

Experimental Models of Transcription Factor–Associated Maturity-Onset Diabetes of the Young

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Six monogenic forms of maturity-onset diabetes of the young (MODY) have been identified to date. Except for MODY2 (glucokinase), all other MODY subtypes have been linked to transcription factors. We have established a MODY3 transgenic model through the β -cell-targeted expression of dominant-negative HNF-1 α either constitutively (rat insulin II promoter) or conditionally (Tet-On system). The animals display either overt diabetes or glucose intolerance. Decreased insulin secretion and reduced pancreatic insulin content contribute to the hyperglycemic state. The conditional approach in INS-1 cells helped to define new molecular targets of hepatocyte nuclear factor (HNF)-1 α . In the cellular system, nutrient-induced insulin secretion was abolished because of impaired glucose metabolism. Conditional suppression of HNF-4 α , the MODY1 gene, showed a similar phenotype in INS-1 cells to HNF-1 α . The existence of a regulatory circuit between HNF-4 α and HNF-1 α is confirmed in these cell models. The MODY4 gene, IPF-1 (insulin promoter factor-1)/PDX-1 (pancreas duodenum homeobox-1), controls not only the transcription of insulin but also expression of enzymes involved in its processing. Suppression of Pdx-1 function in INS-1 cells does not alter glucose metabolism but rather inhibits insulin release by impairing steps distal to the generation of mitochondrial coupling factors. The presented experimental models are important tools for the elucidation of the β -cell pathogenesis in MODY syndromes. *Diabetes* 51 (Suppl. 3):S333–S342, 2002

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DN, dominant negative; Dtr, double transgenic; HNF, hepatocyte nuclear factor; K_{ATP} channel, ATP-sensitive K^+ channel; L-PK, liver-type pyruvate kinase; MODY, maturity-onset diabetes of the young; OGDH, 2-oxoglutarate dehydrogenase; PDX-1, pancreas duodenum homeobox-1; RIP, rat insulin promoter; rTA, reverse tetracycline-dependent transactivator; TCA, tricarboxylic acid; UCP, uncoupling protein.

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Maturity-onset diabetes of the young (MODY) is a monogenic form of type 2 diabetes characterized by early age of onset and autosomal dominant transmission and is usually not associated with insulin resistance (1,2). MODY is a clinically heterogeneous group of genetic disorders that may account for 2–5% of type 2 diabetes. Using the candidate gene screening approach, mutations in the gene encoding for the enzyme glucokinase were first defined as the genetic cause of MODY2 (3). Although glucokinase is expressed mainly in hepatocytes and islet cells, its mutations primarily cause a defect in glucose sensing by the pancreatic β -cells. MODY2 patients are usually mildly hyperglycemic throughout life and rarely require therapy (1). MODY1, -3, -4, -5, and -6 were discovered by positional cloning and have been associated with mutations in genes coding for the transcription factors hepatocyte nuclear factor (HNF)-4 α , HNF-1 α , IPF-1 (insulin promoter factor-1)/PDX-1 (pancreas duodenum homeobox-1)/IDX-1 (islet duodenum homeobox-1), HNF-1 β , and NeuroD/BETA2, respectively (1,2,4). Although mutations in these transcription factors display heterogeneous clinical phenotypes, the primary cause of the various MODY subtypes is β -cell dysfunction (2,4). To delineate the molecular mechanisms underlying β -cell pathology, we established in vivo and in vitro models of several forms of MODY.

EXPERIMENTAL MODELS OF MODY3

MODY3-associated mutations in the HNF-1 α gene are highly penetrant and constitute the most common form of MODY in many countries (2). MODY3 patients progressively develop severe hyperglycemia (5) and may exhibit the full spectrum of complications of diabetics if not treated with either oral hypoglycemic agents or insulin (2). In fact, 5–10% of antibody-negative diabetics, previously classified as type 1, are due to mutations in HNF-1 α (6). Chronic hyperglycemia in MODY3 patients is due to a primary defect in β -cells rather than in insulin action on target tissues (5,7). Although HNF-1 α is expressed in both liver and pancreas, liver function is unaffected in MODY3 patients. Insulin secretion in response to glucose and arginine is severely impaired, and this β -cell defect can already be detected in nondiabetic MODY3 carriers (7–9). Mutations linked to MODY3 have been localized throughout the different functional domains of the HNF-1 α protein

as well as in the promoter region of the HNF-1 α gene (2,10). However, the exact molecular mechanism by which these mutations impair HNF-1 α function remains unclear. Both dominant-negative actions and haploinsufficiency have been suggested to participate in the development of the MODY3 phenotype (8,11,12).

Mice with targeted disruption of HNF-1 α display the diabetic phenotype but also suffer from multiple organ manifestations such as renal dysfunction with massive glucosuria, pathological liver tests, and hepatomegaly (13,14). Pancreatic islets isolated from these animals exhibit attenuation not only in the generation of ATP but also in the rise of cytosolic Ca²⁺—two signals essential for glucose-stimulated insulin secretion (15,16). However, in contrast to the situation in MODY3 patients who harbor a single mutated HNF-1 α allele, insulin secretion in mice heterozygous for the disrupted allele of *hnf-1 α* is normal (14,16). This may be related to compensatory overexpression of HNF-1 β in this mouse model (14). Although the HNF-1 α ^{-/-} mice yielded important information (13,14,16,17), the pleiotropic effects observed in these mice have complicated analysis of the precise role of this transcription factor in determining normal pancreatic β -cell function. To further characterize the basis of the β -cell dysfunction induced by HNF-1 α deficiency, we established both *in vivo* and *in vitro* experimental models for MODY3 (11,12,18). The rationale of the *in vivo* study is to specifically suppress HNF-1 α function by targeted overexpression of a DN mutant of HNF-1 α (DNHNF-1 α) (11) in β -cells of transgenic mice. Two approaches were followed to achieve β -cell-specific expression of DNHNF-1 α in the transgenic mice. First, the rat insulin promoter (RIP) II was used to directly drive the expression of the DNHNF-1 α transgene (RIP-DNHNF-1 α mice). Second, the binary Tet-On system was used to achieve doxycycline-inducible expression of this transgene (Tet-DNHNF-1 α and RIP-rtTA mice).

RIP-DNHNF-1 α males became markedly diabetic around 6 weeks of age (Fig. 1A) and were already hyperglycemic in the fasted state (18). The RIP-DNHNF-1 α females were glucose intolerant (Fig. 1B) but never developed diabetes. Females also exhibited elevated nonfasting blood glucose values at weaning (134% of controls, $P < 0.001$) similar to those of males (138% of controls, $P < 0.001$). This was associated with comparable decreases in pancreatic insulin content (~40% of control), which thereafter increased in females and declined further in males (18). The males showed a blunted insulin response to intraperitoneal glucose injection at 5 weeks of age and failed to increase plasma insulin at 6 weeks. To examine the consequences of β -cell-specific expression of DNHNF-1 α on insulin secretion, we also perfused the pancreas of 5-week-old males (before the onset of diabetes). The first phase of glucose-stimulated insulin secretion was diminished by 50%, whereas the small second phase was less affected (69.4% of control). Arginine potentiation of glucose-induced insulin secretion was even more inhibited (26.9% of control). Progressive β -cell dysfunction was also demonstrated by age-related reduction in pancreatic insulin content and severely damaged β -cell structure. There was swelling of the mitochondria and dilatation of the endoplasmic reticulum (18). The latter is a sign of

apoptosis, which has been documented in INS-1 cells expressing the same transgene (19). Islet GLUT2 expression was severely reduced in 6-week-old RIP-DNHNF-1 α males. Unfortunately, further studies of gene expression were impossible because we were unable to isolate islets from these transgenic animals because of disrupted islet structure. The islet disorganization could be due, at least in part, to downregulation of the cell adhesion molecule E-cadherin, observed in a similar mouse model (20) exhibiting a more severe phenotype because the animals required insulin therapy. In that model, glucose-stimulated insulin secretion in isolated islets from glucose-intolerant female mice was also markedly attenuated.

As an alternative approach, we used an inducible system that allows conditional expression of DNHNF-1 α . Two independent transgenic mouse lines were generated. The first carries the reverse tetracycline-dependent transactivator (rtTA) driven by RIP (RIP-rtTA mice). The second harbors the DNHNF-1 α transgene under the control of the Tet operator (Tet-DNHNF-1 α mice). Two Tet-DNHNF-1 α founder mice were identified by PCR and Southern blotting and used to establish two transgenic families, termed Tet-18 and Tet-23. Tet-DNHNF-1 α mice from both families were then crossed with RIP-rtTA mice to obtain double-transgenic (Dtr) RIP-rtTA/Tet-DNHNF-1 α mice. Dtr mice constitute ~25% of each litter, whereas the other littermates are either nontransgenic or single-transgenic. The latter were subdivided into two control groups: 1) those positive for the RIP-rtTA transgene (rtTA⁺) and 2) those carrying the Tet-DNHNF-1 α transgene or negative for both transgenes (controls). To induce expression of DNHNF-1 α , pregnant mice and their offspring were given doxycycline in their drinking water (0.8 g/l); these doxycycline-treated animals were then compared with untreated mice. To assess glucose homeostasis in mice from both Tet-DNHNF-1 α families crossed with the RIP-rtTA mice, glucose tolerance tests were performed on ~9-week-old mice. The results are summarized in Fig. 2A and B (-dox) and Fig. 2C and D (+dox). Unexpectedly, all RIP-rtTA⁺ mice, whether treated with doxycycline or not, exhibited mild glucose intolerance. Because there is high expression of rtTA in the β -cells of the RIP-rtTA family, normal β -cell function may be altered. Given the glucose intolerance of the rtTA⁺ mice, these animals were used as controls in this study. As expected, in the absence of activation by doxycycline, the glucose intolerance of Dtr mice, whether male or female, was not further increased when compared with rtTA⁺ mice (Fig. 2A and B). This result indicates that although rtTA expression by itself affected β -cell function, it remained unable to transactivate the Tet-DNHNF-1 α transgene in the absence of doxycycline. In contrast, when mice from the Tet-18 and Tet-23 families were treated with doxycycline, Dtr males became more severely glucose intolerant (Fig. 2C and D), statistically significant at 60, 90, and 120 min; this result was apparently much more pronounced in males of the Tet-18 family. Interestingly, in this family, the fasting blood glucose values were also significantly higher than those in controls, which was due to two Dtr males exhibiting mild hyperglycemia (~12 mmol/l). Together these results indicate that treating RIP-rtTA/Tet-DNHNF-1 α Dtr mice with doxycycline further aggravates the glucose intolerance observed in the rtTA

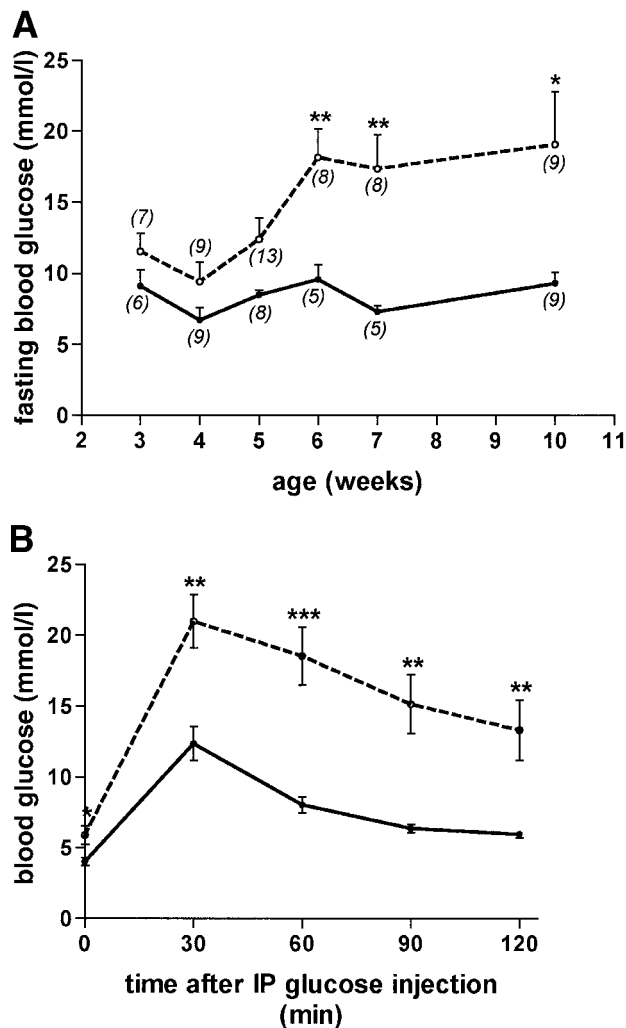


FIG. 1. Overexpression of DNHNH-1 α in β -cells of transgenic mice induces diabetes in males and glucose intolerance in females. **A:** Evolution of fasting (6 h) blood glucose levels in control and RIP-DNHNH-1 α males (●). RIP-DNHNH-1 α males become overtly diabetic around the age of 6 weeks, with a mean fasting glycemia of 18.1 ± 2.01 vs. 9.58 ± 1.03 mmol/l in controls (○). **B:** The 13-week-old females were subjected to an intraperitoneal (IP) glucose tolerance test after an overnight (15-h) fast. At all time points measured, blood glucose values were significantly higher in RIP-DNHNH-1 α females (○; $n = 7$) than in control females (●; $n = 6$). Reprinted with permission from Hagenfeldt-Johansson et al. (18).

males. The intolerance was more pronounced in males than in females (data not shown) and in Dtr males of the Tet-18 than in the Tet-23 family (Fig. 2). Interestingly, this difference between males and females parallels the sexual dimorphism observed in RIP-DNHNH-1 α mice (18,20). It should also be noted that although doxycycline-treated RIP-rtTA/Tet-DNHNH-1 α animals never became overtly diabetic, males from both families occasionally exhibited fasting blood glucose >10 mmol/l. This relative hyperglycemia was never observed in mice not subjected to doxycycline treatment.

Using immunostaining with an antibody against the NH₂-terminus of HNF-1 α , we showed that anti-HNF-1 α staining of islets isolated from untreated animals did not notably differ between control and glucose-intolerant Dtr

males of the Tet-23 family (Fig. 3A and B). However, DNHNH-1 α overexpression could be demonstrated in the islets of the doxycycline-treated Tet-23 Dtr males that exhibited some degree of hyperglycemia (Fig. 3C and D). In support of this, insulin staining was attenuated in pancreas sections from Tet-23 Dtr males (Fig. 3F), although not to the same extent as in RIP-DNHNH-1 α males. Taken together, the phenotype of the Dtr males is reminiscent of the clinical characteristics reported for marker-positive nondiabetic MODY3 patients (7). This inducible HNF-1 α animal model will provide a powerful tool to study the pattern of gene expression over time and determine the primary and secondary effects of DNHNH-1 α in MODY3 pathology.

TARGETS FOR HNF-1 α

To identify HNF-1 α target genes and elucidate the molecular mechanisms underlying β -cell dysfunction after its suppression, we also established INS-1 stable clones permitting expression of the artificial rat HNF-1 α mutation DNHNH-1 α (11) and the most common human mutation HNF-1 α P291fsinsC (21), respectively. As summarized in Table 1, HNF-1 α targets multiple genes implicated in metabolism-secretion coupling. Induction of either DNHNH-1 α or HNF-1 α P291fsinsC resulted in severely impaired glucose- and leucine-stimulated, but not KCl-evoked, insulin secretion (11,21). Accordingly, glucose and leucine oxidation was severely reduced in cells expressing DNHNH-1 α (11). This defect lies in the mitochondria because [2-¹⁴C]pyruvate oxidation and glucose-induced mitochondrial hyperpolarization (reflecting electron transport chain activity) were inhibited. The carbon in the [2-¹⁴C]pyruvate can only be lost to ¹⁴CO₂ at either the isocitrate dehydrogenase or 2-oxoglutarate dehydrogenase (OGDH) steps in the tricarboxylic acid (TCA) cycle. Interestingly, the mRNA and protein levels of the catalytic OGDH E1 subunit, which is involved in conversion of [2-¹⁴C]pyruvate to ¹⁴CO₂, were reduced in HNF-1 α P291fsinsC cells. Furthermore, the expression of uncoupling protein (UCP)-2 was increased. UCP-2 may act as a protonophore and dissipate the mitochondrial membrane potential, thereby uncoupling respiration from ATP synthesis (21). In addition, the generation of ATP by nutrients was abolished in both the doxycycline-treated DNHNH-1 α and HNF-1 α P291fsinsC cell lines (11,21). Together, these results strongly point to defective mitochondrial metabolism as a primary mechanism underlying the diminished cytosolic Ca²⁺ rise (11) and impaired insulin secretion in cells with induced HNF-1 α deficiency. It is tempting to speculate that β -cell mitochondrial dysfunction and morphological changes in β -cells of RIP-DNHNH-1 α mice may not be mutually exclusive. In addition to its apparent importance in regulating mitochondrial function, HNF-1 α was also shown to regulate genes involved in glucose transport and glycolysis, such as GLUT2, aldolase B, and liver-type pyruvate kinase (L-PK). In DNHNH-1 α cells, the glycolytic flux was indeed decreased, despite unaltered expression of glucokinase, the rate-limiting glycolytic enzyme (11). Our findings contradict those of Shih et al. (22), who showed by RT-PCR that glucokinase expression was reduced in islets of *hnf-1 α ^{-/-}* mice and hypothesized that diminished glycolysis rather than defective mitochondrial function impairs glucose-stimulated insulin secretion. In-

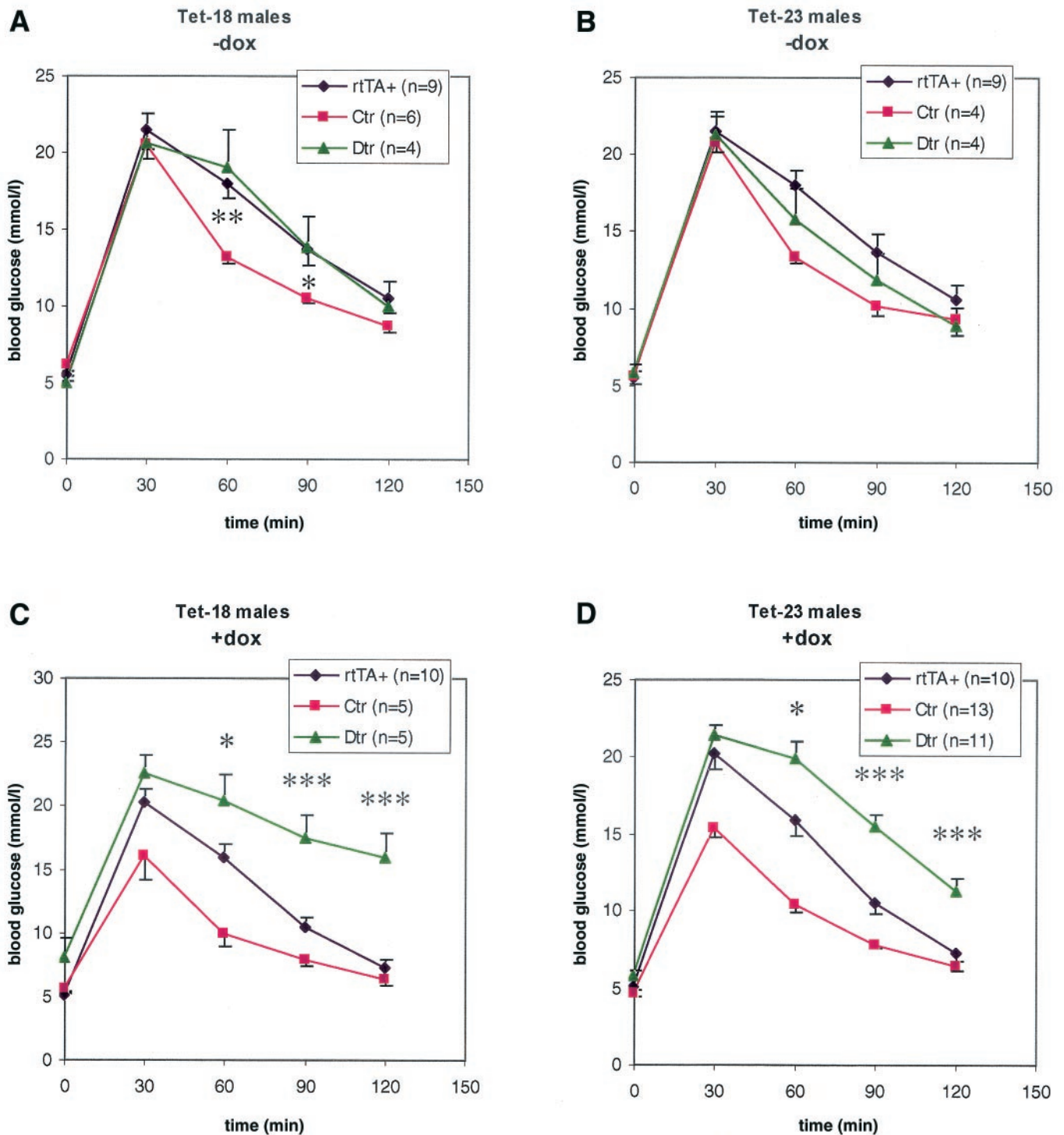


FIG. 2. *A* and *B*: The RIP-rtTA transgene induces glucose intolerance in the absence of doxycycline. The 9-week-old male mice from the Tet-18 (*A*) and Tet-23 (*B*) families, not treated with doxycycline (-dox), were subjected to an intraperitoneal glucose tolerance test (IPGTT) after an overnight (15-h) fast. The rtTA⁺, control (Ctr), and Dtr groups are as defined in the text. Values are means ± SE. Male rtTA⁺ mice are significantly glucose intolerant compared with control mice (the 60- and 90-min time points are the most important ones in an IPGTT, reflecting the efficiency of glucose disposal). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by unpaired two-tailed *t* test, comparing rtTA⁺ with control mice. *C* and *D*: Doxycycline treatment induces glucose intolerance in RIP-rtTA/Tet-DNHNF-1α Dtr males. The 9-week-old male mice from the Tet-18 (*C*) and Tet-23 (*D*) families, treated with doxycycline (+dox) from conception, were subjected to IPGTTs as in *A* and *B*. Dtr males from both families (*C* and *D*) were clearly glucose intolerant when compared with the rtTA⁺ group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by unpaired two-tailed *t* test, comparing Dtr with rtTA⁺ mice.

terestingly, Northern blot analysis revealed increased rather than decreased glucokinase mRNA in islets isolated from the same transgenic animals (15). The hypothesis proposed by Shih et al. suggests that MODY3 and MODY2

should display similar clinical phenotypes. However, there are distinct clinical and pathophysiological profiles between these two genetic subtypes of diabetes, reflecting the clear differences in β-cell dysfunction (5). Loss-of-

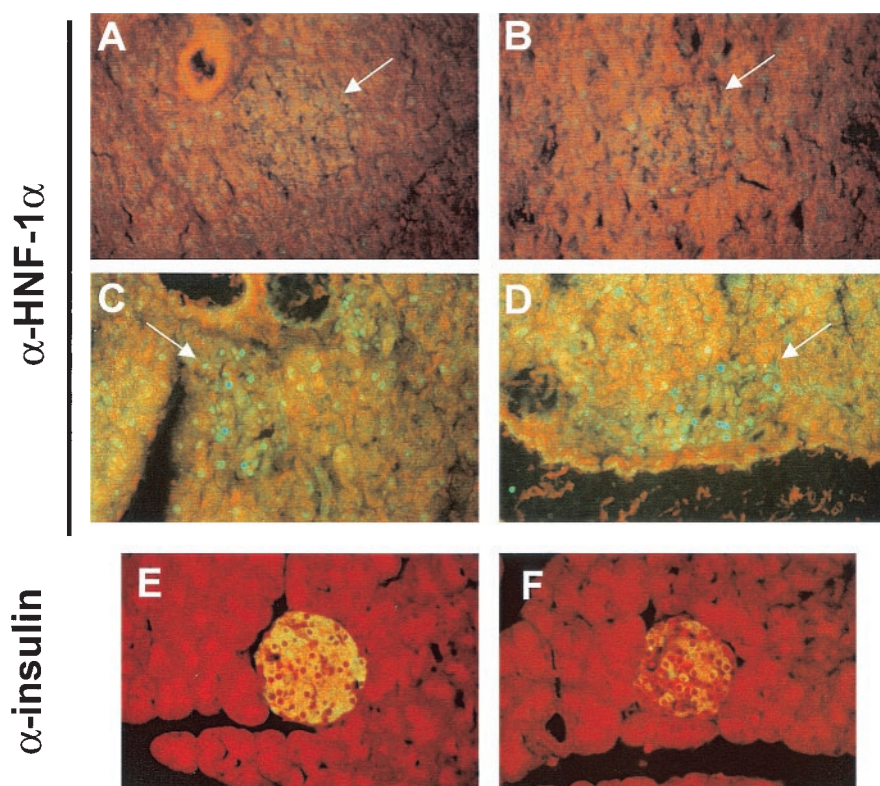


FIG. 3. DNHNf-1 α is expressed in the islets of Dtr Tet-DNHNf-1 α males and affects insulin staining. The 7 μm -thick cryostat sections (A–D) or 5- to 7 μm -thick paraffin sections (E and F) of pancreas from 10-week-old control (A and E) and Dtr (B–D and F) doxycycline-treated Tet-DNHNf-1 α males from the Tet-23 family were stained with an antibody against the NH₂-terminus of HNF-1 α (A–D) or insulin (E and F). Expression of DNHNf-1 α (indicated by arrows) in the islets of doxycycline-treated Dtr mice was only detected in the few males that developed mild hyperglycemia (compare B with C and D). A slight decrease in insulin staining was always observed in the islets of Dtr males, as shown in F.

function mutations in glucokinase lead to a stable defect of glucose sensing, whereas the HNF-1 α mutations cause progressive impairment of glucose-stimulated insulin secretion rather than glucose sensing (5). Therefore, the abolished insulin secretion in response to glucose in MODY3 patients supports our conclusion that HNF-1 α deficiency causes both reduced glycolysis and diminished mitochondrial metabolism.

Dominant-negative suppression of HNF-1 α function markedly reduced cellular insulin, both at the mRNA and protein levels, by direct inhibition of RIP I activity (11,21). It has been demonstrated that HNF-1 α regulates the PDX-1 promoter activity (23). We found that induction of HNF-1 α P291fsinsC for 96 h caused 60% reduction of PDX-1 mRNA levels (Fig. 4A). However, the suppressive effect of HNF-1 α P291fsinsC on insulin precedes its action on PDX-1 expression (21). Using our DNHNf-1 α and HNF-1 α P291fsinsC cell models, genes such as cyclin E, p27^{KIP1}, and Bcl-xL, which regulate cell proliferation and apoptosis, have been identified as HNF-1 α targets (19,24). The altered expression of these genes may also be implicated in the progressive deterioration of β -cell function and cell mass in MODY3 patients. HNF-1 α has also been shown to be an essential transcriptional regulator of bile acid and HDL cholesterol metabolism (17) and to control renal glucose reabsorption (25).

CELL MODELS FOR MODY1

MODY1, caused by mutations in the HNF-4 α gene, is much less prevalent than other subtypes of MODY. Although it is less penetrant than MODY3 and the average age of patients with MODY1 at diagnosis is slightly higher, clinical fea-

tures of MODY1 are very similar (1,2). Primary β -cell dysfunction in MODY1 patients also results in impaired insulin secretion and leads to the development of a severe form of diabetes (1,2). HNF-4 α acts upstream of HNF-1 α in a transcriptional cascade that mediates hepatic gene expression and differentiation (26). A mutation in the HNF-4 α binding site of the HNF-1 α promoter has been reported in a MODY3 family, suggesting that this transcriptional hierarchy could also exist in β -cells (10). Targeted deletion of HNF-4 α results in embryonic lethality that prevents further analysis of HNF-4 α function in β -cells (27). Our INS-1 cell model, permitting conditional expression of HNF-4 α or its dominant-negative mutant DNHNf-4 α , represents a unique system for this analysis (28). Similar to DNHNf-1 α , induction of DNHNf-4 α leads to defective mitochondrial metabolism and, as a consequence, impaired insulin secretion. Decreased TCA cycle activity, as well as partial uncoupling of the mitochondrial electron transport chain, could be demonstrated. In addition, we also showed that the expression of the OGDH E1 subunit and UCP-2, as well as GLUT2, aldolase B, and L-PK, is regulated by HNF-4 α . Induction of DNHNf-4 α indeed eliminated endogenous HNF-1 α expression and severely reduced HNF-1 α promoter activity in a reporter gene assay (28), suggesting that HNF-4 α is an upstream regulator of HNF-1 α .

Two recent reports showed that a distant upstream promoter in the HNF-4 α gene (P2) is specifically used in INS-1 and pancreatic β -cells (29,30). The P2 promoter containing an HNF-1 α binding site is functional (29) and is occupied by HNF-1 α selectively in pancreatic islets in vivo (30). Accordingly, HNF-4 α transcription is severely de-

TABLE 1
Effects of DN suppression of HNF-1 α on β -cell function

Insulin secretion	
Glucose stimulated	90% inhibition
Leucine induced	70% inhibition
KCl elicited	Unaffected
Metabolism	
Glycolysis	30% reduction
Mitochondrial oxidation	
Glucose	45% reduction
Leucine	70% reduction
Pyruvate	50% reduction
ATP generation	
Glucose produced	90% decrease
Leucine evoked	90% decrease
Plasma membrane depolarization	
Glucose induced	80% inhibition
Leucine stimulated	80% inhibition
KCl evoked	Unaltered
[Ca ²⁺] _i rises	
Glucose stimulated	56% inhibition
Leucine induced	45% inhibition
KCl depolarization evoked	Unchanged
Mitochondrial membrane hyperpolarization	
Glucose induced	66% decrease
Target genes	
Insulin	Reduced expression
GLUT2	Reduced expression
L-PK	Reduced expression
Aldolase B	Reduced expression
HMGCoA reductase	Reduced expression
OGDH E1	Reduced expression
UCP-2	Enhanced expression
Pdx-1	Reduced expression
HNF-4 α	Reduced expression
E-cadherin	Reduced expression
IGF-1	Reduced expression
MIF	Reduced expression
Cyclin E	Reduced expression
P27 ^{KIP1}	Enhanced expression
Bcl-xL	Reduced expression

creased in islets, but not in liver, of *hnf-1 α ^{-/-}* mice (30). These studies thus indicate that HNF-1 α can also act upstream of HNF-4 α in β -cells, revealing a complex transcription factor circuit operating to maintain normal β -cell function (30). To verify this hypothesis, we analyzed the expression pattern of HNF-4 α in our INS-1 stable clones after induction of HNF-1 α P291fsinsC for 96 h. As shown in Fig. 4A, dominant-negative suppression of HNF-1 α resulted in 40% reduction in the HNF-4 α mRNA level. The effect is specific because the transcript of the housekeeping gene cyclophilin remains unaltered. This finding supports the notion of a regulatory loop between HNF-4 α and HNF-1 α transcription (30). A MODY1 family was reported in which the PDX-1 binding site in the HNF-4 α P2 promoter was mutated, suggesting a regulatory circuit between PDX-1, HNF-4 α , and HNF-1 α . We therefore investigated the consequence of upregulation and downregulation of PDX-1 function on HNF-4 α expression in INS-1 cells, which permit inducible expression of PDX-1 or a dominant-negative mutant of PDX-1 (DN-PDX-1) (31). Induction

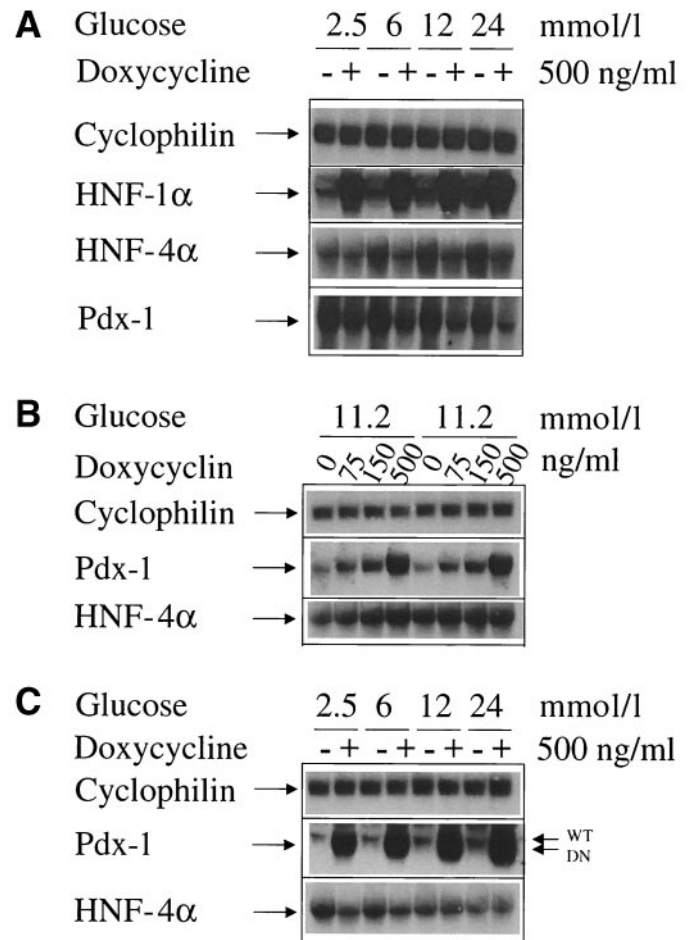


FIG. 4. A: Induction of HNF-1 α P291fsinC suppresses the mRNA levels of PDX-1 and HNF-4 α . PDX-1 and HNF-4 α mRNA levels were quantified by Northern blotting. There was 20 μ g of total RNA analyzed by hybridizing with PDX-1 and HNF-4 α cDNA probes. The same membrane was stripped and rehybridized with cyclophilin and HNF-1 α cDNA probes. HNF-1 α P291fsinsC number 32 cells (21) were cultured with or without 500 ng/ml doxycycline in standard glucose medium (11.2 mmol/l) for 72 h and continued for a further 16 h in 2.5 mmol/l glucose medium before incubation at indicated glucose concentrations for an additional 8 h. B: Overexpression of PDX-1 causes a modest increase in HNF-4 α expression. Northern blotting was performed as indicated in A. PDX-1 number 6 cells (31) were cultured with 0, 75, 150, and 500 ng/ml doxycycline in standard glucose medium (11.2 mmol/l) for 96 h. C: Induction of DN-PDX-1 results in a slight reduction of HNF-4 α expression at lower glucose concentrations. Northern blotting was performed as indicated in A. DN-PDX-1 number 28 cells (31) were cultured with or without 500 ng/ml doxycycline in standard glucose medium (11.2 mmol/l) for 72 h and continued for a further 16 h in 2.5 mmol/l glucose medium before incubation at indicated glucose concentrations for an additional 8 h.

of PDX-1 caused a mild increase in HNF-4 α mRNA (Fig. 4B), whereas dominant-negative suppression of PDX-1 resulted in a slight reduction in this transcript (Fig. 4C). These results suggest that PDX-1 does not play a major role in the regulation of HNF-4 α expression, at least in our in vitro system. The regulatory loop between PDX-1 and HNF-4 α /HNF-1 α remains to be established.

EXPERIMENTAL MODELS OF MODY 4

PDX-1 is required for pancreatic development and for maintaining the β -cell phenotype (31,32). Homozygosity

for a mutation in the *PDX-1/IPF-1* gene results in failure of pancreas formation, whereas heterozygosity for an inactivating mutation and missense mutations in this gene are associated with MODY4 and late-onset type 2 diabetes in humans, respectively (33,34). The primary defect in MODY4 is impaired glucose-stimulated insulin secretion (35). Targeted disruption of *PDX-1* in mice results in pancreatic aplasia (32), whereas mice with β -cell-specific deletion of *PDX-1* develop diabetes (36). Brissova et al. (37) recently reported impaired glucose tolerance caused by defective glucose-stimulated insulin secretion in the conventional heterozygous *PDX-1* mutant mouse. Reduced GLUT2 expression and decreased glucose-evoked NAD(P)H generation in islets were also observed, suggesting impaired glucose metabolism. It is noteworthy that glucokinase expression is unaffected in islets isolated from these mice, supporting our previous conclusion that *PDX-1* does not regulate the glucokinase gene (31), in contrast to previous claims by Watada et al. (38). Unaltered glucokinase levels were also demonstrated in the islets of mice with β -cell-specific deletion of *PDX-1* (36). Furthermore, *PDX-1* does not bind to the β -cell glucokinase promoter in vivo (39).

The in vivo study on mice with β -cell-specific deletion of *PDX-1* (36) and our in vitro work on INS-1 cells expressing DN-Pdx-1 (31) indicate that *PDX-1* promotes the β -cell phenotype by maintaining the expression of β -cell-specific genes while suppressing glucagon levels. Suppression of glucagon expression by *PDX-1* requires synergism with other β -cell-specific transcription factors because ectopic expression of *PDX-1* in glucagonoma cells failed to eliminate glucagon (40). We also showed that maximum induction of Brain-4, an α -cell-specific transcription factor, initiated detectable levels of glucagon but did not attenuate the expression of β -cell-specific genes in INS-1 cells (31). This result is in good agreement with a recent in vivo study by Hussain et al. (41), where *PDX-1* promoter-driven expression of Brain-4 in pancreatic precursors transactivates glucagon in β -cell lineage without altering β -cell gene expression. Therefore, both Brain-4 expression and loss of *PDX-1* function are necessary for differentiation of the α -cell lineage. The transcription factors that switch on and off Brain-4 and *PDX-1*, respectively, remain to be defined.

We further investigated the mechanism by which *PDX-1* deficiency impairs insulin secretion using our INS-1 cell models established for inducible expression or suppression of *PDX-1* (31). After a 4-day induction of DN-Pdx-1, glucose-stimulated insulin secretion was markedly inhibited, whereas overexpression of *PDX-1* had no effect (42). In contrast to the dominant-negative suppression of HNF-1 α and HNF-4 α , loss of *PDX-1* function also impaired K⁺-induced insulin release. This was also observed in the pancreas perfusion experiment in *PDX-1*^{+/-} mice (37). Glucokinase enzyme activity and mRNA level were not altered by dominant-negative suppression of *PDX-1* function. Although GLUT2 expression was decreased by 90% after induction of DN-Pdx-1, glycolysis was unaltered, explained by the large excess of the transporter. Similarly, induction of DN-Pdx-1 did not affect mitochondrial metabolism. We found that induction of DN-Pdx-1 caused downregulation of insulin, prohormone convertases (PC1/3 and

2), ATP-sensitive K⁺ (K_{ATP}) channel subunits (Sur1 and Kir6.2), fibroblast growth factor receptor 1, and glucagon-like peptide 1 receptor. The molecular basis of the global inhibition of insulin secretion remains undefined at present, but decreased insulin processing combined with decreased K_{ATP} channel activity may contribute to the secretory defect.

OTHER TRANSCRIPTION FACTORS IMPLICATED IN β -CELL FUNCTION

The predominant feature of MODY5 patients, carrying mutations in the gene encoding HNF-1 β , appears to be renal dysfunction, usually diagnosed before diabetes (43). HNF-1 β -deficient mice die shortly after implantation by abnormal visceral endoderm (44). HNF-1 β is highly homologous to HNF-1 α , and the two transcription factors can form heterodimers for cognate DNA binding (45). Overexpression of DN-HNF-1 α and the human mutation HNF-1 α P291fsinsC, whether in mice or in cell lines, also suppresses HNF-1 β function. To our knowledge, specific studies on the role of HNF-1 β in β -cells have not been performed.

Mutations in the gene encoding the transcription factor NeuroD 1/BETA2 have been linked to MODY6 as well as type 1 diabetes (4,46). Mice with targeted disruption of NeuroD 1 died of diabetes shortly after birth (47). The NeuroD 1-deficient pancreas displayed reduced endocrine cell mass, affecting especially the β -cells (47). However, in an alternative genetic background, the *NeuroD 1*^{-/-} mice display neonatal diabetes but become normoglycemic through pronounced β -cell neogenesis (48). The role of NeuroD 1 in the regulation of pancreatic β -cell function remains to be established.

Foxa2 (HNF-3 β), which is expressed in islets, has been suggested as the upstream transactivator of HNF-4 α , HNF-1 α , *PDX-1*, and HNF-1 β in the transcriptional hierarchy (49,50). Because these experiments were performed in embryonic stem cell-derived embryoid bodies, this transcriptional cascade may not apply to differentiated β -cells and hepatocytes. Indeed, genetic analysis has failed to link *Foxa2* mutations to MODY pedigrees (51). In addition, Sund et al. (52) showed that HNF-1 α and HNF-1 β mRNA levels are unchanged, whereas HNF-4 α mRNA is slightly elevated in mice with liver-specific deletion of *Foxa2*. Most recently, Tan et al. (53) demonstrated that adenovirus-mediated overexpression of *Foxa2* in mouse liver led to a drastic decrease in the expression of HNF-4 α and HNF-1 α . The phenotype of mice with β -cell-specific deletion of *Foxa2* is severe hyperinsulinemic hypoglycemia (54) rather than diabetes. We found that *PDX-1* expression in INS-1 cells was not regulated by *Foxa2* (55). Overexpression of *Foxa2* suppressed rather than activated the expression of HNF-4 α and HNF-1 α in INS-1 cells. These results confirm that *Foxa2* is not a candidate gene for MODY (51).

DISCUSSION

The principal sites of action of HNF-1 α , HNF-4 α , and *PDX-1* in β -cell function are illustrated in Fig. 5. Insulin expression is regulated by all three genes. HNF-1 α and HNF-4 α regulate metabolism-secretion coupling and generation of ATP, whereas *PDX-1* appears to act beyond

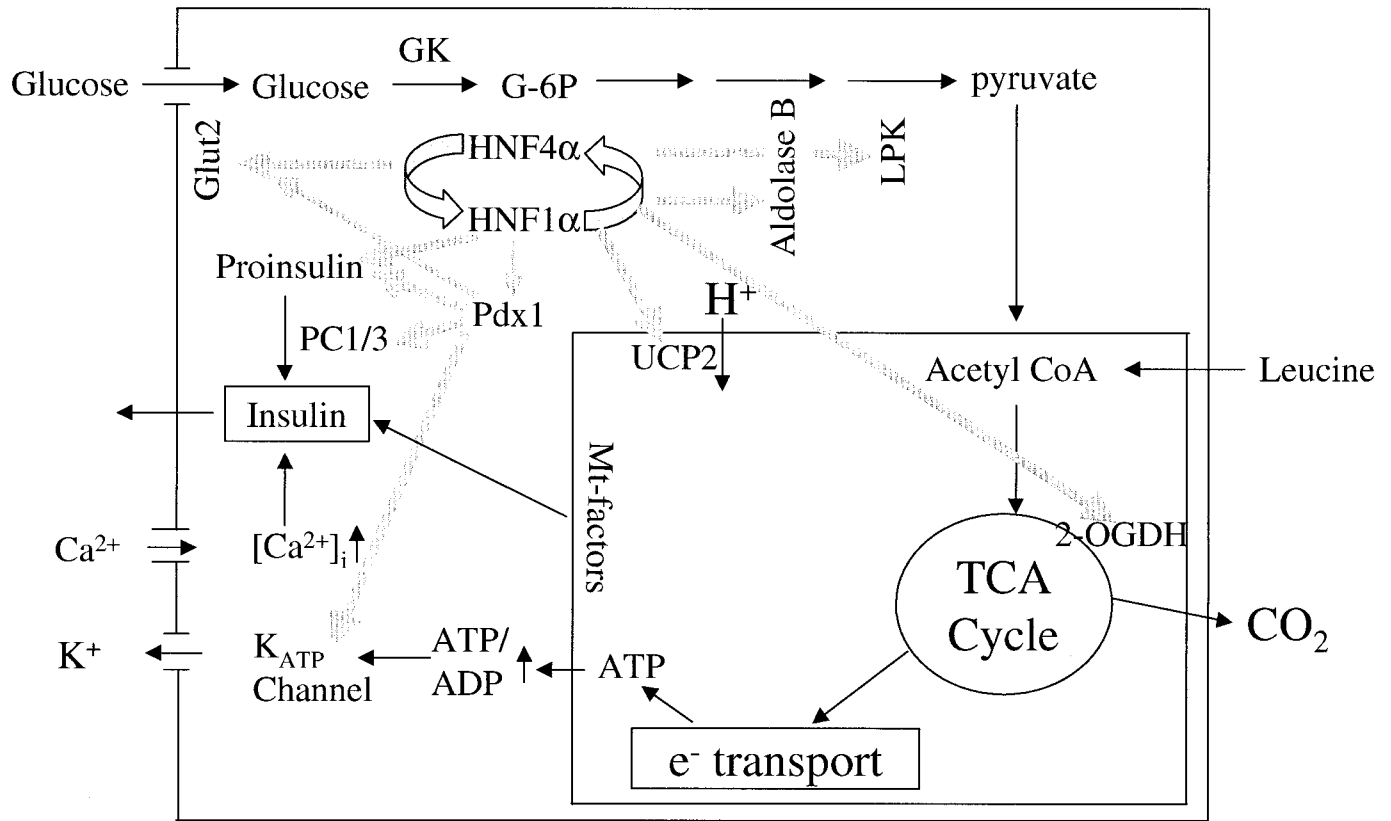


FIG. 5. The principal sites of action of HNF-4 α , HNF-1 α , and PDX-1 in the β -cell. Glucose is converted into pyruvate through glycolysis and is transported into the mitochondria to provide substrates to the TCA cycle. The generation of ATP, or rather the ATP:ADP ratio, increases and causes closure of K_{ATP} channels, depolarization of the plasma membrane, and opening of voltage-sensitive Ca^{2+} channels. The rising of $[Ca^{2+}]_i$ evokes exocytosis of insulin. HNF-4 α and HNF-1 α , which act as a transcriptional circuit, regulate the expression of genes implicated in glycolysis and mitochondrial metabolism. PDX-1 does not play a significant role in regulation of genes essential for glucose metabolism. PDX-1 mediates the expression of proinsulin and enzymes involved in its processing; it also targets the expression of the K_{ATP} channel subunits Sur1 and Kir6.2. The regulatory loop between PDX-1 and HNF-4 α /HNF-1 α remains to be established.

mitochondrial metabolism, affecting insulin exocytosis. The stable diabetic phenotype of the RIP-DNHN-1 α males and the glucose intolerance of the females make these mice attractive models of MODY3, amenable to molecular biological and pharmacological investigation. The INS-1 cell lines engineered to manipulate the MODY genes have helped to identify new target genes and to define the regulatory circuit of β -cell transcription factors.

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