yeast cells and can be selectively inhibited by the drug triclosan (3, 6), we sought to test whether an analogous construct would rescue the Mecr deficiency in mice. Therefore, a sequence encoding the bacterial fabI ETR was introduced into exon 2 of mouse Mecr. The construct was C-terminally tagged with a double HA epitope allowing visualization of fabI expression using western blot analysis. The splicing acceptor sites of exon 2 of Mecr as well as 54 nucleotides of the exon 2 itself were maintained in frame with the fabI coding region. As the Mecr mitochondrial targeting sequence (MTS) is encoded by exon 1, splicing between the latter and the modified exon 2 is predicted to yield in a chimeric mRNA encoding HA-tagged fabI N-terminally extended with the Mecr MTS. This construct is followed by the strong transcriptional terminator, BGH (13) and was designed to exclude the possibility of producing intact Mecr (Fig. 1C). The corresponding cDNA, encoding a fabI variant N-terminally appended with the Mecr mitochondrial targeting sequence and a C-terminal double HA tag, complemented the respiratory growth defect of a yeast etr1Δ strain (Supplementary Material, Fig. S2). The indirect immunofluorescence studies demonstrated that the MecrfabI protein chimera expressed in cultured mammalian HEK 293T cells localized to
mitochondria (Supplementary Material, Fig.S3A). Furthermore, when tested in separate experiments, plasmid borne fabI activity was observed in mitochondrial enriched fraction prepared from pcDNA6 MmMecr-fabI HAHA transfected HEK 293T cells. Taken together, these results show that this fabI variant can be expressed and targeted into mammalian mitochondria in active form (Supplementary Material, Fig.S3B).

**Mecr and fabI in the Mecr mutant mouse lines**

Production of the \( \text{fabI} \) transcript in E 9.5 embryos of homozygous mutants, heterozygous and wild type embryos from heterozygous \( \text{Mecr}^{\text{tm(fabI)}} \) females was studied by RT-PCR. We robustly detected the \( \text{fabI} \) transcript in homozygous knock-in embryos. In comparison, the \( \text{fabI} \) mRNA levels were about 25% in heterozygous embryo and \( \text{fabI} \) transcription was essentially absent in wild type embryos (Fig. 2A). In addition, \( \text{Mecr} \) mRNA expression in all the embryos derived from pregnant heterozygous \( \text{Mecr}^{\text{tm(fabI)}}, \text{Mecr}^{\text{tm1a}} \) and \( \text{Mecr}^{\text{tm1d}} \) were analyzed by RT-PCR and found reduced to background levels in all the homozygous mutants compared to heterozygous or wild type controls (Supplementary Fig.S4).

Embryos of identical genotype were pooled to obtain cell extract material sufficient for immunoblot analysis. The expression of fabI in these extracts was analyzed by western blotting probing for the C terminal HA tag to confirm presence of the chimeric protein in \( \text{Mecr}^{\text{tm(fabI)}} \) embryos. A strong signal was observed when probing extract of homozygous \( \text{fabI} \) knock-in embryos with the HA antibody, a less intense signal was observed in extracts from heterozygous embryos. A weak signal corresponding to a protein of similar molecular mass was also observed in samples from homozygous wild type (Fig. 2B). However, the signals from samples containing the fabI construct were consistently and reproducibly much stronger in repeated experiments. Therefore, we suggest that the band observed in wild type embryo extract is a signal from an unspecific interaction with a protein of similar size as fabI-HA. An identical signal could be seen from extracts prepared from
wild type embryos developing in Mecr<sup>+/+</sup> mothers (data not shown), indicating that the cross-reacting band is not a result of contamination with maternal tissue expressing fabI-HA.

Immunoblotting for Mecr expression demonstrated the presence of Mecr protein in protein extract from wild type and heterozygous embryos, whereas no Mecr protein could be visualized in samples from mutant embryos homozygous for any one of the three types of Mecr modification (Fig. 2C-E).

**Embryonic lethality studies of mecr mutants**

The breeding of heterozygous Mecr<sup>tm1a</sup>, Mecr<sup>tm1d</sup> and Mecr<sup>tm(fabI)</sup> lines did not produce any homozygous mutant pups, indicating that the mutant embryos died during embryonic development and that fabI complementation did not rescue the embryonic lethality phenotype. Therefore, triclosan inhibition of fabI activity for induction of a phenotype as initially planned was unnecessary and the analysis of the breedings was extended to embryonic lethality studies of all three mutant lines.

When pregnant heterozygous Mecr<sup>tm1a</sup> females were dissected between E 7.5 to E 18.5 (Fig. 1D, Supplementary Material, Table S1, Figure S1), we found that homozygous knock-out embryos were smaller in size, displayed opaque yolk sac membranes and were adherent to membranes by E 8.5, whereas wild type yolk sac membranes were translucent and clear and embryos were easy to separate from membranes. Follow-up dissections indicated that the time of embryonic death was variable, occurring between E 9.5 to E 15.5 as determined by visible heart beats or pulsing blood in umbilical vessel and necrotic signs. Long-surviving homozygous knock-out embryos of the Mecr<sup>tm1a</sup> line found alive at E 14.5 were pale and had hemorrhages (Fig. 3A). Due to the variability in embryonic lethality, we suspected that Mecr<sup>tm1a</sup> construct could not completely inactivate the Mecr gene. Although the Mecr<sup>tm1a</sup> construct inserts a strong transcriptional terminator between exons 1 and 2, the exons themselves as well as the Mecr ORF remain intact.
The apparent leakiness of the $\text{Mecr}^{\text{m1a}}$ allele was the motivation for the generation of the $\text{Mecr}^{\text{m1d}}$ mouse line (described above and in Materials and Methods). In our initial studies of embryonic lethality of $\text{Mecr}^{\text{m1d}}$ embryos, we observed that the window of period of embryonic death was much narrower than for $\text{Mecr}^{\text{m1a}}$ mutants. The homozygous $\text{Mecr}^{\text{m1d}}$ embryos were dead (based on absence of heart beat) and at varying stages of resorption at E 11.5. Dissections focusing on the period E 7.5 to E 12.5 (Fig. 1D, Supplementary Material, Table S2, Figure S1) confirmed the time window of embryonic death ranged from E 9.5 to E 11.5. The homozygous $\text{l1d}$ knock-out embryos displayed abnormal morphology similar to $\text{l1a}$ knock-out embryos at E 8.5. At E 9.5, some of homozygous mutants were dead. The live homozygous $\text{Mecr}^{\text{m1d}}$ mutant embryos were smaller in size and displayed a developmental delay characterized by retention of dorsal curvature, resembling the morphology of wild type E 8.5 embryos and adherent fetal membranes (Fig. 3B). The live $\text{l1d}$ knock-out embryos at E 10.5 were also paler than control littermates (Fig. 3C).

Dissections of pregnant females at specific time point of gestation varying from E 8.5 to E 13.5 were also carried out for $\text{Mecr}^{\text{m(fabl)}}$ mice (Fig. 1D, Supplementary Material, Table S2, Figure S1). The homozygous knock-in embryos were found to develop normally and undistinguishable from wild type and heterozygous littermates until E 8.5. At E 10.5, all the homozygous knock-in embryos were paler, fragile and smaller than their heterozygous or wild type littermates (Fig. 3D), with pale and empty yolk sac vessels (Fig. 3E) and their heart beats were weak or absent. All the homozygous $\text{Mecr}^{\text{m(fabl)}}$ embryos were dead and at varying stages of resorption at E 11.5, establishing a period of embryonic lethality in knock-in mice from E 10.5 to E 11.5.

Chi square test analysis of embryos at E 8.5 indicated that the numbers of genotypes in each of generated modified mouse lines were not significantly different from expected Mendelian segregation. These results show that there was no genotype specific death among the experimental lines before E 8.5. Despite numerous efforts, we were unable to produce mouse embryonic fibroblasts from either the homozygous $\text{Mecr}^{\text{m1a}}$ or $\text{fabl}$ knock-in embryos.
Protein lipoylation

Total protein extracts from all the Mecr knock-out and fabI gene replacement embryos (either E 11.5 or E 9.5) were probed with LA antibody. Presence of lipoylated proteins was used as indicator for functionality of the mtFAS pathway (5). The lipoylated E2 subunits of PDH (Dlat) and KGD (Dlst)) were present in extracts from all wild type samples as well as in corresponding heterozygotes (Fig. 4A). Interestingly, a clear lipoylated Dlat signal was observed in protein extracts from homozygous Mecr\textsuperscript{tm1a} embryos at E 11.5 (Fig. 4A). The observation is in line with the proposed leakiness of the Mecr\textsuperscript{tm1a} construct in homozygous embryos, allowing for synthesis of low levels of LA used for modification of Dlat. The result also agrees with the observations of the embryonic lethality studies (Supplementary Material, Table S1 and Fig. 4A). No lipoylated proteins could be detected in homozygous Mecr\textsuperscript{tm1d} knock-out embryos, consistent with complete loss of Mecr function in this embryo line (Fig.4 and Supplementary Material, Fig. S5A, lane 1). Extracts from the Mecr\textsuperscript{tm(fabI)} repeatedly revealed a faint band cross-reacting with the anti-LA antibody in homozygous mutants. This band corresponds to lipoylated Dlat and the presence of this signal indicated a mild rescue of the enoyl-reductase deficiency in these specimens (Fig. 4A and Supplementary Material Fig. S5B, lane 1). This is in line with the brief extension of embryonic survival of these fabI knock-in embryos.

Mitochondrial respiratory chain (RC) activities in Mecr\textsuperscript{tm(fabI)} embryos

Mitochondrial dysfunction has been shown to lead to embryonic lethality (14,15,16,17), and mutations in mtFAS of S. cerevisiae have been reported to result in severe respiratory chain defects (6). We therefore investigated whether Mecr mutations are linked to mitochondrial respiratory chain dysfunction. As Mecr\textsuperscript{tm1d} embryos that were available at E 8.5 were too small for this type of analysis and were frequently already dead or dying at E 9.5, we turned to the Mecr\textsuperscript{tm(fabI)} model for
which embryos were robustly alive at E 9.5. Mitochondrial respiratory function was analyzed from homogenized embryonic materials with an Oxygraph-2k.

Incubation of homogenized, permeabilized embryos in a mitochondrial respiration media supplemented with glutamate, malate, and pyruvate revealed similar basal O$_2$ consumption rates per embryo with 11.34 ± 2.98 pmol/s per embryo, 11.96 ± 2.04 pmol/s per embryo, 9.14 ± 2.22 pmol/s per embryo for wild type, heterozygous and $fabI$ knock-in, respectively (values in means ± SD). The rate of oxygen consumption increased to 17.92 ± 4.64 pmol/s per embryo, 22.94 ± 5.10 pmol/s per embryo after addition of ADP in wild type and heterozygous embryos whereas the increase in rate in homozygous $fabI$ knock-in was not statistically significant (Fig. 4C). All the embryo preparations were unresponsive to the addition of cytochrome c, confirming the integrity of the outer mitochondrial membrane in these preparations. Further addition of succinate, wherefrom electrons are transferred to coenzyme Q bypassing the complex I in the respiratory chain, led to a further increase in oxygen consumption in wild type (28.42 ± 6.28 pmol/s per embryo) and heterozygous (33.46 ± 9.88 pmol/s per embryo) embryos. The $fabI$ knock-in embryos also responded (12.90 ± 4.24 pmol/s per embryo), but the rate of oxygen consumption did not reach the level found in wild type and heterozygous embryos. The maximum rates of respiration determined by titration with FCCP were 40.76 ± 5.58 pmol/s per embryo in wild type, 43.02 ± 11.68 pmol/s per embryo in heterozygotes and 15.72 ± 1.30 pmol/s per embryo in homozygous mutants.

**Placental defects in homozygous $Mecr^{tm1d}$ and $Mecr^{tm(fabI)}$ mutant embryos**

Placental insufficiency at E 10.5 is known to result in developmental failure (18). Because homozygous $Mecr^{tm1d}$ and $Mecr^{tm(fabI)}$ embryos showed pale vitelline vessels and experienced death at a matching time period, histological analyses of placenta were carried out at E 9.5. We found that the fetal part of the placentas of homozygous $Mecr^{tm1d}$ and $Mecr^{tm(fabI)}$ mutants was much thinner compared to wild type controls (Fig. 5A-D). Moreover, the organization of trophoblast giant cells,
spongiotrophoblasts and the labyrinthine layer of the fetal part of placenta in these specimens was poor and maternal and fetal blood spaces in labyrinth were not evident (Fig. 5C and D).

Analyses of electron micrographs of placenta from \( Mecr^{m(fabI)} \) embryos further confirmed failure of differentiation of labyrinthine trophoblasts as well as maternal and fetal blood spaces in labyrinth, which were indistinguishable. Furthermore, electron microscopic analyses revealed evenly distributed mitochondria of oval shape in wild type cells with clearly visible and organized cristae (Fig. 5E). In contrast, mitochondria in labyrinthine trophoblasts of homozygous mutant embryos were clustered, irregular in shape and electron-lucent without clear cristae structure (Fig. 5F).

**Mecr deficiency is associated with disturbed transcriptional control by PPAR in fetal placenta**

The histological and ultrastructure evidence clearly pointed to a role of Mecr in placental development. Immunohistochemical analysis revealed that Mecr expression was very weak in the fetal part of placentas connected to wild type and heterozygous embryos, and undetectable in the corresponding structures of homozygous \( fabI \) knock-in embryos. A strong immunohistochemical staining for Mecr was found in decidual sites of placentas irrespective of embryo genotype. Because the decidual sites are a maternal contribution to the placenta, the data indicate that the genotype of the embryos (wild type, heterozygous or \( fabI \) knock-in) does not affect Mecr expression in maternal decidua (Supplementary Material, Fig. S6D).

To obtain further information on effects of Mecr mutations on placental development, we analyzed the population of trophoblast giant cells and spongiotrophoblast cells in fetal placenta associated with wild type and homozygous \( fabI \) knock-in embryos. We carried out RNA *in situ* hybridization using probes specific for trophoblast giant cells (Pl1) as well as spongiotrophoblast cells (Tpbpa or 4311). The cellular linings of both trophoblast cell types were thinner in fetal placenta of homozygous knock-in embryos (Fig. 6A-D). In addition, folliculotropin was also undetectable by
immunohistochemical means in fabI knock-in placenta (Fig. 6F), whereas a strong signal was observed in wild type and heterozygous placenta (Fig. 6E).

PPARγ and PPARβ/δ mediated signaling are essential for placental development (19, 20) while no placental abnormalities in PPARα knock-outs were reported (21). Mecr has recently been implicated in PPARα and γ mediated signaling (11). Therefore, we analyzed PPAR target genes expression by RT-PCR using total RNA samples isolated from whole placentas of embryos of Mecr<sup>tm(fabI)</sup> breedings. The Muc1 transcript was chosen as a representative PPARγ target gene (22), while the Mdfi transcript is a marker specific for PPARβ signaling (23). Both transcripts (Fig. 6G and H) were found to be reduced to 50 % in homozygous mutant placenta compared to WT controls. This result suggests that a negative effect of Mecr on placental development is mediated in part by disturbed PPAR signalings.

**Cardiac developmental defects in Mecr<sup>tm(fabI)</sup> embryos at E 10.5**

The histological analysis of hearts from wild type embryos revealed a clearly distinguishable atrial chamber, ventricular chamber, and endocardial cushion associated with atrio-ventricular canal at E 10.5 (Fig. 7A). In homozygous fabI knock-in mutants, hearts were underdeveloped and resembled embryonic heart at E 8.5- 9.5. The primitive atrial chamber and ventricular chamber were observed in homozygous mutants (Fig. 7B). The trabeculation in myocardial wall of ventricular chambers was visible in both wild type and mutant heart (arrows, Fig. 7A and B).

The embryos in uterine swelling were taken for micro computed tomography (µCT) imaging. Based on the phenotypes of more than 100 genetically confirmed embryos from Mecr<sup>tm(fabI)</sup> breeding at E 10.5, the small and pale uterine swellings were taken to present homozygous fabI knock-in mutants and large and red uterine swellings were chosen as controls (wild type / heterozygous). The µCT analysis revealed that in mutant embryos heart and cavity volumes were 19 % and 21 % smaller
than in controls, respectively, and the fabI knock-in embryo had a two chambered heart (Fig. 7D) whereas control embryo had 3-4 chambered heart (Fig. 7C).

**Discussion**

Mitochondrial defects are associated with many diseases like obesity, diabetes, cancer, cardiovascular and neurodegenerative disorders, demonstrating the vital role of these organelles in cellular function. Functional mitochondria are also essential for embryogenesis (14, 15, 16, 17).

Aiming to understand the physiological role of mtFAS in mammals, we here generated several mutated Mecr mouse lines and analyzed the causes for the ensuing developmental defects. Unlike the mutations that cause MEPAN, which results from compromised function of human MECR (10), our knock-out and fabI knock-in constructs were unable to support development of an organism sufficiently healthy to survive until birth. While the value of our results is limited in terms of in understanding the progression of MEPAN in patients, which may be more appropriately addressed in neuron-specific Mecr knock-out models, they yielded in some intriguing insights in the role of Mecr and mtFAS in embryonic development.

The surprisingly wide time window of embryonic death of the homozygous Mecr<sup>tm1a</sup> mouse line embryos, varying from E 9.5 to E 15.5, was indicative of aborted embryonic development due to a variety of etiological factors (18). The live homozygous Mecr<sup>tm1a</sup> “knock-out-first” embryos at E 14.5 had hemorrhages and were paler. Paleness can be attributed to defective erythropoiesis (24) and hemorrhage may be due to failure in either platelet development (25) or angiogenesis (26). These defects may all contribute to embryonic death at this stage of development. Based on several observations, we propose that homozygous Mecr<sup>tm1a</sup> embryos still have residual Mecr function. In this model, embryonic survival was prolonged compared to embryos lacking exon 2 or fabI replacement mutants. In addition, although neither a Mecr transcript nor Mecr protein can be detected by qPCR or visualized by western blots of homozygous Mecr<sup>tm1a</sup> embryo extracts,
respectively, the clear presence of lipoylated Dlat indicated mitochondrial synthesis of LA in these mutants. Because octanoic acid synthesized by mitochondria is used for synthesis of LA and all the exons of Mecr are intact, we conclude that this knock-out is leaky. This conclusion is supported by the complete absence of lipoylated Dlat in the Mecr<sup>tm1d</sup> knock-out lacking exon 2 (Fig. 4A). If maternally derived LA could explain this observation, no difference in lipoylation should be detected between Mecr<sup>tm1a</sup> and Mecr<sup>tm1d</sup> lines.

Our studies on Mecr<sup>tm1d</sup> and Mecr<sup>tm(fabI)</sup> embryonic lethality show more narrow windows of embryonic death. Mecr<sup>tm1d</sup> embryos died between E 9.5 to E 11.5, while the knock-in Mecr<sup>tm(fabI)</sup> embryos perished between E 10.5 and E 11.5. Notably, also the retardation of embryonic growth was delayed in Mecr<sup>tm(fabI)</sup> embryos compared to homozygous Mecr<sup>tm1d</sup> embryos (E 9.5 and E 8.5, respectively). This difference in growth delay suggests that partial complementation of Mecr function is due to the ETR activity of fabI. Congruently, weak lipoylation of the Dlat in Mecr<sup>tm(fabI)</sup> mutants could be detected by western blotting. Earlier studies by Yi and Maeda reported that LA synthase knock out embryos die between E 7.5 to E 9.5(27). The slight difference in period of embryonic lethality between Mecr<sup>tm1d</sup> KO (C57BL/6) and Lias<sup>−/−</sup> (129S) may be due to differences in the genetic background of mice strain used in these studies.

The growth delay and time point of death of homozygous mutants of Mecr<sup>tm1d</sup> and Mecr<sup>tm(fabI)</sup> embryos, match symptoms described as typical for indication of placental developmental defects in mice (28,29). Our investigation of the placental development in our mouse lines confirmed that these organs were hypoplastic and disorganized. The trophoblast cells have many functions in developing placenta, as for example secretion of hormones regulating the growth of fetal and maternal components of this organ (23, 30, 31, 32). The overall smaller size and the irregular arrangement of the fetal part of the placenta (Fig. 5A-D) can be the result of trophoblast differentiation defects (23, 33). Our RNA in situ hybridization studies for markers of trophoectoderm (Pl1) and ectoplacental cone descendants (Tpbpa/4311) (23, 34, 35) indicated a
highly reduced cell population expressing PI1 and Tpbpa/4311 in homozygous mutant embryonic placenta (Fig. 6B and D). The diminished trophoblast lining is congruent with the thinning of placenta we observed in histological analysis, as is the apparent absence of folliculotropin in trophoblast cells of placenta associated with fabl knock-in embryos.

The PPAR signaling pathways play important roles in placental development (23, 29, 33, 36, 37). The transcriptional control of PPAR regulated genes by Mecr has been reported to be mediated by an mtFAS product (11) or a cytosolic isoform of Mecr (12). The evidence presented in the latter investigation suggests that the cytosolic Mecr variant arises from a splicing variant of the Mecr transcript that results in translation initiation in exon 2 further downstream of the coding region, producing a Mecr isoform devoid of the mitochondrial targeting signal. The expression of non-mitochondrial variant of Mecr might not be completely abolished by insertion of a transcriptional terminator after exon 1 in our Mecrtm1a model. The presence of this Mecr variant in the embryos, which cannot be produced in either Mecrtm1d or Mecrtm(fabl) mutants, may explain in part the survival of “knock-out-first” embryos until E 14.5. However, as protein lipoylation occurs in the Mecrtm1a embryos, a certain amount of Mecr protein must be targeted also to mitochondria also in these mutants.

Both the PPARβ/δ and PPARγ knock-out embryos die around E 10 with evident placental defects (23, 36, 38). In both of these mutants, disturbed trophoblast differentiation was reported as cause of death (23, 33). Our analysis revealed a reduction of PPARγ and β/δ target gene expression in placentas of fabl knock-in embryos compared to wild type controls. As the RNA samples were isolated from placenta carrying both maternal and fetal contributions, the actual reduction of PPAR target gene expression is likely to be much more severe in the fetal fraction than evident in our data. While our results present no definite proof of a role of Mecr in the PPAR signaling pathway, they are consistent with the previous reports of a possible regulatory role of Mecr mediated by PPAR pathways (11, 12). It is therefore not completely ruled out that the possible disrupted PPAR
signaling might contribute to placental developmental failure in the mutants. If such a link exists, it is worth of noting that the physiological ligands of PPARs are fatty acids and eicosanoids (39, 40) and PPARs are located in the extramitochondrial space. However, free standing ACP of mtFAS is localized in mitochondria. Therefore, a role for a non-mitochondrial isoform of Mecr protein or unknown downstream events would be more plausible as effector to PPAR signaling than a product of mtFAS.

Another possible cause for embryonic lethality between E 9–12 is a cardiovascular developmental defect (25). In line with the generalized retarded growth, the heart was found underdeveloped in the homozygous mutants compared to controls. Further histological analysis of homozygous fabI knock-in embryos at E 10.5 showed delayed cardiac development in comparison to wild type controls (Fig. 7A and B). The μCT analysis confirmed that heart appeared tubular in homozygous Mecr<sup>tm(fabI)</sup> mutants, whereas chamber-like organization appeared in wild type controls at E 10.5 (Fig. 7C and D).

An important finding of our study was the effect of Mecr inactivation on mitochondrial function in embryos and placentas of Mecr<sup>tm(fabI)</sup> mutants. The results of our electron microscopy studies on placentas of mutant embryos, revealing an abnormal mitochondrial structure in labyrinthine trophoblast, in conjunction with our observation of a reduced rate of mitochondrial respiration in permeabilized cells from fabI knock-in embryos indicate that absence of Mecr or diminished ETR activity yields in respiratory dysfunction also in mammals. This conclusion is in line with data reported for experiment on knocking down ACP in cultured HEK293T cells and in mitochondria isolated from tamoxifen inducible conditional Mcat knock out mice (7, 41) and congruent with results reported for yeast (6).

Several mouse models deficient in mitochondrial energy metabolism and embryonic development have been described in the literature. For instance, Johnson et al. reported that a knock-out of the
Pdha1 subunit of PDH yields in embryos with disrupted mitochondrial energy metabolism and developmental delay at E 9.5 stage followed by subsequent death (42). This time period is close to the findings reported here, suggesting that oxygen dependent mitochondrial metabolism plays an important role at this stage of development. This is in agreement with Larsson et al., who reported embryonic death at E 8.5 to E 10.5 with loss of mitochondrial DNA and cytochrome c oxidase activity in mitochondrial transcription factor A (Tfam) knock-out embryos (14). Another example for mitochondrial dysfunction associated embryonic lethality is embryonic death at mid-gestation caused by cytochrome c deficiency (15). Although the conceptus is able to oxidize pyruvate during pre-implantation, there are several reports indicating that this stage of development can be successfully completed in spite of a respiratory defect (43). Energy metabolism of early post implantation embryos depends on anaerobic glycolysis which is maintained until days E 6 to 7.5 (44).

Our current work on mtFAS applying three different genetic modifications in MeCr gene showed that disruption of activity of this pathway leads to embryonic lethality due to pleiotropic effects including disturbances in fetal placental and cardiovascular developments and retardation of embryonic growth at the stage when the embryos become depending on aerobic metabolism. These defects coincided with disturbed PPARγ and PPARβ/δ-mediated signaling. Based on the analysis of several factors involved in mitochondrial function, we propose that diminished mitochondrial respiratory capacity is upstream of this pantropic morphological phenotype. In view of the data presented in this work, mtFAS defects should be considered as yet another potential factor for embryonic loss also in humans.

**Materials and Methods**

**Mouse strains**
Mice were maintained in the Experimental Animal Center of University of Oulu according to accepted criteria for humane care and use of experimental animals. The experimental protocols were approved by the Animal Care and Use Committee of National Animal Experiment Board, Finland (license number- ESAVI/7696/04.10.03/2012).

**Production of $\text{Mecr}^{\text{tm1a}}$ and $\text{Mecr}^{\text{tm1d}}$ mice**

“Knock-out-first” type $\text{Mecr}$ mutant mice, B6Dnk; B6Brd; B6N-Tyr$c$Brd $\text{Mecr}^{\text{tm1a(EUCOMM)Wtsi}}$ (EM: 04825) were derived from cryopreserved heterozygous embryos, obtained from the European Mouse Mutant Archive (EMMA) as a free of charge Transnational Access service. These animals harbor a construct where an FRT-flanked neo selection cassette, followed by a strong transcriptional terminator signal was placed between exons 1 and 2 of the $\text{Mecr}$ gene, with loxP sites introduced 5’ and 3’ of exon 2 (Fig. 1A). The pups born after embryo transfer were back-crossed with C57BL/6JOlaHsd. Genotyping was done according to the supplier’s instructions.

To generate the $\text{Mecr}^{\text{tm1d}}$ mice, heterozygous $\text{Mecr}^{\text{tm1a}}$ mice were bred with transgenic mice Tg(ACTFLPe)9205Dym/J, expressing the $\text{FLP1}$ recombinase gene under the direction of the human $\text{ACTB}$ promoter (45) (backcrossed to C57BL/6JOlaHsd), to delete the FRT flanked selection cassette. The resulting mice ($\text{Mecr}^{\text{tm1c}}$) carry a conditional knock-out allele with $\text{Mecr}$ exon 2 flanked by loxP sites. The heterozygous $\text{Mecr}^{\text{tm1c}}$ strain mice were bred with Tg(CAG-Cre)13Miya mice with ubiquitous Cre expression (46) to generate $\text{Mecr}^{\text{tm1d}}$ mice in which the floxed exon 2 was deleted (Fig. 1B). Genotyping was done using primers 5’-CAGGGCTGACCCAGAGTTTC- 3’ and 5’-GACCCTGCTCTCATGAGCTGTCC- 3’. The mutant amplicon was about 500 bp and wild type about 1000 bp.

**Generation of $\text{Mecr}^{\text{fm(fabI)}}$ mice**

Starting with a commercial knockout cassette (loxP-FRT-PGK-gb2-neo-FRT, Gene Bridges, Heidelberg, Germany), a knock-in cassette for insertion of $\text{fabI}$ in frame with base pair sequence of 5’ region of $\text{Mecr}$ exon 2 was constructed. *In vitro* gene synthesis was used to produce a cassette
with a *fabI* double HA tag replacing the loxP site in this construct, as well as homologous arms (Gene Script USA, Piscataway, NJ). The construct includes a NotI site followed by 26 bp from intron 1 and 54 bp from exon 2 of mouse *Mecr*, whole *fabI* with double HA tag, *BGH* transcription terminator and FRT flanked neomycin selection cassette having another transcription terminator, and BamHI restriction site (Supplementary Material, Fig. S6A). The construct was verified by sequencing and then recombineered into a BAC containing *Mecr* (clone coordinate RP 24-76P5, BAC PAC, Children’s Hospital Oakland Research Institute, Oakland, CA). BAC clones harboring the insert were selected by chloramphenicol and kanamycin resistance and confirmed by PCR and agarose gel electrophoresis. The construct was then recombineered into targeting vector p15AmpDTA (47). The targeting construct (Fig. 1C) harbored by the p15AmpDTA vector contained 1.2 kb of *Mecr* sequence homology upstream of the insertion site (5’ arm) and 8 kb *Mecr* sequence 3’ of the insertion point (3’ arm). A NotI site was introduced to allow linearization of the vector for electroporation into ES cells. The correct clones were selected by resistance to ampicillin (targeting vector) and kanamycin (knock-in selection cassette) and further confirmed by PCR/agarose gel analysis (Supplementary Material, Fig. S6B) as well as sequencing of the final construct.

The NotI-linearized targeting vector was electroporated into PRX-B6N ES cells (Primogenix, Boulevard, MO) in the Biocenter Oulu Transgenic Core Facility. The ES cell clones containing the proper recombination product were first screened by PCR, followed by Southern blotting (Supplementary Material, Fig. S6C). The primers used for PCR screening of ES cell clones were i) 5’- ATATGCCAGAGCCTGACATC- 3’, ii) 5’- CTAATCCTGAGCACTTCAGAC- 3’ and iii) 5’- GCGTTAACATAGTCACCATCC- 3’. i) and ii) yield in about 1400 bp product from wild type, and i) and iii) about 1600 bp product from *fabI* inserted DNA. Two ES cell clones were selected for injection into C57BL/6JOlaHsd blastocysts.
The male chimeras harboring a knock-in insert were bred with C57BL/6JolaHsd females to generate heterozygous mice. The heterozygotes appeared healthy and were interbred to generate homozygous Mecr<sup>tm(fabI)</sup> mice. A Mecr<sup>tm(fabI)</sup> mouse line devoid of the selection cassette was also generated by deleting the FRT-flanked selection cassette by breeding with Tg(ACTFLPe)9205Dym/J (45) as above. The primer sequences used for genotyping of Mecr<sup>tm(fabI)</sup> mice were i) 5’- ATGATTTTTAGAACCAAGCAGGTTG- 3’, ii) 5’-AATAGTAACGGTGACGGAATCG- 3’ and iii) 5’-CTAATCCTGAGCAGGTTG- 3’.
i) and ii) yield in a 960 bp product from mutant allele, i) and iii) a 450 bp product from wild type allele.

**Southern blotting**

A 200 bp fragment of the upstream region of the 5’ homologous arm of Mecr is labeled by random priming was used as a probe (Random primed DNA labelling kit, Roche, Espoo, Finland). The restriction enzymes used were HindIII and EcoRV and routine procedure was done (48). The wild type bands ran close to the 3 kb whereas mutant DNA bands were almost 2.4 kb (Supplementary Material, Fig. S6C).

**Western blot analysis of embryos**

Total proteins were extracted using T-Per tissue protein extraction reagent (Thermo Scientific, Waltham, MA) according to manufacturer instructions. Immunoblotting was done with antibodies recognizing the HA epitope (1:4000, Sigma Aldrich, St. Louis, MO), or Mecr antibody (1:2000, Cat. Nr 51027-2-AP; Proteintech, Chicago, IL), or anti lipoic acid antibody (1:1000, EMD Millipore, San Diego, CA). The anti Mecr antibody specifically recognizes mouse and human Mecr protein as judged by detection of human MECR expressed in yeast, disappearance of the cross reacting band in patients with destabilizing MECR mutations (10) or upon shRNAi mediated knock-down of Mecr in mouse NIH3T3 cells (Supplementary Material, Fig.S7). The same anti
Lipoic acid antibody has been used successfully in previous publications by us (6, 10) and others. For loading controls either β actin (Abcam, Cambridge, UK) or Ponceau S (Sigma Aldrich) solutions were used.

**RT-PCR studies**

Total RNA from $Mecr^{tm1d}$ and $Mecr^{tm(fabl)}$ mouse embryos at E 9.5 and $Mecr^{tm1a}$ at E 10.5 were isolated with an RNaseasy Mini Kit (Qiagen, Hilden, Germany) and all RNA samples were DNase-treated with RNase- Free DNase Set (Qiagen). cDNA was produced with a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific). qPCR was performed with a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) using fluorogenic probe-based Taqman chemistry with the Taqman probes for mouse Mecr and Taqman Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. For relative quantification, the results were normalized with actin, $Actb$, as endogenous controls. To study PPAR target gene expression, RNA samples were isolated from placenta at E 9.5 as described above. cDNA was synthesized from 1µg RNA. $Muc1$ (22) and $Mdf1$ (23) probes were used as markers for PPARγ and PPARβ/δ target genes, respectively. The protocol was same as described above. For the $fabl$ transcription level analysis, qPCR was done in CFX 96 Real-Time PCR system (Bio-Rad, Helsinki, Finland) using iTaq™ Universal SYBR® Green Supermix and specific primers. The $fabl$ primers were 5'-CATCGACACCATGTTGCGC- 3’ and 5’- GCGTTAACATAGTCACCATC - 3’. The endogenous control, β-Actin, primers were 5’-GGCTGTATTCCCCTCCATCG-3’ and 5’-CCAGTTGTAACATGCCCATGT-3’.

**Mitochondrial respiratory complex activity studies**

The respiratory complex activity measurements were done by modification of the protocol described previously (49). Briefly, the embryos were dissected from pregnant mothers and kept in PBS on ice until the measurements were carried out. Embryo material was gently homogenized in
mitochondrial respiration medium MiR05 (0.5 mM EGTA; 3 mM MgCl₂; 60 mM lactobionic acid; 20 mM taurine; 10 mM KP; 20 mM HEPES; 110 mM D-sucrose; 1 g/l BSA, essentially fatty acid free; pH 7.1) before measurement.

The homogenate prepared from each embryo was placed into the Oxygraph-2k chamber (OROBOROS Instruments, Innsbruck, Austria) in a total volume of 2 ml. The chamber was closed and the cells were permeabilized by titration of 5 µg digitonin into the chamber. Glutamate (final concentration of 10 mM), malate (2 mM) and freshly prepared pyruvate (5 mM) were titrated into the chamber and the baseline respiration of the permeabilized cells in the homogenate was measured. Further substrates were titrated in the chamber in the following order: ADP (5 mM), cytochrome c (10 µM), succinate (10 mM) and FCCP (titrated into the incubation mixture in 0.5 µM steps until maximum respiration is reached) as uncoupler. The change of oxygen consumption in the chamber after each titration was recorded.

Embryonic lethality studies

The heterozygous females and males of “knock-out-first” (Mecr<sup>tm1a</sup>), Mecr null mutants (Mecr<sup>tm1d</sup>) and Mecr<sup>tm(fabI)</sup> lines were bred in this study. The period of embryonic lethality in Mecr<sup>tm1a</sup>, Mecr<sup>tm1d</sup> and Mecr<sup>tm(fabI)</sup> mice were determined by timed mating and dissection of pregnant heterozygous females of all lines, at various days of gestation. The morphological appearance and viability of the embryos were assessed. To determine the genotype of embryos, a tail sample or whole embryos (if they were E8.5 or younger) were collected. Embryos from E9.5 and older were collected for histological, biochemical and molecular studies. Placenta samples were collected for molecular, ultrastructural and histological analysis.

Histological analysis
Each uterine swelling was separated. The outer layers of the uterus were removed and the embryos were harvested. The rest of myometrium with placenta was fixed in 4 % paraformaldehyde in 100 mM NaPi buffer (pH 7.4) over night and the uterus was cut into halves mid-sagittally. One of the half was used for regular histology processing and embedded in paraffin. Sagittal sections of 5 µm were used for H&E staining or immunohistochemistry with Mecr antibody (1:250). Sections of 2.5 µm were used for immunohistochemistry probing for folliculotropin (Fol) with antibody raised against human chorionic gonadotropin ((1: 4000, Dako, Glostrup, Denmark) and for RNA in situ hybridization respectively. The 5 µm sections from wild type and fabI knock-in embryos at E 10.5 from Mecr<sup>tm(fabI)</sup> breedings were also processed similarly to analyze the heart morphology. The stained sections were analyzed with Olympus BX 51 microscope equipped with Cell^ M imaging software (Olympus, Planegg, Germany).

Transmission Electron Microscopy (TEM)

Placenta samples were fixed in a glutaraldehyde (1 %), formaldehyde mixture (4 %) in 100 mM NaPi. They were post fixed in 1 % osmium tetroxide, dehydrated in acetone and embedded in Epon LX 112 (Ladd Research Industries, Williston, VT). Thin sections (70 nm) were cut with Leica Ultracut UCT ultramicrotome, stained with uranyl acetate and lead citrate and examined in Tecnai G2 Spirit 120 kV transmission electron microscope (FEI Europe, Eindhoven, The Netherlands). Images were captured by Quemesa CCD camera operated with iTEm software (Olympus Soft Imaging Solutions, Munster, Germany).

Micro computed tomography (μCT)

The uterine swelling with embryos at E 10.5 were dissected and prepared for high-resolution μCT imaging. For microstructural imaging samples were paraformaldehyde fixed, dehydrated with graded ethanol series and moved to hexamethyldisilazane (HMDS). HMDS treated uterine swellings were attached with dental wax to holder and placed in μCT scanner (Skyscan 1272,
Brüker microCT, Kontich, Belgium). For imaging low energy X-rays with 40 kV and no filtering was applied. Pixel size was set to 1.75 µm and 1800 projections were collected over 360 degrees. Each projection was collected for 1815 ms and averaged three times. Projection images were reconstructed with NRecon-software (v.1.6.9.8, Brüker microCT) and ring artefact and beam hardening corrections were applied. 3D data sets were visualized with CTVox (3.2.0 r1294). Heart morphology was analyzed using CTAn software, where heart was first manually segmented by drawing regions of interest (ROIs). ROIs were refined with shrink-wrap function and original image. Chambers were extracted with threshold and morphological operations and 3D model and analyses were performed. This provided measures of heart, heart muscle volume and chambers. Results from analysis were rendered in CTVol software.

**RNA in situ hybridization**

The 7 µm sagittal sections of myometrium with placentas from E 9.5 embryos were used. Briefly, antisense and sense riboprobes for Pl1 (34, 35) and Tpbpa or 4311 (23), were generated by *in vitro* transcription of the corresponding cDNA clones with SP6, T7 or T3 RNA polymerase, using DIG RNA labeling mix (Roche). *In situ* hybridizations were done following the routine method (50). After hybridization and post hybridization washing, digoxigenin labelled sections were detected by alkaline phosphatase conjugated anti-DIG antibody and Fast red as substrate (Roche). Counterstaining was done with Mayer’s hematoxylin and coverslips mounted with ImmuMount (Thermo Scientific).

**Statistical analysis**

Chi square test was applied to analyze whether the genotypes of embryos in each *Mecr* modified mouse lines followed Mendelian segregation. The transcription levels are given by means and standard deviations (SD) and the statistical significances of RT-PCR analysis were evaluated with independent samples t-test. The outcomes of the oxygen consumption variables are grouped by
genotype and presented graphically. Independent samples t-test was used to evaluate the statistical significances of differences in outcome levels across genotype groups. To analyze the change in outcome levels of substrate titrations within the genotypes, we applied t-test for repeated measurements. IBM SPSS Statistics version 22 and GraphPad Prism version 5.00 were used for the statistical analyses.

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Conflict of Interest statement: None declared.

References


ortholog of YBR026p/MRF1’p of the yeast mitochondrial fatty acid synthesis type II. *J. Biol. Chem.*, 278, 20154-20161.


Legends to Figures

Figure 1. Modification of mouse Mecr gene. (A) A FRT flanked selection cassette inserted in intron 1 and lox P site on both side of exon 2 (Mecr\textsuperscript{tm1a}). (B) The Mecr\textsuperscript{tm1d} - line was generated from Mecr\textsuperscript{tm1a} mice as described in Materials and Methods. The selection cassette and exon 2 were deleted by breeding with the appropriate recombinase-expressing mice. (C) For the Mecr modification 54 nucleotides from 5' end of exon 2 was fused with \textit{fabI} sequence appended with double HA tag (Mecr\textsuperscript{tm(fabI)}). This sequence is followed by transcription terminator BGH attached to an FRT flanked selection cassette. The figure is not drawn to scale.

Figure 2. Mecr and fabI expression in Mecr mutants. (A) qPCR analysis for \textit{fabI} expression in total RNA samples from Mecr\textsuperscript{tm(fabI)} embryos. The amount of mRNA was normalized using actin (Actb) as reference gene. Results are shown as means ± SD, n= 5 per group, independent samples t-test, *** p< 0.001, ** p< 0.01, * p< 0.05. (B- E) Western blot analysis of different Mecr embryos. HA expression in Mecr\textsuperscript{tm(fabI)} embryos (B) and Mecr protein expression in embryos from Mecr\textsuperscript{tm(fabI)} (C), Mecr\textsuperscript{tm1a} (D) and Mecr\textsuperscript{tm1d} (E) breedings. Mecr protein expression was not detected in any of the three homozygous mutants.
Figure 3. Morphology of control and mutant embryos. (A) A wild type (left) and a homozygous Mecr<sup>tm1a</sup> mutant (right) which was paler and had multiple hemorrhages at E 14.5. (B, C) Mecr<sup>tm1d</sup> embryos control (left) and knock-out (right) embryos at E 9.5 (B) and E 10.5 (C). (D) A small homozygous Mecr<sup>tm(fabI)</sup> mutant (right) and regular-sized control littermate (left) at E 10.5 (E) A homozygous Mecr<sup>tm(fabI)</sup> embryo at E 10.5 with pale vitelline vessels. The homozygous mutants in B, C and D were smaller and developmentally delayed than control littermates. All the embryos in this figure were alive at the time of dissection as justified by pulsing blood in umbilical vessel (A) or beating heart (B-E). Scale bar 1mm.

Figure 4. Protein lipoylation in modified Mecr embryos and cellular respiration in fabI knock-in embryos. (A) Western blot analysis for lipoylated E2 subunits of PDH (Dlat) and KGD (Dlst) in protein samples from embryos of different genotype. Mecr<sup>tm1a</sup> (WT/WT and 1a/1a), Mecr<sup>tm1d</sup> (WT/WT, 1d/WT, 1d/1d), 6 Protein molecular mass marker, Mecr<sup>tm(fabI)</sup> (WT/WT, fabI/WT, fabI/fabI). The arrow points to the lipoylated Dlat band visualized weakly in samples from fabI/fabI embryos. (B) Ponceau staining for protein loading control of A. (C) Titration of oxygen consumption by permeabilized homogenized embryos at E 9.5 from Mecr<sup>tm(fabI)</sup> breedings with various substrates. G+M+P is referring to respiratory rate in incubations using glutamate (G), malate (M) and pyruvate (P) as described in Materials and Methods. Note that basal rates of oxygen consumption were similar in all embryos tested at E 9.5. Relative respiratory rates after addition of ADP (D), cytochrome c (c), succinate (S) and F max after addition of the uncoupler FCCP into the incubations are indicated. The mutant knock-in embryo was unresponsive between titration of each substrate compared to wild type and heterozygous embryos. *p < 0.05, ** p < 0.01, ***p < 0.001.

Figure 5. Histological and ultrastructural characterization of placentas of control and Mecr mutants (A-D) Histology of placenta at E 9.5. Placentas associated with wild type (A), heterozygous (B) and homozygous fabI knock-in (C) embryos from Mecr<sup>tm(fabI)</sup> breedings and homozygous Mecr<sup>tm1d</sup>
knock-out (D). The fetal placenta is thinner and disorganized in homozygous mutant of $Mecr^{tm(fabI)}$ and $Mecr^{tm1d}$ mouse lines. Scale bar 200 μm. Dotted lines demarcate the placental layers; de, decidua, sp, spongiotrophoblast and la, labyrinth. (E and F) TEM image of mitochondria (arrows) in labyrinthine trophoblast of wild type (E) and mutant (F) placenta from $Mecr^{tm(fabI)}$ breedings. Scale bar 1 μm.

**Figure 6.** Fetal placental analysis. RNA *in situ* hybridization (A-D) and immunohistochemistry (E and F) of placentas associated with wild type (top) and *fabI* knock-in (bottom) embryos from $Mecr^{tm(fabI)}$ breedings. Riboprobes for a trophoblast giant cell marker (Pl 1) and a spongiotrophoblast marker (Tpbpa) are used. Trophoblast giant cell (A and B) and spongiotrophoblast (C and D) layers are markedly thinner in the mutants (B and D) than in wild type (A and C). Dotted lines demarcate maternal and fetal placenta. Scale bar 500 μm. (E and F) The folliculotropin (Fol) expression was negligible in fetal placenta of homozygous mutant (F) compared to wild type (E). Scale bar 200 μm. (G and H) RT-PCR analysis for PPAR target gene expression in total RNA samples from $Mecr^{tm(fabI)}$ placenta. (G) *Muc1*, PPARγ target gene, n= 5 per group. (H) *Mdfi*, PPARβ target gene, n = 6 per group. The amounts of mRNA were normalized using actin (Actb) as reference gene. Results are shown as means ± SD, independent samples t- test, * p< 0.05.

**Figure 7.** Morphology of heart at E10.5 in wild type and *fabI* knock-in embryos. (A and B) Histology of the embryo hearts. The atrium (a), ventricle (v) and endo-cardial cushion (e) are distinguishable in wild type embryos (A). The primitive atrial chamber (a) and ventricle (v) are the only distinguishable chambers in the heart of mutant embryos (B). Even though ventricular trabeculations can be seen in both embryos (arrows), heart development in mutant embryo was delayed compared to wild type control. Scale bar 200 μm. (C and D) μCT analysis of heart. The embryonic heart had 3-4 chambered appearance in control embryos (C) whereas 2 chambered in
mutant hearts (D). The encircled heart region is shown in insert. The distance between each purple dot is 500 µm.

**Abbreviations:**

ETR 2-enoyl-thioester reductase  
FAS Fatty acid synthesis  
KGD Ketoglutarate dehydrogenase  
LA Lipoic acid  
Mecr Mitochondrial enoyl-CoA/ACP reductase  
mtFAS Mitochondrial fatty acid synthesis  
PDH Pyruvate dehydrogenase  
PPAR Peroxisome proliferator-activated receptors  
RC Respiratory chain
Table S1: Embryonic dissections from E 7.5 to E 18.5 in *Mecr<sup>tm1a</sup>* line. The genotype of embryos is confirmed by PCR. Homozygous knock-out embryos were found dying between E 9.5 to E 15.5. Parenthesis shows percentage of embryos of each genotype.

<table>
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<tr>
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<td>5(17%)</td>
<td>14(48%)</td>
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<tr>
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<tr>
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<td>28</td>
<td>5(18%)</td>
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<tr>
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Table S2: Embryo dissections in $\text{Mecr}^{\text{imld}}$ and $\text{Mecr}^{\text{imfab}}$ mouse lines. Parenthesis shows percentage of embryos of each genotype. Some of the homozygous knock-out and knock-in embryos were dead at E. 9.5 or E 10.5 (a), and all of the homozygous mutants were dead and at varying resorption stage at E 11.5 (b).

<table>
<thead>
<tr>
<th>Day</th>
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<th>$\text{Mecr}^{\text{imfab}}$</th>
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<td>WT/1d</td>
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<tr>
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<td>3(16 %)</td>
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<td>E 13.5</td>
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Yeast studies

Generation of different yeast models

Plasmids YEplac195 (1), pYE352-CTA1 (2), pYE352::mtFabI (3) and *S. cerevisiae* strain W1536 8betrlΔ (4) have been described previously. The respiratory deficiency of *etr1Δ* yeast strains is partially rescued by expression of Mecr or mitochondrially localized *E. coli* fabI. In order to test the functionality of the double HA-tagged fabI chimera N-terminally appended with the Mecr mitochondrial targeting sequence (MTS) encoded by exon1/2 (MmMecr-fabI HAHA), strain W1536 8betrlΔ, was transformed with a construct expressing the corresponding protein, along with controls. Growth rescue by the YEpl195 (pCTA1)MmMecr-fabI HAHA was assessed by testing respiratory growth of these yeast transformants on SCG (synthetic complete glycerol), containing only a non-fermentable carbon source. Total cell extracts from the *etr1Δ* yeast strains carrying YEpl195 (pCTA1)MmMecr-fabI HAHA and control constructs were analyzed by western blotting and probed with anti HA antibody to visualize fabI-HAHA expression levels.

Construction of the yeast plasmids expressing the MmMecr-fabI chimera

The *E. coli fabI* gene was amplified from plasmid pYE352::mtfabI using oligos EcfabI fndXbaI: 5’-CATCTAGACCATGGGTTTTCTTTTCCG-3’ and EcfabIrevXhoI: 5’-GACTCGAGTTATTTTCAGTTGAGTTCGT-3’, cut with XbaI and XhoI and ligated into XbaI-XhoI-digested pYE-352-CTA1, from which the CTA1 ORF had been removed, to generate pYE-pCTA1-fabI.

The pYE-CTA1-fabI plasmid was digested with XbaI and PstI to remove a fragment of *fabI* from the ATG to the internal PstI site. DNA sequence containing sequences from mouse Mecr exon 1 and exon 2, fused to a sequence containing the *E. coli fabI* coding region from the ATG start codon (preceded by an XbaI restriction site) to the PstI site of the *fabI* ORF was synthesized by Mr Gene
Mr Gene, Regensburg, Germany, see sequence and structure below). This DNA was digested with XbaI and PstI and ligated into the XbaI/PstI – digested pYE-CTA1-fabl plasmid to generate YEp(pCTAI)MmMecrfabl, expressing a chimera consisting of the N-terminus of Mecr (Mecr MTS followed by several amino acids) fused to fabl.

The CTA1 terminator sequence was amplified from plasmid pYE352::mtfabl (3) using the following primers: 5’SphI-HA-STOP-tCTA1: 5’-ACATGCATGCTACCCCTACGACGTGCCCGACTACGCCTGATGAAAGTAACAAATGGCG-3’ and 3’ HindIII tCTA1: 5’-TAATAAGCTTTATGATGATCAAGAC-3’. This PCR produces a DNA fragment with a sequence encoding the HA tag, preceded by an SphI site on the 5’ end and two stop codons on the 3’ end followed by the yeast CTA1 terminator. This product was digested with SphI and HindIII and inserted into the SphI/HindII sites of plasmid YEplac195.

In a second PCR reaction, using primer 5’-EcoRI CTA1: 5’-TATAGAATTCTTAGAAGGTGAAGAAATAG-3’ and primer 3’ SphI HA XhoI fabl: 5’-ACATGCATGCGGCGTAGTCGGGCACGTCGTAGGGGTACTCGAGTTTCAGTTCGAGTTCGTTC-3’ and pYE352::mtFabI as template, a chimera was produced consisting of the EcoRI-preceded CTA1 promoter fused to a sequence encoding E. coli fabl enoyl reductase appended N-terminally with the yeast COQ3 mitochondrial targeting signal and C-terminally with an in-frame HA tag, followed by an SphI site. This PCR product was digested with EcoRI and SphI and ligated into the intermediate plasmid construct described in the previous paragraph. The resulting plasmid, YEp195 (pCTA1)mtfablHAHA expresses double-HA tagged, mitochondrially localized fabl from the yeast CTA1 regulatory sequences.

YEp(pCTAI)MmMecrfabl, sequence was cut with EcoRI and PstI and ligated into YEp195(pCTA1)mtfablHAHA, where the corresponding EcoRI/PstI fragment had been removed by restriction digest. The resulting plasmid YEp195(pCTA1)MmMecr-fablHAHA, expresses fabl
appended on the N-terminus by the *Mecr* mitochondrial targeting signal and tagged C-terminally by a double HA epitope. The sequence of the expressed chimera is identical to the sequence predicted to be expressed in the *fabl* knock-in mouse.

**DNA/protein sequences of the MmMecr-fabl HAHA construct**

**MmMecr-fabl complete**

TCTAGATCTAGAATGTTGGTCAGCCAGCGAGTGACAGGGGCGAGCCCGGGCACCCTCT
AGCTTGGCGGGTGCTCTCTCGAGGCTTGGTACCGCCAGCCGGCAGCCCGGGCACCATCTCTCTACTCC
GCTCTCTCTGACCGCACTACGGGGTGCGGGGCGCTGGTCTATGGCAACCATGGGGATCCAG
CCAAGGTCTGCTCAAGCTGAAGAACCTGGAGCTCACTGCTGTGGAAGGATCTGACGTCCA
CGTGAGGATGATGTTTCTTTTCTCCCGGTAAGCGCATTTCTGTGTAACCCCGGTGTTGCGGAGAATCC
TATCCATCGGCGTACGGATTCTCGAGGCATGCACCAGCGAACCCGGGCTCAATTGGGT
TCTGACATCGTTCTGCGAGTGCGATGTTGCAGAAAGATGCCACAGCATGCAACCCATGTTCCGC
TGAACCGGGAAGGTTTGCGCAGAAAATCTGAAAGGCGGGCGTAGACATCCGAGTCTGACGTCCA
CTGGGCGATACGCTGGATGGTGACTATGTTAACCCCGGTGAAGGCTTCCACCAAAAT
GCCACAGCACATCGCTCTACAGCTTCTGGTCAGAAGGCTTCCCTCCGCTCCAGAGTCG
GAATCCGGGATGCTGAGCTGGAGCTGGAGTTAAGCGGTGGAGGCTTCTCTATTGGTCCAG
ACTACACGGGGTGTCTGGGACGGATGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT GG
**Yellow**: Mecr exon 1 sequence

**Green**: Mecr exon 2 sequence

**Turquoise**: HA tag encoding sequence

**Underlined**: synthesized sequence

RMLVSQRVTGARARAPQLAGLLEAWYRHGRTTSSYSALSEPSRVRALVYGNHGDPAK

VVQ

LKNLELTAVEGSDVHVRMGFLSGKRILVTGVASKLSIAYGIQAQMHRGAEELAFTYQNDK

LKGRVEEFAAQLGSDIVALQCDVAEDASIDTMFAELGKVWPKFDGFVHSIGFAPGDQLDGD

YVNAVTREGFKIAHDISSYSFVAMAKACRSMLNPGSALLTLSYLGAERAIPNYVNMGLAK

ASLEANVRYMANAMGPEGVRVNAISAGPIRTLAAASGKDFRKMHLHCEAVTPRRTVTIE

DVGNAAFLCSDLSSAGISGEVHVDDGFSIAAMNELELKLEYPYDVPDYAACPYDVPDY

A STOP STOP

**Yellow**: Polypeptide sequence encoded by Mecr exon 1

**Green**: Polypeptide sequence encoded by Mecr exon 2

**Turquoise**: HA tags

**Underlined**: predicted mitochondrial targeting sequence

**Mitochondrial targeting prediction for Mecr-fabIHAHA construct**

**targetP analysis**

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<td>0.019</td>
<td>0.105</td>
<td>M</td>
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MitoProt analysis

Probability of export to mitochondria: 0.3795

Cleavage site: 54

Cleaved sequence:
MLVSQRVTGARARAPQLAGLLEAWYRHGRTSSYSALSEPSRVRALVYGNHG

Construction of MmMecrfabI HAHA expression plasmids for cell culture studies

The YEp195(pCTA1)MmMecr-fabIHAHA plasmid used in yeast studies was used to construct
mecr::fabIHAHA expression plasmid for cell culture studies. The Mecr-fabIHAHA in the
YEp195(pCTA1)MmMecr-fabIHAHA was amplified with primers 5’-
CACTGTAATGTCCAAATCG

ATTAGATATCGCTAGCAAAACAACGCCACTCATTTG-3’. The PCR product was digested with
XbaI and EcoRV and inserted to pcDNA6/myc-His plamid (Addgene) and the resulting plasmid
pcDNA6 MmMecr-fabIHAHA was used for studies. A pcDNA3 control plasmid expressing the HA
epitope (pcDNA3-HA) was constructed by inserting a double stranded linker into the XbaI and
ApaI sites of the plasmid polylinker. The sequences of the oligos constituting the linker construct
were (sense): 5’-
CTAGAGGAGGAGGAATGTACCATACGATGTCCAGATTACGCTTAGGGCC

(antisense): 5’-CTAAGCGTAATCTGGAACATCGTATGGGTACATTCCTCCTCCTCCT-3’.

Localization and enzyme activity of Mecr-fabIHAHA fusion protein in cell culture

To study the mitochondrial localization of the Mecr-fabI fusion protein, HEK293T cells grown on
Dulbecco’s Modified Eagle’s media (Gibco, Finland) supplemented with 10% foetal bovine serum
(GE Healthcare, Pasching, Austria) and 100 U/ml penicillin and 100µg/ml streptomycin (Sigma).
The cells grown on coverslips were transfected with the plasmid pcDNA6 MmMecr-fabI HAHA
and the pcDNA3-HA control plasmid by calcium phosphate precipitation method (5). On the next day, cells were incubated with 200 nM MitoTracker Red CMXRos (Molecular Probes Europe, Leiden, Netherlands) at 37°C for 20 mins and fixed with 4% paraformaldehyde in PBS at room temperature for about 10 minutes. The cells were treated with 0.1% saponin and 1% BSA in PBS, followed by incubation with monoclonal HA antibody (1:1000, Sigma) and anti-mouse Alexaflour 488 (1:500, Thermo Scientific) secondary antibody. The stained cells are mounted with ImmuMount (Thermo Scientific). Samples were imaged using Zeiss LSM 700 confocal microscope, Zen Software (Carl Zeiss, Oberkochen, Germany) with 40X Apo oil immersion objectives.

For the Mecr-fabI fusion protein activity assays, 100 mm plates of HEK 293 cells were transfected either with pcDNA6 MmMecr-fabI HAHA or empty pcDNA6/myc-His plasmid by the calcium phosphate precipitation method as described above. After 24 hours, cells from four plates with respective plasmids were collected and pooled together. The cell pellets were washed once with PBS and homogenized with Potter homogenizer in 0.22M mannitol, 70 mM sucrose, 10 mm HEPES, 1 mM EGTA, pH 7.5 containing protease inhibitor cocktail (cOmplete Tablet EDTA-free, Roche). The homogenate was centrifuged at 560g for 10 min at 4°C twice. The supernatant was recentrifuged at 10,000g for 10 mins at 4°C. The resulted pelleted mitochondrial fractions were suspended in 50mM NaPi buffer pH 7.4 containing 0.05% Triton X-100.

The pcDNA6 MmMecr-fabI HAHA-borne enoyl reductase activity in mitochondrial preparation was measured by monitoring the oxidation of NAD(P)H using a JASCO V-660 spectrophotometer at 22°C. The assay mixture consisted of 50 μM NADH or NAD(P)H in 50mM NaPi pH 7.4 with 0.05% Triton X-100 and the reaction was started by adding 2E-hexenoyl-CoA to the final concentration of 60 μM.
Generation of shRNA mediated knock down of mouse NIH3T3 cell lines

Mouse NIH3T3 cell line was used to generate a Mecr RNAi mediated knock down cell lines. A retroviral vector was used to generate two different constructs that produce shRNAs each targeting mouse Mecr encoded mRNA in different locations, as described previously (6). Using this method, two shRNA knock down cell lines were generated ( Mecr shRNA 1 and Mecr shRNA 2), as well as a negative control cell line transfected with the empty retroviral vector (RVH1 control). The oligonucleotide pairs (Sigma-Aldrich, St. Louis, MO) used to construct the expression cassette were as following:

For the cell line  Mecr shRNA 1, (sense) 5’-
GATCTCCGCACTGATTGGAATCCCTAAGTTCAAGAGACTTAGGGATTCGAATTCATCAGTGCTTTTGGAAC-3’ and (antisense) 5’-
TCGAGTTCCAAAAAGCACTGATTGGAATCCCTAAGTTCTCTTTGAACAGTTCTTCAATGCGGA-3’, and for cell line  Mecr shRNA 2 (sense) 5’-
GATCTCCGGATCTAGGAGCTGATTATGTTTCAAGAGAACAATCAGCTCTTAGATCCTTTTGGAAC- 3’ and (antisense) 5’-
TCGAGTTCCAAAAAGGATCTAGGAGCTGATTATGTTTCTTTGAAACATAATCAGCCTCCTAGATCCGGA-3’.

Whole cell protein lysate from the cell lines was extracted using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) and separated by SDS-PAGE and transferred to nitrocellulose membrane as described in Material and Methods in the main text. In the western blot assay for Mecr level, the same Mecr antibody (Cat. Nr 51027-2-AP, Proteintech, Chicago, IL) as in our embryonic extract studies was used. In the loading control assay for actin, monoclonal actin antibody (Novus Biologicals, Littleton, CO) was used.
Results

Yeast studies

When the \textit{etr1Δ} yeast cells were transformed with \textit{fabI} alone, double HA tagged \textit{fabI} or \textit{MmMecr-fabI} HAHA construct, the cells regained the ability to grow in SCG media, indicating that the rescue of the respiratory competence had occurred (Supplementary Figure, Fig. S2A). The western blotting studies showed that extract from cells expressing \textit{fabI} appended N-terminally with the yeast Coq3 mitochondrial targeting signal (MTS) (3) revealed a single band of the expected size (Fig.S2B, lane 1). Extract from \textit{etr1Δ} cells transformed with \textit{YEpl95 (pCTA1)MmMecr-fabI} HAHA exhibited multiple bands, with a faint band corresponding to HA-tagged \textit{fabI} and several stronger bands of lower molecular mass (Fig. S2B, lane 3). This apparent degradation is likely to be due to inefficient recognition of the mouse MTS by the yeast mitochondrial import machinery. We have found that appending human \textit{Mecr} or the components of mammalian mitochondrial ketoreductase HSD8 and CBR4 (7, 8) vastly improves their potency of complementing mtFAS mutants with yeast MTS. The degradation was not visible in the \textit{fabI} knock-in embryos.

Mecr-fabI expression in cultured mammalian cells

The immunostaining of HEK 293T cells transfected with pcDNA6 MmMecr-fabI HAHA plasmids with HA antibody displayed punctuated signaling pattern in cytoplasm. The superimposition of this signal with signal arising from Mitotracker staining of the same cells showed an overlapping pattern suggesting the mitochondrial localization of MmMecr-fabI HAHA in the cells. The immunostaining of HEK 293T cells transfected with control pcDNA3-HA plasmids with HA antibody visualized large focal intracellular fluorescence areas. This signal did not colocalize with the Mitotracker fluorescence and therefore we suggest that in these cells the signal detected with the HA antibody arose from oligopeptide aggregates (Supplementary Figure, Fig.S3A). The \textit{fabI} from \textit{E. coli} shows high preference towards NADH over NADPH in reduction of 2E-enoyl-CoA substrate to their
saturated counterparts (9). NADH-dependent enoyl reductase activity in mitochondria enriched fractions derived from HEK 293T cells transfected with MmMecr-fabI HAHA and control plasmids were 0.353±0.17 µmol/min per mg of protein and 0.075±0.04 µmol/min per mg of protein (values in means ± SD, n=5, p=0.014), respectively (See this data presented as box whisker plot, Supplementary Figure S3B). If NADH was replaced by NADPH, the 2-enoyl reductase activity was below detection limits of the assay system used.

Validation of polyclonal Mecr antibody

To validate Mecr antibody, two approaches are used. In the first approach, western blot assay was applied to determine the effect of the Mecr - transcript-specific shRNA mediated knock downs on the intensity of the visualized protein band with predicted molecular mass (36 kDa). There was clear reduction in the signal of visualized 36 kDa band in Mecr shRNA 1 and Mecr shRNA 2 cell lines (Supplementary Figure, Fig. S7A). In the second approach, samples from Mecr\textsuperscript{tm1a} embryos gave no signal for homozygous mutants whereas in the western blotting of wild type and heterozygous embryos, the size of the visualized band matched with the band of predicted molecular mass and also obtained from yeast extract expressing recombinant human MECR (5) (Supplementary Figure, Fig. S7B).

Supplementary Material References


Legends to Supplementary figures

**Figure S1:** Percentage of living homozygous mutant mice embryos during embryonic development.

The percentages of live homozygous mutant embryos start to decline sharply after E 9.5. However, few live embryos could be recovered until day E 14.5 in *Mecr*<sup>tm1a</sup>. No live homozygous mutant embryos were found for *Mecr*<sup>tm1d</sup> and *Mecr*<sup>tm(fabI)</sup> after E 11.5.

**Figure S2.** Studies on complementation of *etr1Δ* yeast strain by bacterial *fabI*. (A) The yeast strains grown on SCG media to assess the complementation of respiratory activity by constructs. i, wild type yeast cells; ii, *etr1Δ* strain as negative control; iii, *etr1Δ* strain complemented with *fabI* construct; iv, *etr1Δ* strain complemented with double HA tagged *fabI* construct; v, *etr1Δ* strain complemented with double HA tagged *MmMecr-fabI* construct. All the constructs rescued respiratory deficiency in the *etr1Δ* strain (partially if not completely). (B) Western blotting for HA tag expression. Lane 1, *etr1Δ* strain complemented with double HA tagged *fabI* construct; lane 2, *etr1Δ* strain complemented with *fabI* construct and lane 3, *etr1Δ* strain complemented with double HA tagged *MmMecr-fabI* construct.

**Figure S3:** Studies on subcellular localization and enoyl reductase activity borne from *MmMecr-fabI* HAHA construct in HEK 293T cells. (A) Indirect immunofluorescence studies of HA tagged protein localization in cells carrying construct and control plasmids are shown in left panels. The middle panels display the cellular mitochondrial network visualized with Mitotracker. In the panels on the right, the images of the left and middle panels are merged, showing colocalization of Mecr-fabI fusion protein signal with Mitotracker staining. (B) The NADH-dependent fabI activity in HEK 293T cells transfected with either *MmMecr-fabI*HAHA construct or control plasmid. Box whisker plot shows the data from 5 independent biological experiments. The p-value = 0.014.

**Figure S4:** A qPCR for *Mecr* mRNA expression in *Mecr* mutants. (A, B, C) *Mecr* mRNA in embryos produced from *Mecr*<sup>tm1a</sup>, *Mecr*<sup>tm1d</sup> and *Mecr*<sup>tm(fabI)</sup> heterozygous mothers, respectively. The
amount of mRNA was normalized using actin (Actb) as reference gene. Results are shown as means ± SD, n= 5 per group, independent samples t-test, **** p< 0.0001 *** p< 0.001, ** p< 0.01, * p< 0.05. The apparent increase of Mecr transcript in the Mecr<sup>tm1d</sup> derived samples compared to Mecr<sup>tm1a</sup> and Mecr<sup>tm(fabI)</sup> samples is likely due to contamination from maternal tissue, as it was very difficult to cleanly separate these embryos from the yolk sac.

**Figure S5.** Protein lipoylation example in Mecr<sup>tm1d</sup> and Mecr<sup>tm(fabI)</sup> embryos. Western blot analysis for lipoylated proteins in protein extract from embryos at E 9.5. (A) Protein extract from Mecr<sup>tm1d</sup> embryos lane (1 WT/WT, lane 2 1d/WT, lane 3 1d/1d). (B) Protein extract Mecr<sup>tm(fabI)</sup> embryos (lane 1 WT/WT, lane 2 fabI/WT, lane 3 fabI/fabI). The lack of lipoylated proteins in homozygous 1d mutants indicates that LA synthesis is impaired in the mutants. The lack of Mecr and presence of some amount of lipoylated Dlat protein in homozygous fabI knock-in mutants, indicating partial complementation of 2-enoyl thioester reductase activity by fabI.

**Figure S6.** Generation of Mecr<sup>tm(fabI)</sup> construct. (A) Nucleotide sequence of modified exon 2 of Mecr<sup>tm(fabI)</sup> construct. The sequence contains 54 nucleotides at 5’- end of exon 2 of Mecr (pink) followed by whole fabI with double HA tag (cyan), stop codons (red) a small linker sequence (grey) and a poly A signal. (B) Genotyping of DNA from ES cells. The upper band represents the amplicon from mutant DNA and the lower band corresponds to the wild type DNA PCR product. (C) Southern blotting of ES cells to confirm recombination. Lanes 1-5, DNA from heterozygous ES cells and lane 6- wild type control DNA. (D) Immuno histochemistry for Mecr expression in foetal part of placenta associated with wild type (left), heterozygous (center) and homozygous Mecr<sup>tm(fabI)</sup> embryos (right) at E 9.5. Mecr expression is negligible in foetal placenta for all genotypes compared to maternal placenta. Dotted line demarcate maternal and foetal placenta. Scale bar 200 µm.

**Figure S7.** Validation of Mecr antibody by western blotting. (A) Total protein isolated from NIH3T3 cells. Lane 1, control cell line; lanes 2 (Mecr shRNA 1) and 3 (Mecr shRNA 2), Mecr
knock down cell lines. The blot was probed with Mechr antibody and actin antibody. (B) Total proteins from embryos of different genotypes from Mechr<sup>tm1a</sup> breedings and yeast expressing human MECR were analyzed by western blotting probing with Mechr antibody. The shown data are from same blot, but the exposure time for visualization of the embryonic protein signals were 5 min, while the signal from the recombinant MECR expressed in yeast was 5 sec.
A

B

C
Nair RR et al 2017 Fig. 5
Fig. 1: Graph showing the percentage of live embryos observed on a particular embryonic day.

Fig. 2: HA blot showing bands at 28 kDa and 42 kDa with Actin as a control.
A

Nair RR et al 2017 supplementary Fig. 3

mecr::fabI

Control

B

p=0.014

NADH-dependent fabI activity (μmol/min per mg of protein)
Nair RR et al 2017 supplementary Fig. 4

A

Transcription level of Mecr

Genotype

1a/1a  100NT  WT/WT

B

Transcription level of Mecr

Genotype

1d/1d  100NT  WT/WT

C

Transcription level of Mecr

Genotype

tau/tau  tau/WT  WT/WT