The EMBO Journal (2005) 24, 3313–3324 | © 2005 European Molecular Biology Organization | All Rights Reserved 0261-4189/05
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THE EMBO JOURNAL

The SWI/SNF chromatin-remodeling complex subunit SNF5 is essential for hepatocyte differentiation

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Regulation of gene expression underlies cell differentiation and organogenesis. Both transcription factors and chromatin modifiers are crucial for this process. To study the role of the ATP-dependent SWI/SNF chromatin-remodeling complex in cell differentiation, we inactivated the gene encoding the core complex subunit SNF5/INI1 in the developing liver. Hepatic *SNF5* deletion caused neonatal death due to severe hypoglycemia; mutant animals fail to store glycogen and have impaired energetic metabolism. The formation of a hepatic epithelium is also affected in SNF5-deficient livers. Transcriptome analyses showed that *SNF5* inactivation is accompanied by defective transcriptional activation of 70% of the genes that are normally upregulated during liver development. These include genes involved in glycogen synthesis, gluconeogenesis and cell–cell adhesion. A fraction of hepatic developmentally activated genes were normally expressed, suggesting that cell differentiation was not completely blocked. Moreover, *SNF5*-deleted cells showed increased proliferation and we identified several misexpressed genes that may contribute to cell cycle deregulation in these cells. Our results emphasize the role of chromatin remodeling in the activation of cell-type-specific genetic programs and driving cell differentiation.

The EMBO Journal (2005) **24,** 3313–3324. doi:10.1038/sj.emboj.7600802; Published online 1 September 2005
Subject Categories: chromatin & transcription; differentiation & death
Keywords: cell differentiation; Cre-loxP; INI1; proliferation; transcriptome

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Received: 8 December 2004; accepted: 5 August 2005; published online: 1 September 2005

Introduction

During the past decade, it has become clear that modulation of the chromatin state plays a major role in the regulation of gene expression in eukaryotes (see Khorasanizadeh, 2004). The chromatin state is affected by covalent modification of histone tails which are associated with either activated or repressed transcription (see Fischle *et al*, 2003). Such modifications can influence both the accessibility of nucleosomal DNA and the recruitment of regulatory proteins. Chromatin structure can also be affected by large remodeling complexes that are well conserved between eukaryotes, such as the SWI/SNF (switching defective/sucrose nonfermenting) complex (see Becker and Horz, 2002; Martens and Winston, 2003). First described in the yeast *Saccharomyces cerevisiae*, the 2 MDa SWI/SNF complex includes a DNA-dependent ATPase subunit, SNF2, that functions as the motor of the complex. Yeast contains a single *SNF2* gene, whereas two homologous genes have been characterized in mammals, *Brm/SNF2*α and *Brg1/SNF2*β. These two subunits are mutually exclusive in mammalian complexes. In addition to the catalytic subunit, two other proteins form the core complex: SNF5/INI1/BAF47 and SWI3 (BAF170 or BAF155 in mammals) (Phelan *et al*, 1999). These core subunits are thought to be present in all SWI/SNF complexes that contain in addition roughly 10 distinct subunits.

The SWI/SNF complex plays an important role in transcriptional regulation in the yeast *S. cerevisiae*. Whole-genome transcription analysis revealed that roughly 6% of the genes are SWI/SNF-dependent (Holstege *et al*, 1998; Sudarsanam *et al*, 2000). Several studies suggest that the SWI/SNF complex does not only act as a transcriptional activator, but can also be a repressor (Sif *et al*, 2001; Martens and Winston, 2002).

In multicellular organisms, cell differentiation requires extensive reprogramming of gene transcription. In addition to cell type-specific transcription factors, chromatin modifiers are thought to be involved in this process. Nevertheless, few studies have addressed the *in vivo* role of chromatin-remodeling complexes in cell differentiation. The first evidence for such a role in mammalian cells came from the use of homologous recombination in mice: targeted inactivation of several core subunits of the SWI/SNF complex, namely Brg1, SNF5 and BAF155, results in embryonic lethality at the peri-implantation stage (Bultman *et al*, 2000; Klochendler-Yeivin *et al*, 2000; Roberts *et al*, 2000; Guidi *et al*, 2001; Kim *et al*, 2001). In all the three knockout mice, inner cell mass development is altered, and Brg1 and SNF5 inactivation also affect the trophectoderm lineage. However, the transcriptional targets of SWI/SNF activity at this early stage are unknown. Using cell-specific inactivation systems, Brg1 has recently been shown to be required for T-cell development (Chi *et al*, 2003; Gebuhr *et al*, 2003). These studies suggest that Brg1 (and therefore the SWI/SNF complex) is essential

to allow gene activation in response to signaling pathways that drive successive steps of T-cell differentiation. The use of *in vitro* models of cell differentiation has also provided some insights into the role of SWI/SNF complex. For example, SWI/SNF activity is required for the activation of several muscle-specific genes upon MyoD induction (de la Serna *et al*, 2001). However, the consequences of SWI/SNF inactivation on global genome reprogramming have not been investigated in these systems.

Here, we studied the role of the SWI/SNF complex in cell differentiation using the hepatoblast–hepatocyte lineage as a model. We conducted specific inactivation of the complex core subunit SNF5 in these cells and evaluated its impact at the cellular and molecular levels. We show that liver-specific *SNF5* inactivation impairs glycogen storage and epithelial morphogenesis, which are characteristics of hepatocyte differentiation. Transcriptome analyses revealed that these defects are associated with defective transcriptional activation of 70% of the genes that are normally upregulated during liver development. Our results provide a link between chromatin remodeling and development and show that SWI/SNF-dependent chromatin remodeling is required for the genetic programming of differentiated cells.

Results

Liver-specific inactivation of SNF5 in SNF5$^{flox/-}$ mice results in perinatal lethality

We generated a *SNF5* conditional allele in which two loxP sites flanked exons 1 and 2 of the gene (*SNF5^{flox}*). Cre-mediated recombination results in deletion of the two first exons of *SNF5*, including the initiation ATG codon, and generates a null allele. *SNF5* was inactivated in the developing liver using the AlfpCre transgene. AlfpCre-driven recombination begins at the onset of liver bud formation and leads to complete recombination in liver cells of endodermal origin: hepatocytes and cholangiocytes (Kellendonk *et al*, 2000; Coffinier *et al*, 2002). Our breeding strategy (*AlfpCre; SNF5$^{+/-}$* × *SNF5$^{flox/flox}$*) generated embryos with deleted *SNF5* in Cre-expressing cells (*AlfpCre; SNF5$^{flox/-}$* refered to as 'mutants'), and wild-type or heterozygous embryos ((*SNF5$^{flox/+}$*), (*SNF5$^{flox/-}$*) and (*AlfpCre; SNF5$^{flox/+}$*), collectively referred to as 'controls'). At postnatal day 10, no mutant mice were found ($n = 65$), while at embryonic day (E) 18.5, mutants were present at normal Mendelian ratios ($n = 27$). Close examination revealed that mutant mice were born normally, but died within the first 12 h after birth ($n = 26$). Nonetheless, neither growth retardation nor macroscopic liver defects were observed at birth.

PCR analysis confirmed that the predicted *SNF5^{deleted}* allele was detected in livers expressing Cre recombinase (Figure 1A). *SNF5^{flox}* deletion was not complete. This was expected as hematopoietic cells represent more than 50% of total liver cells during fetal life (Paul *et al*, 1969) and these cells do not express the AlfpCre recombinase. Quantitative RT–PCR carried out on total liver RNAs showed that *SNF5* mRNA level was reduced by 2.2-fold in mutant livers (Figure 1B).

To confirm the cell specificity and efficiency of AlfpCre-driven recombination, we also generated mice that included the *ROSA26R* reporter allele in addition to the floxed *SNF5*

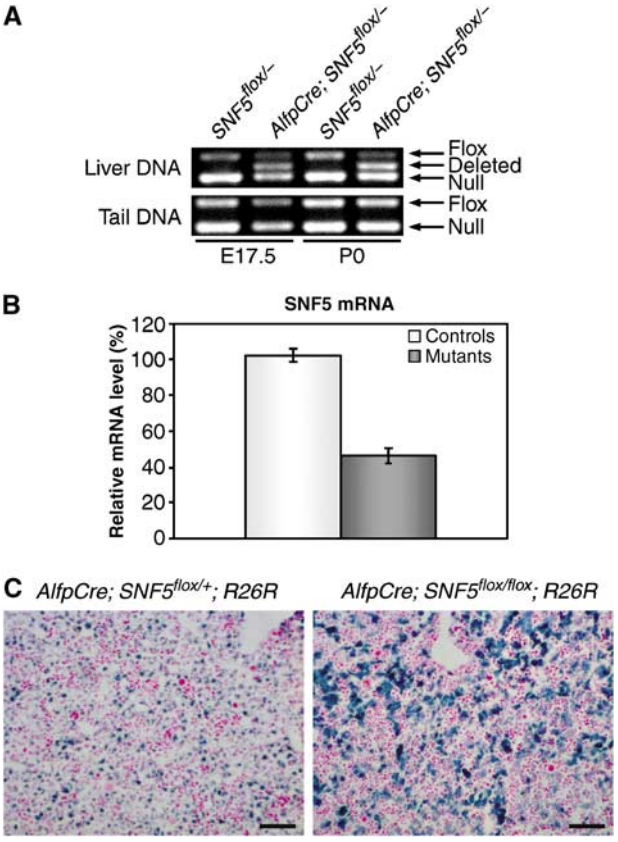

Figure 1 Liver-specific inactivation of *SNF5*. (**A**) Detection of Cre-mediated *SNF5* deletion in the liver. PCR analysis using primers that amplify the null, flox and deleted alleles. Mice genotypes are indicated on the top. The deleted allele was detectable in the livers expressing Cre recombinase both before (E17.5) and after (P0) birth. (**B**) Quantitative RT–PCR analysis of *SNF5* expression. SNF5 mRNA level was reduced by 2.2-fold in mutant livers at E18.5. Error bars represent standard error of the mean ($n_{control} = 4$, $n_{mutant} = 4$). (**C**) AlfpCre-mediated recombination is specific of liver parenchymal cells. X-gal staining on liver sections of control *AlfpCre; SNF5$^{flox/+}$; ROSA26R* (left panel) and mutant *AlfpCre; SNF5$^{flox/flox}$; ROSA26R* (right panel) at E18.5. β-gal activity is an indicator of Cre-driven recombination on the *ROSA26* locus. In both controls and mutants, most parenchymal cells were β-gal-positive and hematopoietic cells were β-gal-negative. For unclear reasons, the staining intensity of control parenchymal cells was weaker than that of mutant cells. Scale bars: 200 μm.

allele (*AlfpCre; SNF5$^{flox/flox}$; ROSA26R*). This allele contains a *lacZ* gene, whose activity is induced by the Cre-driven recombination (Soriano, 1999). These mice had the same phenotype as *AlfpCre; SNF5$^{flox/-}$* animals (data not shown). *SNF5* heterozygous littermates (*AlfpCre; SNF5$^{flox/+}$; ROSA26R*) were also obtained. X-gal-stained liver sections revealed that in both mutants and controls over 95% of the parenchymal cells were β-galactosidase (β-gal)-positive at E18.5 (Figure 1C). As expected, hematopoietic cells were β-gal-negative. These data indicate that most liver parenchymal cells underwent Cre-mediated recombination.

Glucose metabolism is strongly impaired in SNF5-deleted livers

To unravel the cause of the early postnatal morbidity of mutant mice, we followed the glycemia within hours of birth (P0) and found that mutant mice were strongly

Table I Liver-specific inactivation of *SNF5* leads to decreased glycemia and hepatic glucose production

Genotype	Glycemia ($mg\,dl^{-1}$)	Hepatic glucose production ($\mu mol\,min^{-1}\,g^{-1}$)		
		Total	Gluconeogenesis	Glycogenolysis
Control	58 ± 13 ($n=19$)	2.5 ± 0.5 ($n=5$)	0.56 ± 0.14 ($n=5$)	1.9 ± 0.5 ($n=5$)
Mutant	<10 ($n=13$)	0.8 ± 0.2 ($n=8$)	0.27 ± 0.08 ($n=8$)	0.6 ± 0.3 ($n=8$)

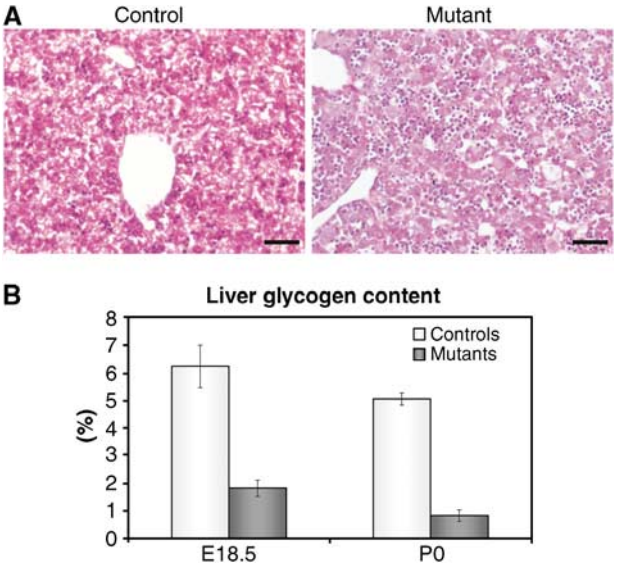

Figure 2 Mice carrying a liver-specific inactivation of *SNF5* fail to store glycogen. (**A**) PAS staining for the presence of glycogen in control (left panel) and mutant (right panel) liver sections at E18.5. Control livers showed extensive glycogen deposition. Staining was almost absent in mutant livers. Scale bars: 200 μm. (**B**) Glycogen content of mutant livers (w/w) was significantly reduced ($P<0.05$) when compared to control livers. Error bars represent standard error of the mean (E18.5: $n_{control}=5$, $n_{mutant}=4$; P0: $n_{control}=5$, $n_{mutant}=3$).

hypoglycemic (Table I). The average glycemia of control mice was 58 mg/dl, while all 13 mutants tested presented a glycemia lower than 10 mg/dl. This defect was not linked to a feeding problem, as mutant and control mice digestive tracts both contained milk. Hypoglycemia was also seen in E18.5 mutant mice 2 h after caesarian delivery (data not shown). As these levels of glycemia are not compatible with survival, we conclude that hypoglycemia is the likely cause of perinatal lethality of mutant mice.

Glycogen synthesized from maternal glucose in the liver during gestation is essential to support life during the first hours after birth. To determine if hypoglycemia was linked to a glycogen storage defect, we stained liver sections for glycogen using the Periodic Acid-Schiff (PAS) reaction. Glycogen-specific staining was decreased in mutants at E17.5, E18.5 and P0 (Figure 2A and data not shown). To quantify this defect, we measured glycogen levels in E18.5 and P0 control and mutant livers. Glycogen levels were strongly reduced in mutant livers both before and after birth (Figure 2B).

In order to evaluate the actual capacity of mutant livers to synthesize glucose, we measured the hepatic glucose production in newborn liver slices. We found a three-fold reduction ($P<0.05$) of total glucose production in mutant livers (Table I). Interestingly, this defect was due to a combined significant reduction of both glycogenolysis and gluconeogenesis rates, respectively by three- and two-fold ($P<0.05$) (Table I). These results suggest that mutant hypoglycemia is the consequence of reduced glycogenolysis due to lower glycogen accumulation, but also to a concomitant decreased in gluconeogenesis, indicating that both pathways supporting liver glucose production are impaired upon *SNF5* inactivation.

To gain more insights on the defects in glucose metabolism and on other potential functions of SNF5, we conducted a comparative transcriptome analysis of control versus mutant liver RNAs (see Materials and methods). The data obtained unravel the molecular basis of the drastic reduction in liver glycogen storage. First, expression of the liver glycogen synthase (*Gys2*) was downregulated by 2.1-fold. Second, a regulatory subunit of protein phosphatase 1 (PP1), Ppp1rc3 (PP1 regulatory subunit 3C, also known as PTG for protein targeting to glycogen), was also expressed at lower levels in mutant livers. This protein targets PP1 to the glycogen particle, and allows it to activate glycogen synthesis and inhibit glycogen breakdown (see Brady and Saltiel, 2001). Finally, we observed a downregulation of the glucose transporter Slc2a2 (Glut2), which should affect glucose uptake from maternal sources before birth (Figure 3).

Hydrolysis of glucose 6-phosphate (G6P) into glucose and phosphate is a common and important step in both glycogenolysis and gluconeogenesis. It is accomplished by two proteins: the G6P ER translocator (G6pt1) and the phosphatase (G6pc) (see van Schaftingen and Gerin, 2002). Both genes were strongly downregulated in mutant livers (Figure 3). Five additional genes involved in gluconeogenesis were also downregulated. This should result in the inactivation of the two irreversible reactions that allow pyruvate to be converted back to G6P: the pyruvate carboxylase (Pcx) step and the fructose-1,6-bisphosphatase 1 (Fbp1) step (Figure 3) (see Hers and Hue, 1983).

Other genes involved in energy metabolism were also affected in mutant livers (Figure 3). The downregulation of the two first enzymes involved in galactose catabolism (galactokinase (Glk) and galactose-1-phosphate uridyl transferase (Galt)) probably affects galactose usage as an energy source. Consequences of downregulation of three other genes, *glucose phosphate isomerase 1* (*Gpi1*), *6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1* (*Pfkfb1*) and *pyruvate dehydrogenase kinase, isoenzyme 4* (*Pdk4*), are difficult to predict. Finally, *isocitrate dehydrogenase 2* (*Idh2*) and *ATPase, Na^+/K^+ transporting, alpha 1 polypeptide* (*Atp1a1*) downregulation could reduce ATP synthesis in mutant hepatocytes (Figure 3).

Taken together, these data show that SNF5 is essential for glycogen synthesis, glycogenolysis and gluconeogenesis in the liver.

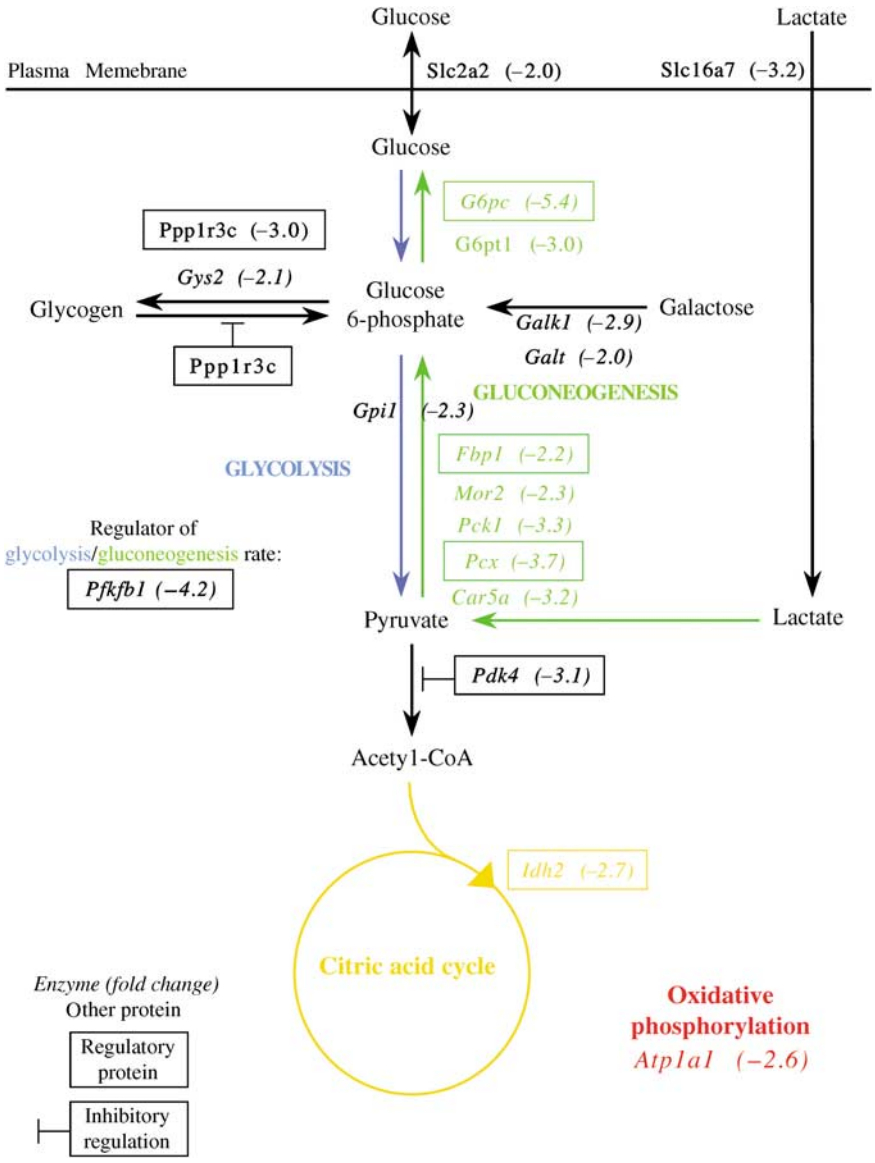

Figure 3 Impaired glucose metabolism upon loss of *SNF5* in fetal livers. The scheme shows genes involved in energy metabolism that were significantly downregulated ($P<0.05$) more than two-fold in E18.75 mutant livers. Key enzymes of gluconeogenesis (Gpc, Fbp1, Pcx) and glycogen synthesis (Gys2) were strongly downregulated. For each gene, the mutant versus control fold change is indicated. Genes encoding enzymes are italicized. Boxed names represent genes encoding regulatory proteins. Gene products involved in gluconeogenesis, citric acid cycle and oxidative phosphorylation are represented in green, yellow and red, respectively.

SNF5 is essential for epithelial differentiation of hepatocytes

Establishment of cell–cell junctions is one of the most important steps during terminal hepatocyte differentiation (Feracci *et al*, 1987). To explore this process, we performed immunodetection of proteins that participate to these junctions. The Zonula occludens 1 (ZO-1) protein is normally localized to tight junctions (Stevenson *et al*, 1986), as observed in control animals (Figure 4A). In contrast, almost no ZO-1 protein was detected in mutants (Figure 4B), suggesting that tight junctions were absent. E-cadherin is also involved in cell–cell interactions and is normally localized at both adherens junctions and desmosomes (Johnson *et al*, 1993). The expression of this protein was also downregulated in mutant livers (Figure 4C and D). Furthermore, mutant livers presented ultrastructure abnormalities. Electron microscopy observations showed that intermembrane space between adjacent cells was larger than in controls and that cell–cell junctions were disrupted (Figure 4E and F). Mutant cells also showed abnormal cytoplasms with lower organelle and mitochondria content, again suggesting a defect in terminal hepatocyte differentiation (Figure 4F).

Transcriptome data are consistent with a defect in cell–cell junction formation in mutant livers (Table II). The gene encoding E-cadherin, *Cadherin 1*, was downregulated 1.8-fold in mutant livers. Furthermore, genes encoding proteins of desmosomes (*Desmocollin 2* and *Desmoglein 2*), gap junctions (*Gjb1/Connexin-32* and *Gjb2/Connexin-26*) and adherens junctions (*Ceacam1*) were also downregulated. Interestingly, *Tjp1*, the gene encoding ZO-1, was normally expressed at the mRNA level.

Taken together, these data show that SNF5 is essential for the assembly of all types of epithelial cell–cell junctions, namely tight junctions, adherens junctions, gap junctions and desmosomes. Moreover, this phenotype is underlied by a defective transcriptional activation of several genes encoding proteins that participate in these junctions.

SNF5 is required for the expression of a specific set of developmentally regulated and liver-specific genes

Our global transcriptome analysis showed that a considerable number of genes are dependent on SNF5 activity

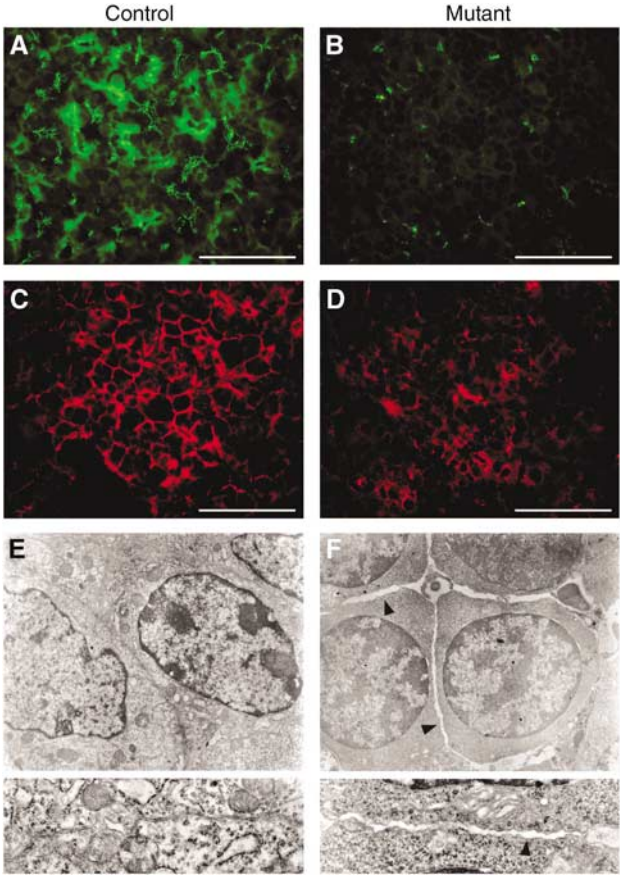

Figure 4 *SNF5* inactivation results in the disruption of epithelial architecture of liver parenchyma. (**A, B**) ZO-1 immunodetection (green) on liver sections in control (A) and mutant (B) mice at E18.5. Tight junctions are stained in control livers. In mutants, almost no signal is detected. (**C, D**) Loss of E-cadherin (red) in mutant livers (D) when compared to controls (C). Scale bars (A–D): 75 μm. (**E, F**) Electron micrographs of control (E) and mutant (F) livers at E18.5. Gaps in between cells were observed in mutant livers (arrowheads). Insets show details of cell–cell contacts.

(ArrayExpress accession number: E-MEXP-241). We detected 9929 probe sets out of 36 000 present on the DNA chips (see Materials and methods). Of these, 412 (4.1%) were significantly decreased more than two-fold and 860 (8.7%) more than 1.5-fold in mutant livers (Supplementary Table I). The number of increased probe sets was smaller: only 71 (0.7%) were significantly increased more than two-fold and 259 (2.6%) more than 1.5-fold (Supplementary Table II). This broad effect could have been due to the lack or underrepresentation of hepatic cell types in mutant livers. However, histological examination (see Figures 2A and 4F) and lineage analysis (see Figure 1C) demonstrated that hepatic cells were indeed present. In addition, several specific markers of this lineage, including *albumin 1* and *alpha-fetoprotein*, were normally expressed in mutant livers (data not shown).

To further assess the effect of *SNF5* inactivation on hepatocyte differentiation, we analyzed the transcriptome more thoroughly. We compared wild-type E18.75 livers with transcriptome data obtained from wild-type 3T3 immortalized fibroblasts (Gerald and Mechta, ArrayExpress accession number: E-MEXP-239) and from wild-type E14.5 livers (Chéret and Pontoglio, ArrayExpress accession number: E-MEXP-240). Using these comparisons, we defined two groups of genes according to tissue-specific expression ('liver-enriched genes' and 'ubiquitous genes'), and two others according to temporal expression pattern ('developmentally activated genes' and 'not developmentally regulated genes') (see Materials and methods). We monitored the effect of *SNF5* inactivation by calculating the number of genes downregulated ('decreased genes'), upregulated ('increased genes') and unchanged ('not changed genes') upon *SNF5* inactivation in these different groups of genes. Interestingly, the 'decreased' probe sets represented a large proportion of the 'liver-enriched genes' and the 'developmentally activated genes' (Figure 5A and B). In contrast, no gene was 'developmentally activated' and 'increased' upon *SNF5* inactivation (Figure 5B). We also found that, out of 235 genes that were both 'liver-enriched' and 'developmentally activated', 164 (70%) were downregulated and 71 (30%) were not affected by *SNF5* inactivation (Figure 5C). These 71 probe sets included genes involved in different hepatic functions, indicating that *SNF5* inactivation did not completely block hepatocyte differentiation (a partial list is given in Table III). As stated above, a major fraction of genes involved in energy metabolism were downregulated, while half of the serum proteins were normally expressed. Finally, the expression of the majority of cytochrome P450 genes was reduced.

We next verified the expression levels of liver-enriched transcription factors in mutant livers (Supplementary Table III). Interestingly, none of the transcription factors required

Table II Genes involved in cell–cell adhesion that were significantly downregulated ($P<0.05$) in E18.75 mutant livers

UniGene cluster	Gene symbol	Gene name	Alternate name	Type of junction	Fold change
280547	Dsc2	Desmocollin 2		Desmosome	−2.5
21198	Gjb1	Gap junction membrane channel protein beta 1	Connexin-32	Gap junction	−2.0
34118	Gjb2	Gap junction membrane channel protein beta 2	Connexin-26	Gap junction	−1.9
360512	Ceacam1	CEA-related cell adhesion molecule 1		Adherens junction	−1.8
345891	Dsg2	Desmoglein 2		Desmosome	−1.8
35605	Cdh1	Cadherin 1	E-cadherin	Adherens junction	−1.8

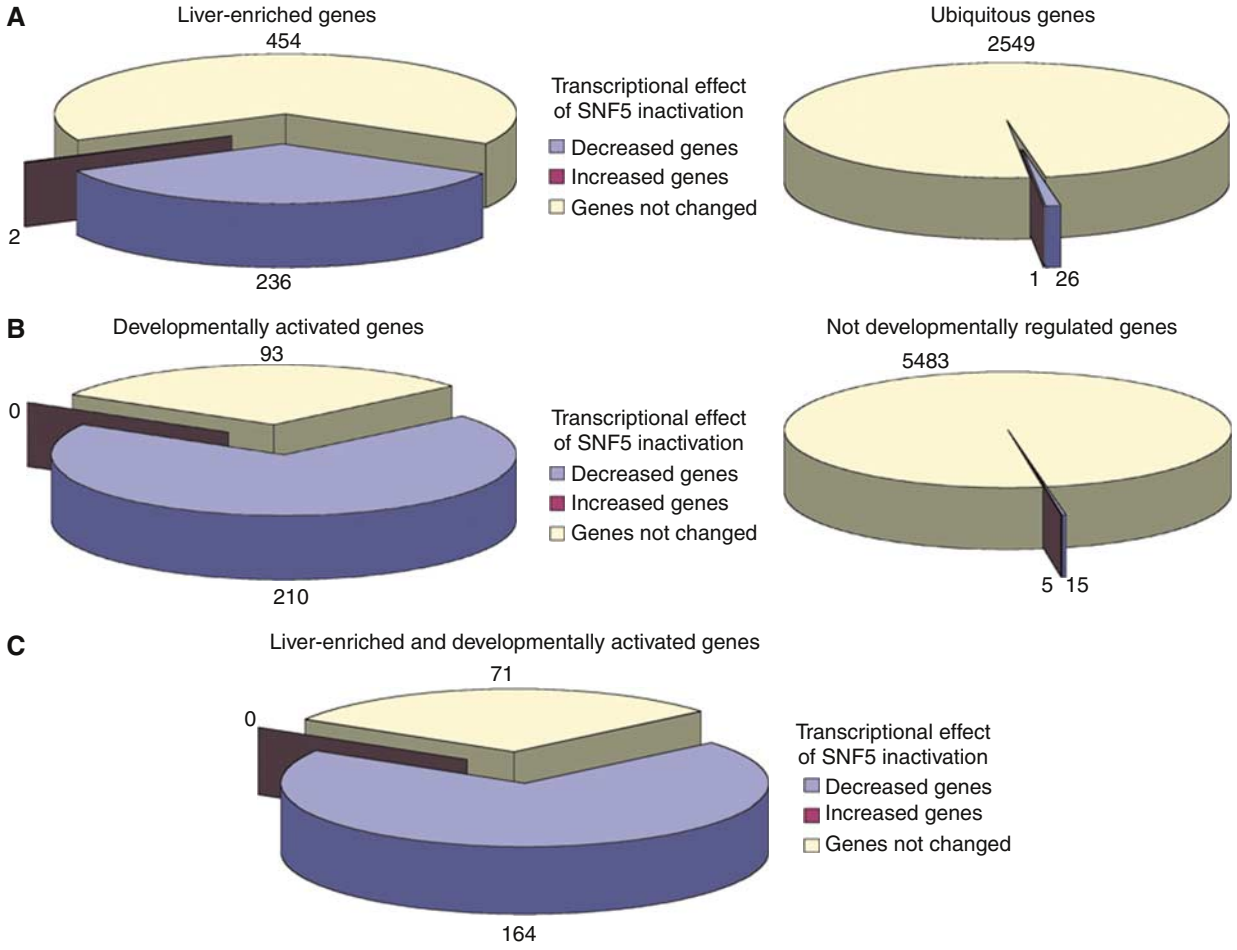

Figure 5 SNF5 is required for the establishment of the genetic program of differentiated hepatocytes. Transcriptional effect of *SNF5* inactivation on different groups of genes: (**A**) 'Liver-enriched genes' versus 'ubiquitous genes'; (**B**) 'developmentally activated genes' versus 'not developmentally regulated genes'; (**C**) intersection of 'liver-enriched genes' and 'developmentally activated genes'. (A, B) An important proportion of 'liver-enriched genes' (A) and 'developmentally activated genes' (B) were downregulated upon *SNF5* inactivation. (C) Almost 70% of the genes that are both 'liver-enriched' and 'developmentally activated' were downregulated in the absence of SNF5. In all, 30% of those genes did not require SNF5 activity for their activation.

for hepatocyte differentiation was affected by *SNF5* inactivation. On the contrary, some transcription factors involved in the induction of metabolic changes in response to environmental changes were downregulated. However, inactivation of these transcriptions factors does not compromise liver development. These data suggest that the broad transcriptional effect of *SNF5* inactivation is not the consequence of the lack of expression of tissue-specific transcription factors.

The 'ubiquitous' and 'developmentally not regulated' groups were mainly composed of 'not changed' genes (Figure 5A and B). However, this effect may be overestimated as, by definition, the 'ubiquitous' and 'not developmentally regulated' groups probably contain several genes that are also highly expressed in the hematopoietic cells that represent an important fraction of the total liver and that do not undergo Cre-driven recombination.

To test whether *SNF5* inactivation affected hematopoietic genes by an indirect effect, we screened the group of 'liver-enriched genes' and found 40 genes with known hematopoietic expression or homologous to hematopoietic genes. This was not surprising since the reference RNA for the definition of 'liver-enriched genes' was fibroblast RNA. From these genes, 39 were not affected by *SNF5* inactivation, including genes of erythroid cells (*Ankyrin 1*, *protein band 4.1* and *4.2*, *hemoglobin a1*), lymphocytes (*Rag1*, *lymphotoxin B*), megacaryocytes (*Fli1*) and macrophages (*lysozyme*) (Supplementary Table IV).

Taken together, these results show that during hepatocyte differentiation SNF5 acts positively on the expression of roughly two thirds of liver-specific and developmentally activated genes. However, another subset of such genes is independent from SNF5 activity.

SNF5 inactivation leads to increased cell proliferation and misexpression of cell cycle-related genes

Hepatocyte differentiation at the end of gestation is accompanied by a strong decrease in cell proliferation (Gruppuso *et al*, 1997). Using S (PCNA) and M (phosphorylated serine 10 histone H3; Mateescu *et al*, 2004) phase markers, we found that proliferation was significantly ($P<10^{-3}$) higher in mutant livers when compared to controls (Table IV). The rise in proliferation was of 1.6-fold at E17.5 and 4–5-fold at E18.5. A consistent, significant ($P<10^{-3}$) 40% increase in parenchy-

Table III Specific subsets of genes are dependent or independent on SNF5 activity to be correctly activated during terminal hepatocyte differentiation. Examples of liver-enriched and developmentally activated genes that were decreased (left) or not changed (right) upon *SNF5* inactivation

Liver-enriched and developmentally activated genes			
Decreased genes upon *SNF5* inactivation		Genes not changed upon *SNF5* inactivation	
Gene symbol	Gene name	Gene symbol	Gene name
	Energy metabolism		
Acat1	Acetyl-coenzyme A acetyltransferase 1	Abcd2	ATP-binding cassette, subfamily D (ALD), member 2
Car5a	Carbonic anhydrase 5a, mitochondrial	Fabp1	Fatty acid-binding protein 1, liver
Car8	Carbonic anhydrase 8	Gyk	Glycerol kinase
Fabp4	Fatty acid-binding protein 4, adipocyte	Hao1	Hydroxyacid oxidase 1, liver
Facl2	Fatty acid coenzyme A ligase, long chain 2	Itih4	Inter-alpha-trypsin inhibitor, heavy chain 4
Fbp1	Fructose bisphosphatase 1	Lcat	Lecithin cholesterol acyltransferase
G6pc	Glucose-6-phosphatase, catalytic		
G6pt1	Glucose-6-phosphatase, transport protein 1		
Pck1	Phosphoenolpyruvate carboxykinase 1, cytosolic		
Pcx	Pyruvate carboxylase		
Pfkfb1	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 1		
Ppp1r3c	Protein phosphatase 1, regulatory (inhibitor) subunit 3C		
	Serum proteins		
A2m	Alpha-2-macroglobulin	Afp	Alpha fetoprotein
Apoa5	Apolipoprotein A-V	C4bp	Complement component 4-binding protein
Apoc4	Apolipoprotein C-IV	Cfh	Complement component factor h
C4	Complement component 4 (within H-2S)	Cfhl1	Complement component factor h-like 1
Crp	C-reactive protein, petaxin related	Cfi	Complement component factor i
F10	Coagulation factor X	Cpb2	Carboxypeptidase B2 (plasma)
F11	Coagulation factor XI	F13b	Coagulation factor XIII, beta subunit
Hp	Haptoglobin	Ttr	Transthyretin
Igfbp2	Insulin-like growth factor-binding protein 2		
Mup1	Major urinary protein 1		
	Antimicrobial peptides		
Defb1	Defensin beta 1	Camp	Cathelicidin antimicrobial peptide
Lbp	Lipopolysaccharide-binding protein		
Pglyrp	Peptidoglycan recognition protein		
	Calcium regulation		
S100a1	S100 calcium-binding protein A1	Rgn	Regucalcin
		S100a8	S100 calcium-binding protein A8 (calgranulin A)
		S100a9	S100 calcium-binding protein A9 (calgranulin B)
	Cell–cell interactions		
		Cldn1	Claudin 1
	Cytochrome P450		
Cyp2c37	Cytochrome P450, family 2. subfamily c, polypeptide 37	Cyp2f2	Cytochrome P450, family 2, subfamily f, polypeptide 2
Cyp2c38	Cytochrome P450, family 2. subfamily c, polypeptide 38	Cyp3a11	Cytochrome P450, family 3, subfamily a, polypeptide 11
Cyp2c70	Cytochrome P450, family 2, subfamily c, polypeptide 70	Cyp3a16	Cytochrome P450, family 3, subfamily a, polypeptide 16
Cyp2d9	Cytochrome P450, family 2, subfamily d, polypeptide 9		
Cyp2d10	Cytochrome P450, family 2, subfamily d, polypeptide 10		
Cyp2d26	Cytochrome P450, family 2, subfamily d, polypeptide 26		
Cyp2e1	Cytochrome P450, family 2, subfamily e, polypeptide 1		
Cyp3a13	Cytochrome P450, family 3, subfamily a, polypeptide 13		
Cyp3a25	Cytochrome P450, family 3, subfamily a, polypeptide 25		
Cyp4a10	Cytochrome P450, family 4, subfamily a, polypeptide 10		
Cyp4a14	Cytochrome P450, family 4, subfamily a, polypeptide 14		
Cyp4f15	Cytochrome P450, family 4, subfamily f, polypeptide 15		
Cyp8b1	Cytochrome P450, family 8, subfamily b, polypeptide 1		
Cyp39a1	Cytochrome P450, family 39, subfamily a, polypeptide 1		

mal cell number was also observed. We confirmed this result by specifically analyzing the lineage of Cre-recombined cells in control and mutant mice carrying an *ROSA26R* allele (see Figure 1C).

The transcriptome analysis of $SNF5^{-/-}$ livers at E18.75 showed that several genes involved in cell cycle control were deregulated (Table V). The strongest affected gene was $p21^{Cip1}$ (*Cdkn1a*), with a −3.7-fold change. Western blot analysis confirmed this downregulation (data not shown). $p21^{Cip1}$ is an essential regulator of cell cycle progression at G1, as it inhibits the activity of cyclin–CDK2 and 4 complexes (el-Deiry *et al*, 1993; Harper *et al*, 1993). Other modifications in gene expression might also contribute to the bypass of G1 control. S-phase kinase-associated protein 2 (Skp2 or p45),

involved in $p27^{Kip1}$ degradation, was upregulated in mutant livers (Carrano *et al*, 1999). $p27^{Kip1}$ is another member of the Cip/Kip family of cyclin-dependent kinase inhibitors (Toyoshima and Hunter, 1994). In addition, two positive regulators of G1/S transition, cyclin E1 (*Ccne1*) and B-Myb (*Mybl2*), were upregulated.

Other genes involved in S, G2 or M phases of the cell cycle were also upregulated in mutant livers. Assembly of prereplicative complexes at origins is the first step in DNA replication and consists of the sequential binding of the origin recognition complex (ORC), cell division cycle 6 homolog (Cdc6) and the mini-chromosome maintenance protein complex (MCM) (see Takisawa *et al*, 2000). Two of the genes encoding these factors were upregulated in mutant livers: *Cdc6* and mini-chromosome maintenance deficient (*Mcmd*). After assembly of prereplicative complexes, replication proceeds via the action of several enzymes. Two of these showed increased expression in mutants: *Prim1* (DNA primase, p49 subunit) and *Pola1* (DNA polymerase alpha 1, 180 kDa subunit) (Table V). In addition, several antiproliferative genes, such as *Col18a1* (the gene encoding endostatin, an inhibitor of cell proliferation and tumor growth; O'Reilly *et al*, 1994), *Ets2* and *Btg1*, were downregulated more than two-fold in mutant livers (Table V).

Finally, several genes involved in apoptosis were also downregulated in mutant livers (Table V). Interestingly, most of them were proapoptotic genes, as for example *Fas/Tnfrsf6* and *Bim/Bcl2l11* (see O'Connor *et al*, 1998; Nagata, 1999).

Taken together, our data show that *SNF5* deletion leads to increased cell proliferation. This defect could be due to a partial inactivation of the G1/S checkpoint.

Table IV Proportion of PCNA and mitotic phosphorylated serine 10 histone H3 (H3pS10)-positive parenchymal cells in control and mutant livers

	PCNA		H3pS10
Genotype	E17.5 (%)	E18.5	E18.5
Control	11.1±1.2	2.4±0.4	1.8±0.3
Mutant	17.9±0.9	11.9±0.7	7.1±0.5

Discussion

We show here that liver-specific inactivation of the SWI/SNF complex core subunit *SNF5* leads to perinatal lethality associated with impaired glucose metabolism. Transcriptome analysis of *SNF5*-deleted livers revealed that the SWI/SNF complex is necessary for correct expression of a large number of genes during liver development. We demonstrate that hepatocyte differentiation is affected in mutants, as a subset of liver-specific genes is not correctly activated during liver development. In addition, we show that $SNF5^{-/-}$ hepatocytes proliferate at higher rates than control cells.

Inactivation of *SNF5* resulted in altered expression of 5–10% of expressed genes. Such a broad effect of the SWI/SNF complex has previously been observed in yeast, where it controls the expression of 6% of the genes (Holstege *et al*, 1998; Sudarsanam *et al*, 2000). This result illustrates the importance of chromatin remodeling in the regulation of gene expression.

The SWI/SNF complex and cell differentiation

We used a global approach to understand the role of SNF5 during liver development. Using transcriptome comparisons, we identified two partially overlapping groups of genes: liver-enriched genes and genes activated during liver development (E14.5–E18.5 transition). We show that *SNF5* inactivation had a strong effect on these genes. In particular, up to 70% of the developmentally activated genes were dependent on SNF5 activity for their transcriptional activation. This global analysis demonstrates that SNF5 is required for the activation of the genetic program in differentiated hepatocytes.

Table V *SNF5* inactivation leads to misexpression of key regulators of cell cycle and survival

UniGene cluster	Gene symbol	Gene name	Alternate name	Function	Fold change
195663	Cdkn1a	Cyclin-dependent kinase inhibitor 1A	p21/Cip1	G1/S checkpoint	−3.7
16110	Ccne1	Cyclin E1		G1/S checkpoint	+2.8
35584	Skp2	S-phase kinase-associated protein 2	p45	G1/S checkpoint	+2.4
4594	Mybl2	Myeloblastosis oncogene-like 2	B-Myb	G1/S checkpoint	+1.7
20912	Cdc6	Cell division cycle 6 homolog		Prereplicative complex	+1.6
4502	Mcmd	Mini chromosome maintenance deficient		Prereplicative complex	+1.5
2903	Prim1	DNA primase, p49 subunit		DNA synthesis	+1.9
1923	Pola1	Polymerase (DNA directed), alpha 1		DNA synthesis	+1.5
4352	Col18a1	Procollagen, type XVIII, alpha 1	Endostatin	Antiproliferative	−2.5
290207	Ets2	E26 avian leukemia oncogene 2, 3' domain		Antiproliferative	−2.4
272183	Btg1	B-cell translocation gene 1, antiproliferative		Antiproliferative	−2.1
1626	Tnfrsf6	Tumor necrosis factor receptor superfamily, member 6	Fas	Proapoptotic	−2.6
21109	Gsn	Gelsolin		Proapoptotic	−2.5
141083	Bcl2l11	BCL2-like 11 (apoptosis facilitator)	Bim	Proapoptotic	−2.2
2159	Bnip3	BCL2/adenovirus E1B 19 kDa-interacting protein 1, NIP3		Proapoptotic	−2.0
84073	Bag3	Bcl2-associated athanogene 3		Antiapoptotic	−2.0
10737	Cideb	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector B		Proapoptotic	−1.6
24103	Dapk1	Death-associated protein kinase 1		Proapoptotic	−1.5

However, the absence of SNF5 did not completely block hepatocyte differentiation; 30% of the liver-specific and developmentally activated genes were expressed at normal levels in mutant livers. The persistence of normal transcription rates for these genes may be due to residual SWI/SNF activity in the inactivated cells. A recent study suggests that *SNF5* inactivation is not sufficient to disrupt all Brm- and Brg1-related activities (Doan *et al*, 2004). Moreover, our preliminary studies showed that liver-specific inactivation of *Brg1* presents a stronger phenotype than *SNF5* mutation. At birth, only one Brg1 mutant was recovered alive out of the expected 10.75 ($P<0.05$). Three dead mutants were also observed. Liver-specific inactivation of *Brg1* in a $Brm^{-/-}$ background resulted in a more drastic effect. At birth, no mice lacking both genes in the liver were found either alive or dead (expected: 6, $P<0.05$). These results demonstrate that Brm and Brg1 still function partially in $SNF5^{-/-}$ cells. This may explain the normal expression rates of some developmentally activated genes in *SNF5*-deleted livers. Alternatively, non affected genes may depend on other chromatin-remodeling activities or on histone modifications for expression.

Taken together, our results support an *in vivo* requirement of SWI/SNF-dependent chromatin remodeling in the establishment of the genetic program of differentiated cells and clearly link chromatin remodeling and development.

Targeting of the SWI/SNF complex to specific promoters

SWI/SNF complex has no intrinsic DNA-binding specificity. How is targeting of the complex to a specific promoter achieved? Accumulating evidence suggests that transcription factors can recruit the SWI/SNF complex to specific genes (see Hassan *et al*, 2001; Hsiao *et al*, 2003; Debril *et al*, 2004). In this way, transcription factor-dependent activation would require SWI/SNF chromatin-remodeling properties. To test this hypothesis, we compared SNF5 target genes with genes downregulated upon inactivation of two well-characterized, liver-enriched transcription factors: HNF1α (see Pontoglio, 2000) and 4α (see Watt *et al*, 2003). Transcriptome data were obtained from E18.5 $HNF1\alpha^{-/-}$ livers (Chéret and Pontoglio, unpublished results) and E18.5 liver-specific inactivation of *HNF4α* (Duncan, personal communication, 2004). We found that 17% of genes downregulated upon *HNF1α* inactivation were also decreased in $SNF5^{-/-}$ livers. This proportion was higher, 69%, for downregulated genes in $HNF4\alpha^{-/-}$ livers. These results indicate that SNF5 activity is necessary for few HNF1α targets and for an important proportion of HNF4α targets. Moreover, liver-specific inactivation of *HNF4α* also results in hepatocyte differentiation defects and perinatal lethality (Parviz *et al*, 2003). Therefore, HNF4α may be one of the targeting factors of SWI/SNF in hepatocytes. Other candidates for this function include the transcription factor C/EBPα (CCAAT/enhancer-binding protein). This factor has been shown to interact with the SWI/SNF complex, at least in adipocytes and aging livers (Pedersen *et al*, 2001; Iakova *et al*, 2003). Furthermore, the phenotype of $C/EBP\alpha^{-/-}$ mice resembles that of liver-specific inactivation of *SNF5*. Actually, $C/EBP\alpha^{-/-}$ mice die just after birth from hypoglycemia and present reduced hepatic glycogen levels, defective gluconeogenesis, disruption of normal liver architecture and increased hepatocyte proliferation (Wang *et al*, 1995; Flodby *et al*, 1996).

The SWI/SNF complex, cell cycle control and tumor suppression

Numerous studies have connected the SWI/SNF complex with proliferation control (see Roberts and Orkin, 2004). In particular, *SNF5* acts as a tumor suppressor gene in both humans and mice. The first genetic evidence of this link was the identification of biallelic inactivation of *hSNF5/INI1* as the cause of most cases of malignant rhabdoid tumors (MRT), a highly aggressive pediatric cancer (Versteege *et al*, 1998; Sevenet *et al*, 1999). Similar tumors were also observed in $SNF5^{+/-}$ mice (Klochendler-Yeivin *et al*, 2000; Roberts *et al*, 2000; Guidi *et al*, 2001). Reintroduction of SNF5 in MRT-derived cell lines induces G1 arrest, and, in some cell lines, senescence or apoptosis (Ae *et al*, 2002; Betz *et al*, 2002; Versteege *et al*, 2002; Zhang *et al*, 2002; Reincke *et al*, 2003). Moreover, inducible inactivation of *SNF5* in adult mice results in a very rapid tumor development compared to other tumor suppressor genes such as *p53*, $p16^{Ink4a}$ or $p19^{Arf}$ (Roberts *et al*, 2002).

Here we show that *SNF5* inactivation leads to increased cell proliferation. Furthermore, transcriptome analysis revealed that several key regulators of the cell cycle were misexpressed upon *SNF5* inactivation. Increased proliferation of $SNF5^{-/-}$ cells could be linked to the cellular differentiation defect. Indeed, cell differentiation is often associated with growth arrest and upregulation of cell cycle inhibitors, such as $p21^{Cip1}$ (see Zhu and Skoultchi, 2001). In this case, gene expression modifications that we observed could be an indirect consequence of *SNF5* inactivation. Alternatively, increased cell proliferation could be the direct result of SNF5-dependent transcriptional effects on regulators of cell cycle and apoptosis. Two recent studies showed that $p21^{Cip1}$ is a direct transcriptional target of the SWI/SNF complex, at least *in vitro* (Hendricks *et al*, 2004; Kang *et al*, 2004). These results correlate with our observation that $p21^{Cip1}$ expression is downregulated in $SNF5^{-/-}$ livers. Finally, we cannot rule out that the effect of *SNF5* inactivation on cell proliferation results from both direct and indirect mechanisms.

In conclusion, we show here that the SWI/SNF complex is essential for establishment of the genetic program of differentiated cells. Moreover, we have identified several misexpressed genes that may contribute to SWI/SNF-driven cell cycle regulation. Whole-genome analysis of gene expression modifications upon SWI/SNF inactivation in mammalian non malignant cells provides an important tool to understand the role of chromatin-remodeling complexes in cell differentiation and proliferation.

Materials and methods

Generation of mice with a lox-conditional allele of SNF5

A 20 kb murine SNF5 genomic clone spanning 4.5 kb of the 5′ untranslated region, exons 1 and 2 and intron 2 was isolated from a 129SVJ mouse l phage library (Klochendler-Yeivin *et al*, 2000). A conditional allele was generated by flanking the first two exons with loxP sites. A loxP site was inserted at an *Eco*RV site 1 kb upstream of exon 1 and a loxP–neo–loxP cassette was introduced into an *Sac*I site 600 bp downstream of exon 2. A thymidine kinase gene was added downstream of the right recombination arm for negative selection. Following electroporation and selection of CK35 ES cells, recombination into the SNF5 locus was confirmed by Southern blot hybridization to external probes. Subsequently, the neo cassette was deleted from the correctly targeted cells by transient transfection with pIC-Cre. DNA from G418-sensitive clones was analyzed by

PCR using primers flanking the loxP–neo–loxP cassette (415F-CTTG CCAGGTGAGTCTG; 416R-GCCACCAGCCAGATGTCATAC). The correctly targeted ES cells were injected into C57BL/6 blastocysts and chimeras that showed germ-line transmission of the targeted allele were selected. The mice were genotyped by PCR using the primers described above. The deleted allele was detected using primers flanking the first loxP site (407F-GTTGTTAGTCCCTTTGCTCC; 408R-TGTAGTCTAGGCTGGGTGTG) and 416R.

Mice

AlfpCre (Kellendonk *et al*, 2000) and *SNF5*$^{+/-}$ (Klochendler-Yeivin *et al*, 2000) mice were previously described. All animals received humane care and the institutional review committee approved the study protocol.

Biochemical analysis, histological analysis and X-gal staining

Glucose levels were measured using a Glucotrend analyzer (Roche). Glycogen levels were determined as described (Roe and Dailey, 1966). For hepatic glucose production measurements, see Supplementary data. For histological analysis, organs were fixed in paraformaldehyde (PFA) 4% and embedded in paraffin. Sections were stained with hematoxylin and eosin or PAS. X-gal stainings were performed as described (Pontoglio *et al*, 1996).

Microarray analysis

Total RNAs were obtained from E18.75 frozen livers ($n_{control}=4$, $n_{mutant}=8$) using TRIzol® procedure (Invitrogen). RNA preps were purified with Rneasy columns (Quiagen) and pooled according to their genotype. Control and mutant pools were used to synthesize cRNA and hybridize it to Affymetrix Murine Genome U74v2 A, B and C GeneChip® arrays that contain about 36 000 probe sets. These steps have been carried out in the Affymetrix facility at the Institut Curie (Paris) according to manufacturer's procedures. All hybridizations were carried out in triplicates (ArrayExpress accession number: E-MEXP-241).

We defined pertinently detected probe sets as probe sets that were not declared 'absent' more than once (out of the three hybridization replicates) by the Microarray Suite Software MAS 5.0 (Affymetrix). For these probe sets, expression level fold changes were obtained using the dChip 1.3 software (Li and Wong, 2001). Only significantly changed probe sets (paired *t*-test, $P<0.05$) were taken into account. For the definition of 'ubiquitous' and 'liver-enriched' genes, we only used U74v2 A and B chips. We compared wild-type E18.75 livers with transcriptome data obtained from 3T3 immortalized fibroblasts (Gerald and Mechta, ArrayExpress accession number: E-MEXP-239). Probe sets expressed at the same levels in both samples were included in the 'ubiquitous genes' group. Probe sets preferentially expressed in the liver when compared to fibroblasts (with at least a five-fold increase) were included in the 'liver-enriched genes' group. For temporal specificity, we compared wild-type E18.75 livers with wild-type E14.5 livers (Chéret and Pontoglio, ArrayExpress accession number: E-MEXP-240). Probe sets expressed at higher levels at E18.75 (at least five-fold increase) were included in the group of 'developmentally activated genes'. Probe sets expressed at the same levels were termed 'not developmentally regulated genes'. Only few probe sets were expressed at lower levels at E18.75 when compared to E14.5 ($n=7$); thus we did not analyze their behavior.

For several genes, dChip 1.3-fold change estimations were confirmed by quantitative RT–PCR (correlation coefficient = 0.949) (Supplementary Figure 1).

Immunohistochemical procedures

For ZO-1 and E-cadherin immunodetection, livers were frozen in isopentane cooled in a liquid nitrogen bath, and sectioned at 7 μm in a cryostat. Sections were air-dried, fixed in PFA 4% for 5 min, and blocked for 30 min in Tris buffer saline (TBS)/normal goat serum 10%/BSA 1%/Triton X-100 0.3%. Thereafter, sections were incubated with primary antibody (ZO-1: Developmental Hybridoma Bank R26.4C, 1/200 dilution; E-cadherin: BD Transduction Laboratories 610181, 1/500) in TBS/normal goat serum 1%/BSA 0.1%/Triton X-100 0.3% overnight at 4°C. Sections were then incubated with secondary antibody (fluorescein linked, 1/200 dilution) for 1 h at room temperature (RT).

For PCNA and H3pS10 staining, paraffin sections were microwaved (two times for 5 min, 500 W) in 10 mM citric acid pH 6. Thereafter, sections were treated using for PCNA: UltraVision Mouse Tissue Detection System Anti-Mouse HRP/DAB (LabVison) and PCNA (Ab-1) Cat#NA03 antibody (Oncogene Research, 1/80 dilution); for H3pS10: H3pS10 antibody (gift of B Mateescu, 1/3000) overnight at 4°C and secondary antibody (anti-rabbit Texas red, 1/250) 1 h at RT. At least 1200 parenchymal cells *per* genotype and *per* marker were counted.

Quantitative RT–PCR

Total RNAs were obtained as described below. RNAs were DNase treated (DNase I Rnase-free, Roche) and reverse transcribed (oligo dT primer) according to the manufacturer's protocols (Superscript II, Invitrogen). Quantitative RT–PCR was carried out on an ABI PRISM® 7000 sequence detection system using SYBR® Green. Primers were designed using PrimerExpress 2.0® software (Applied Biosystems). For primer sequences see Supplementary Table V. All genes have been tested in E18.5 control ($n=8$) and mutant ($n=8$) mice.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We are grateful to M Tanguy, V Komnenovic and A Doyen for technical assistance and advice, to K Wanherdick and D Gentien for Affymetrix GeneChips hybridizations, to N Cayet and MC Prévost for electron microscopy studies, to P Chambon for the *Brg1* floxed allele, to S Medjkane and O Delattre for helpful discussions, to B Mateescu for the H3pS10 antibody. We thank J Weitzman for critical reading of the manuscript. The monoclonal antibody R26.4C against ZO-1 developed by DA Goodenough was obtained from Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. LG was supported by a fellowship from the 'Association pour la Recherche contre le Cancer', the French Ministry of Research and the Pierre et Marie Curie University. AR was supported by Boehringer Ingelheim Fonds and the 'Fondation pour la Recherche Médicale'. The use of Affymetrix technology was supported by the 'Subvention à l'achat de puces Affymetrix' program of the French Ministry of Research. This work was supported by 'La Ligue contre le Cancer-Comité de Paris', the 'Association pour la Recherche contre le Cancer', HFSP (grant RGP0310/2001-M302 'Functional and structural studies of SWI/SNF-related complexes'), AICR (research grants 00-221 and 03-109 to AK-Y) and ICRF (project grant to AK-Y).

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