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MAP4K4 impairs energy metabolism in endothelial cells and promotes insulin resistance in obesity

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Abstract

The blood vasculature responds to insulin, influencing hemodynamic changes in the periphery, which promotes tissue nutrient and oxygen delivery and thus metabolic function. The lymphatic vasculature regulates fluid and lipid homeostasis, and impaired lymphatic function can contribute to atherosclerosis and obesity. Recent studies have suggested a role for endothelial cell (EC) Mitogen activated protein kinase kinase kinase kinase 4 (Map4k4) in developmental angiogenesis and lymphangiogenesis as well as atherosclerosis. Here, we show that inducible EC Map4k4 deletion in adult mice ameliorates metabolic dysfunction in obesity despite the development of chylous ascites and a concomitant striking increase in adipose tissue lymphocyte content. Despite these defects, animals lacking endothelial Map4k4 were protected from skeletal muscle microvascular rarefaction in obesity, and primary ECs lacking Map4k4 displayed reduced senescence and increased metabolic capacity. Thus, endothelial Map4k4 has complex and opposing functions in the blood and lymphatic endothelium post-development. Whereas blood endothelial Map4k4 promotes vascular dysfunction and impairs glucose homeostasis in adult animals, lymphatic endothelial Map4k4 is required to maintain lymphatic vascular integrity and regulate immune cell trafficking in obesity.

67   **Introduction**

68   Type 2 diabetes (T2D) is associated with impaired glucose homeostasis, insulin

69   resistance and an increased inflammatory state (16, 35, 43, 46). Though much work has

70   sought to investigate the mechanisms by which T2D occurs and how to ameliorate this

71   disease, there is still a lack of mechanistic understanding of the contribution of individual

72   physiological systems to disease progression. In particular, the vascular endothelium

73   has been investigated as a contributor to T2D pathology (18, 19, 41). Endothelial cells

74   line the blood and lymphatic vasculature within every tissue, and the vascular

75   endothelium has a unique role to communicate nutritional and inflammatory status of

76   underlying tissue to the systemic circulation by delivering nutrients, hormones and

77   oxygen (1). Conversely, the lymphatic endothelium regulates fluid balance, intestinal

78   lipid absorption, and immune function (7, 25). Recent human and mouse genetic studies

79   have suggested that the endothelium plays a multifaceted role in whole body

80   metabolism (4, 18, 19, 34, 37).

81   In obesity the endothelium becomes resistant to the hormone insulin, which

82   impairs blood flow and thus insulin and nutrient transport to tissues such as skeletal

83   muscle resulting in increased plasma glucose levels and type 2 diabetes (19, 47).

84   Furthermore, lymphatic vessels within adipose tissue can become leaky and further

85   exacerbate obesity by promoting adipogenesis (17, 32). Finally, type 2 diabetes is also

86   associated with low-grade inflammation in multiple tissues including adipose tissue and

87   liver (16), which is mediated in part by immune cell recruitment from the peripheral

88   vasculature (22, 23, 29, 30). Thus, identifying molecular targets within the vasculature

89   that mediate these dysfunctions are important for improving human health.

90   Our laboratory recently demonstrated that inducible, systemic loss of the protein

91   kinase Mitogen activated protein kinase kinase kinase kinase 4 (Map4k4) in adult obese

92   mice improved insulin sensitivity and regulated insulin secretion (8, 26). Using

93   constitutive endothelial-specific knockout animals, we demonstrated that Map4k4 has a

94   profound and complex role to control lymphatic vascular development (27); however,

95   inducible endothelial Map4k4 deletion was beneficial in *Apoe*^{-/-} mice, as *Apoe*^{-/-} mice

96   lacking Map4k4 (28) demonstrated reduced atherosclerotic plaque development and

97   reduced leukocyte recruitment. We thus hypothesized that obese C57BL6/J mice

98   lacking endothelial Map4k4 might also display reduced obesity-induced adipose tissue

99   inflammation and therefore improved insulin sensitivity.

100   The present studies were designed to bypass the developmental phenotype

101   recently reported in constitutively expressed Cdh5 cre (Ve-Cadherin cre) Map4k4

102   endothelial-specific knockout mice (27) by generating inducible endothelial-specific

103   Map4k4 deletion in adult C57BL6/J mice using the tamoxifen-induced Cdh5(PAC)ERT2-

104   cre (44). Interestingly, chow-fed animals displayed no overt phenotype; however, when

105   challenged with a high fat diet (HFD), inducible endothelial-specific Map4k4 knockout

106   mice (M4K4 iECKO) displayed a non-significant trend to improved glucose tolerance

107   and significantly enhanced insulin sensitivity compared with controls. Despite this

108   metabolic improvement in M4K4 iECKO mice, these animals also displayed lymphatic

109   defects as noted by chyle leakage in the abdomen (chylous ascites) and increased

110   immune cell content in epidiymal adipose tissue. Despite these lymphatic defects,

111   skeletal muscle capillary density was maintained after HFD in M4K4 iECKO mice

112   compared with controls, and isolated endothelial cells derived from these animals

113 displayed enhanced energy metabolism and protection from senescence. Taken

114 together, these results demonstrate a complex and critical role for endothelial Map4k4

115 to maintain lymphatic vascular integrity yet promote systemic insulin resistance in

116 obesity.

117

118 **Materials and Methods**

119

120 **Mouse models:** The University of Massachusetts Medical School Institutional Animal

121 Care and Use Committee approved all of the animal procedures. Map4k4 Flox/Flox

122 animals and M4K4 iECKO mice (Cdh5(PAC)-CreERT2) were maintained on a pure

123 C57Bl6/J background after at least 8 backcrosses and have been previously described

124 (28). At 6-8 weeks of age, male Flox/Flox and Flox/Flox/cre+ littermates were injected

125 with 1 mg tamoxifen/day in corn oil (Sigma) for 5 days; 2 weeks after tamoxifen

126 injection, the mice were fed standard chow or high fat diet (60% fat, Research diets

127 RD12492i) for 16 weeks. Mice were euthanized by CO_2 inhalation followed by bilateral

128 pneumothorax. Mice were fasted for 16 h for GTTs or for 4 h for ITTs. Fasted mice were

129 i.p. injected with glucose (1 g/kg) or insulin (1 IU/kg). Blood glucose levels were

130 determined from the tail vein using a Breeze-2 glucose meter (Bayer). No statistical

131 methods were used to predict sample size, no randomization was performed, and the

132 investigations were not blinded.

133

134 **Miles assay:** 4 weeks after tamoxifen injections, mice were injected intravenously (i.v.)

135 with 1.5% Evans Blue Dye (50 mg/kg) into the tail vein. 30 mins post-injection, mice

136   were sacrificed by cervical dislocation and perfused with saline for 10 minutes. Pieces

137   of inguinal white adipose tissue (WAT), epididymal WAT, lung and liver were excised,

138   weighed and incubated in formamide overnight at 55°C with shaking to extract the dye.

139   Absorbance of the extravasated dye was quantified by spectrophotometry at 620 nm

140   and normalized to tissue weight.

141

142   **RNA isolation and quantitative RT-PCR:** Total RNA was isolated, cDNA was

143   prepared, and quantitative RT-PCR was performed as previously described (28).

144   Primer sequences are detailed in **Table 1.**

145

146   **Immunostaining:** Whole mount staining was performed on tissues that had been fixed

147   in 10% formalin for 2-6 hours. Adipose tissue, skeletal muscle or aortic rings were

148   blocked overnight in 10% BSA and 0.3% triton X-100 in PBS at 4C, stained overnight

149   with Isolectin B4 (Life Technologies I21411; 1:40) in 100 mM MgCl2, 100 mM CaCl2, 10

150   mM MnCl2, and 1%Triton X-100 in PBS at 4C, and washed 3x 20 minutes in 5% BSA,

151   0.15% triton X-100 in PBS at room temperature. Tissues were mounted in ProLong

152   Gold (Life Technologies). Whole mount images were visualized in flattened 25 um z-

153   stacks with a Solamere Technology Group modified Yokogawa CSU10 spinning disk

154   confocal system with a Nikon TE-2000E2 inverted microscope at 10x or 20x. Images

155   were acquired with MetaMorph Software, version 6.1 (Universal Imaging, Downingtown,

156   PA). A Zeiss Axiovert 100 inverted microscope with a 5x or 10x objective and AxioCam

157   HRm camera was used for aortic ring assay images. At least 3 muscle or adipose tissue

158   images were quantified and averaged for average vascular density per mouse. Images

159 were quantified using Image J Analysis Software. Different mice were used for the

160 analyses of different tissues.

161

162 **Senescence Assays:** Primary mouse lung endothelial cells (MLECs) were prepared

163 by digestion and immune-isolation using CD31 and ICAM-2-coated magnetic beads as

164 previously described (28). MLECs were used on passage 3 for all experiments.

165 Senescence was assessed in MLECs that had grown to confluence with the beta

166 galactosidase senescence assay kit (Cell Signaling) according to manufacturer's

167 instructions. Percent positive staining was determined in at least 3 5x fields using image

168 J software. Nuclei were stained with Hoechst (Sigma), and the entire well was imaged

169 using the In Cell high content imager and counted using Columbus Image data analysis

170 software. Percent positive area was normalized to nuclei number.

171

172 **Flow Cytometry:** The epididymal adipose tissue stromal vascular fraction (SVF) was

173 isolated by digestion in Hanks balanced salt solution, 2.5% BSA and 2 mg/mL

174 collagenase for 45 minutes and strained through a 70 µm filter followed by red blood cell

175 lysis (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA). Cells were blocked with mouse

176 IgG in FACS buffer (1% BSA/PBS). Cells were stained with antibodies directed towards

177 F4/80 (APC, ABd serotec), CD11b (Percp 5.5, BD), Siglec F (PE, BD), GR-1 (APC-Cy7,

178 BD), Ly6c (PE-Cy7, BD), Galectin 3 (FITC, BioLegend), and CD11c (V450, BD) or CD3

179 (PE-Cy7 or APC-Cy7, BD), CD4 (FITC, BD), CD8 (Percp, BD), CD25 (APC-Cy7, BD),

180 Foxp3 (PE, BD), CD19 (V450, BD), or NK1.1 (APC, BD). Foxp3 staining was performed

181 using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to

182   manufacturer's instructions. All antibodies were used at a 1:200 dilution. The data were

183   collected on an LSRII (BD) and were analyzed with FlowJo software.   Samples were

184   gated for scatter and single cells. Lymphocyte populations were gated first on low size

185   and scatter prior to gating for positive staining. Gates were drawn based on

186   fluorescence minus one (FMO) controls.   A total of at least 100,000 events were

187   recorded.

188

189   **Cellular metabolism measurements:** Confluent cell monolayers were obtained by

190   seeding 60,000 MLECs overnight on 0.2% gelatin-coated Seahorse XF24 tissue culture

191   plates (Seahorse Biosciences).   Seahorse glycostress or mitostress tests were

192   performed using the standard protocol according to manufacturer's instructions on an

193   XF24 Seahorse extracellular flux analyzer. Drug concentrations were as follows:

194   Glucose (10 mM), Oligomycin (2.5 uM), 2-DG (50 mM), FCCP (1 uM), Antimycin A and

195   Rotenone (0.87 uM). Oxygen consumption rate (OCR) or extracellular acidification rate

196   (ECAR) values were normalized to protein content as assessed by BCA assay

197   (Thermo-Pierce). Each OCR or ECAR value represents an average from duplicate

198   wells.

199

200   **Statistical Analysis:** A two-tailed Student's t-test was used to compare two groups in

201   GraphPad Prism 6.0 or 7.0. Where indicated, experiments comparing multiple groups

202   were analyzed with two-way ANOVA with repeated measures. $P < 0.05$ was considered

203   to be statistically significant, and $P = 0.05$-0.09 was considered to be a non-significant

204   trend. Variance was estimated using the standard error of the mean.

205

206 **Results**

207   **Inducible EC Map4k4 loss in adult obese mice improves glucose tolerance.**

208 To bypass the lethal effects of Map4k4 deletion in development, Map4k4 was deleted

209 inducibly with tamoxifen in adult C57Bl6/J mice between 6-8 weeks of age (M4K4

210 iECKO; Fig. 1A). The deletion pattern of these mice using this protocol has been

211 previously reported and is nearly 100% after 18 weeks as assessed in primary mouse

212 lung ECs (MLECs) (28). Inducible Map4k4 deletion in these mice on a chow diet

213 revealed no obvious phenotypes, and no change in body weight was observed between

214 genotypes (Fig. 1B). Both Flox/Flox and M4K4 iECKO animals gained weight to a

215 similar extent on HFD, and no alteration in body weight or tissue weights was observed

216 and weights of all tissues including subcutaneous adipose tissue (SAT), epididymal

217 white adipose tissue (eWAT), liver and spleen were unchanged between HFD-fed

218 control and M4K4 iECKO mice (Fig. 1B-F).

219   Glucose tolerance and insulin sensitivity were next assessed in chow- and HFD-

220 fed Flox/Flox and M4K4 iECKO mice. In chow-fed Flox/Flox and M4K4 iECKO animals,

221 no alterations were observed in glucose tolerance or insulin sensitivity (Fig. 1G-H).

222 However, after HFD, Map4k4 iECKO animals demonstrated a non-significant trend to

223 improved glucose tolerance compared with Flox/Flox controls (Fig. 1G) and a significant

224 improvement in HFD-induced insulin resistance (Fig. 1H). We have previously reported

225 that mice inducibly lacking whole body Map4k4 had dramatic reductions in insulin levels

226 after HFD, which contributed to improvements in insulin sensitivity (26). Interestingly, a

227 non-significant trend to reduced insulin levels was also observed in M4K4 iECKO mice

228   after HFD compared with Flox/Flox controls (Table 2), suggesting that these animals

229   are in fact less insulin resistant than control littermates. Taken together, these data

230   suggest that M4K4 iECKO mice on HFD demonstrate enhanced insulin sensitivity

231   compared with control Flox/Flox littermates.

232   **Reduced expression of inflammation genes in liver of HFD-fed M4K4 iECKO**

233   **mice.** The intriguing improvement in insulin sensitivity in M4K4 iECKO mice suggested

234   that M4K4 expression within the vasculature was detrimental to metabolic homeostasis.

235   We had previously demonstrated that endothelial Map4k4 promoted vascular

236   inflammation in atherosclerosis by promoting leukocyte recruitment (28). Thus, we

237   hypothesized that similar mechanisms may be at play in obesity, and assessed whether

238   loss of endothelial Map4k4 ameliorated HFD-induced inflammation.

239   Histological assessment of liver from Flox/Flox and M4K4 iECKO mice revealed

240   no remarkable differences between genotypes after HFD (Fig. 2A). However, reduced

241   mRNA expression of adhesion molecule *Icam-1* was observed, and there was a non-

242   significant trend to reductions in levels of adhesion molecules *Vcam-1*, and *Selp* as well

243   as immune cell marker *F4/80* in whole liver of HFD-fed iECKO mice compared with

244   controls (Fig. 2B,C), which is consistent with previous observations that endothelial

245   Map4k4 promotes immune cell recruitment and inflammation (28).

246   **HFD-induced chylous ascites and eWAT immune cell content in M4K4**

247   **iECKO mice**. Visual inspection, flow cytometry assessment and histological

248   assessment of eWAT from chow-fed control and M4K4 iECKO mice did not reveal any

249   significant changes in appearance or immune cell content (Figure 3 and not shown).

250   However, chylous ascites was observed in approximately 50% of HFD-fed M4K4 iECKO

251   mice (Fig. 3A). In addition, the HFD-fed M4K4 iECKO mice displayed a striking increase

252   in adipose tissue immune cell infiltration in eWAT after HFD, even in animals where no

253   noticeable chyle leakage was present (Fig. 3B). This observation was surprising

254   because increased eWAT inflammation in obesity is associated with glucose intolerance

255   and insulin resistance in humans and in mice (16), and M4K4 iECKO mice displayed

256   enhanced insulin sensitivity on HFD (Fig. 1G-H). This phenotype seemed to be confined

257   to eWAT, as retroperitoneal white adipose tissue (rWAT), mesenteric WAT (mWAT),

258   subcutaneous WAT (SAT), and intrascapular brown adipose tissue (BAT) histology

259   revealed no significant alterations in tissue morphology between the genotypes after

260   HFD (Fig. 3C).

261   Numerous studies have demonstrated that macrophages are the predominant

262   immune cell type in obese AT and accumulate in crown-like structures that surround

263   adipocytes (45, 48). However, the inflammatory phenotype observed within the M4K4

264   iECKO eWAT was not characteristic of crown-like structures and instead resembled

265   dense cell clusters with small, dark nuclei (Fig. 3B). This observation coupled with the

266   chyle leakage observed in M4K4 iECKO mice (Fig. 3A) suggested that this cell

267   population might be atypical. To elucidate what cell types comprised the immune cells

268   within the adipose tissue, the stromal vascular fraction (SVF) was isolated from eWAT,

269   and flow cytometry was performed. Interestingly, no significant differences in the SVF

270   CD11b+/F4/80+ macrophage populations were observed between Flox/Flox and M4K4

271   iECKO mice, nor were there differences in the CD11b+/F4/80+/Cd11c+ pro-

272   inflammatory macrophage population (Fig. 4A, C). Furthermore, mRNA expression of

273   macrophage markers *F4/80*, *Itgam*, *Itgax* and *Cd68* and macrophage-derived cytokines

274   *Ccl-2, Il-1β, Il-6, and Tnf-α* were not altered in control and iECKO HFD-fed mice (Fig.

275   4D).

276   Lymphocyte populations were then assessed within eWAT of Flox/Flox and

277   M4K4 iECKO mice. Flow cytometry of eWAT-derived SVF revealed a significant

278   increase in lymphocyte populations, as a 72% increase in Cd19+ B lymphocytes (3%

279   vs. 5.1% of SVF for Flox/Flox or M4K4 iECKO mice, respectively, Fig. 4E) and a 47%

280   increase in total Cd3+ T lymphocyte content (12.5 vs. 18.4% of SVF for Flox/Flox or

281   M4K4 iECKO mice, respectively, Fig. 4E) was observed in M4K4 iECKO mice. Further

282   assessment of T lymphocyte subsets within SVF revealed that pro-inflammatory Cd8+

283   as well as anti-inflammatory Cd4+ and Treg (Cd25+/Foxp3+) lymphocyte populations

284   were significantly enhanced in M4K4 iECKO SVF by a similar extent (Fig. 4B, E).

285   Analysis by qRT-PCR of whole eWAT also demonstrated a significant enhancement of

286   T lymphocyte markers *Cd4* and *Cd8* and a non-significant trend to an increase in *Foxp3*

287   gene expression (p=0.09) in M4K4 iECKO eWAT after HFD, but interestingly there was

288   no concomitant increase in T-cell derived cytokines *Il-4, Il-10, Ifn-γ, Il-13, Il-17*, or *IL-21*

289   (Fig. 4F). These data suggest that the lymphocyte accumulation in M4K4 iECKO eWAT

290   after HFD may be a passive accumulation of naïve lymphocytes a consequence of

291   chyle leakage into the abdominal cavity. These data also suggest that although chyle

292   leakage is only visually present in 50% of animals, there is likely lymphatic leakage and

293   dysfunction in all of the M4K4 iECKO animals, even if no chyle is observed by eye.

294   **Maintained blood vascular integrity and reduced microvascular rarefaction**

295   **in skeletal muscle of M4K4 iECKO mice.** Lymphatic dysfunction promotes obesity

296   and is associated with metabolic disease in mice (17, 32). Thus, it is intriguing that

297 despite their lymphatic chyle leakage defects, M4K4 iECKO mice are glucose tolerant

298 and insulin sensitive compared with controls. However, the VE Cadherin promoter used

299 to generate M4K4 iECKO mice deletes genes in both blood and lymphatic endothelial

300 compartments (36). Thus, we cannot exclude the possibility that the improved

301 metabolism in the M4K4 iECKO mice is due to changes in blood vascular ECs. Previous

302 studies using cell culture models demonstrated that Map4k4 loss reduces endothelial

303 cell barrier function (24, 28). Thus, vascular permeability was assessed in vivo by

304 injecting Evan's blue dye i.v. into chow fed Flox/Flox and Map4k4 iECKO mice, and no

305 differences were observed in the amount of dye that had leaked into adipose tissue,

306 liver or lung between genotypes (Fig. 5A-D). Thus, Map4k4 may not be critical to

307 maintain baseline vascular permeability in healthy mice. Angiogenesis and blood

308 vascular function is critical for metabolic homeostasis, as humans and animals with

309 angiogenic defects display metabolic dysfunction, and angiogenesis is critical to proper

310 adipose tissue expansion and health (5, 6, 14, 15, 38). To assess angiogenic potential,

311 aortic ring angiogenesis assays were performed; however, no difference was observed

312 in the number of isolectin B4-stained sprouts from Flox/Flox and M4K4 iECKO aortas

313 (Fig. 5E-F).

314   Capillary density was next assessed in tissues of the HFD-fed Flox/Flox and

315 M4K4 iECKO mice. eWAT was isolated, and whole-mount samples were

316 immunostained with isolectin B4 as a measure of capillary density. However, only a

317 non-significant trend in isolectin B4 staining was observed in HFD-fed M4K4 iECKO

318 mice (Fig. 5G-H). In skeletal muscle, capillary density is paramount for insulin-mediated

319 hemodynamic changes and reflects insulin sensitivity, and a loss of capillary density, or

320   capillary rarefaction, occurs in obesity (4, 13). To assess this, soleus muscle was

321   isolated from chow and HFD-fed Flox/Flox and M4K4 iECKO mice and stained whole-

322   mount with isolectin B4. In chow-fed mice, there was no difference in microvascular

323   density between genotypes (Fig. 5I-J). However, HFD-fed M4K4 iECKO animals were

324   resistant to capillary rarefaction compared with controls (Fig. 5I-J). These observations

325   are reminiscent to what has been previously reported in the retina using the constitutive

326   Cdh5 promoter (27). This protection from capillary rarefaction could explain why M4K4

327   iECKO mice are insulin sensitive, as capillary rarefaction is associated with insulin

328   resistance and metabolic disease (4, 34).

329   Because loss of endothelial Map4k4 promoted enhanced lymphatic vascular and

330   blood vascular density phenotypes in development and protected against capillary

331   rarefaction in HFD (Fig. 5) (27), we hypothesized that Map4k4 may affect EC growth.

332   Indeed, our previous reports describe that ECs derived from M4K4 iECKO mice

333   displayed enhanced proliferation, which is relevant in development, tissue expansion,

334   and response to injury (27). In normal, uninjured tissues, microvascular ECs are mostly

335   quiescent; however, they are subject to senescence in obese states and with aging

336   (42). To assess whether replicative senescence was altered by loss of Map4k4, primary

337   MLECs were isolated from chow-fed Flox/Flox or M4K4 iECKO mice that had been

338   injected with tamoxifen as demonstrated in Fig. 1A. ECs senesce rapidly in culture (11);

339   thus, endogenous β-galactosidase (β-gal) activity was assessed at passage 3 in

340   confluent MLECs. Whereas Flox/Flox MLECs demonstrated abundant β-gal staining

341   consistent with senescence, M4K4 iECKO MLECs displayed a 55% reduction in

342   endogenous β-gal activity as assessed by stained area and normalized to total number

343   of nuclei (Fig. 6 A-B).

344   **Enhanced mitochondrial and glucose metabolism in ECs Lacking Map4k4.**

345   Recent studies have demonstrated that glycolysis and fatty acid oxidation are critical for

346   EC proliferation (10, 33). The reduced senescence observed in M4K4 iECKO MLECs

347   (Fig. 6A-B) suggested that these cells might be more metabolically active than Flox/Flox

348   controls (49). Our laboratory originally identified Map4k4 as a negative regulator of

349   glucose uptake in adipocytes (40); thus, ECs lacking Map4k4 may also display

350   increased glycolysis and metabolic flux. Mitochondrial and glycolytic function was

351   assessed in Flox/Flox and M4K4 iECKO MLECs using the Seahorse extracellular flux

352   analyzer. In a mitochondrial stress test, the basal oxygen consumption rate (OCR) and

353   mitochondrial coupling efficiency were similar between genotypes (Fig. 6C-D). However,

354   when   the   mitochondria   were   uncoupled   with   Carbonyl   cyanide-4-

355   (trifluoromethoxy)phenylhydrazone (FCCP), a dramatic increase in oxygen consumption

356   was observed indicating increased spare capacity of the mitochondria in M4K4 iECKO

357   MLECs compared with Flox/Flox controls (Fig. 6C-D). When subjected to a glycolysis

358   stress test, no changes in acidification rates in response to glucose alone were

359   observed, suggesting that basal glycolysis was similar between genotypes (Fig. 6E-F).

360   However, when M4K4 iECKO MLECs were treated with oligomycin to inhibit

361   mitochondrial ATP production, the increased acidification rate demonstrated a non-

362   significant trend to enhanced glycolytic capacity, as well as a significant increase in

363   glycolytic reserve (Fig. 6E-F). These data suggest that loss of Map4k4 in ECs allows for

364   a better response to energy stress and could therefore contribute to the improved

365 metabolic phenotype and the protection from capillary rarefaction that was observed in

366 M4K4 iECKO mice despite lymphatic abnormalities.

367

368 **Discussion**

369   These studies reveal a complex role of endothelial protein kinase Map4k4 to

370 promote whole-body metabolic dysfunction. Using tamoxifen-inducible

371 Cdh5(PAC)ERT2-cre to delete endothelial Map4k4 in adult C57Bl6/J mice, we observed

372 that loss of endothelial Map4k4 improved insulin sensitivity in obesity (Fig. 1). Mice

373 lacking endothelial Map4k4 displayed unaltered adipose tissue size or hepatic lipid

374 content but reduced inflammatory gene expression in liver (Fig. 2). Loss of endothelial

375 Map4k4 also caused chyle leakage and immune cell infiltration in eWAT (Figs. 3-4),

376 presumably due to lymphatic vascular deficiencies. These phenotypes seem to

377 counteract each other; while Map4k4 expression is detrimental in the blood vasculature,

378 it is required for normal lymphatic vascular function. These observations are consistent

379 with previous observations in development, in which loss of endothelial Map4k4 through

380 use of constitutively expressed Ve Cadherin-Cre led to postnatal lethality due to fluid

381 leakage and chylothorax (27). These observations are also consistent with our previous

382 study in *Apoe*^{-/-} mice, in which mice lacking endothelial Map4k4 were protected from

383 atherosclerosis development (28). Chyle leakage was not observed in *Apoe*^{-/-} mice

384 lacking endothelial Map4k4 after deletion with tamoxifen using the same cre driver

385 (Cdh5(PAC) Ve-ERT2) and injection protocol described here; however, the lymphatics

386 in *Apoe*^{-/-} mice are dysfunctional, which could account for this difference (20, 21, 39).

387   Though apparent lymphatic defects were observed in M4K4 iECKO mice, ECs

388   derived from these animals remarkably demonstrated improved metabolic

389   characteristics (Figs. 3, 6). This complex metabolic phenotype observed in M4K4

390   iECKO mice after HFD may be explained by examining the blood and lymphatic

391   vascular systems as independent systems: though loss of Map4k4 is detrimental to the

392   lymphatic vascular system, it could be beneficial to the blood vascular system. Recent

393   studies have demonstrated that glycolysis and fatty acid oxidation are critical for blood

394   vessel angiogenesis and EC proliferation (10, 33), and we demonstrate here that

395   primary ECs lacking Map4k4 are resistant to senescence in culture and display

396   enhanced glycolytic and mitochondrial respiration (Fig. 6). The increase in endothelial

397   metabolism and reduced senescent phenotype is consistent with previously published

398   observations describing enhanced proliferation in ECs lacking Map4k4 (27). These

399   observations could partially explain why mice lacking endothelial Map4k4 were resistant

400   to capillary rarefaction in obesity (Fig. 5), which likely contributes to the improvement

401   observed in insulin sensitivity in HFD-fed M4K4 iECKO mice (Fig. 1). These data more

402   broadly suggest that the improved metabolic phenotype in M4K4 iECKO mice may be

403   due to ameliorating the detrimental effects of obesity on the blood vasculature. One

404   limitation to the interpretation of these metabolic data from isolated MLECs is that these

405   cultures represent a mixed population of lymphatic and blood ECs as they were isolated

406   using the pan-endothelial cell marker CD31. Future studies will separate out

407   phenotypes within the lymphatic and blood EC populations to address mechanistically

408   how Map4k4 regulates EC specification and function.

409   Chyle leakage can reportedly cause obesity; however, M4K4 iECKO mice

410   displayed similar weights as their Flox/Flox littermates (17, 31). Although not all M4K4

411   iECKO mice displayed visible chyle leakage after HFD, it is likely that all displayed

412   some degree of lymphatic dysfunction, as pathophysiological changes in lymphatic

413   vascular integrity can occur even if chyle leakage is not visible (17, 32). Furthermore,

414   despite chyle leakage, HFD-fed M4K4 iECKO mice demonstrated improved insulin

415   sensitivity (Fig. 1). One might expect that in the setting of dysfunctional lymphatic

416   vessels that lipid absorption from the intestine may be compromised in M4K4 iECKO

417   mice, which could contribute to the improved metabolic function in these animals.

418   Though excreted lipids were not examined, no alteration in plasma triglyceride or NEFA

419   content was observed between M4K4 iECKO mice and control littermates (Table 1);

420   furthermore, no change in weight or adiposity was observed. Future studies will be

421   conducted using Prox-1 ERT2 cre mice to delete Map4k4 in the lymphatic endothelium,

422   although these animals do have some cre expression in non-lymphatic tissues. (2).

423   Alternatively, Cx40-CreERT2 animals could be used to delete Map4k4 in arterial

424   endothelium only (3); these animal models will be critical to understand the contribution

425   of the blood vs. lymphatic endothelium to the metabolic phenotype that was observed

426   here.

427   In conclusion, we have demonstrated a complex role for protein kinase Map4k4

428   in developed vasculature to mediate the responses to high fat diet and thus regulate

429   glucose homeostasis in adult animals. Furthermore, these data suggest that Map4k4 is

430   not only required for vascular development, but also plays a significant role in vascular

431   inflammation and remodeling in pathology in adulthood. As the complexities of signaling

432 cascades are beginning to be understood in the blood vs. lymphatic vascular

433 endothelium, future studies assessing the contribution of these metabolic signaling

434 pathways in blood vs. lymphatic vascular compartments will provide great insight into

435 the mechanisms by which the vasculature contributes to glucose homeostasis and

436 insulin signaling in T2D.

437

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449

450 **References**

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612   **Conflict of Interest:** The authors declare no financial conflicts of interest.

Figure Legends

Figure 1. Improved glucose tolerance in the context of no change in body weight in HFD-fed M4K4 iECKO mice. A. Tamoxifen injection and deletion scheme of endothelial Map4k4. **B-F.** Mice were fed chow or HFD for 16 weeks starting 2 weeks post-tamoxifen injections. **B.** Weight curves of chow and HFD-fed Flox/Flox and M4K4 iECKO mice (N=9-11). **C-F.** Tissue weights of HFD-fed Flox/Flox and M4K4 iECKO mice. **C.** SAT, **D.** eWAT, **E.** Liver, **F.** Spleen (N=9-11). **G.** Blood glucose levels during a glucose tolerance test. **H.** Percent glucose remaining during an insulin tolerance test. (ANOVA *; p<0.05, N=8-9 chow, 13-15 HFD).

Figure 2. Reduced expression of genes encoding proteins in inflammation pathways in HFD-fed M4K4 iECKO livers. Flox/Flox or M4K4 iECKO mice were fed HFD for 16 weeks starting 2 weeks post-tamoxifen injections. **A.** Representative H&E stained histological liver sections of at least 5 mice per genotype. Scale bars represent 100 um. **B-C.** Whole liver was isolated, and quantitative rtPCR was performed. **B.** leukocyte adhesion moecules, **C.** leukocyte markers (*; p<0.05, N=8-9).

Figure 3. Chyle leakage and eWAT inflammation in HFD-fed M4K4 iECKO mice. Flox/Flox or M4K4 iECKO mice were fed chow or HFD for 16 weeks starting 2 weeks post-tamoxifen injections. **A.** Representative image of HFD-fed

636   M4K4 iECKO mouse displaying chyle in the abdominal cavity. **B.** H&E staining of

637   eWAT from Flox/Flox or M4K4 iECKO mice after chow or HFD. **C.** H&E staining

638   of SAT, rWAT, mWAT and intrascapular BAT from Flox/Flox or M4K4 iECKO

639   mice. Scale bar represents 100 um. Images are representative of at least 5

640   animals per genotype.

641

642   **Figure 4. Lymphocyte accumulation in adipose tissue of HFD-fed M4K4**

643   **iECKO mice. A-F.** Flox/Flox or M4K4 iECKO mice were fed chow or HFD for 16

644   weeks starting 2 weeks post-tamoxifen injections. Flow cytometry was performed

645   from HFD-fed Flox/Flox or M4K4 iECKO mouse SVF. **A-B.** Representative flow

646   cytometry panels. **A.** Cd11b and F4/80-positive macrophages. **B.** Cd3+ and Cd4

647   or Cd8 positive lymphocytes. **C.** Quantitation of flow cytometry for Cd11b and

648   F4/80-positive macrophages or Cd11b, F4/80 and Cd11c-positive macrophages

649   as a percentage of cells in SVF (N=8-12). **D.** qRT-PCR for macrophage marker

650   genes and macrophage-derived cytokines from whole adipose tissue as

651   normalized to *36b4* (N=8-11). **E.** Quantitation of flow cytometry for Cd3, Cd4-,

652   Cd8-, Treg (foxp3+/Cd25+) and Cd19- positive lymphocytes as a percentage of

653   cells in SVF (*; $p<0.05$, **; $p<0.005$, N=3-6, 7-11). **F.** qRT-PCR for lymphocyte

654   marker genes and lymphocyte-derived cytokines from whole adipose tissue as

655   normalized to *36b4* (*; $p<0.05$, N=8-11).

656

657   **Figure 5. Protection from capillary rarefaction in skeletal muscle of M4K4**

658   **iECKO mice.** Mice were fed chow (**A -F, I-J**) or HFD (**G-H, I-J**) for 16 weeks

659   starting 2 weeks post-tamoxifen injections. **A-D.** Chow-fed mice were injected i.v.

660   with Evans blue dye for one hour, dye was perfused out with PBS, extracted from

661   tissues overnight, and assessed spectrophotometrically (N=5-9). **E-J** Tissues

662   were stained with isolectin B4 as a measure of vascular density. **E-F.** Aortas

663   were isolated, embedded in collagen, and treated with Vegf-A. **E.** Representative

664   images. Scale bar represents 250 um. **F.** Quantification of the average number of

665   iB4-positive sprouts after 6 days (N=10-11, average of 7-16 technical replicates

666   per animal). **G-H.** eWAT was isolated and stained with iB4. **G.** Representative

667   images of HFD-fed Flox/Flox an M4K4 iECKO eWAT. Scale bar represents 100

668   um. **H.** Quantitation of iB4-stained area (N=8-9). **I-J.** Soleus muscle was isolated

669   and stained with iB4. **I.** Representative images of chow-fed (upper) and HFD-fed

670   (lower) Flox/Flox and M4K4 iECKO soleus muscle. Scale bars represent 100 um.

671   **J.** Quantitation of iB4-stained area (*; $p < 0.05$, N=5-8).

672

673   **Figure 6. Reduced senescence and enhanced metabolism in ECs lacking**

674   **Map4k4. A-F.** Primary MLECs were derived from chow-fed Flox/Flox or M4K4

675   iECKO mice. **A-B.** Confluent MLECs were stained for endogenous β-

676   galactosidase activity and normalized to nuclei number as measured by Hoechst

677   staining. **A.** Representative image; scale bar represents 100 um. **B.** Quantitation

678   of stained area (**; $p < 0.005$, N=9-10). **C.** Oxygen consumption rate profile of

679   MLEC mitochondrial respiration. Vertical lines indicate the time of addition of

680   oligomycin, FCCP, or antimycin A and rotenone. **D.** Quantitation of mitochondrial

681   respiration; values are normalized to protein content (*; $p < 0.05$, N=5). **E.**

682   Extracellular acidification rate profile demonstrating glycolytic function in MLECs.

683   Vertical lines indicate the time of glucose, oligomycin, and 2-DG addition. **F.**

684   Quantitation of glycolytic function; values are normalized to protein content (*;

685    $p < 0.05$, N=7).

686

687

688   **Table 1. RT-PCR primer sequences.**

Gene	Forward	Reverse
F4/80	CCCCAGTGTCCTTACAGAGTG	GTGCCCAGAGTGGATGTCT
Cd68	CCATCCTTCACGATGACACCT	GGCAGGGTTATGAGTGACAGTT
Ccl-2	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
36b4	TCCAGGCTTTGGGCATCA	CTTTATCAGCTGCACATCACTCAGA
Itgam	ATGGACGCTGATGGCAATACC	TCCCCATTCACGTCTCCCA
Itgax	CTGGATAGCCTTTCTTCTGCTG	GCACACTGTGTCCGAACTCA
Il-1b	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Il-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Cd3	AGTGCAGTTCGGGAACAGAAG	GATTGGCTACTCTGCTGGGT
Cd4	TCACCTGGAAGTTCTCTGACC	GGAATCAAAACGATCAAACTGCG
Cd8	CCGTTGACCCGCTTTCTGT	CGGCGTCCATTTTCTTTGGAA
Foxp3	GGTACACCCAGGAAAGACAGC	AAGACCTTCTCACAACCAGGC
Il-4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
Il-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Ifng	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
Il-13	CCTGGCTCTTGCTTGCCTT	GGTCTTGTGTGATGTTGCTCA
Il-17	TCAGCGTGTCCAAACACTGAG	CGCCAAGGGAGTTAAAGACTT
Il-21	GGACCCTTGTCTGTCTGGTAG	TGTGGAGCTGATAGAAGTTCAGG
Pepck	CTGCATAACGGTCTGGACTTC	CAGCAACTGCCCGTACTCC
G6pc	CGACTCGCTATCTCCAAGTGA	GTTGAACCAGTCTCCGACCA
Gck	TGAGCCGGATGCAGAAGGA	GCAACATCTTTACACTGGCCT
Fasn	GGAGGTGGTGATAGCCGCTAT	TGGGTAATCCATAGAGCCCAG
Cidec	ATCAGAACAGCGCAAGAAGA	CAGCTTGTACAGGTCGAAGG
Cidea	TGACATTCATGGGATTGCAGAC	GGCCAGTTGTGATGACTAAGAC
Cd14	CTCTGTCCTTAAAGCGGCTTAC	GTTGCGGAGGTTCAAGATGTT
Ccl-3	TTCTCTGTACCATGACACTCTGC	CGTGGAATCTTCCGGCTGTAG
Ccl-5	TCGAGTGACAAACACGACTGC	GCTGCTTTGCCTACCTCTCC
Tnf-a	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
Icam-1	GTGATGCTCAGGTATCCATCCA	CACAGTTCTCAAAGCACAGCG
Vcam-1	AGTTGGGGATTCGGTTGTTCT	CCCCTCATTCCTTACCACCC

Sele	ATGAAGCCAGTGCATACTGTC	CGGTGAATGTTTCAGATTGGAGT
Selp	CATCTGGTTCAGTGCTTTGATCT	ACCCGTGAGTTATTCCATGAGT

689

690 **Table 2. Plasma analysis.**

	Flox/Flox	M4K4 iECKO
Insulin (ng/mL)	4.22 ± 0.63	2.93 ± 0.22 $
NEFA (nmol/L)	0.37 ± 0.07	0.29 ± 0.07
Triglycerides	0.90 ± 0.09	1.26 ± 0.32

691

692 **Table 2.** Flox/Flox and M4K4 iECKO mice were fed HFD for 16 weeks. Animals

693 were fasted overnight, and plasma insulin, non-esterified fatty acids (NEFA), and

694 triglyceride levels were assessed ($; p=0.06, N=3-8).

695
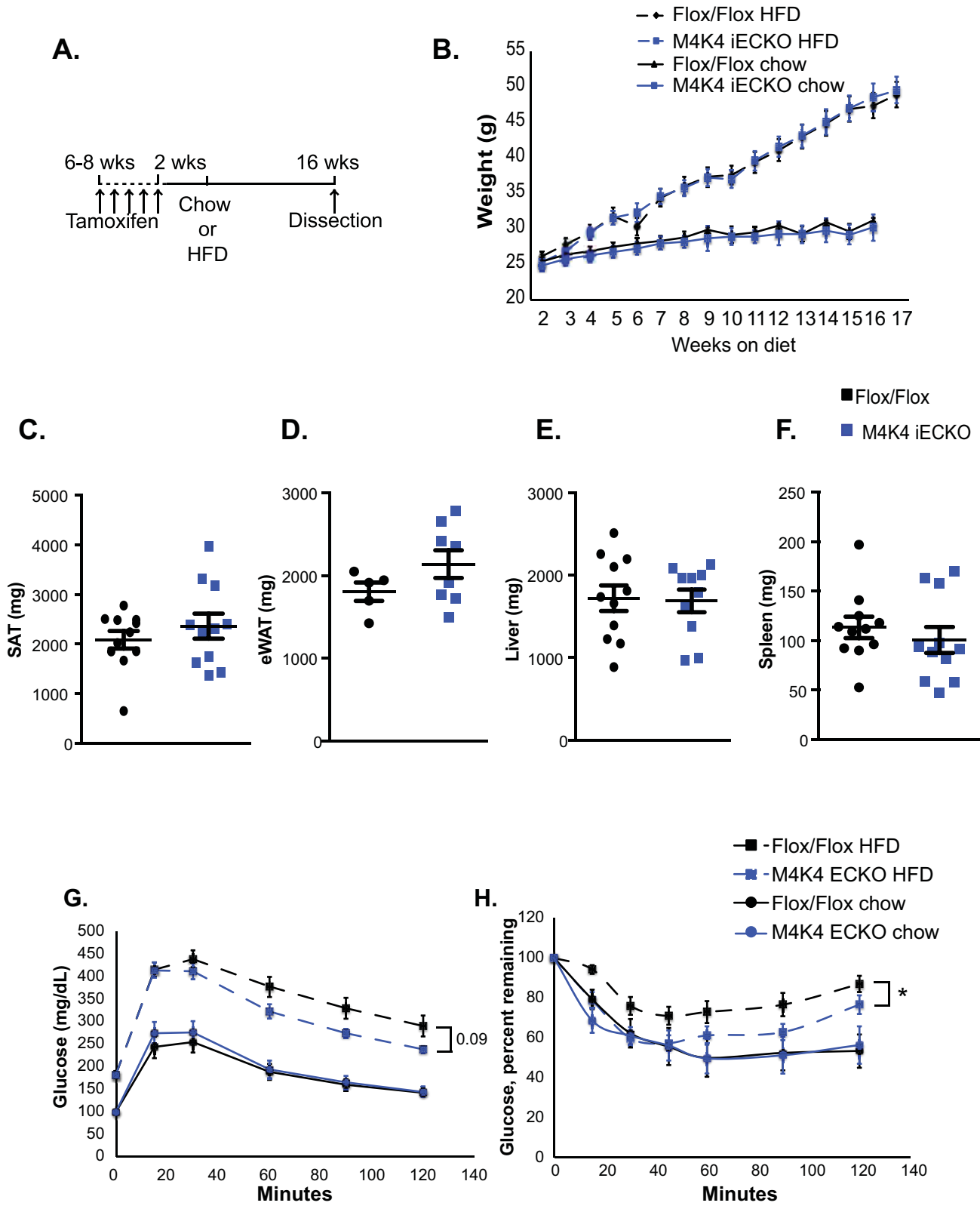
Figure 1

Figure 2

A.

B.

C.

Figure 3

Figure 4

A. eWAT

B. SAT

C. Liver

D. Lung

● Flox/Flox
■ M4K4 iECKO

E. Isolectin B4 — Flox/Flox, M4K4 iECKO

F. Number of Sprouts

● Flox/Flox
■ M4K4 iECKO

G. Isolectin B4 — Flox/Flox, M4K4 iECKO

H. Percent stained — 0.09

● Flox/Flox
■ M4K4 iECKO

I. Isolectin B4 — Flox/Flox, M4K4 iECKO — Chow, HFD

J. Chow, HFD — Percent stained — *

● Flox/Flox
■ M4K4 iECKO

Figure 5

Figure 6