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3 **An integrative transcriptional logic model of hepatic insulin resistance**

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25 ***Highlights***

26 • Foxo1 regulates liver metabolism through active enhancers, and hepatocyte
27 maintenance through core promoters

28 • Foxo1 regulates glucose genes through fasting-dependent intergenic enhancers

29 • Bipartite intron regulation of lipid genes is partly fasting-independent

30 • Ppar α contributes to the transcriptional resiliency of Foxo1 metabolic targets

31 • Insulin resistance causes de novo recruitment of Foxo1 to active enhancers

32 • A stepwise model of insulin resistance

33 **ABSTRACT**

34 Abnormalities of lipid/lipoprotein and glucose metabolism are hallmarks of hepatic insulin
35 resistance in type 2 diabetes. The former antedate the latter, but the latter become progressively
36 refractory to treatment and contribute to therapeutic failures. It's unclear whether the two processes
37 share a common pathogenesis and what underlies their progressive nature. In this study, we
38 investigated the hypothesis that genes in the lipid/lipoprotein pathway and those in the glucose
39 metabolic pathway are governed by different transcriptional logics that affect their response to
40 physiologic (fasting/refeeding) as well as pathophysiologic cues (insulin resistance and
41 hyperglycemia). To this end, we obtained genomic and transcriptomic maps of the key insulin-
42 regulated transcription factor, FoxO1, and integrated them with those of CREB, PPAR α , and
43 glucocorticoid receptor. We found an enrichment of glucose metabolic genes among those
44 regulated by intergenic and promoter enhancers in a fasting-dependent manner, while lipid genes
45 were enriched among fasting-dependent intron enhancers and fasting-independent enhancer-less
46 introns. Glucose genes also showed a remarkable transcriptional resiliency, i.e., an enrichment of
47 active marks at shared PPAR α /FoxO1 regulatory elements when FoxO1 was inactivated.
48 Surprisingly, the main features associated with insulin resistance and hyperglycemia were a
49 "spreading" of FoxO1 binding to enhancers, and the emergence of target sites unique to this
50 condition. We surmise that this unusual pattern correlates with the progressively intractable nature
51 of hepatic insulin resistance. This transcriptional logic provides an integrated model to interpret
52 the combined lipid and glucose abnormalities of type 2 diabetes.

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56 ***Significance Statement***

57 The liver is a source of excess lipid, atherogenic lipoproteins, and glucose in patients with type 2
58 diabetes. These factors predispose to micro- and macrovascular complications. The underlying
59 pathophysiology is not well understood, and mechanistic insight into it may provide better tools
60 to prevent, treat, and reverse the disease. Here we propose an alternative explanation for this
61 pathophysiologic conundrum by illustrating a transcriptional “logic” underlying the regulation of
62 different classes of genes. These findings can be interpreted to provide an integrated stepwise
63 model for the coexistence of lipid and glucose abnormalities in hepatic insulin resistance.

64 **Main Text**

65 **INTRODUCTION**

66 An impairment of the physiologic response to insulin, or insulin resistance, remains the central
67 cause of type 2 diabetes together with declining insulin secretory capacity, and its principal
68 unmet treatment need (1). The pleiotropic nature of insulin resistance poses a therapeutic
69 challenge by having different effects on different organs, and different biological consequences
70 within the same cell type, not to mention evidence of genetic heterogeneity (2). Nowhere is this
71 challenge more apparent than at the liver, a central organ in the pathogenesis of two key
72 abnormalities in diabetes: increased production of atherogenic lipoproteins that increase the
73 diabetic's susceptibility to heart disease (1); and increased glucose production, predisposing to
74 microvascular complications (3). In addition, the progressive nature of the latter defect (4),
75 together with declining β -cell function (5), likely underlies the therapeutic failure of antidiabetic
76 drugs (6). Among drugs directly targeting hepatic glucose production, the diabetic pharmacopeia
77 remains woefully limited to metformin (7).

78 Understanding whether the two central defects of hepatic insulin resistance harken back
79 to a shared mechanism, or arise independently, has obvious implications for the discovery of new
80 treatments (8). A useful conceptualization that has gained some consensus separates insulin
81 signaling into FoxO1-dependent and Srebp1c-dependent branches, the former emanating from
82 activation of Akt and allied kinases to regulate glucose metabolism, and the latter being relayed
83 through mTOR to supervise lipid synthetic and turnover pathways (2). However, while the case
84 for FoxO1 regulation of specific genes is strong, its genome-wide regulatory function in the
85 broader context of the nutrient response has only been marginally addressed (9, 10). Therefore,
86 the extent to which the lipid and glucose metabolic branches of insulin signaling share a common

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87 regulatory network remains unknown. Moreover, transcriptional networks are integrated,
88 redundant units with overlapping functions. During fasting, as glucagon, catecholamine, and
89 FFA levels rise, a host of factors is activated to modulate glucose and lipid mobilization. Besides
90 FoxO, they include CREB, PPARs, CEBPs, and nuclear receptors (11). To address these
91 questions, we undertook to generate a liver FoxO1 cistrome in different physiologic and
92 pathophysiologic states and compare it with the CREB, PPAR α , and glucocorticoid receptor
93 cistromes. By leveraging a new mouse model developed for genome-wide interrogation of
94 FoxO1 function (12), we discovered a FoxO1 transcriptional logic that provides insight into
95 hepatic insulin action and resistance.

96

97 **RESULTS**

98

99 ***In vivo* features of hepatic FoxO1 translocation**

100 There is a dearth of primary data on the kinetics of hepatic FoxO1 localization in response to
101 hormones and nutrients in the living organism. To optimize conditions for genome-wide ChIP-
102 seq, we performed immunohistochemistry in wild-type mice to determine the time- and dose-
103 dependence of FoxO1 nucleocytoplasmic translocation in response to insulin. Insulin injection
104 into the inferior vena cava triggered rapid FoxO1 translocation that reached a plateau by 15
105 minutes (Fig. S1a), with an ED₅₀ of 0.02U/kg (plasma level 0.4 ng/mL) (Fig. S1b). In contrast,
106 HNF4A remained nuclear throughout (Fig. S1a). Thus, FoxO1 translocation is rapid and
107 sensitive to physiological levels of insulin.

108 Next, we investigated translocation in response to fasting and refeeding. Following a
109 physiologic 4-hr fast, 1-hr refeeding induced complete FoxO1 translocation (Fig. 1a). In contrast,
110 a prolonged, 16-hr fast resulted in decreased FoxO1 immunoreactivity. Subsequent refeeding for
111 up to 4 hr failed to translocate residual FoxO1 to the cytoplasm, while FoxO1 immunoreactivity
112 increased and HNF4A immunoreactivity decreased after 2-hr refeeding (Fig. S1c). The reduced
113 protein levels and delayed translocation are likely secondary to FoxO1 deacetylation (13-15).
114 FoxO1 localization correlated with plasma glucose and insulin levels, as well as liver Akt
115 phosphorylation. Thus, rapid nuclear exclusion in the 4-hr-fast/1-hr-refeed design was associated
116 with a modest rise of glucose and insulin levels (Fig. S1d) and increased Akt phosphorylation
117 (Fig. S1e), whereas persistent nuclear localization in the 16-hr fast/4-hr refeed design was
118 associated with hyperglycemia, hyperinsulinemia (Fig. S1d), and reduced Akt phosphorylation
119 (Fig. S1e). Based on these findings, we selected the 4-hr fast and 1-hr refeed time points to
120 assess the hepatic FoxO1 regulome.

121

122 **FoxO1 regulome during fasting and refeeding**

123 To study the genome-wide regulation of FoxO1 with fasting and refeeding, we interrogated
124 genome occupancy by FoxO1 using an anti-GFP antibody in FoxO1-Venus knock-in mice (12)
125 for chromatin immunoprecipitation (ChIP), to overcome the limitations of anti-FoxO1
126 antibodies. As reported (12), anti-FoxO1 antibodies detected the FoxO1-Venus fusion protein
127 encoded by the modified *Foxo1* locus (Fig. S2a-b). Comparison between the two antibodies at
128 known FoxO1 target genes (*Igfbp1*, *G6pc*, and *Pck1*) confirmed the specificity and superior
129 sensitivity of the GFP antibody (Fig. S2c) (16). We next compared ChIP-qPCR and ChIP-seq
130 using GFP antibody in FoxO1-Venus mice in the same conditions (Fig. S2d-h). Both approaches
131 demonstrated similar decreases of FoxO1 binding to *Igfbp1*, *G6pc*, and *Pck1*, and the lack of
132 effects on the unrelated *Fkbp5*. As the results were internally consistent, we performed further
133 analysis with GFP antibody.

134 Genome-wide FoxO1 ChIP peak calling detected ~15,000 peaks; ~8,000 unique peaks in
135 fasting, ~1,000 in refeeding, and 5,000 in both conditions but to different extents (Fig. 1b). >30%
136 of FoxO1 sites localized to promoters/transcription start sites (TSS) (Fig. 1c). Signal intensity
137 plots demonstrated that refeeding cleared FoxO1 binding to autosomes (Fig. 1d and S3a),
138 regardless of the distance from TSS (Fig. S3b). Known (Fig. S3c) and *de novo* motif analyses
139 (Fig. 1e, Fig. S4) retrieved the FoxO1 motif TGTTTAC (12). This motif was found in 17% and
140 29.3% of FoxO1 sites in fasted and refed conditions, respectively. The same motif was found in
141 fasted and refed conditions (Fig. S3d), and was evenly distributed between 1 and -5Kb from TSS
142 (Fig. S3e) (17).

143 Next, we integrated ChIP-seq and RNA-seq data into a hepatic FoxO1 regulome. To
144 identify FoxO1-regulated mRNAs, we induced somatic ablation of FoxO1 in liver by injecting
145 *Foxo1*^{lox/lox} mice with AAV-Cre (A-FLKO) and documented its completeness and specificity by
146 mRNA measurements and western blotting of different tissues (Fig. S3f-g). After 3 weeks, we
147 isolated livers from 4-hr-fasted A-FLKO and control (A-WT) mice and performed RNA-seq. We
148 plotted the log₂ difference in DNA binding (FoxO1 ChIP-seq peak number in fasted *vs.* refed
149 animals) *vs.* the log₂ difference in gene expression between A-WT and A-FLKO mice
150 (differentially expressed genes, DEGs). Thus, the effect of genotype lies along the vertical axis,
151 and that of fasting along the horizontal axis (Fig. 1f and Table S1).

152 Contingency analyses showed the strongest association between DEGs in the fasted state
153 and FoxO1 DNA binding sites at promoters/TSS (183 of 198, or 92.4%), followed by introns
154 (260 of 344, 75.6%), and intergenic sites (181 of 281, 64.4%), respectively (*p* < 0.0001). Since
155 DEGs are more likely to be FoxO1 targets, these data provide initial, suggestive evidence of a
156 FoxO1 transcriptional logic, *i.e.*, genes regulated by FoxO1 in a fasting/refeeding-dependent
157 manner have a greater frequency of FoxO1 sites in their promoter/TSS, introns, and intergenic
158 regions.

159

160 **FoxO1 regulates metabolic genes through active hepatic enhancers**

161 In addition to metabolism, FoxO1 regulates cellular maintenance functions in a fasting-
162 independent manner (18). We sought to understand the transcriptional logic of these diverging
163 functions. We hypothesized that basic cellular functions would be regulated through core
164 promoters, which are generally found within 1 kb from TSS and are associated with
165 housekeeping genes and developmental TFs (19). Conversely, we surmised that metabolic genes

166 would be regulated through tissue-specific enhancers (11, 20). To test the hypothesis, we mapped
167 active enhancers using H3^{K27ac} and H3^{K4me1} ChIP-seq (21) in fasting and refeeding, and
168 determined their overlap with FoxO1 sites (Fig. S5a).

169 Of 5,303 active enhancers co-localizing with FoxO1 sites genome-wide, 2,975 were
170 unique to fasting, 1,022 to refeeding, and 1,306 were found in both conditions (Fig. S5b). FoxO1
171 enhancers localized mostly to intergenic regions and introns, and to a lesser extent to
172 promoter/TSS (Fig. 2a). The rate of clearance in response to refeeding varied according to
173 genomic annotation: 59.6% in intergenic regions (804/1348); 67.9% in introns, (1085/1597); and
174 81.5% in promoters/TSS (564/692) ($p < 0.0001$).

175 Next, we performed ontology analyses of genes associated with FoxO1 sites in active
176 enhancers vs. core promoters and visualized causal relationships among enriched terms in
177 directed acyclic graphs (DAG) (22). FoxO1 sites in active enhancers were overwhelmingly
178 enriched in metabolic genes, with the top three fundamental ontologies being glucose
179 metabolism, lipid homeostasis, and insulin response (FDR 10^{-40} to -70) (Fig. 2b, c, S5c). These
180 gene ontologies showed a strong correlation between the fasting/refeeding ratio of FoxO1 DNA
181 binding (Fig. 2d, e and S5d) ($b = 0.09, p < 0.0001$), and changes to mRNA expression following
182 FoxO1 ablation, especially in fasting conditions (Fig. 2f). In contrast, enhancer-less FoxO1 sites
183 in promoter/TSS included gene ontologies related to intracellular transport, DNA repair, ncRNA
184 processing, and protein modification by small protein conjugation (Fig. 2g, h, S5e) (FDR 10^{-20} to
185 -40). These sites showed a lesser correlation between the fasting/refeeding ratio of FoxO1 binding
186 (Fig. 2i-j and S5f) ($b = 0.29, p < 0.0001$). More importantly, mRNAs encoded by genes lacking
187 active enhancers were largely unaffected by FoxO1 ablation (Fig. 2k). The active enhancer
188 marker, H3^{K27ac}, was unaffected by fasting and refeeding ($b = 0.91, p < 0.0001$) (Fig. S5g).

189 These results indicate that the cell maintenance and metabolic functions of FoxO1 are
190 ruled by distinct transcriptional logics: the former are governed by core promoters in a
191 fasting/refeeding-independent manner, whereas the latter are governed by active enhancers and
192 show a strong dependence on nutritional status (18).

193

194 **Enrichment of FoxO1 sites in introns of triglyceride and cholesterol genes**

195 The second most common genomic annotation of FoxO1 binding sites mapped to introns (Fig.
196 1c). The corresponding genes showed changes to their mRNAs following FoxO1 ablation (Fig.
197 1f and Table S1). To understand the functional correlates of FoxO1 binding to introns, we
198 compared expected and actual distribution of FoxO1 sites across the genome for different gene
199 ontology groups. Interestingly, triglyceride metabolism genes showed a skewed distribution,
200 with FoxO1 binding sites occurring at two- to three-fold the expected frequency at two locations:
201 5 to 50kb and –50 to –5kb from TSS (proximal introns and distal promoters), and 30 to 50% of
202 the expected frequency at 0 to 5kb and 50 to 500kb from TSS (Fig. 3a). In contrast, glucose
203 metabolism genes showed an enrichment 50 to 500kb from TSS, followed by the 5 to 50kb
204 regions (Fig. 3b). Statistical analyses of annotation distribution demonstrated that triglyceride
205 metabolism genes were significantly enriched in introns, while glucose metabolism genes were
206 enriched in intergenic and promoter/TSS sites ($p = 0.03$) (Fig. 3c).

207 The ontology groups of intron-enriched genes included a nearly exclusive assortment of
208 lipid, lipoproteins, and cholesterol genes (Fig. 3d). Nearly half of intron sites were associated
209 with active enhancers (Fig. 2a). Next, we analyzed the FoxO1 regulome by intron enhancer
210 status. Linear regression analysis of FoxO1 tags in the fasted vs. refed state demonstrated that
211 introns marked by active enhancers showed a three-fold lower coefficient of variation than

enhancer-less introns ($b = 0.19$ vs. 0.06) (Fig. S6a–b), and were more frequently associated with variations of the encoded mRNAs in A-FLKO. For example, ScarB1 (23) (Fig. 3e), Angptl4, and Angptl8 (24) (Table S2) showed fasting-induced FoxO1 binding to active intron enhancers, as well as altered mRNA levels upon FoxO1 ablation. In contrast, the *ApoB*, *ApoA1/C3/A4/A5* and *C2/C4/C1/E* clusters (the latter syntenic with the human *APOCII* enhancer) (25) showed fasting-independent FoxO1 binding to enhancer-less introns, and preserved mRNA expression following FoxO1 ablation (Fig. 3f-g, Table S2).

The transcriptional logic of the FoxO1 regulome emerging from the preceding analyses suggests that a majority of glucose metabolism genes are governed by an intergenic/proximal promoter/TSS active enhancer-logic in a fasting-inducible manner, whereas a majority of triglyceride, lipoprotein, and cholesterol genes are ruled by a bipartite intron-logic: fasting-dependent active intron enhancers and fasting-independent enhancer-less introns.

We hypothesized that this differential logic underlies hepatic insulin resistance. We tested the hypothesis using three different conditions: (i) resilience analysis to determine whether these two regulatory modalities affect the ability of these genes to undergo compensatory changes in response to variations in FoxO1 function, as a surrogate measure of insulin action (Fig. S1); (ii) comparative genomic analyses with other fasting-induced TFs to identify functional partners and redundancies; and (iii) genome-wide FoxO1 ChIP-seq in insulin-resistant/hyperglycemic mice.

230

231 **Transcriptional resiliency of glucose metabolic genes**

First, we sought to determine whether different modalities of FoxO1 regulation (intergenic and promoter/TSS vs. intron) were associated with differential compensation by other TFs that may affect the pathophysiology of insulin resistance. To this end, we compared gene expression

235 differences between constitutive *vs.* adult-onset somatic ablation of FoxO1 in liver (26-28) and
236 correlated these differences with ChIP-seq data.

237 We generated *Alb-Cre:FoxO1^{f/f}* mice to induce constitutive hepatic FoxO1 ablation (C-
238 FLKO) and compared gene expression differences between adult-onset (A-FLKO, described in
239 Fig. 1) and constitutive (C-FLKO) knockouts according to nutritional state (fast *vs.* refeed),
240 genotype (WT *vs.* FoxO1 ablation), and timing of ablation (A-FLKO *vs.* C-FLKO) using RNA-
241 seq (Fig. S7). t-SNE plots showed large differences in fasted *vs.* refed gene expression patterns
242 between A-FLKO and their matched controls (A-WT). In contrast, the differences between C-
243 FLKO and C-WT were considerably blunted (Fig. 4a). We calculated fold-change and average
244 gene expression in each WT/knockout pair to draw MA-plots of log-intensity ratios (M-values)
245 *vs.* averages (A-values). The number of differentially regulated genes in fasted C-FLKO mice
246 decreased by 60% compared to A-FLKO (227 *vs.* 585), whereas it was similar in refed
247 conditions (301 *vs.* 243) (Fig. 4b-e, Table S3). Thus, a first conclusion is that chronic
248 compensatory changes partially mask the effect of FoxO1 ablation on the fasting response.

249 Next, we determined the ontologies of genes undergoing compensatory changes as a
250 function of nutritional status (fast *vs.* refeed), genotype (knockout *vs.* WT), and timing-of-
251 ablation (A-FLKO *vs.* C-FLKO) (Fig. 4f). We identified four ontology groups (A-D). Group A
252 was comprised of genes induced by fasting, and group C of genes induced by refeeding, neither
253 of which was affected by FoxO1 ablation in either A-FLKO or C-FLKO mice. These groups
254 included cellular, immune, chemical, and stress response genes. In contrast, group B was
255 comprised of genes affected by genotype (A-FLKO *vs.* A-WT and C-FLKO *vs.* C-WT),
256 regardless of the timing of ablation. This group included primarily lipid and fatty acid
257 metabolism genes whose expression decreased with fasting in FoxO1 knockouts. Group D was

258 enriched in genes regulated by fasting, genotype, and timing of ablation. These genes were
259 induced by fasting in WT, but not in A-FLKO mice. However, the differences between WT and
260 A-FLKO were virtually lost in C-FLKO mice. This group included metabolic pathways, retinol
261 and PPAR signaling, and steroid function genes (Fig. 4g). In contrast, only a small number of
262 genes, primarily linked to extracellular matrix-receptor interaction and protein digestion and
263 absorption, were uniquely affected following constitutive ablation.

264 We examined group D at a more granular level to identify genes in which the effect of
265 FoxO1 ablation became less marked in C-FLKO (i.e., lower fold-change and higher FDR value
266 between control and KO mice in C-FLKO than those in A-FLKO). These genes involved
267 classical FoxO1 targets regulating insulin signaling (*Irs2*), gluconeogenesis (*G6pc*, *Pck1*,
268 *Ppargc1a*), glycolysis (*Gck*, *Pfkfb1* and 3, *Ldh*), ketogenesis (*Hmgcs1*), and glucose/fatty acid
269 partitioning (*Pdk4*) (Table S3). Other genes undergoing compensation included 17 members of
270 the *Cyp2* family and 6 members of the *Cyp4* family of drug metabolizing enzymes, *Angptl8*,
271 *Fgf21*, *Gdf15*, *Klf15*, *Slc13a5* (encoding INDY), *Enho* (encoding Adropin), *Fmo3*, and *Asns*.

272 Among genes involved in fatty acid synthesis or oxidation, apolipoproteins, and
273 cholesterol trafficking, only *Vldlr* and *Lpin1* showed >50% compensation. Thus, FoxO1-
274 regulated glucose metabolism genes as well as several metabolically important genes undergo a
275 compensatory response following constitutive FoxO1 ablation, whereas the majority of lipid
276 metabolism genes don't. We termed this finding transcriptional resiliency.

277

278 **A FoxO1/PPAR α signature of fasting-inducible enhancers**

279 Transcriptional regulation of the fasting response involves several TFs, including CREB, GR,
280 and PPAR α (11). To understand the integration of these networks with FoxO1 and their potential

281 role in the transcriptional resiliency observed after FoxO1 ablation, we compared the present
282 dataset with published genome-wide ChIP-seq of these three factors (29, 30). Analyses of peak
283 distribution demonstrated that CREB peaks are enriched at promoters, while GR and PPAR α are
284 enriched in introns and intergenic regions (Fig. 5a). When overlaid with FoxO1 sites, we found
285 that co-localization of FoxO1/PPAR α (Fig. 5b) prevailed at active intergenic and intron
286 enhancers, where approximately half of FoxO1 sites are shared with PPAR α (Fig. 5c, e). In
287 contrast, trinomial combinations FoxO1/CREB/PPAR α prevailed at enhancer-less promoters
288 (Fig. 5d, e). At active enhancer sites, 11.2% of unique FoxO1 sites were associated with changes
289 in gene expression following FoxO1 ablation, whereas only 5.4% of shared sites (FoxO1 and
290 CREB or PPAR α) did ($p < 0.0001$, Table S4). This difference was not seen in non-active
291 enhancer sites (6.09% vs. 5.93%, respectively) ($p = \text{NS}$, Table S4). Gene ontology analysis (Fig.
292 5f) showed that abnormal gluconeogenesis is the most significant annotation of
293 FoxO1/PPAR α shared intergenic peaks ($FDR = 2.22 \times 10^{-31}$), while lipid homeostasis is the most
294 significant in introns ($FDR = 2.01 \times 10^{-21}$).

295 Next, we asked whether co-regulation by FoxO1 and PPAR α can explain the resiliency
296 of gene expression. We plotted each FoxO1/PPAR α shared peak with active enhancer marks vs.
297 changes to mRNA encoded by associated genes in A-FLKO and C-FLKO (Fig. 5g-h). In both
298 intergenic (Fig. 5g) and intron (Fig. 5h) sites, >80% of FoxO1/PPAR α co-regulated genes
299 showed a compensatory response to constitutive FoxO1 ablation (75 of 92 and 68 of 76,
300 respectively). In intergenic sites, we found notable resilient glucose metabolism genes, such as
301 *Pck1*, *G6pc*, *Irs2*, *Ppargc1a* and *b*, *Ppp1r3g*, *Cry1*, *Gdf15* (31) and *Klf15* (32) (Fig. 5g). In
302 introns, we found lipid genes, such as *Gdf15*, and *Lipc* (Fig. 5h, Table S5). Thus, shared
303 FoxO1/PPAR α enhancers are more likely to undergo compensation when FoxO1 is inactive.

304

305 **Enhancer spreading of FoxO1 binding in insulin resistance/hyperglycemia**

306 To evaluate the effects of insulin resistance and hyperglycemia on the FoxO1 regulome, we
307 subjected FoxO1-Venus mice to high fat diet (HFD) or treatment with the insulin receptor
308 antagonist, S961 (33). Both interventions impaired refeeding-induced FoxO1 translocation (Fig.
309 6a) and caused hyperglycemia (not shown). However, as the effects of S961 were more marked,
310 we performed genome-wide ChIP-seq in livers of 4-hr-fasted/1-hr-refed mice treated with S961
311 vs. vehicle.

312 Regression analysis of FoxO1 tags under fasted and refed conditions showed a two-fold
313 higher coefficient in S961-treated mice than in vehicle controls ($b = 0.28$ vs. 0.62 , Fig. 6b),
314 consistent with impaired translocation (Fig. 6a). We examined FoxO1 binding to representative
315 genes of the two main transcription logics identified above, intergenic/promoter/TSS (glucose)
316 vs. intron (lipid) genes. We found the emergence of novel FoxO1 binding patterns at active
317 enhancers of both classes. Examples included intergenic/promoter enhancers of glucogenic
318 (*G6pc*, *Pck1*, *Klf15*) (Fig. 6c-d, S8a) and glucose–lipid metabolic partitioning genes (*Pdk4*) (Fig.
319 6e), as well as intron enhancers of lipid/cholesterol genes (*ApoA1/C3/A4*, *Scarb1*) (Fig. 6f, S8b-
320 c). These novel FoxO1 peaks were unaffected by fasting/refeeding, and included both FoxO1
321 binding motif-containing sites and sites without FoxO1 motifs. In contrast, novel FoxO1 marks
322 at enhancer-less sites occurred less frequently. Thus, insulin resistance and hyperglycemia bring
323 about an ectopic, dysregulated binding of FoxO1 at enhancer sites, which we term enhancer
324 spreading.

325

326 **DISCUSSION**

327

328 The present study provides transcriptional logic insight into the differential regulation of glucose
329 and lipid metabolism in response to nutrient changes, and in insulin resistance. There are
330 obviously non-transcriptional components to this pathophysiologic state that are partly cell-
331 nonautonomous (34), but the present study was designed to establish genome-wide map that
332 integrates multiple TFs, including FoxO1, with the salient pathophysiologic features of hepatic
333 insulin action and resistance. The main conclusions are: (i) the transcriptional logic of FoxO1 is
334 compatible with the bifurcating model of insulin signaling to lipid vs. glucose metabolism (35),
335 whereby glucose metabolic genes are governed by intergenic and promoter/TSS enhancers, and
336 lipid genes by a bipartite intron logic that includes fasting-dependent intron enhancers and
337 fasting independent enhancer-less introns. (ii) Active enhancers of glucose metabolic genes show
338 transcriptional resiliency, likely through shared PPAR α /FoxO1 regulatory elements. (iii) Insulin
339 resistance and hyperglycemia result in the spreading of FoxO1 binding to enhancers, resulting in
340 quantitative and qualitative abnormalities of FoxO1 marks (12).

341 Based on these findings, we propose this model (Fig. 7): in the physiologic
342 fasting/refeeding transition, FoxO1 is cleared more efficiently from enhancer-containing sites
343 than from enhancer-less sites. As the former are more tightly associated with glucose genes, and
344 the latter with lipid/lipoprotein genes, in the initial stages of insulin resistance glucose genes can
345 still be regulated, while regulation of lipid genes is impaired. This differential sensitivity can
346 explain why lipid/lipoprotein abnormalities chronologically precede hyperglycemia in the
347 progression of diabetes (36). Further work will be required to functionally interrogate different
348 classes of sites. As insulin resistance progresses, the gradual compensation of glucose vs. lipid
349 genes in response to chronic vs. adult-onset FoxO1 ablation (transcriptional resiliency at

350 intergenic and promoter/TSS enhancers) can be interpreted to suggest that glucose genes can
351 gradually become FoxO1-independent, allowing transcription factors (likely PPAR α) to induce
352 their expression. In the clinically overt stage of the disease, as insulin resistance increases,
353 activation of FoxO1 at ectopic (or low-affinity) enhancers leads to worsening fasting
354 hyperglycemia, and may possibly underlie therapeutic failures. The proposed model integrates *in*
355 *vivo* pathophysiological and cell biological data with genome-wide assessments to explain a
356 clinical conundrum that has important practical implications for treatment and drug development
357 (1). This model also addresses two criticisms leveled at the FoxO-centric view of insulin action:
358 (i) that FoxO1 sensitivity to insulin makes it an unlikely candidate as a mediator of insulin
359 resistance (37); and (ii) that transcription of candidate glucogenic genes alone does not fully
360 explain increased hepatic glucose production (38). Indeed, the gamut of FoxO1 targets includes
361 most genes involved in insulin action, and the failure to detect abnormalities in their expression
362 following constitutive somatic ablation of FoxO1 can be explained by their resiliency.

363 To demonstrate a distinctive FoxO1 transcriptional logic, we decisively leveraged the
364 ability to examine FoxO1 targets by genome-wide ChIP-seq (12). Previous studies have been
365 limited by the sensitivity of available FoxO1 antibodies, and have therefore detected fewer
366 FoxO1 binding sites (9, 10, 39). There is a partial dissociation between the ChIP results,
367 indicating that FoxO1 is still bound at several sites after refeeding, and the immunofluorescence
368 that shows FoxO1 nuclear exclusion. However, ChIP is more sensitive than
369 immunohistochemistry, being based on PCR amplification, and can detect lower levels of FoxO1
370 protein. The formation of different molecular complexes likely underlies the different modes of
371 FoxO1 action. In this regard, we have previously shown that SIN3a is the FoxO1 corepressor at
372 glucokinase, providing a mechanistic precedent for gene-specific targeting (8). The preferential

373 regulation of FoxO1 by fasting/refeeding at active enhancers likely results from intrinsic and
374 extrinsic factors, such as higher DNA accessibility at active enhancers (40), and active enhancer-
375 promoter interactions (41) that affect assembly of pre-initiation complexes, initiation of
376 transcription by RNA polymerase II, or transcription bursting (19).

377 Following FoxO1 ablation, expression of its targets can be compensated for by
378 transcription factors acting synergistically, through its parologue FoxO3, or reorganization of
379 chromatin accessibility at sites where FoxO1 acts as pioneer transcription factor (42), as shown
380 with other FoxO isoforms (27). Interestingly, genes associated with glucose metabolism (*G6pc*,
381 *Pck1*, *Ppargc1a*, *Pdk4* and *Klf15*), but not those regulating general cellular responses, are
382 selectively compensated for following FoxO1 ablation. FoxO1 peaks in these genes are cleared
383 by refeeding, but not in insulin-resistant conditions. These genes have been shown to play a role
384 in diabetes in studies with insulin-resistant mice (26, 43-45).

385 There are parallels between our findings and recent evidence that immunocyte
386 differentiation is controlled by an enhancer- or core promoter-driven logic, with a striking
387 partition between the two gene sets (46). The former activity is cued by the overall activity
388 pattern of distal enhancers, while the latter is aligned with promoters. Although it is disputed
389 whether core promoters and enhancers represent different entities or synergistically regulate
390 transcriptional bursting, enhancers are thought to be tissue-specific, and thus more likely to
391 confer specificity on the tissue-specific metabolic functions of FoxO1 (20).

392 Our comparative analysis provides evidence of cooperative and non-cooperative
393 interactions with GR, CREB and PPAR α , the latter involving up to half of the FoxO1 sites in
394 active enhancers. The extensive sharing of intergenic active enhancers of glucose genes by
395 FoxO1 and PPAR α is a novel finding of this study that dovetails with the different physiologic

396 cues regulating these two TFs. During fasting, glycogenolysis precedes gluconeogenesis and the
397 generation of FFA substrates that activate PPAR α (47). Thus, we envision that FoxO1 and
398 PPAR α act in a physiologic relay to ensure continuity between the early and late fast. The
399 significant overlap between FoxO1 and PPAR α may also provide an explanation for the
400 relatively mild phenotypes of liver-specific inactivation of FoxO1 (26) and PPAR α (48).
401 Functional elucidation of their interactions will be important to determine key targets in glucose
402 metabolism and their role in diabetes pathogenesis.

403 **Data sharing**

404 Further information and requests for resources and reagents should be directed to and will be
405 fulfilled by Takumi Kitamoto (tk2752@cumc.columbia.edu).

406 **Data and Code Availability**

407 The ChIP-seq and RNA-seq datasets generated during this study are available at the NCBI GEO
408 [GSE151546]

409 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

410 **Animals**

411 Mice were housed in a climate-controlled room on a 12h light/dark cycle with lights on at 07:00
412 and off at 19:00, and were fed standard chow (PicoLab rodent diet20, 5053; PurinaMills). Male
413 mice of C57BL/6J background aged 8-12 weeks were used. FoxO1-Venus mice have been
414 described (12, 49). Briefly, To express GFP (Venus), we
415 obtained the pCAG:myr-Venus plasmid. A 15-amino acid linker sequence was placed between
416 the C terminus of FoxO1 and N terminus of Venus to alleviate steric hindrance. We used BAC
417 recombineering to generate FoxO1-Venus ES cells. To generate constitutive liver-specific
418 FoxO1 knockouts, we crossed FoxO1^{lox/lox} and Albumin-cre (50) transgenic mice. Adult onset
419 liver-specific FoxO1 knockout mice were generated by injection of 1×10¹¹ purified viral particles
420 (AAV8.TBG.eGFP or AAV8.TBG.Cre, Penn Vector Core) per mouse via tail vein. We
421 performed metabolic analysis or killed animals on day 21 post-injection. To assess FoxO1
422 localization and other liver parameters, we took organs from 4-hr-fasted (10:00 to 14:00) or 4-hr-
423 fasted/1-hr-refed mice. For prolonged fasting experiments, we removed food overnight (18:00 to
424 10:00). Mice were killed 0, 1, 2, or 4 hr after refeeding. For insulin treatment, we anesthetized
425 16-hr-fasted mice with ketamine (100mg/kg) and xylazine (10mg/kg) i.p., followed by injection

426 of 1U/kg insulin (NovoLog®, Novo Nordisk, Denmark) in the inferior vena cava (IVC). We
427 collected blood and took the liver before and after insulin injection. Blood glucose was measured
428 using (CONTOUR®NEXT ONE, Ascensia, USA), and insulin with a mouse-specific ELISA kit
429 (Mercodia, USA). All animal experiments were in accordance with NIH guidelines, approved
430 and overseen by the Columbia University Institutional Animal Care and Use Committee.

431 **Primary hepatocyte isolation**

432 Primary hepatocyte isolation was performed as described (51). We anesthetized male mice with
433 ketamine (100mg/kg) and xylazine (10mg/kg) i.p., cannulated the IVC with a 24-gauge catheter
434 (Exel international), and infused 50 cc EGTA-based perfusion solution followed by 100 cc type I
435 collagenase solution (Worthington Biochemicals). Following cell dissociation, we filtered cells
436 with 100 mm mesh cell strainers, and gradient centrifugation steps to purify cell suspension.
437 Then, we suspended hepatocytes at 5×10^5 cells / mL in Medium 199 (Sigma), 10% FBS (Life
438 Technologies), antibiotics (plating medium). After plating for 2 hr on collagen-coated plates, we
439 exchanged plating medium for 4 hr.

440

441 **METHOD DETAILES**

442 **Chemicals and antibodies**

443 Ketamine was from KetaSet® and Xylazine from AnaSed®; medium 199, HBSS, EGTA,
444 HEPES, PenStrep and Gentamycin from Life Technology; collagen type 4 from Worthington;
445 Insulin (NovoLog®) and S961 from Novo Nordisk A/S; sodium orthovanadate from New
446 England Bio; Bovine Serum Albumin (BSA) from Fisher Scientific. 16% paraformaldehyde
447 (PFA) was from Electron Microscopy Sciences, and was diluted in sterile phosphate buffer
448 solution to 4% final concentration. Anti FoxO1 (for Western Blot and immunohistochemistry,

449 C29H4), anti panAkt (for Western Blot, 40D4) and phosphor-Akt (Ser473) (for Western Blot,
450 D9E), normal Rabbit IgG (for chromatin immunoprecipitation, 2729) were from Cell Signaling.
451 HNF4A (for immunohistochemistry, ab41898), GFP (for chromatin immunoprecipitation, ab
452 290), FoxO1 (for chromatin immunoprecipitation, ab39670) were from Abcam. H3K27ac (for
453 chromatin immunoprecipitation, 39133) was from Active motif. Anti GFP
454 (immunohistochemistry, A-6455) was from Invitrogen.

455 **Protein analysis**

456 Livers were lysed in sonication buffer containing 20 mM HEPES pH7.5, 150 mM NaCl, 25 mM
457 EDTA, 1% NP-40, 10% glycerol, 1 mM Na vanadate, 1 mM phenylmethylsulphonyl fluoride
458 (PMSF), and protease and phosphatase inhibitors cocktail (Cell Signaling). We sonicated lysates
459 for 100 sec (5×, output 70%, 20sec/20sec) and centrifuged them for 15 min at 14,000 rpm. 30 µg
460 protein (Pierce BCA, Thermo scientific) was subjected to SDS-PAGE. We used the following
461 antibodies: Akt (1:2,000), phosphor-Akt (Ser473) (1:2,000), β-actin (1:1,000), FoxO1 (1:1,000)
462 (all from Cell Signaling), and GFP (1:1,000) (Abcam, ab290).

463 **Immunohistochemistry**

464 We anesthetized 8- to 12-week-old mice fasted or refed for various lengths of time and perfused
465 them with 4% PFA through the IVC. Livers were collected, fixed in 4% paraformaldehyde for 2-
466 hr, dehydrated in 30% sucrose overnight at 4°C, embedded in OCT (Sakura, Torrance, CA),
467 frozen to -80°C, and cut into 7-µm sections. We used primary antibodies to FoxO1 (1:100; Cell
468 signaling technology, Boston, MA) and HNF4A (1:100; Abcam, Cambridge, MA), and
469 secondary anti-IgG antibodies conjugated with Alexa Fluor 488 and 555 for each of the species
470 (1:1,000; Life Technologies). Immunofluorescence was visualized by the TSA fluorescence
471 system (PerkinElmer, Waltham, MA).

472 **Real-time qPCR**

473 We lysed livers in 1 mL of TRIzol, purified RNA using RNeasy Mini Kit (Qiagen, Germantown,
474 MD), reverse-transcribed it with qScript cDNA Synthesis Kit (QuantaBio, Beverly, MA), and
475 performed PCR with GoTaq® qPCR Master Mix (Promega, Madison, WI). Primer sequences are
476 available upon request. Gene expression levels were normalized to 18S using the 2-DDCt
477 method and are presented as relative transcript levels.

478 **RNA-seq library constructions and data analysis**

479 We prepared the samples from three mice for each group, and generated the libraries
480 individually. Libraries for RNA-seq were prepared using the TruSeq Stranded mRNA Sample
481 Prep Kit (Illumina), following the manufacturer's protocol. Deep sequencing was carried out on
482 the Illumina NextSeq 500 platform using the NextSeq 500/550 high-throughput kit v2.5
483 (Illumina) in 75-base single-end mode according to the manufacturer's protocol. Sequenced
484 reads from the RNA-seq experiment were aligned to mouse genome mm10 using HISAT2.
485 Cufflinks was used for transcript assembly. Gene expression levels were expressed as fragments
486 per kilobase of exon per million mapped sequence reads and Cuffdiff was used for statistical
487 comparison.

488 **Chromatin immunoprecipitation assays and ChIP-seq library construction**

489 The ChIP-IT® High Sensitivity kit (Active Motif, Carlsbad, CA) was used for chromatin
490 immunoprecipitation (ChIP) following the manufacturer's protocol. We anesthetized 8- to 12-
491 week-old mice after 4-hr fasting followed or not by 1-hr refeeding and perfused them with 10
492 μM orthovanadate through the IVC. We harvested samples from left lobe of liver tissues and
493 pooled 100mg of samples from three individual replicates for further experiments. We obtained
494 sheared chromatin from 300 mg of liver extract using a S220 Focused-ultrasonicator (Covaris).

495 Immunoprecipitation was performed using 4 µg of anti-GFP antibody for 10 µg of sheared
496 chromatin. The specificity of the anti-GFP antibody was confirmed by western blotting of liver
497 extract. ChIP-seq libraries were constructed using KAPA Hyper Prep Kit (KAPA Biosystems)
498 according to the manufacturer's instructions. ChIP-seq libraries were quantified by Tapestation
499 (Agilent) and sequenced on an Illumina NEXTseq (Illumina, San Diego, CA, USA) with 75-base
500 single-end mode.

501 **ChIP-qPCR**

502 Real-time ChIP-qPCR was carried out as described above. The signal of binding events was
503 normalized against input DNA for primer efficiency (Active Motif). Quantitative PCR primers
504 used are listed. *G6pc* forward: GCCTCTAGCACTGTCAAGCAG and reverse:
505 TGTGCCTTGCCCCTGTTTATATG; *Pck1* forward: TCCACCAACACACCTAGTGAGG and
506 reverse: AGGGCAGGCCTAGCCGAGACG; *Igfbp1* forward:
507 ATCTGGCTAGCAGCTTGCTGA and reverse: CCGTGTGCAGTGTCAATGCT; *Fkbp5*
508 forward: TTTTGTGTTGAAGAGCACAGAA and reverse: TGTCAGCACATCGAGTTCAT.

509 **ChIP-seq data analysis**

510 Reads were aligned to mouse genome mm10 using Bowtie2 software (52). The reads used in
511 subsequent analysis passed Illumina's purity filter, aligned with no more than 2 mismatches, and
512 mapped uniquely to the genome. Duplicate reads were removed with Picard tools. The tags were
513 extended at their 3'-ends to 200-bp. Technical information of sequencing depth and aligned reads
514 is summarized in Table S6. Peak calling was performed by MACS 2.1.0 (53) with the *p*-value
515 cutoff of 10^{-7} for narrow peaks and with the *q*-value cutoff of 10^{-1} for broad peaks against the
516 input DNA control sample. The transcription start site (TSS) determined on mouse genome
517 mm10 was used as measurement of the distance of each peak. HOMER software suite (54) was

518 used to perform motif analysis, annotate peaks, such as promoter/TSS, introns, exons, intergenic,
519 5' UTR, non-coding RNA, and 3' UTR, merge files, and quantify data to compare peaks. For the
520 detection of active enhancers, we used bedtools (55) by collecting the intersection of the peaks of
521 TF and histone marks.

522 **In vivo insulin-resistant model**

523 For high-fat diet-induced insulin resistance, animals were fed either standard or High-fat chow
524 (Rodent Diet with 60kcal% fat, D12492i; Research diets Inc.) beginning at 8 weeks of age for 4
525 weeks. For S961 treatment, vehicle (normal saline) or 10nmol S961 was loaded into Alzet
526 osmotic pumps 2001 and implanted subcutaneously on the back of mice. Mice were euthanized 3
527 days after implantation.

528 **Additional Data Sets**

529 The following public source data were used in this work: ChIP-seq data from adult mouse liver
530 [H3^{K4me1}] (56) (GEO: GSE31039), PPAR α (30) (GEO: GSE35262), GR and CREB (29) (GEO:
531 GSE72084).

532 **QUANTIFICATION AND STATISTICAL ANALYSES**

533 Values are presented as means \pm SEM, and analyzed using Prism 8.2.1 (GraphPad Software,
534 Inc.). We used unpaired Student's *t*-test for normally distributed variables for comparisons
535 between two groups, one-way ANOVA followed by Bonferroni post-hoc test for multiple
536 comparisons, and Pearson's correlation coefficient to investigate the relationship between two
537 variables. Chi-square tests are applied for contingency analysis. We used a threshold of $p < 0.05$
538 to declare statistical significance.

539

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546

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- 674
- 675

676 FIGURE LEGENDS

677

678 Figure 1. Distribution of genome-wide FoxO1 binding sites in the fast-refeed transition
679 (a) FoxO1 and HNF4 α immunohistochemistry in liver. Scale bar = 50 μ m. (b) Venn diagram of
680 the number of FoxO1 peaks in fasted or refed conditions. (c) Distribution of FoxO1 peaks
681 relative to annotated RefSeq genes (color-coded) compared with mouse genomic background. (d)
682 Signal intensity plots of ChIP-seq data for FoxO1 compared to input chromatin. The highest
683 level of binding occupancy of chromatin is at the top. (e) De novo motif analysis of the FoxO1
684 ChIP-seq. Logos of the recovered FoxO1 motif shows position-specific probabilities for each
685 nucleotide ($p = 1e-185$ in fast, $1e-195$ in refeed). (f) Scatterplots of FoxO1 ChIP-seq peaks,
686 expressed as \log_2 fold-change of FoxO1 tags between fast and refeed (horizontal axis) vs. \log_2
687 fold-change of mRNA levels between wild type and liver-specific FoxO1 knockout mice
688 (vertical axis) for each genomic site. FoxO1 peaks detected in fasted or refed conditions were
689 included in this analysis, and their number at each genomic annotation is shown inside each
690 graph. Detailed information on peaks associated with genes whose FDR < 0.05 is in Table S1.
691 Red= FDR < 1%; Blue= 1% \leq FDR < 5%; Green= 5% \leq FDR < 10%; Black= 10% \leq FDR. See
692 also Figure S1 and S2.

693

694 Figure 2. Comparison of the features of FoxO1 sites in active enhancers vs. non-enhancers in
695 promoter/TSS
696 (a) Bar diagram of FoxO1 active enhancers (red) and FoxO1 non-active enhancers (green) in
697 each genomic location. The number of active enhancer/non-active enhancer at each genomic
698 location is: Intergenic=1795/849, 5' UTR= 128/966, Promoter/TSS= 760/4303, exon= 384/1258,

699 intron= 2034/2501, non-coding= 44/158), TTS= 105/17), 3' UTR= 53/22. (b) Directed acyclic
700 graph derived from gene ontology analysis (GO) of biological processes associated with 5,305
701 FoxO1 active enhancers by GREAT GO tools. Letters correspond to the groups shown in (c) and
702 Fig. S3c. Numbers indicate the term's fold-enrichment. Red circles: fundamental ontologies in
703 the hierarchy listed in (c). Blue circles: additional enriched ontologies. Gray circles: parent
704 ontologies. (c) List of GO in (b) and their $-\log_{10}$ FDR. (d–f) Heatmap alignments of ChIPseq
705 FoxO1 binding in fast (d), fast/refeed ratio (e), and FDR of gene expression changes between
706 wild type and liver FoxO1 knockout mice (f) in GO related to glucose metabolic processes, lipid
707 homeostasis, and cellular response to insulin genes as listed in (b, c). (g, h) Same GO analysis as
708 in (b, c) applied to 4,303 FoxO1 sites lacking active enhancer marks in promoter/TSS. (i–k)
709 Heatmap alignments as in (d–f) of GO related to ncRNA processing, DNA repair, and protein
710 modification as listed in (g, h). See also Figure S3

711

712 Figure 3. Different FoxO1 binding logic between triglyceride and glucose metabolism genes
713 (a, b) Comparison between region-gene associations of triglyceride homeostasis (yellow bar) (a),
714 or glucose metabolic process (orange bar), with set-wide FoxO1 binding sites (blue bar) as
715 detected by FoxO1 ChIP-seq in fasted or refed conditions, binned by orientation and distance
716 from TSS. * $= p < 0.05$; ** $= p < 0.01$; **** $= p < 0.0001$ by chi-square test. (c) Distribution of
717 FoxO1 binding sites associated with triglyceride homeostasis or glucose metabolic process genes
718 according to genomic annotation as in Fig. 1c. $p = <0.03$ by contingency analysis. (d) GO
719 analysis of biological processes associated with 4,535 FoxO1 binding sites in introns using
720 GREAT GO tools. (e–g) IGV Genome browser views of FoxO1 peaks and associated H^{3K27ac} and
721 H3^{K4me1} histone marks at selected apolipoprotein clusters (Apob and ApoC2/C4/C1/E Apob,

722 Apoc4-c2, Apoc1, Apoe) and ScarB1. Signals are normalized for the comparisons between
723 fasted and refed conditions. FoxO1 signals are aligned with peak regions. Red arrows indicate
724 active enhancers as detected by H^{3K27ac} and H3^{K4me1} signals. FoxO1 peaks in introns are listed in
725 Table S2. See also Figure S4.

726

727 Figure 4. Resilience analysis of FoxO1-regulated genes

728 (a) t-SNE plot of RNA-seq data (n= 8). Circles indicate fasted and triangles refed animals. Filled
729 red symbols: AAV-GFP-injected animals (A-WT in the text); empty symbols with red border:
730 AAV-CRE-injected animals (A-FLKO in the text); green filled symbols: *Foxo1*^{loxp/loxp} (C-WT in
731 the text); empty symbols with green border: Alb-Cre/*Foxo1*^{fl/fl} (C-FLKO in the text). (b-e)
732 MA-scatterplots of average expression levels vs. log₂ fold-change induced by FoxO1 ablation in
733 tag count within exons of Ensemble gene bodies in fasted (b) or refed (c) A-FLKO, and fasted
734 (d) or refed (e) C-FLKO. Red dots represent differentially expressed genes (DEGs) (FDR ≤
735 0.05). The number of DEGs is indicated in each box. (f) Enrichment analysis of k-Means clusters
736 with molecular pathways underlying each category with top 1,000 variable genes among all
737 samples used in (a) by iDEP tools. (g) GO analysis of DEGs in fasted conditions, shown in (b)
738 and (d), by Shiny GO tools. Red heatmap shows FDR of genes in A-FLKO or C-FLKO. Violin
739 plots show log₂ fold-change of gene expression between control and A-FLKO (red) or C-FLKO
740 (green) for DEGs. Number of DEGs is indicated at the top. Purple heatmap shows FDR of each
741 ontology described next to it. Red- colored ontologies indicate the top enriched term in each
742 category. The number of genes in each ontology is shown in parenthesis in (f, g). DEGs are listed
743 in Table S3. See also Figure S5.

744

745 Figure 5. Comparative analysis among fasting inducible transcriptional factors
746 (a) Distribution of PPAR α , CREB and GR binding sites in fasted conditions. (b) Peak plot
747 mapping the overlap of the FoxO1 (Fig. 1e) and PPAR α , CREB and GR peaks. (c-d) Intersection
748 analyses of active (c), and non-active (d) FoxO1 and PPAR α , CREB or GR enhancer peaks in
749 fasting conditions. (e) Proportion of PPAR α peaks with/without active enhancer marks in FoxO1
750 active enhancers in fasting conditions according to genomic annotation. (f) Heatmap with
751 associated FDR of phenotype ontology terms of shared FoxO1/PPAR α active enhancers (red
752 bars) in intergenic regions and introns. (g, h) Resiliency plots of genes associated with shared
753 FoxO1/PPAR α active enhancers in intergenic regions (g) and introns (h). Plot show log₂ fold-
754 change induced by adult-onset vs. constitutive liver FoxO1 ablation. Resilient genes (FDR ≤ 0.05
755 in AFKO or CFKO mice, showing lower fold-change and higher FDR value in CFKO mice than
756 AFKO mice) are indicated by blue dots, non-resilient genes (FDR ≤ 0.05 in AFKO or CFKO
757 mice) are marked by red dots, FDR > 0.05 in both mice by white dots. DEGs are listed in Table
758 S5.
759

760 Figure 6. The transition of FoxO1 binding sites under insulin resistant condition
761 (a) Immunohistochemistry of FoxO1 and HNF4 α after 4hr fasting or following 1hr refeeding in
762 high fat diet (HFD)-fed mice or insulin receptor antagonist (S961)-treated mice. Scale bar = 20
763 μ m. (b) Scatterplots showing linear regression analysis of FoxO1 tag count between fasted and
764 refed conditions. Green: vehicle; red: S961-treated mice. (c-f) IGV Genome browser views of
765 FoxO1 peaks with or without S961 treatment and associated H3 K^{27ac} and H3 K^{4me1} marks of at
766 *G6pc*, *Pdk4*, *Angptl4/8*, and *ApoA1/C3/A4*. See also Figure S6.

767

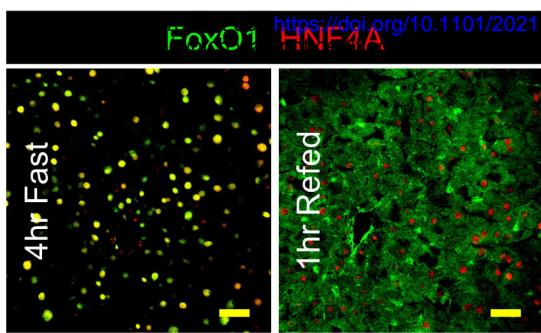
768 Figure 7. Model of FoxO1 transcriptional logic in the pathogenesis of selective insulin resistance

769 In normal conditions, FoxO1 is cleared upon refeeding from resilient enhancers, enriched in
770 glucose metabolism genes, but not in introns, enriched in lipid metabolism genes. With the onset
771 of insulin resistance-induced hyperinsulinemia, FoxO1 can be cleared from resilient enhancers,
772 but not from introns, increasing serum lipoprotein and triglyceride levels. As insulin resistance
773 progresses, compensation by PPAR α and spreading of FoxO1 binding to additional sites bolsters
774 expression of glucose metabolic genes, inducing fasting hyperglycemia with dyslipidemia.

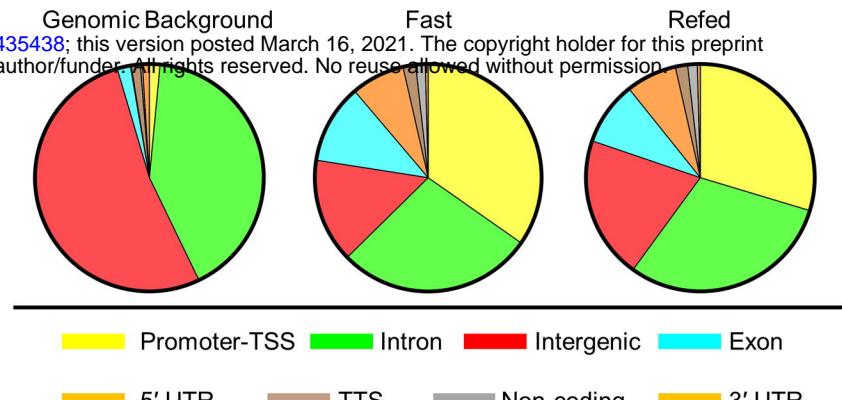
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Figure 1

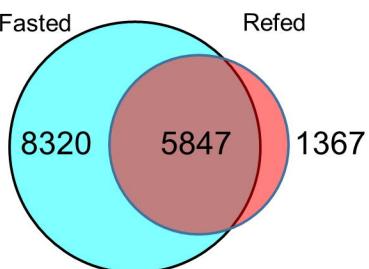
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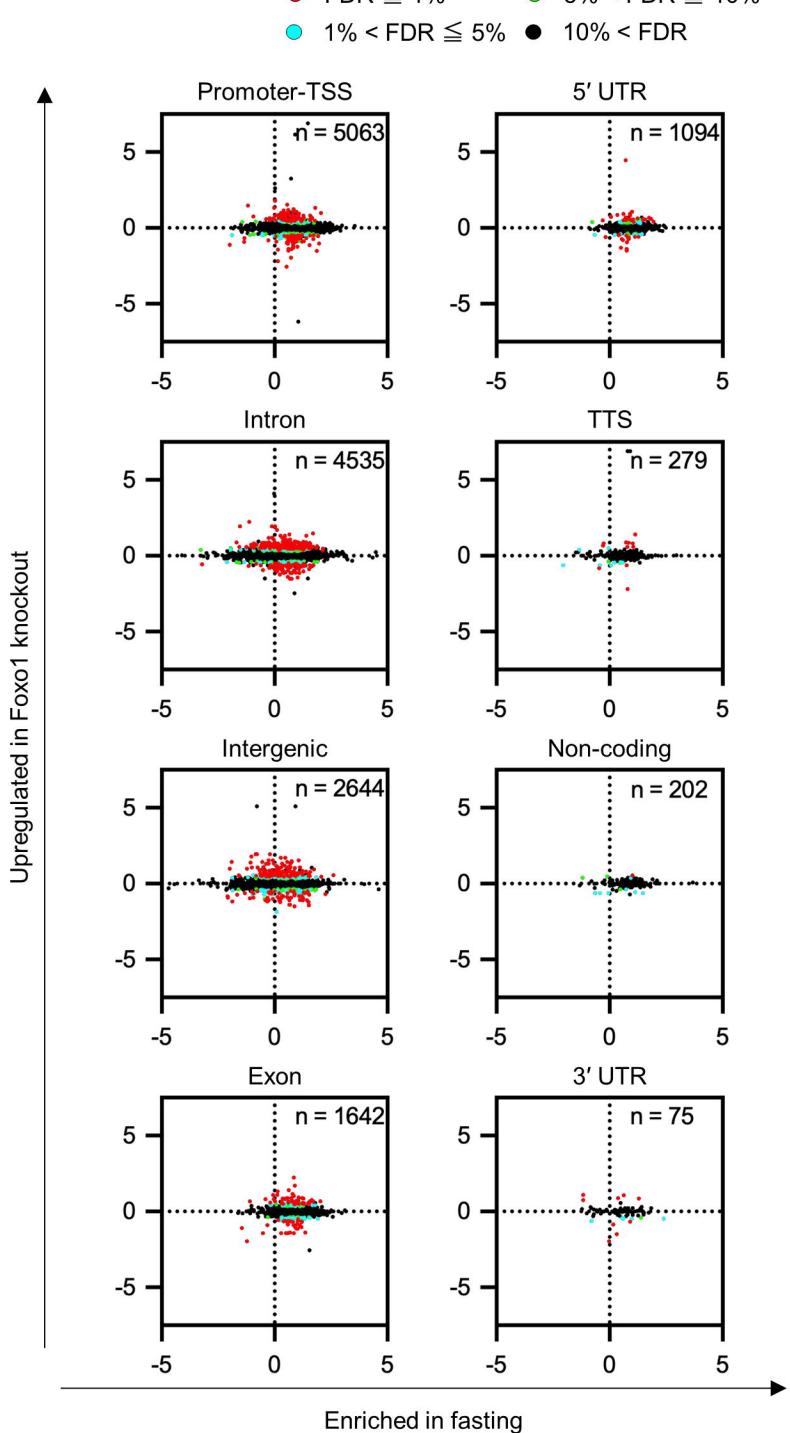
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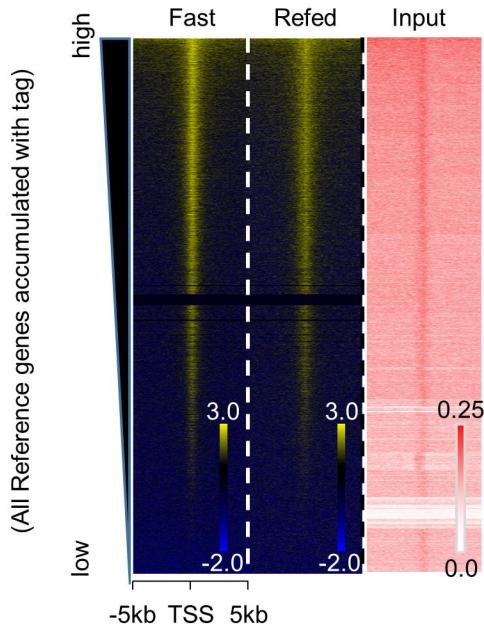
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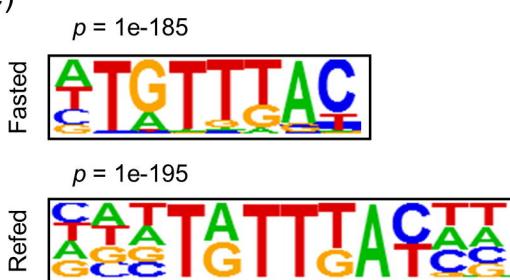


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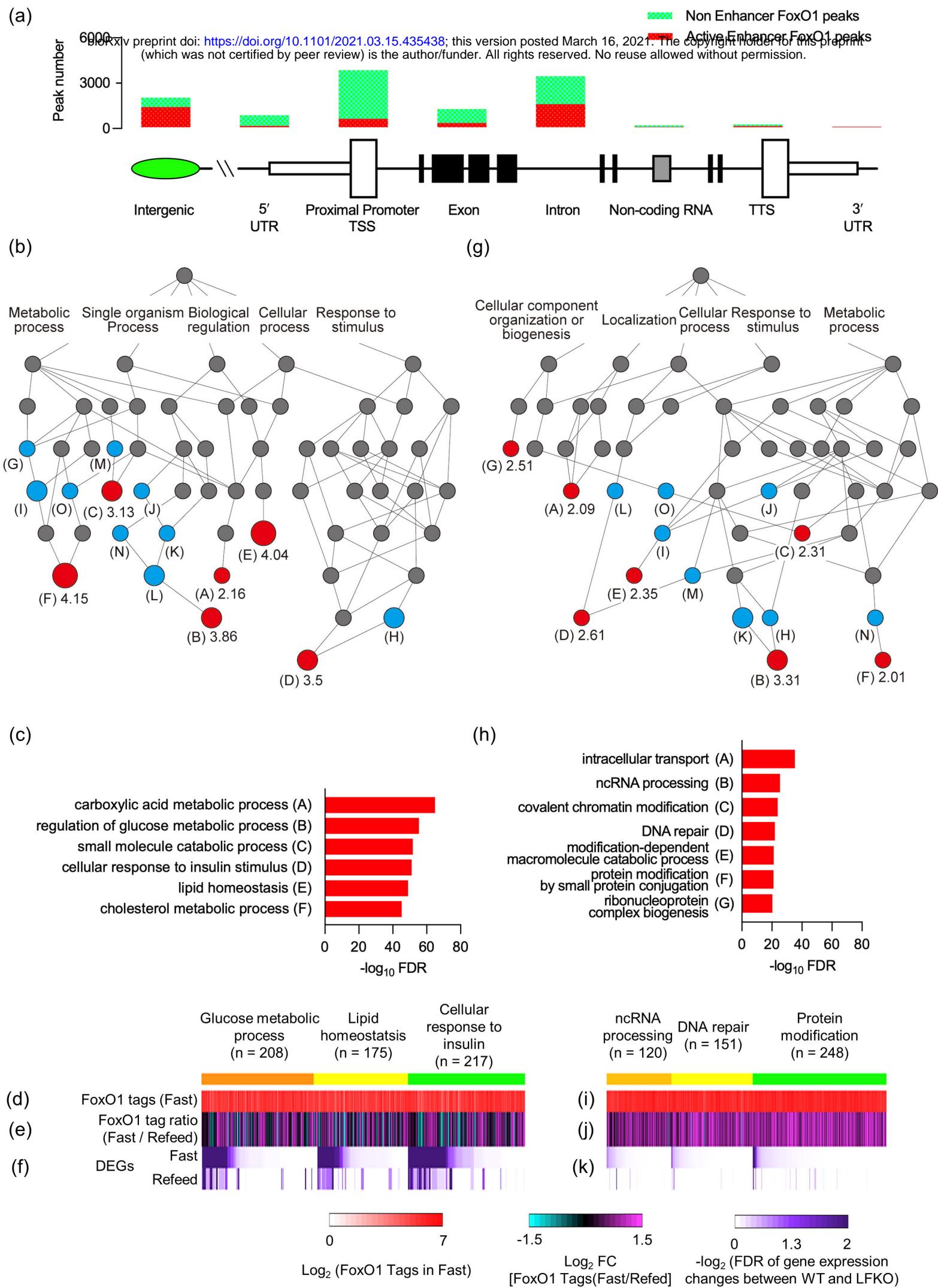


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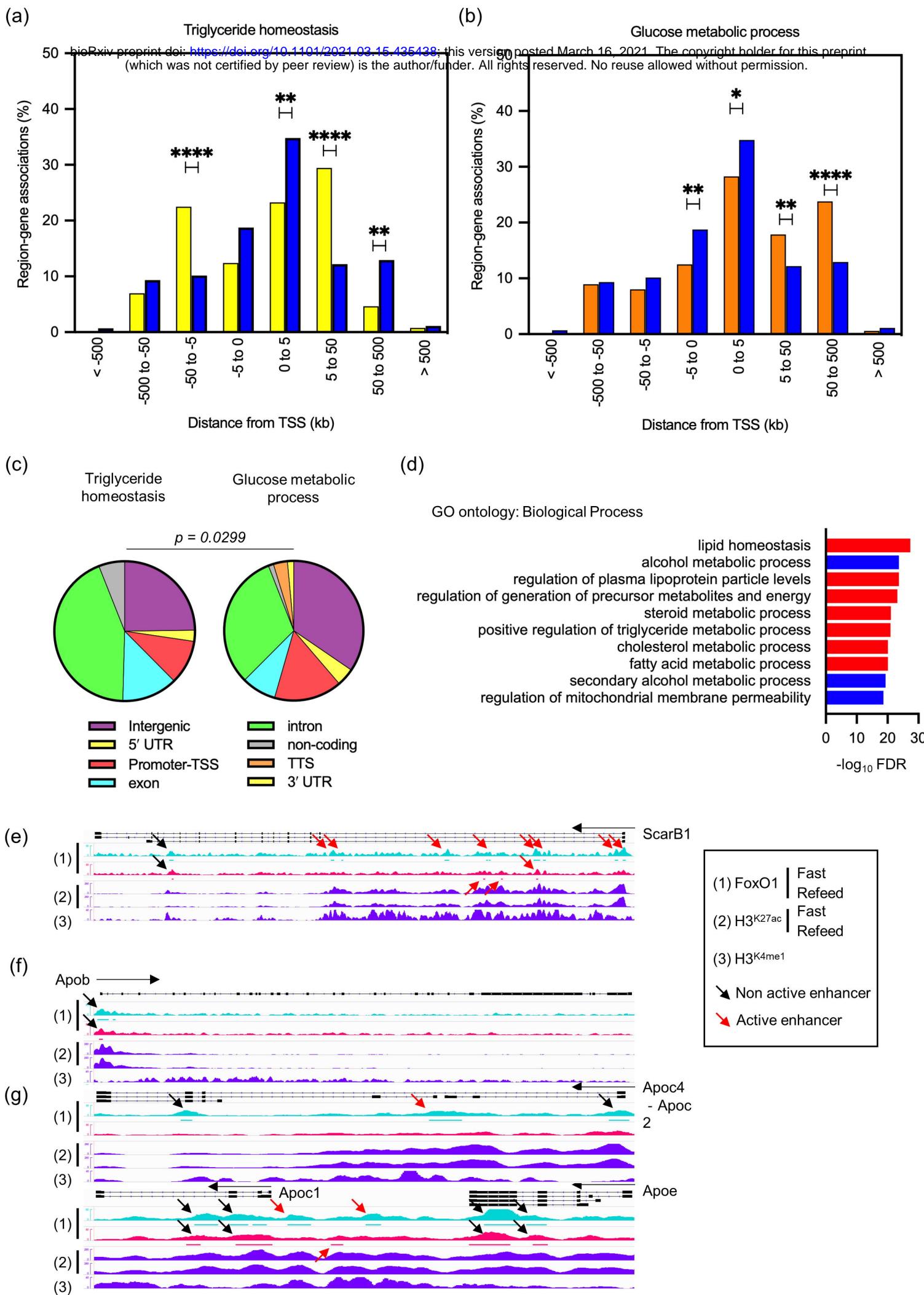


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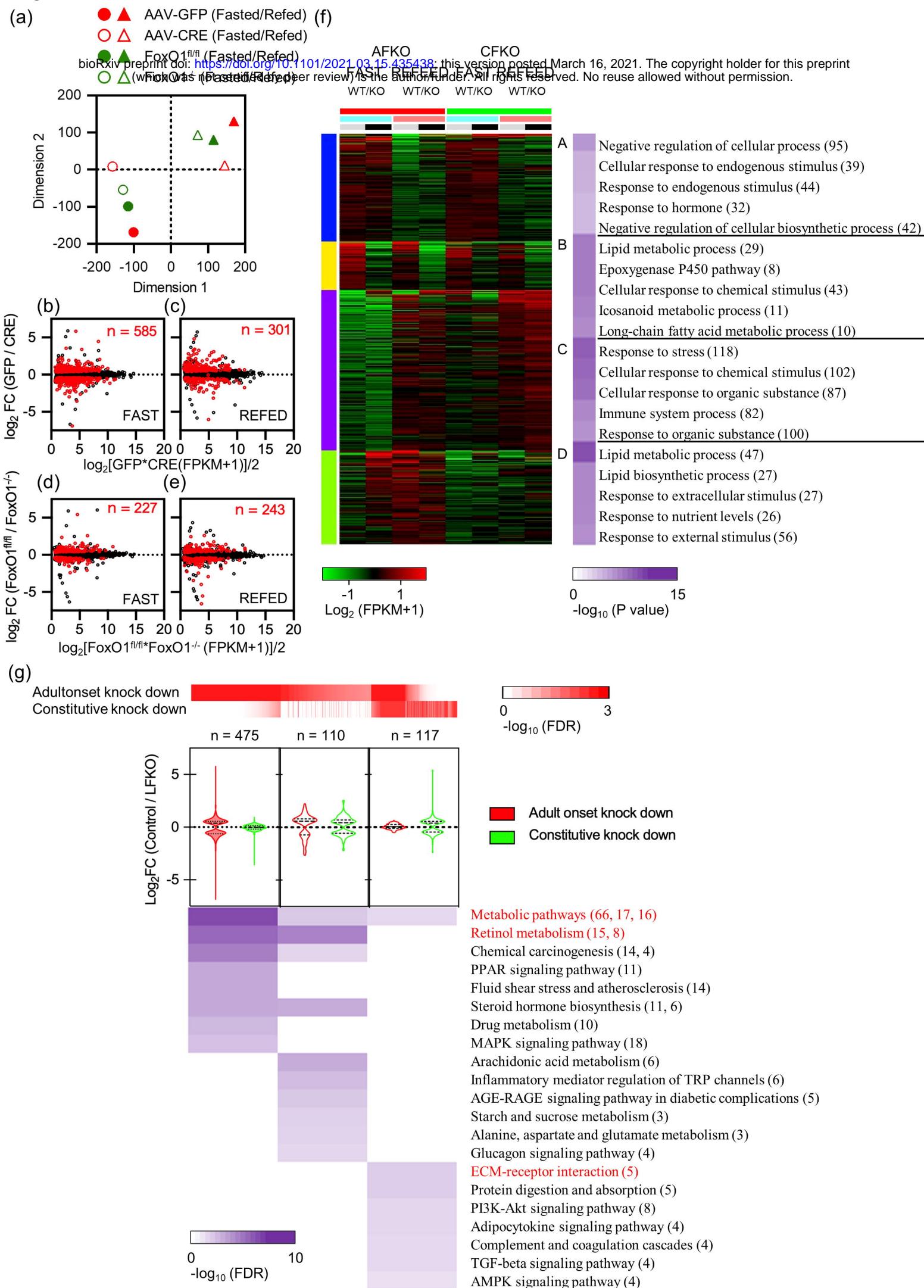
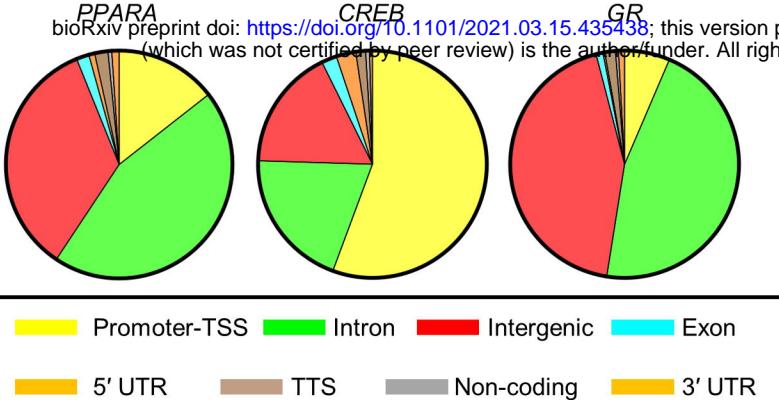
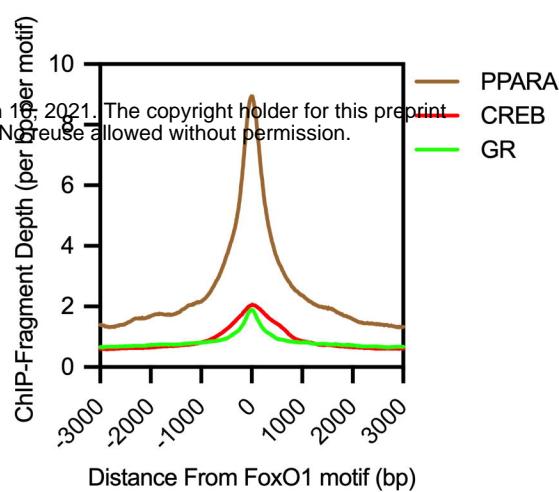


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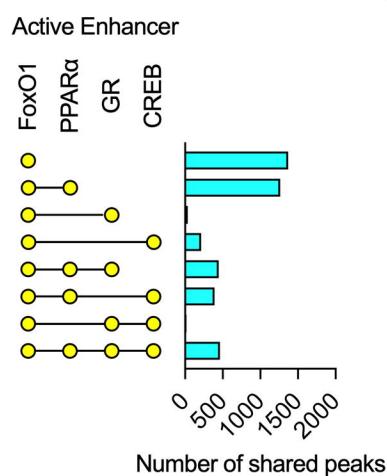
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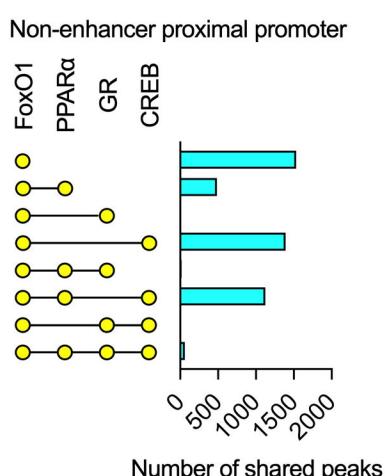
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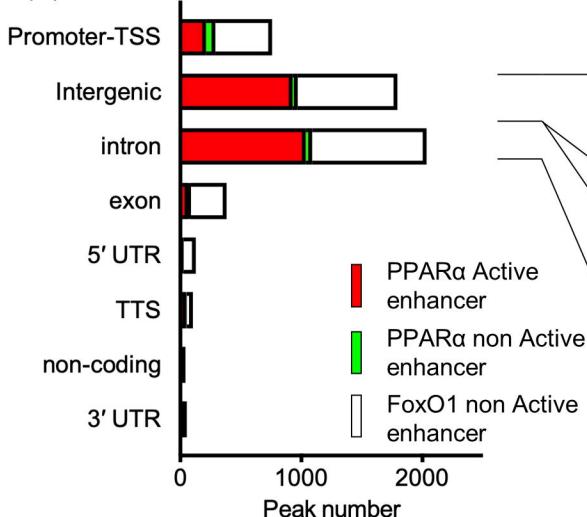
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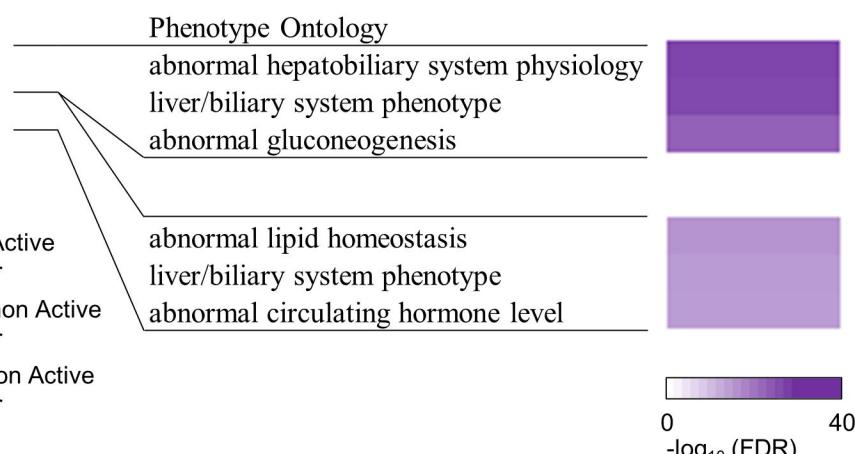
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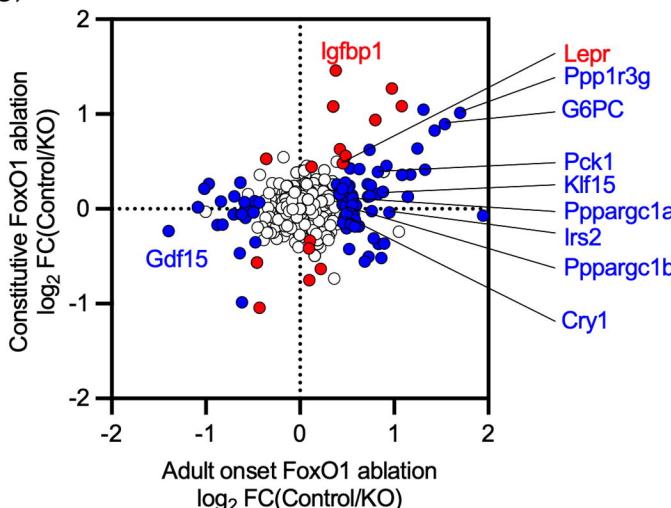
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(f)



(g)



(h)

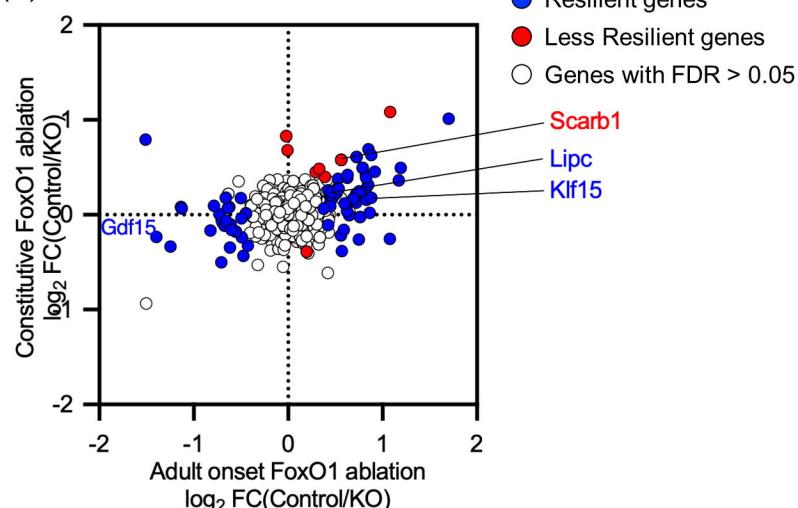


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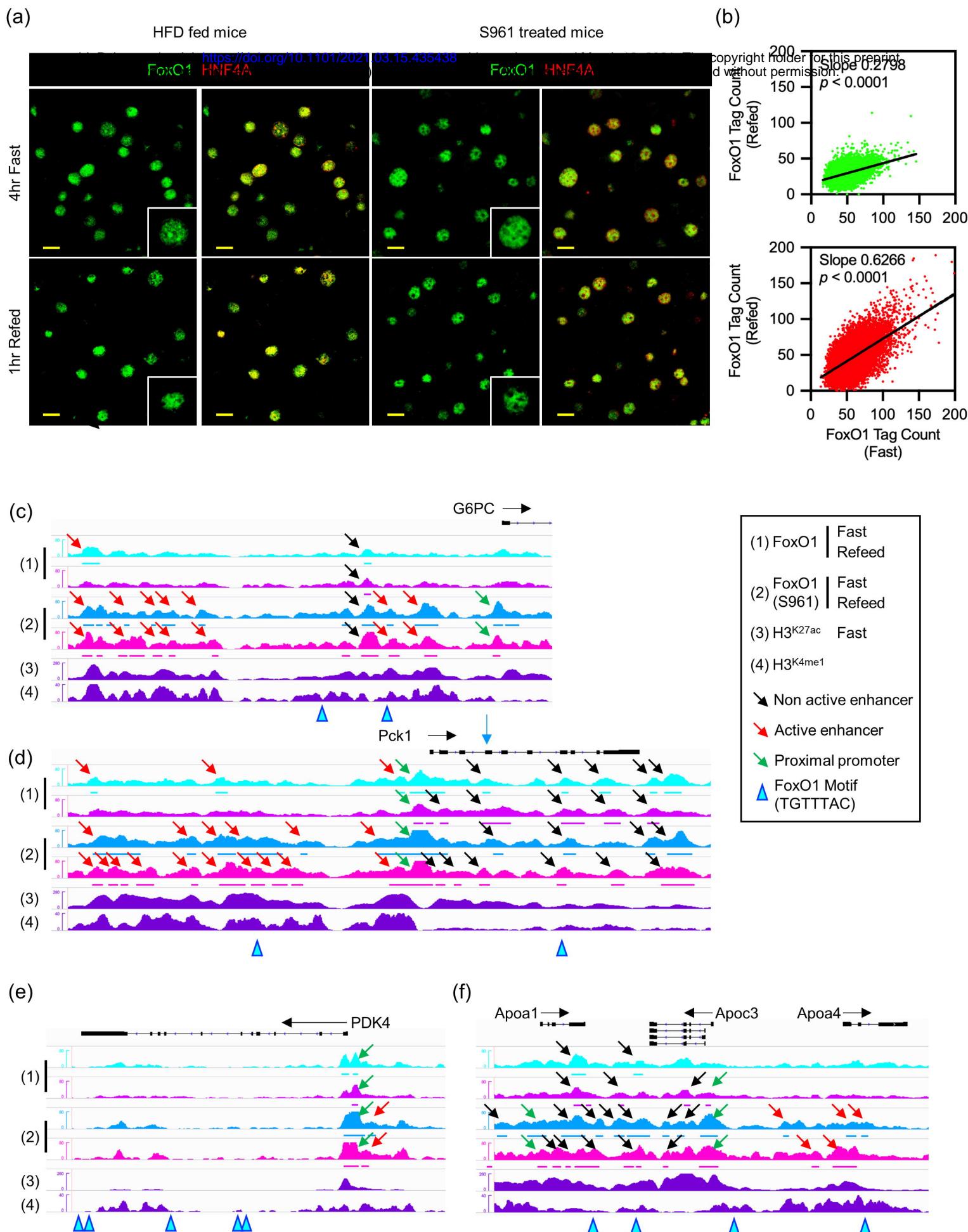


Figure 7.

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