

**A mouse model of human liver disease due to inherited mutations
in the *ATP8B1 (FIC1)* gene.**

by

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DISSERTATION

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Ap

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by

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ACKNOWLEDGMENTS

Art is I; science is we.
Claude Bernard

The seven years I have spent in San Francisco and at UCSF constitute the longest continuous period I have ever lived in a single country or a single city, even if one accounts for my year in Amsterdam. These have been the richest but also the most challenging years of my life. Graduate school is a lonely endeavor, but also one that cannot be accomplished without the support of various communities: of science, of scholarship and of the heart. I now wish to acknowledge those, whose wisdom, guidance, assistance and companionship helped me bring this journey to completion.

The above quotation succinctly expresses my personal view of science and the place it has in my life. Though some find in science individualistic and personal rewards, for me its joys are greatest when shared and accomplished together with others. It is so much more rewarding to yell “Eureka, we found it!” if there’s someone else there, not just to hear you, but also to share those rare, precious moments of discovery and jubilation.

Of those I hereby must acknowledge, first and foremost I must thank Nelson Freimer, my dissertation advisor and principal investigator of my thesis project. Nelson was instrumental in bringing me to UCSF. He offered me a project that suited my interests extremely well and gave me an amazing amount of autonomy and independence, which, while challenging to the extreme, was also an invaluable learning experience. I am not

the only person to observe that being a graduate student in Nelson's lab was like being a postdoc elsewhere, so in a way I got a valuable head start into the realities of working in science. Nelson stood by my very long-winded project through thick and thin, and was understanding and supportive even when the road hit a bumpy patch, for which I am very grateful. His passion for genetics and great scientific confidence were always inspiring.

I am particularly grateful to have been part of the unique laboratory group he created. It was admittedly rather strange that I found myself studying genetics of liver disease in a neurogenetics lab located in the basement of a psychiatric institute... but in the past two years since it disbanded, I've missed NGL very much. It was an exciting place, where lab meetings careened wildly from liver biology to the arcana of perfect pitch to elephant seal evolution to defining schizophrenia and Tourette's syndrome, with the vagaries of the bipolar project repeatedly teaching us all difficult and valuable lessons about the genetics of complex disease. Such amazing breadth of investigation and of sheer scientific curiosity was something I greatly cherished and will probably be hard pressed to find elsewhere, though I think I must try.

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when my project had to find a new physical home, and thus provided the continuity that allowed me to conclude my graduate career smoothly even after Nelson left for Los Angeles. I treasure her friendship, both within and beyond the realms of science.

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My sojourn in the Experimental Hepatology lab immersed me in the intricacies of cell and organ biology, taught me many new skills and provided me with valuable exposure

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Finally, I must acknowledge the support and affection I have received from my family and friends, most of them so very far away from here, which enabled me to get through the tough times and persevere. I am grateful to my family for supporting my then brave decision to go off to college on the other side of the world all these long 11 years ago, and especially to my mother Maria and my sister Ewa, for making sure I never really got too far away in spirit. My best friends: Magda Orzeszyna, Magda Sobieszczyk, Małgosia Leszczyńska and Haneen Rabie lent their support, mostly long-distance with the grand help of the lifeline called email. The excellent epistolary skills of Magda O were particularly key at all the most difficult moments, and hey, that gargantuan surprise

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To all of you – my most sincere thanks.

* * *

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Chapter 3 is a manuscript in preparation included here by permission of Leo W. J. Klomp, the principal author. I made substantial contributions to the determination of intron-exon boundaries and to mutation screening in PFIC1 and BRIC patients described in this manuscript. The work of the other authors: Leo W. J. Klomp, Laura N. Bull, Saskia van Mil, Michiel van Eijk, Jenneke Juijn, Carlos Pabón-Peña, Lauren Smith, Joseph A. DeYoung, Justijn Gombert, Gerda van der Brugge, Alexander S. Knisely, Roderick Houwen, Nelson B. Freimer and Ruud Berger is hereby acknowledged.

I performed all of the work in Chapter 4 myself, with scientific guidance from Nelson B. Freimer and Nigel P. Killeen, and excellent advice and technical assistance from members of the Killeen laboratory.

Chapter 5 is a manuscript in preparation; I designed and performed a substantial majority of the work presented therein, and wrote the manuscript. Of the other authors, Nelson Freimer, Ronald Oude Elferink, Nigel Killeen and Laura Bull are hereby acknowledged as principal investigators and for scientific guidance of the project, Elaine Eppens for Western Blot analysis, Roel Ottenhoff and Norbert Looije for mouse surgery, and Alexander Knisely for histopathological expertise and advice.

Roel Ottenhof and Norbert Looije also performed much of the mouse surgery described in chapter 6, while Ronald Oude Elferink provided guidance and scientific mentorship and the use of his laboratory resources at the Academic Medical Center, Amsterdam.

ABSTRACT

A mouse model of human liver disease due to inherited mutations in the *ATP8B1 (FIC1)* gene.

by Ludmila Pawlikowska

Bile secretion is an essential function of the vertebrate liver. Cholestasis, the impairment of bile formation or flow, is a common manifestation of liver disease. This dissertation describes the identification of *FIC1*, a novel gene mutated in a spectrum of inherited cholestasis syndromes, and the analysis of the systemic effects of *Fic1* loss in a mouse model.

Genomic mapping had previously identified a locus on chromosome 18q21-22 as the probable location of a gene defective in two inherited diseases: benign recurrent intrahepatic cholestasis (BRIC) and progressive familial intrahepatic cholestasis type 1 (PFIC1). Fine genetic analysis and physical mapping narrowed the locus to 300 kb. cDNA library screening and sequence scanning identified a novel gene, *FIC 1* (ATP8B1) with strong homology to a P-type ATPase subfamily with predicted aminophospholipid translocation activity. *FIC1* mutations were found in both BRIC and PFIC1 patients.

FIC1 was broadly expressed in epithelial tissues, particularly in the ileum. Hepatic expression of *FIC1* was surprisingly low. The mouse orthologue of *FIC1* was isolated and found to share high sequence homology with human *FIC1*.

In order to create a mouse model of *FIC1*-related liver disease, I generated mice homozygous for the G308V (G923T) substitution found in Amish PFIC1 patients. The mutant mice had profoundly diminished Fic1 levels in the liver and ileum. This confirmed that Fic1^{G308V/G308V} acts as a functional null mutation.

In contrast to human PFIC1 patients, Fic1^{G308V/G308V} mice were not cholestatic and suffered no liver damage, but did manifest signs of dysregulated bile salt (BS) homeostasis, including elevated serum BS levels. Furthermore, Fic1^{G308V/G308V} mice showed enhanced biliary BS secretion, but nevertheless accumulated up to 100x normal levels of serum BS when fed a BS-supplemented diet. Their high retention of orally administered ³H-taurocholate revealed excessive BS absorption in the intestine upon cholate feeding. We concluded that Fic1^{G308V/G308V} mice had a defect in the regulation of intestinal BS absorption.

Analysis of the *Fic1* mutant mice thus suggests that the primary etiological insult in *FIC1*-related human liver disease may arise in the intestine. These results also reveal the importance of regulation of intestinal BS resorption for BS homeostasis.



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DISSERTATION ADVISOR'S STATEMENT REGARDING PUBLICATIONS

This dissertation includes two manuscripts on which Ludmila Pawlikowska is not the principal (first) author, but for which she performed a substantial proportion of the work. Her contributions to these publications are indicated below.

Bull *et al* (1998):

Ludmila performed the direct screen of the liver cDNA library that first identified a *FIC1* partial cDNA. She cloned the cDNA fragments, generated and analyzed sequence scanning data to identify an additional exon in *FIC1*, and extended the cDNA sequence 5' by database searches and RACE. She also screened this gene to detect mutations in cholestasis patients, characterized a large deletion, isolated and analyzed RNA, and conducted bioinformatic analysis of *FIC1* sequence (identification of transmembrane domains, conserved domains, BLAST comparisons, phylogenetic alignments).

Klomp *et al* (in preparation):

This manuscript is to be submitted for publication at approximately the same time as the submission of this dissertation. Ludmila performed sequence analysis of *FIC1* to identify intron-exon boundaries, and performed PCR, DNA sequencing and sequence analysis for identification of additional mutations. She also contributed to the writing and editing of the manuscript.

Nelson B. Freimer, Dissertation Advisor to Ludmila Pawlikowska
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LIST OF ABBREVIATIONS INCLUDING GENETIC NOMENCLATURE

ABC	ATP binding cassette
ALAT (SGPT)	alanine aminotransferase (serum glutamic pyruvic transaminase)
ALP	alkaline phosphatase
ASAT (SGOT)	aspartate aminotransferase (serum glutamic oxaloacetate transaminase)
ASBT/IBAT (<i>SLC10A2</i>)	apical sodium-dependent bile acid transporter/ileal bile acid transporter
ATP	adenosine triphosphate
BA	bile acid(s)
BDL	bile duct ligation
bp	base-pairs
BRIC	benign recurrent intrahepatic cholestasis
BS	bile salt(s)
BSEP (SPGP, <i>ABCB11</i>)	bile salt export pump (originally termed sister of p-glycoprotein)
cM	centimorgan
EST	expressed sequence tag
<i>FIC1</i> (<i>ATP8B1</i>)	familial intrahepatic cholestasis 1
FXR/BAR	farnesoid X receptor/bile acid receptor
GGT	gamma glutamyl transferase
IBABP	intestinal bile acid binding protein
ICP	intrahepatic cholestasis of pregnancy

kb	kilo-base-pairs
MDR3/Mdr2 (<i>ABCB4</i>)	multi-drug resistance p-glycoprotein 3/2 (Note: Mdr2 is the murine orthologue of human MDR3)
NTCP (<i>SLC10A1</i>)	Na ⁺ taurocholate cotransporting peptide
OATP2 (<i>SLC21A6</i>) OATP-A (<i>SLC21A3</i>)	organic anion transporting peptide
PCR	polymerase chain reaction
PFIC1, 2, 3,	progressive familial intrahepatic cholestasis type 1, 2, 3
RACE	rapid amplification of cDNA ends
TC	taurocholate
TCDC	taurochenodeoxycholate
TDC	taurodeoxycholate
TMC	tauromuricholate
TUDC	tauroursodeoxycholate
³ H-TC	tritium-labeled taurocholate

CHAPTER ONE

INTRODUCTION

Towards a molecular classification of inherited intrahepatic cholestasis

I never saw a man grown so yellow!

How's your liver?

George Gordon, Lord Byron

Prologue

In the newly dawned genomic, or, as some would have it, post-genomic era of biology, each week brings several reports of genetic variants associated with human disease, or diseases linked to newly pinpointed genetic loci. One of the goals of the human genome project was to make identification of the genetic underpinnings of human phenotypes commonplace, and this promise is certainly being fulfilled (Lander *et al* 2001, Venter *et al* 2001). However, as the genomic enterprise stumbles towards the realization that linking genetic defects with disease provides but the preface of biological understanding, and even the recently burgeoned field of high throughput study of gene expression cannot offer more than an exponentially expanding surfeit of clues, attention turns back to that common bane of biological and investigational processes, the rate limiting step. That, in modern biology, is still physiological research: the painstaking process that tests hypotheses generated from genetic sequences and clinical observations – in *in vivo* models. For all of modern biology's success in finding the simplest possible system, the most tractable cell or tissue or organism that will allow dissection of a specified phenomenon by recapitulation of its critical components, the investigator of human disease can rarely avoid that most sophisticated and complex of experimental systems, the mammalian model, of which the smallest, simplest and most importantly, the easiest one to manipulate genetically is of course the laboratory mouse (Peltonen & McKusick 2001, Jimenez-Sanchez *et al* 2001).

Genetically modified mice are human geneticists' destined companions, and much effort is currently being expended to streamline and accelerate the slow techniques of

manipulating the mouse genome to accommodate pressures from genomics and proteomics to generate and examine whole cohorts of mutants (Zambrowicz *et al* 1998, Zheng 1999, Mitchell *et al* 2001, Rossant *et al* 2001). Yet, historically, so much has been learned by the painstaking but focused generation and examination of one mutation, one strain at a time. Ironically, this dissertation probably belongs among the last studies undertaken the “old-fashioned way”, but the area of biology to which it contributes provides an excellent case history in how much information could be gleaned from the particular interplay of human “forward” genetics and mouse “reverse” genetics that has been typical of the last 20 years of biomedical research, following the advent of positional cloning of human disease genes and gene targeting in the mouse.

The research presented in this dissertation also provides an excellent example of how genetics can inform not only disease diagnosis but also a rational classification of syndrome subtypes and pathological processes, and of how genetic clues prompt the discernment of clinical patterns that, although recorded, were not immediately comprehensible when obscured by descriptive disease paradigms based on clinical manifestations of poorly understood physiological etiology. Put another way, while clinical medicine tends to obscure important phenotypic differences in the interest of diagnostic simplification, genetics provides a framework for rational phenotype classification based on molecular etiology. When genetics deconstructs a previously broadly defined clinical entity into an array of subtypes each with a precise genetic etiology, the way is paved for reclassifying clinical entities in a rational manner that avoids historical biases and points out new avenues of prevention and treatment. Thus, as

detailed below, the study of inherited cholestasis has progressed from a collection of poorly defined syndromes, often named after their original describers (Summerskill, Aegenaes', Alagille's syndrome) (Summerskill 1972, Aegenaes *et al* 1970, Alagille *et al* 1969) or even after the families in which they were first appreciated (Byler disease, Clayton *et al* 1969), through an intermediate phase, where genetic and phenotypic differences between the subtypes were appreciated sufficiently to break the entities into subclasses, somewhat hampered by a less than informative chronological numbering scheme (PFIC 1, 2, 3 and sometimes 4, Oude Elferink & Groen 2002). This evolution of the nomenclature is still in progress, but promisingly, an informative and biologically relevant scheme of classification explicitly based on the underlying genetic defect has emerged. Despite its obvious advantages, the latest nomenclature scheme does run into problems when describing separate clinical entities arising from distinct classes of mutations in the same gene becomes necessary, but this does not negate its value in communicating pertinent information about disease etiology.

Physiological context

Bile formation and secretion is a specialized and essential function of the vertebrate liver. The description of biliary physiology set out below is based primarily on human anatomy, though it also applies to mice and other mammals, with some species-specific idiosyncrasies (rats, for example, have no gallbladder, while mice do; rat bile is closer on composition to the human than the mouse [Coleman *et al* 1979, Alvaro *et al* 1986]).

Bile, a complex aqueous solution of bile salts, lipids, electrolytes, endogenous metabolic waste products (such as bilirubin, the product of hemoglobin degradation) and exogenous toxins, is required for the solubilization, digestion (via activation of digestive enzymes) and absorption of dietary fats, and consequently, also for the absorption of fat soluble vitamins. Secretion of bile is also the body's principal mechanism for excretion of excess cholesterol, predominantly when metabolized into bile salts. Most of the physiological functions of bile are mediated through the action of bile salts, amphipathic molecules of strong detergent properties. Bile secretion itself is an osmotic process that largely depends on active, transporter mediated bile salt secretion, driven against a steep concentration gradient by the energy of ATP hydrolysis (Phillips & Suchy 2001).

Bile acids are synthesized from cholesterol in the parenchymal cells of the liver, the hepatocytes, in a multi-step metabolic process. The rate-limiting step of the predominant, "neutral" pathway of bile acid synthesis is catalyzed by the cholesterol 7 α -hydroxylase (CYP7A1) enzyme (Jelinek *et al* 1990, St.Pierre *et al* 2001). As recently elucidated, bile acid synthesis, including the activity of this enzyme, is under feedback regulation in response to bile salt levels, mediated at the transcriptional level by a family of nuclear receptors including FXR/BAR (Sinal *et al* 2000, Goodwin *et al* 2000). Following conjugation to glycine or taurine, bile acids become primary bile salts and are secreted at the hepatocyte apical membrane, known as the biliary canaliculus. The canaliculi drain into a network of bile ductules, which converge into larger ducts, formed by cholangiocytes, epithelial cells that contribute biliary constituents, mainly water and bicarbonate, and also have the capacity to absorb bile salts and return them via sinusoidal

blood to hepatocytes, forming the recirculation loop known as the cholehepatic bile salt shunt. Bile and its constituents ultimately leave the liver via the hepatic bile duct, and may be stored in concentrated form in the gallbladder. The gallbladder contracts in response to cholecystokinin secretion stimulated by the entry of food into the duodenum, thus releasing bile into the duodenum via the common bile duct (Phillips & Suchy 2001).

Having performed their digestive functions, bile salts are deconjugated and subsequently dehydroxylated (producing the more hydrophobic and toxic secondary bile acid species) by intestinal bacteria, and efficiently reabsorbed in the ileum, and return, via the portal circulation, to the liver, where they are taken up at the basolateral membrane of the hepatocyte, completing the cycle known as enterohepatic circulation of bile salts. A bile acid molecule makes up to 12 such cycles a day; less than 5% of the total bile acid pool is lost from the body through excretion in the feces (0.2-0.6g/day) (Shneider 2001).

Compensation for this loss by precisely regulated *de novo* synthesis in the liver allows maintenance of bile salt homeostasis. Normally, a minimal amount of bile salts (<0.5mg/day) is excreted in the urine, but the kidney is equipped to transport bile salts (Craddock *et al* 1998), and thus, the elevation in serum bile salts seen in some forms of liver disease results in abnormally high urinary excretion and abnormally low fecal excretion of bile salts.

Chemical properties of bile salts

The same chemical properties that make bile salts such excellent detergents, critical for fat solubilization in the digestive process, also make them cytotoxic through damage to

the plasma membrane lipid bilayer, and thus cause directly some of the hepatic pathology that follows any bile salt accumulation (Hofmann & Small 1967). Further hepatotoxicity results from induction of apoptosis by intracellular bile salt accumulation (Jaeschke *et al* 2002).

The degree of cytotoxicity of the various bile salt species closely corresponds to their relative hydrophobicity, which in turn is determined by the number and stereospecific orientation of the hydroxyl groups they carry (Figure 1 and Table 1). Most physiological bile salt species carry between 1 and 3 hydroxyl groups, but more are possible, as demonstrated by appearance of tetrahydroxylated bile salt species in Bsep knockout mice (Wang *et al* 2001). Thus, lithocholate, the secondary bile acid produced through dehydroxylation of chenodeoxycholate by intestinal bacteria in the human colon, has only one hydroxy group, at the bile acid-defining 3α position, and is thus the most hydrophobic and most toxic of the principal endogenous human bile salt species. The dihydroxy bile acids: deoxycholate (3α , 12α ; the other principal secondary bile acid in humans) and chenodeoxycholate (3α , 7α , a primary bile acid in humans and rats) are also quite hydrophobic. Cholate, the principal primary bile salt in humans and mice, falls in the middle of the spectrum, and has three hydroxyl groups (3α , 7α , 12α). All of these bile acids are strongly amphipathic, as all hydroxyl groups cluster on one side of the molecule, leaving the other side with high lipid affinity. In contrast, muricholate (which exists in three different isoforms: the most abundant β , α , and ω), the principal primary murine bile acid, is not only trihydroxylated (3α , 6β and 7β), but also has hydroxyl groups in both the α and the β conformations, resulting in a molecule without an entirely

hydrophobic facet. This is therefore by far the most hydrophilic of all the principal bile acids in humans and rodents. Similarly, ursodeoxycholate, a bile acid endogenously found in bear bile, and used therapeutically in humans, though only dihydroxylated, has its OH groups in opposing spatial conformations (3α , 7β), again resulting in a relatively hydrophilic molecule (Table 1 and Figure 1) (Oude Elferink *et al* 1997).

The relative hydrophilicity of bile acids is further stratified by conjugation to glycine or taurine (to produce bile salts). Either makes the molecule more hydrophilic, but tauroconjugates are more hydrophilic than glycoconjugates because of the presence of the terminal sulphite group. Humans have both tauro- and glycoconjugated bile salts; but murine bile salts are primarily tauroconjugated (Coleman *et al* 1979, Alvaro *et al* 1986). Although the principal primary bile salt species in humans and rats are cholate and chenodeoxycholate, mice have muricholate instead of the latter, and this results in their much more hydrophilic and less toxic bile salt pool.

Furthermore, the most hydrophobic and toxic bile salt species are the secondary ones produced by intestinal bacteria in the colon; but since mice do not have the primary chenodeoxycholate, they also do not have significant amounts of its secondary derivative, lithocholate. These differences in bile salt pool composition profoundly impact the consequences of defects in bile secretion in humans and mice (Coleman *et al* 1979, Alvaro *et al* 1979, Wang *et al* 2001, Chapter 5).

Table 1

The relationship between position and spatial configuration of hydroxyl groups and the relative hydrophobicity of bile salt species.

Bile Salt Tauroconjugated	R1 (C ₃)	R2 (C ₇)	R3 (C ₁₂)	R4 (C ₆)	Hydrophobicity Relative to cholate
Lithocholate	αOH	H	H	H	1.00
Deoxycholate	αOH	H	αOH	H	0.59
Chenodeoxycholate	αOH	αOH	H	H	0.46
Cholate	αOH	αOH	αOH	H	0
Ursodeoxycholate	αOH	βOH	H	H	-0.47
β-muricholate	αOH	βOH	H	βOH	-0.78

Adapted from Oude Elferink, Tytgat & Groen 1997

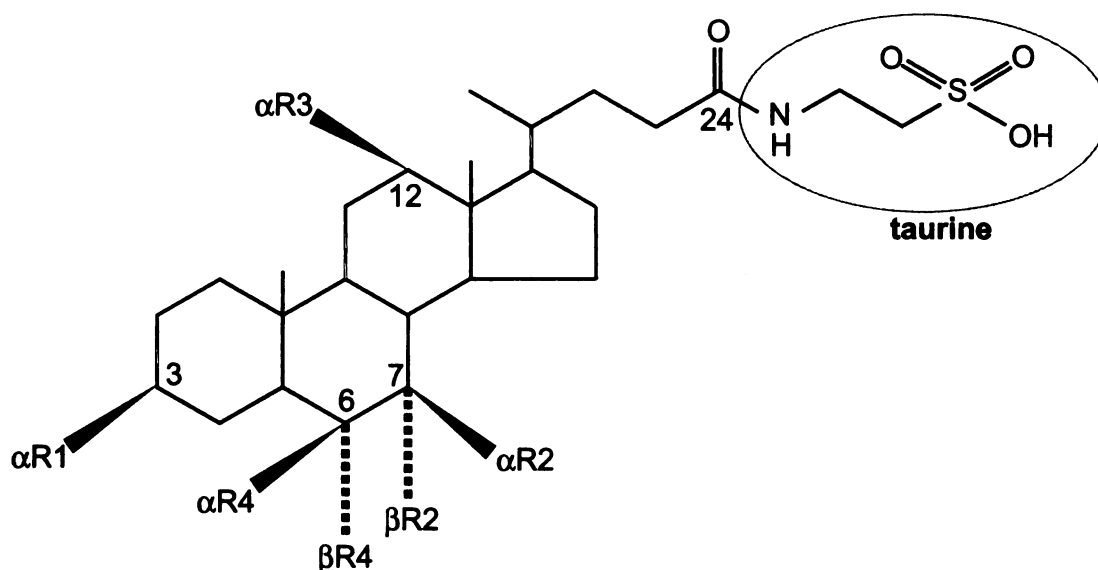


Figure 1

Schematic structure of a tauroconjugated bile salt molecule.

The location and relative spatial orientation of hydroxyl (OH) groups in various physiological bile acid molecules is shown. The R1-R4 designations of the location of the OH groups correspond to those in Table 1. Carbon atoms in the sterol backbone, at which OH groups are substituted, are numbered (3, 6, 7, 12). Carbon 24, the COOH group in a bile acid, is shown amidated by conjugation to taurine. In this representation, OH groups in the α orientation would project above the molecule, out of the plane of the page (black wedge line), while OH groups in the β orientation would project below the plane of the page (dotted line).

Inherited cholestasis syndromes

Ever-evolving nomenclature that reflects advances (and hiccups in those advances) made in biological understanding is a recurring motif in this work, and will vex the compiler of any review. The very name of the clinical entity under consideration is no exception.

Cholestasis is defined as impairment of bile formation or flow. One of the oldest classifications, based primarily on anatomical investigations, divided cholestasis into extrahepatic and intrahepatic. Extrahepatic cholestasis arises from an impediment to bile flow out of the liver and is usually due to physical obstruction or a defect in the extrahepatic bile ducts. Intrahepatic cholestasis has its origin inside the liver, but the wide variety of pathophysiological processes that may result in nominally intrahepatic cholestasis makes this a remarkably uninformative category name. It encompasses cholestasis due to intrahepatic bile duct abnormalities, pharmacological or chemical injury, hormonal imbalance (cholestasis of pregnancy), pathological processes such as inflammation due to autoimmune processes, viral or bacterial infection (both extra- and intrahepatic), and external perturbations such as total parenteral nutrition (Phillips and Suchy 2001). A subset of intrahepatic cholestasis conditions are caused by inherited molecular genetic defects in proteins important for liver and gastrointestinal function (Jansen *et al* 2001, Oude Elferink & Groen 2002). The existence of these syndromes provides an entry route into dissection of the physiological mechanisms of bile secretion, as well as into understanding of the pathophysiological processes that result in cholestasis that ought to be applicable to the diagnosis and treatment of the much more prevalent secondary instances of cholestasis, as well as these rare instances of primary cholestasis, arising from specific molecular defects in the transport systems of the enterohepatic

circulation. In short, the principal tenet of human genetics holds for the study of cholestasis: that explication of the particular and specific instances of disease arising from a single and discrete genetic defect improves understanding of the multifactorial and multigenic processes that lead to most common human diseases.

BRIC

Many syndromes of intrinsic cholestasis with familial distribution are known, most of which present in early childhood or infancy and get progressively more severe (Phillips and Suchy 2001). However, the disorder that provided the starting point for the genetic journey described here proves the exception, as it is characterized by a highly variable age of onset, most commonly before the end of the second decade of life, but sometimes later. As the name suggests, BRIC (benign recurrent intrahepatic cholestasis), first described by Summerskill and Walshe in 1959, is an episodic, usually nonprogressive disease, featuring intermittent, but potentially severe cholestatic episodes of unpredictable onset and variable duration (usually weeks to months; sometimes over a year), with distinct clinical and histological manifestations, which nonetheless resolve spontaneously, are interspersed with prolonged periods of no detectable abnormality, and cause no progressive deterioration of the liver (Summerskill & Walshe 1959, Summerskill 1965). During a cholestatic episode, the patient presents with pruritus (severe itching), a variable degree of jaundice accompanied by pale stools and dark urine, and sometimes with diarrhea. Serum bilirubin, liver enzyme activity and bile salt levels are elevated, indicating liver damage due to impaired bile salt secretion, but, characteristically, serum gamma glutamyl transferase (GGT) levels, elevated in most

forms of cholestasis, are close to normal. Liver biopsies collected during a cholestatic episode usually show histological evidence of cholestasis with no fibrosis and no structural abnormalities. Despite the nonprogressive nature of the disease, the “benign” designation may be something of a misnomer, as clinical symptoms during an attack can be severe, particularly the pruritus, which can profoundly reduce quality of life, as it interferes with sleep, contributes to the anorexia, and to the weight loss associated with fat malabsorption. Liver transplantation has been performed in some patients despite sufficiency of liver function, solely to improve the quality of life; depression, suicidal ideation and suicide attempts have also been reported in patients suffering from particularly severe pruritus (De Koning *et al* 1995, Sinke *et al* 1997, Tygstrup *et al* 1999). It should also be noted that patients initially presenting with BRIC in whom the disease progresses to chronic cholestasis with liver damage have also been reported (van Ooteghem *et al* 2002).

BRIC episodes are usually treated with the same collection of remedies utilized in other forms of cholestasis (Feranchak *et al* 2001). These include bile acid binding resins (cholestyramine), choleric bile salts (ursodiol; UDCA), and treatments aimed to enhance alternative pathways of bile salt clearance from the body (rifampicin, which stimulates catabolism of bile salts into forms that are excreted in the urine). All such treatments aim to reduce the severity of the secondary manifestations of cholestasis by reducing the levels of bile salts in the body, or by manipulating the bile salt pool composition, so as to increase the proportion of less toxic and thus protective hydrophilic species. However, the success of all these treatments has been variable from patient to

patient and also between episodes. At best, the treatments reduce the severity of the symptoms; none prevent episode onset reliably (Bircher 1989, Crosignani *et al* 1991, Cancado *et al* 1998).

Efforts have been made to correlate the onset of BRIC episodes with specific triggers. The extreme variability of the disease clearly implicates both genetic and environmental modifier effects both as triggers and modulators of severity. Factors known to induce other forms of cholestasis have been implicated, such as oral contraceptives and pregnancy in women (de Pagter *et al* 1976). Some correlation has also been observed between BRIC episode onset and viral or bacterial infections, and thus stimulation of the immune system, or perhaps fluctuations in composition and population of intestinal bacterial flora (Bijleveld *et al* 1989A, de Koning *et al* 1995). In some cases, onset of BRIC episodes appeared seasonal, e.g. more frequent in winter, which could reflect seasonal variations in diet, with a high-fat diet consumed in winter (Bijleveld *et al* 1989A).

Various investigations have examined bile salt handling and metabolism in both asymptomatic BRIC patients, as well as in patients currently experiencing an attack. Biliary and serum bile salt composition has been measured. Increased serum levels of lithocholate, the highly toxic secondary bile acid produced by bacterial dehydroxylation of chenodeoxycholate in the colon, have been reported, as has an increased proportion of secondary bile acids in duodenal bile (Kuipers *et al* 1985, Bijleveld *et al* 1989A). This would suggest enhanced absorption of secondary bile acids from the intestine. Bijleveld

et al studied bile salt metabolism in 10 asymptomatic BRIC patients in 1989, and reported a reduced bile acid pool, with an enhanced proportion of chenodeoxycholate and deoxycholate and a reduced proportion of cholate; resulting in a greater overall hydrophobicity of the bile salt pool. Additionally, they observed a greater glycine to taurine ratio among the conjugated bile acid species, which further increased the hydrophobicity of the patients' bile salt pool, as glycoconjugated bile salts are more hydrophobic than tauroconjugated ones (this is also one of the reasons why human bile salts are more hydrophobic than murine ones, which are all tauro-conjugated). Bile salt homeostasis was also affected, with increased fecal bile salt loss and increased bile salt synthesis, which was not sufficient to counteract the loss, as the bile salt pool was contracted (Bijleveld *et al* 1989B).

BRIC patients had long been studied in the Netherlands, and a small group of patients became excellent candidates for population-based genetic mapping analysis when it was appreciated that they came from a somewhat isolated fishing village and were all probably very distantly related and descended from common ancestors. This suggested that all were likely to have inherited the same ancestral mutation, along with a chromosomal segment surrounding it, which would be amenable to genetic identification through a genome screen using polymorphic markers followed by a search for shared segments. Such a screen was performed in three individuals and a 19 cM haplotype shared by five out of six patient chromosomes was identified on chromosome 18q21-22 (Houwen *et al* 1994).

The usual procedure following the identification of a genomic locus associated with disease is to narrow the region by genotyping as many additional patients as possible, and to define the boundaries of the shared region by saturating it with additional polymorphic markers, so as to pinpoint a segment small enough to be tractable for physical mapping and gene identification. This approach was indeed followed. A linkage screen in 14 Dutch families affected with recessively inherited BRIC confirmed the chromosome 18 locus and narrowed it down to a 7 cM region (Sinke *et al* 1997). This study also reported evidence of possible BRIC locus heterogeneity by identification of one family in which BRIC disease was unlinked to the known locus. Simultaneously, however, the project gained in scope and dimension with the re-discovery or renewed appreciation of the observation that another recessively inherited cholestasis syndrome, progressive familial intrahepatic cholestasis (PFIC), albeit more severe, shared certain clinical characteristics with BRIC, in particular the low levels of GGT. This observation inspired the hypothesis that BRIC and PFIC might be allelic disorders caused by mutations of varying severity in the same gene. This intriguing hypothesis was tested by genotyping of markers in the BRIC region in two distantly related PFIC patients and their parents (Carlton *et al* 1995).

Byler Disease/PFIC1

The disorder later known as PFIC1 was originally reported in 1965 and further described in 1969 in seven members of an extended Amish kindred all descended from the same ancestral couple, Jacob Byler and Nancy Kaufman. The syndrome was classified as fatal familial intrahepatic cholestasis, but it was the name Byler Disease, derived from the family surname, that persisted (Clayton *et al* 1965, 1969).

The disease presented in the first months of life with loose, foul-smelling stools (steatorrhea indicative of fat malabsorption), followed by the development of jaundice, pruritus, hepatosplenomegaly, severe growth retardation and failure to thrive. Serum bilirubin and alkaline phosphatase were elevated but cholesterol was normal to low. Complications consistent with malabsorption of fat-soluble vitamins were observed including bone malformations and rickets (vitamin D), gastrointestinal bleeding and nosebleeds due to impaired blood clotting (vitamin K) and seizures (vitamin E). In some of the children the disease was initially episodic, but ultimately became chronic with intermittent exacerbation. All seven children in the original report ultimately died of liver cirrhosis and complications of the disorder by age 14.

Explorative laparotomies and liver biopsies performed in two of the children indicated an essentially normal hepatic architecture and biliary tree. However, some ductular proliferation and slight periportal fibrosis were seen, along with moderate deposition of bile pigment within hepatocytes and in the bile canaliculi. Electron microscopy in one patient showed granular material, identified as bile thrombi, in the canaliculi.

The clinical findings led the authors to postulate that the disorder was due to impaired canalicular secretion of conjugated bile salts and bilirubin, while the segregation pattern of disease in the pedigree was consistent with an autosomal recessive inheritance.

Interestingly, indications of a heterozygote phenotype were seen in the parents. One of the 4 mothers reported severe pruritus in the last trimester of all of her pregnancies, suggestive of cholestasis of pregnancy. In two mothers studied in the last trimester of

pregnancy and also in two of the fathers, sulphobromophthalein loading tests indicated impaired canalicular transport.

Already in this first description of Byler Disease, the unusual correlation of severe clinical symptoms with relatively mild histopathological and biochemical findings prompted the authors to identify similarities between Byler Disease and BRIC, although they did not go as far as suggesting allelic inheritance for the two diseases. Given the limited amount of biochemical and histological data at their disposal, and the profound difference in severity between the two diseases, this was a remarkably astute observation for the time, though three decades would pass before advances in genetic analysis would bring this idea to fruition.

In a followup report in 1972, Linarelli *et al* investigated bile acid metabolism and the enterohepatic circulation of bile salts in another affected child from the Byler kindred and provided important biochemical observations that would become key for the understanding and diagnosis of the disease. They were the first to report elevated total plasma bile acids, low biliary (duodenal) bile salt levels, and an abnormal bile acid composition, in particular high percentages of atypical bile acids and particularly of the highly toxic lithocholic acid, a secondary bile salt absorbed from the intestine and normally not found in infant plasma. They also reported low bile salt excretion in the feces (the normal excretory route) but high excretion in the urine, and replicated previous histopathological findings in the liver. Notably, they were also the first to comment that the unusual particulate appearance of the canalicular bile plugs seen on electron

microscopy was distinct from the fibrillar material seen in other forms of cholestasis, and was a unique characteristic of the disease, which they proposed to call "Byler bile"; a designation that would persist and in the future aid in distinguishing the different subtypes of familial intrahepatic cholestasis.

Simultaneously with the description of Byler Disease, other childhood cholestasis syndromes were being reported and characterized. Some were promptly classified separate clinical entities due to particular extrahepatic symptoms (such as lymphedema in Aegenaes syndrome [Aegenaes *et al* 1968] or developmental abnormalities in Alagille syndrome [Alagille *et al* 1969]), pathological findings (bile duct paucity in Alagille's) and biochemical hallmarks (alpha 1 antitrypsin deficiency [Sharp *et al* 1969]). However, reports of conditions not immediately distinguishable from Byler Disease also appeared; these were sometimes referred to as Byler Syndrome (whereas only patients with proven genealogical links to the Amish Byler family qualified for a diagnosis of Byler Disease) (de Vos 1975, Jacquemin 1994, Kagalwalla 1995, Knisely 2000). An alternative classification of progressive familial intrahepatic cholestasis also emerged, which encompassed both Byler Disease and Byler Syndrome, but allowed inclusion of more vaguely related disorders as well, as long as they were not classifiable as other known clinical entities (Ballow *et al* 1973). The trend towards assembling broad inclusive disease categories would prove problematic in the future; as it would tend to obscure evaluation of the disorder-specific efficacy of therapeutic approaches that were developed in this period, most notably liver transplantation, and later external biliary diversion. Truly detailed phenotyping would emerge again when genetic mapping of rare inherited

diseases became possible, requiring precise delineation of disease subtypes so as to focus on disease arising from particular genetic defect.

Nonetheless, the period of phenotype conflation and coalescence produced further advances in the understanding of biliary physiology and the possible etiological mechanisms of intrahepatic cholestasis. Treatments guided by an understanding of the source of the secondary complications of cholestasis, such as vitamin supplementation, improved survival and ameliorated the quality of life in some patients. It was established that liver transplantation was of immense therapeutic value to PFIC patients, but while, barring complications of transplantation, amelioration of cholestasis and its secondary sequelae was uniformly reported, it was also observed that not all children benefited equally from liver transplantation, and in some, intestinal and other extrahepatic symptoms persisted (Knisely 2000). An alternative surgical therapeutic procedure, external bile diversion, emerged and offered hope of significant improvement of symptoms in some patients while avoiding the trauma of major surgery and the immunological complications of transplantation (Whittington *et al* 1988, 1994, Emond & Whittington 1995). However, again, variability in patient response to the procedure was poorly understood, and rigorously controlled studies of the efficacy of this treatment were lacking, partly due to the rare incidence of the disease. Also, conclusions derived from investigations in distinct patient groups were frequently applied to PFIC as a whole, or extrapolated to imply their applicability to the Amish Byler Disease patients; whereas very few of these interventions were actually performed in Byler Disease patients, partly due to the limited size of the cohort, and partly because of Amish cultural reluctance with

regard to invasive medical procedures. For example, only two of these children ever (to this day, though one more has been listed for transplantation) came to transplant, which may also have something to do with the relatively slow progression of the disease, once secondary manifestations due to vitamin malabsorption are well-controlled (Knisely 2000). Thus, while the general patient census and body of information about broadly-defined PFIC was growing, confusion as to how this information applied to Byler Disease patients was significant, although only truly appreciated in retrospect.

A few additional symptoms associated with PFIC also emerged. Elevated sweat chlorides were reported in at least three groups of patients, prompting evaluation for cystic fibrosis (Bourke *et al* 1996, Lloyd-Still 1981, Knisely *et al* 1997). CFTR involvement was never found, but speculation remained that ATP dependent transport defect in PFIC was not entirely specific to the bile canaliculus. Further support for this intriguing conjecture was provided by reports of pancreatic insufficiency in some patients, also after transplantation (Knisely *et al* 1995, 2000). In many instances, it was difficult to distinguish primary manifestations of the disease from the secondary consequences of chronic malabsorption and malnutrition; for example delayed sexual development and failure to pubesce observed in PFIC children even after reasonably successful therapeutic management of cholestasis. The characteristic of low (for the degree of cholestasis present) serum GGT and cholesterol in PFIC patients was substantiated, and became a defining hallmark of the disease and a cornerstone of its differential diagnosis (Whittington *et al* 1994). The development of HPLC/MS techniques for analyzing bile salt species made defects in bile salt synthesis easier to rule

out as causes of cholestasis, and assisted diagnosis by revealing the characteristic disturbances of bile salt pool composition in PFIC patients: low biliary bile salt with a selective depletion of chenodeoxycholic acid in bile, despite its considerable and abnormal contribution to the elevated serum and urinary bile salts (Tazawa *et al* 1985, Jacquemin *et al* 1994). This finding showed that it was export of chenodeoxycholate from the hepatocyte, and not its synthesis, conjugation or reclamation from the intestine that was impeded in PFIC.

Experimental investigations in rodent models showed that bile salt transport in the canaliculus was an ATP-dependent process (Muller *et al* 1991, Stieger *et al* 1992), as was the uptake of bile salts in the ileum and the uptake of bile salts from the portal blood at the hepatocyte basolateral membrane. As the field of inherited cholestasis was making its leap into the arena of genetic mapping, rodent forms of the hepatocyte basolateral BS transporter (*NTCP*; later *SLC10A1*) (Hagenbuch *et al* 1991, Stieger *et al* 1994) and the ileal bile salt transporter (*IBAT*, later renamed *ASBT*: apical sodium-dependent bile salt transporter, *SLC10B2*, as it is also responsible for bile salt uptake into cholangiocytes and renal duct cells; an alternatively spliced form is present in the cholangiocyte basolateral membrane and probably participates in the cholehepatic shunt pathway) were cloned and characterized (Wong *et al* 1994, Shneider *et al* 1995), as was a plethora of other hepatic transporter proteins. However, the identity of the protein responsible for bile salt export into the canaliculus, the step so severely impacted in rodent experimental as well as various human clinical forms of cholestasis, proved elusive. Thus, with the advent of genetic mapping techniques, attention turned to the PFIC diseases, and the hypothesis

emerged that they were due to a defect in the canalicular, ATP-dependent bile salt transporter, and that genetic analysis in isolated populations would finally pinpoint the long-sought transporter. This hypothesis would inform gene-hunting efforts once the genetic loci associated with PFIC were identified.

Mapping of Byler Disease to the BRIC locus on 18q21-q22

Given that a population based, nonparametric mapping approach was successful in mapping the BRIC genetic locus, using DNA from only three patients presumably descended from common ancestors, appreciation of the clinical similarities between BRIC and PFIC made Byler Disease a very attractive choice for further genetic studies of cholestasis. Many characteristics of the present-day Amish community make it an ideal source of subjects for population genetic mapping approaches: descent from relatively few founder couples, many generations of intermarriage, large family size and strong family ties, low geographical dispersal and very low incidence of marriages outside the community (Knisely 2000) . When an inherited disease manifests within such an isolated population, it is likely that all affected individuals share the same disease allele inherited from a distant common ancestor, and furthermore, that surrounding the disease-causing mutation, a chromosomal segment is also shared in common, detectable by genotyping of polymorphic genetic markers. In the case of a recently founded population such as the Amish, such a segment is likely to be large enough to be detectable, given the spacing of available markers along the genome, but small enough to offer a useful starting point for gene localization (Houwen *et al* 1994).

Patients with Byler Disease had been cared for at the Pittsburgh Children's Hospital in the 1980s and 1990s. When biopsy material became available from two new patients with Byler Disease, genetic material from the affected children and their parents, in the form of immortalized cell lines, was preserved for future genetic studies (Knisely 2000). This resource was utilized in 1994, when the authors of the BRIC mapping paper proposed that BRIC and Byler Disease might be allelic, and went in search of suitable genetic material for testing this hypothesis. No genetic material from other Amish PFIC patients was available at the time, but the success of the BRIC mapping project utilizing just three patients boded well for mapping using just two affecteds in a similarly structured population.

Since the BRIC locus was already characterized, a remarkably quick experiment, by contemporary human genetic standards, sufficed to establish the genetic basis of Byler Disease (Carlton *et al* 1995). Another full genome screen was not necessary. Instead, polymorphic markers spaced at 4 cM intervals in the BRIC locus on 18q21-22 were typed in the two children with Byler Disease and in their parents. For comparison, evenly spaced markers were typed along the remainder of chromosome 18 and on chromosome 17.

A four-marker haplotype shared by all four affected chromosomes was identified, which overlapped with the BRIC disease locus. A similar level of sharing was not seen anywhere else on chromosome 18 or 17, and the likelihood of such sharing occurring by chance was calculated at less than 1×10^{-6} . This strongly suggested that the shared

region on 18q21-22 was inherited identical by descent (IBD) and represented the location of the genetic defect in the Amish form of PFIC. This confirmed that BRIC and Byler disease were probably allelic, arising from mutations, likely of different severity, in the same gene. The possibility that two cholestasis related genes clustered together in a 19 cm region, one mutated in BRIC and the other in PFIC1, though very unlikely, could not be formally ruled out. Nonetheless, the gene(s) involved in BRIC and Byler Disease was expected to have, when found, much to tell about the mechanisms of cholestasis and biliary secretion, and hopes were high that it would prove to be the long sought canalicular bile salt exporter. Ironically, however, but not at all uncharacteristically for science, it was not the most predictable nor paradigmatic story that ultimately revealed itself, and the precise mechanistic role of the BRIC/Byler gene in the etiology of human cholestasis syndromes baffles and tantalizes to this day.

Differentiation of PFIC1 and PFIC2

The mapping of a genetic locus for BRIC and Byler Disease finally allowed the latter to shed its ethically problematic designation and provided the necessary, testable criterion for definitive stratification of the PFIC syndromes based on genetic etiology. The concurrent reexamination of the clinical data put the previously murky notions of heterogeneity in PFIC on firm footing.

Thus, in 1997, Bull *et al* provided the first comprehensive comparison of Byler Disease and the similar disorders usually referred to as Byler Syndrome or PFIC, and proposed a revision of the disease nomenclature, where patients with genetic evidence of linkage to

the 18q21-22 disease locus would be classified as having progressive familial intrahepatic cholestasis type 1 (PFIC1). This group would include all of the patients previously diagnosed with Byler Disease (genotyping of all nine available Amish children with diagnosed Byler Disease or Syndrome revealed homozygosity for the “Byler” haplotype, confirming inheritance of the presumed disease locus), and some patients previously categorized as having Byler Syndrome: those with genetic evidence of homozygosity in the disease locus, or with clinical findings close to the proposed distinguishing features of the PFIC1. The authors showed that certain clinical features distinguished PFIC1, or 18q21-22 linked disease (represented by 9 patients) from other forms of PFIC (represented by 4 patients in this article). These features included the ultrastructural appearance of bile, which was coarsely granular in PFIC1 patients, and amorphous or filamentous in others. Although this observation of “Byler bile” had been made in the initial characterization of the disease (Clayton *et al* 1969), since it was electron microscopy dependent and restricted to a specific technique of sample preparation, it had not been previously given sufficient consideration. Liver histology also proved informative. Upon presentation, PFIC1 patients had a lesser degree of histopathological disturbance, with only bland intracanalicular cholestasis, compared to the neonatal hepatitis and more severe fibrosis and inflammation in the non-PFIC1 patients. Disease progression appeared more rapid in the non-PFIC1 patients, who came to transplantation much earlier (3-6years), than the PFIC1 patients (13-14 years), and showed substantial progression to cirrhosis (however, most recent genotype-phenotype correlation data in PFIC1 and PFIC2 patients have not clearly replicated this finding). This correlated with the serum activities of liver enzymes (ASAT, ALAT and ALP),

diagnostic for liver damage, which were somewhat less elevated in the PFIC1 patients. Levels of GGT and bilirubin were similar in both groups. The validity of these clinical distinctions was confirmed when linkage to the 18q21 locus was excluded in both families studied with non-PFIC1 patients.

Thus the genetic groundwork was laid for a future division of labor in the field of inherited cholestasis. Efforts led by Laura Bull in the Freimer laboratory concentrated on PFIC1 and BRIC, and a large outreach was undertaken to screen patients from all over the world for linkage to the ch18 locus in an attempt to expand the cohort of PFIC1 patients beyond the original Amish kindred. This was important both in ascertaining the prevalence of type 1 disease amongst the entire population of PFIC patients, and in acquiring enough diverse genetic material, rich in the treasured signposts of the geneticist: recombination events that would allow narrowing down the physically large 19 cM locus to a smaller area more tractable for physical mapping. This latter undertaking would of course be aided by parallel genetic analyses of the locus among BRIC patients, also a steadily growing cohort.

The BRIC linkage study of 1997 had narrowed the locus down to approximately 7cM. Ultimately, persistent patient collection and painstaking genotyping and haplotype evaluation in patients with both diseases, as well as the identification of additional polymorphic markers interspersed within the known BRIC/PFIC1 genetic locus allowed the disease locus to be pinpointed to approximately 1cM or 300kb, with a key narrowing

step coming from the identification of a patient carrying a serendipitous chromosomal deletion, bisecting the locus (as well as the gene itself) (Bull 1998, 1999).

Subsequent identification of the gene mutated in BRIC and PFIC1 patients (Bull 1998, Chapters 2 and 3) and investigation of the role its protein product in the enterohepatic circulation of bile acids in a mouse model (Chapters 4, 5 and 6) is the subject of this dissertation. The remainder of this introduction places these findings in the context of genetic advances in understanding of other forms of progressive intrahepatic cholestasis and experimental investigations of biliary physiology that proceeded in parallel.

PFIC2

The conclusions of the Bull *et al* 1997 article establishing PFIC1 as a genetic and clinical entity, were vindicated when then genetic heterogeneity of PFIC was confirmed. First, two collections of patients (Middle Eastern and Swedish) were demonstrated to have PFIC disease unlinked to ch18q21-22 (Strautnieks *et al* 1996, Arnell *et al* 1997).

Subsequently, a second disease locus, designated PFIC-2, was mapped to chromosome 2q24 amongst patients of Arab descent unlinked to ch18q21-22 (Strautnieks *et al* 1997). Examination of genes that mapped to this region identified a likely candidate gene, *SPGP* (sister of p-glycoprotein, renamed *BSEP*, for bile salt export pump, and subsequently classified as *ABCB11*) (Strautnieks *et al* 1998). *BSEP* was a member of the large superfamily of ATP-dependent transporters, whose members had been variously referred to as p-glycoproteins (for pump activity and extensive extracytoplasmic glycosylation), MDR proteins (for their original documented roles in multidrug resistance in cancer) or

ABC transporters (for possession of the ATP-Binding-Cassette) (Childs *et al* 1995, Dean *et al* 2001). As alluded to above, it had been hypothesized that the gene associated with BRIC and PFIC would prove to be an ABC transporter, since most of the then-characterized mammalian membrane transporters, including several hepatocyte and canalicular transporters, belonged to this family. Significant but fruitless efforts had been made to find an ABC-type transporter within the PFIC1/BRIC locus (Bull *et al* 1998). This hypothesis was now vindicated, somewhat ironically to those of us working in the Freimer laboratory on the PFIC1 end of things, not in PFIC1 but in PFIC2.

The full-length sequence of *SPGP/BSEP* was obtained, and many different mutations were found in PFIC2 families (Strautnieks *et al* 1998). At least 35 different mutant alleles of the gene have now been identified in PFIC2 families, including multiple examples of the nonsense, deletion and frameshift mutations that are considered unequivocally “functional nulls”, and many different missense mutations, affecting presumably key protein residues (Thompson & Strautnieks 2001). In the small number of patient liver samples so far studied, *BSEP* mutation is invariably associated with loss of BSEP protein expression, which may reflect structural instability or mistrafficking and subsequent degradation of mutant forms of the polypeptide (Jansen *et al* 1999).

The first clue that the PFIC2 gene would turn out to be the long sought ATP-dependent canalicular bile salt transporter was its presence at the scene of the crime: BSEP was originally detected only in the hepatocyte canalculus, an expression pattern that precisely mirrors its postulated function in bile secretion, a physiological process that occurs only

in the hepatocyte (Childs *et al* 1995). ATP-dependent bile salt transporting activity was first demonstrated by in vitro characterization of the rat orthologue of *BSEP* (Gerloff *et al* 1998), and subsequently with the mouse orthologue overexpressed in a baculovirus system (Noe *et al* 2001). Interestingly, the skate ortholog of the protein has also been studied and proven to transport bile salts (which capacity is abolished upon introduction of sequence alterations analogous to characterized PFIC-2 mutations), which reflects a striking evolutionary conservation (and very ancient lineage, predating the evolution of mammals) given that the skate excretes bile alcohols rather than bile salts in its bile, and additionally has no biliary phospholipids (Cai *et al* 2001).

Since work on functional characterization of the BSEP protein was underway even before its role in PFIC2 disease became known, and interest in proving its function as the canalicular bile salt exporter was high, generation of the *Bsep* mutant mouse followed promptly (Wang *et al* 2001). A “knockout” targeting approach was used, resulting in a mouse strain with defective *Bsep* mRNA and thus no Bsep protein expression, which paralleled the situation seen in PFIC-2 patients. However, examination of the *Bsep*^{-/-} mice offered a set of surprises. The mice did, as expected, have extremely reduced biliary levels of taurocholate, a major bile salt species in humans and mice. However, their overall bile flow and bile salt output was only partially reduced, and output of muricholate as well as of atypical tetrahydroxylated bile salt species was increased, as was cholesterol and phospholipid output in the bile. This overall conservation of biliary function and low degree of cholestasis resulted in a much milder phenotype than that seen in human PFIC2 patients: the mice had no histopathologic evidence of intrahepatic

cholestasis, no progressive liver damage, no jaundice or elevation of serum liver enzymes and were not clinically abnormal beyond some runting early on in life and elevated serum bile salt levels. Nonetheless, they were sensitive to the effects of bile salt feeding, and accumulated intravenously administered radiolabeled taurocholate in the liver, which demonstrated impairment of canalicular bile salt excretion.

Thus the *Bsep* mutant mice reiterated the warning which echoes periodically through the biological community, that murine physiology is not always a perfect model for the human; and that faithful murine models of human syndromes may be quite difficult to construct (Elsa & Lucas 2002). This is vexing to clinicians who look to mouse models to inform therapeutic approaches in human patients, but biologists can nevertheless dissect valuable clues out of mouse-human discrepancies. The difference in the murine and human response to *Bsep* mutation appears in part attributable to the composition of the bile salt pool, and to metabolic differences. The unimpeded excretion of muricholate in the *Bsep* $-/-$ mice revealed that mice must have an alternative canalicular export system, possibly one specific for the uniquely murine hydrophilic bile salt, muricholate.

Furthermore, the appearance of novel tetrahydroxylated BS species in these animals indicated intrahepatic BS modifications at play; suggesting that mice may be able to convert the cholate, accumulating upon *Bsep* abrogation, into either muricholate or into these abnormal, but probably not cytotoxic (due to their high hydrophilicity) BS species. Thus, unlike in humans, *Bsep* loss in mice knocks out one of the BS export pathways, but not the sole one, and further protection is provided by metabolic modification of bile salt species.

Since the exquisite control of BS homeostasis maintained via the enterohepatic circulation of bile salts requires finely-tuned coupling of bile salt synthesis and secretion in the hepatocytes to bile salt loss in the feces and reclamation in the small intestine, the regulatory feedback loops that govern this homeostasis have been of much interest. Recent advances made in the elucidation of the regulatory roles of the (formerly designated) orphan nuclear receptors, in particular FXR (farnesyl x receptor; now also known as BAR, the bile acid receptor) have yielded clues to BSEP regulation as well. The human *BSEP* promoter features an FXR recognition motif and can be transactivated by FXR/BAR in vitro (Ananthanarayanan *et al*, 2001). In vivo, cholate feeding in mice up-regulated canalicular Bsep expression through an FXR/BAR dependent mechanism; while *FXR* $-/-$ mice demonstrated impaired bile acid and lipid homeostasis including an inability to adapt to dietary bile salt or lipid overload (Sinal *et al* 2000). Thus, BSEP expression is regulated via FXR/BAR in response to prevailing bile salt concentrations.

Furthermore, it appears that chronic, externally induced cholestasis is not caused by loss of BSEP expression, which was maintained in cholestatic rats (Lee *et al* 2000). Instead, regulation of BSEP activity probably occurs in a large part on the level of protein trafficking/localization/insertion into the plasma membrane, which was stimulated by taurocholate infusion in the rat liver (Misra *et al* 1998).

These suggestions are important to the consideration of the role of the gene defective in PFIC1 and BRIC in the regulation of bile salt secretion. Although the above discussion emphasizes the distinctions between PFIC1 and PFIC2, from another point of view, the

similarities between the two diseases are striking, if one considers that BSEP is clearly a canalicular bile salt exporter while the PFIC1 protein is probably not (Bull *et al* 1998, Ujhazy *et al* 2001). However, all of the genes implicated in familial cholestasis must be functionally interlinked in the great molecular web of interactions and feedback loops that forms the molecular wiring diagram of the enterohepatic circulation.

It must also be mentioned that the most recent genetic analyses suggest that some low-GGT PFIC disease must result from a defect at yet another locus, as these patients do not show linkage to either the PFIC1 or the PFIC2 locus (Strautnieks *et al* 2001).

Familial cholestasis with high GGT, or PFIC3

The topic of this dissertation is familial cholestasis with low serum GGT. However, the design of these studies was significantly influenced by discoveries relating to the mouse model of the high-GGT form of PFIC, so it is worth summarizing that knowledge here, even though it was possible to exclude those forms of familial cholestasis from consideration at the very start of our genetic journey.

The story of PFIC3 disease, as it is most conveniently named, even though this is a name of very recent vintage, serves also as an interesting counterexample of the direction the flow of knowledge can take between genetics, biology and medicine. The saga of PFIC1 starts with a perplexing, sometimes miscategorized disease, and proceeds to a gene and then to a genetically modified mouse, while the tale of PFIC3 starts with a

miscategorized and therefore perplexing gene, proceeds to the mouse and only then makes the connection to human disease.

The protein product of the gene in question, one of the founding members of the ABC transporter family to which BSEP also belongs, was initially referred to as MDR3 p-glycoprotein (in humans, now renamed *ABCB4*; the mouse orthologue is *Mdr2*).

Although it was originally classified by homology among the multispecific export pumps responsible for the acquired resistance of malignant cells to chemotherapy (MDR stands for multidrug resistance), its unusual expression pattern, essentially confined to the canalicular membrane of the hepatocyte, and the inability to confer an MDR phenotype suggested a different physiological role (Schinkel *et al* 1991, Smit *et al* 1993, 1994).

Generation of an *Mdr2* knockout mouse yielded the unexpected phenotype of progressive liver damage with bile duct abnormalities, and analysis of the bile of the mutant mice revealed a total absence of phospholipid and cholesterol, which explained the hepatic phenotype, since bile salts unaccompanied by lipids cannot form micelles and, being strongly detergent, are cytotoxic through plasma membrane damage (Smit *et al* 1993).

However, this was a surprising result since it had previously been assumed that secretion of lipid into bile is a passive process entirely mediated by the detergent effects of bile salts. Normal cholesterol secretion, but a gene dosage-dependent, 40% reduced secretion of phospholipid in heterozygous animals indicated that phospholipids were the substrate of *Mdr2*. Subsequent investigations with labeled lipid analogs confirmed this substrate specificity and suggested the mechanism of *Mdr2*/MDR3 action (Ruetz & Gros 1995, Smith *et al* 1995). It is now believed to be a phospholipid flippase which translocates

phosphatidylcholine (PC) from the inner to the outer leaflet of the canalicular membrane, where it becomes available for secretion into bile. The lipids present in bile (predominantly PC) do not reflect the lipid composition of the canalicular membrane; thus, the current model postulates that translocase activity of MDR3 provides transient membrane domains high in PC, which are then incorporated into bile as vesicles; while the rest of the canalicular membrane provides the structural rigidity necessary for maintenance of cellular integrity with a less fluid composition. The predominant outer leaflet lipids: sphingomyelin, cholesterol and cholesterol esters make for rather rigid membrane domains, from which lipids cannot be secreted in the absence of PC translocation and creation of more fluid PC-rich subdomains (Oude Elferink, Tytgat & Groen 1997).

In retrospect, given a complete absence of lipid in bile but normal bile salt levels, the phenotype of the *Mdr2* mutant mice appeared relatively mild; this was attributed to the highly hydrophilic and thus not very cytotoxic nature of the mouse bile salt pool. Thus it was postulated that a parallel defect in humans would lead to a much more severe, possibly even fatal, phenotype, since the human bile salt pool is significantly more hydrophobic (Smit *et al* 1993). The search for liver disease patients with a molecular defect in *MDR3* was on. In 1996 a promising patient was identified: a PFIC patient with high serum GGT and histopathological evidence of severe fibrosis and ductular proliferation that echoed the liver pathology in *Mdr2* knockout mice. Gratifyingly, Northern blotting revealed the absence of *MDR3* mRNA in this patient's liver (Deleuze *et al* 1996). In a subsequent study of 31 patients with high GGT, *MDR3* mutations were

found in 17, prompting the reclassification of a subtype of PFIC characterized by high GGT and liver pathology including ductular proliferation and fibrosis and portal hypertension as PFIC-3 (de Vree *et al*, 1998; Jacquemin *et al* 2001). When investigated, the bile of these patients was, as predicted, found to be dramatically depleted in phospholipid, while retaining normal levels of bile salt, which provided a clear criterion for distinguishing PFIC3 from PFIC1 and PFIC2. It also made the very designation of PFIC3 something of a misnomer, or at the very least misleading, since an unimpaired bile salt secretion is somewhat at odds with the concept of cholestasis.

Interesting genotype-phenotype correlations emerged from the identification of a variety of *MDR3* mutations among PFIC3 patients (Jacquemin *et al* 2001). In patients, whose mutation resulted in complete loss of *MDR3* expression from the liver, e.g. in all of the patients with a truncated protein, the disease appeared histopathologically more severe, and clinical symptoms were not markedly improved by administration of UDCA, the hydrophilic bile salt, whose therapeutic principle relies on the replacement of harmful hydrophobic bile salts in the bile salt pool with the less toxic UDCA. On the other hand, some patients with milder, missense mutations, who may have retained some *MDR3* activity, did improve on UDCA. In some of the patients with such missense mutations disease had not presented until the teens or early twenties, and was thus significantly milder than the most often seen form with early childhood onset. The authors proposed that UDCA therapy was only able to alter bile salt pool toxicity sufficiently when some *MDR3* activity and thus lipid secretion into bile was retained.

Furthermore, in a development that echoes the relationship between BRIC and PFIC1, mutations in *MDR3* have recently been implicated in adult liver disease. Heterozygous mutations in *MDR3* have been found in several women who have suffered from intrahepatic cholestasis of pregnancy (ICP), particularly in mothers of several of the studied PFIC-3 patients (de Vree *et al* 1998, Jacquemin *et al* 1999, 2001). ICP manifests with elevated serum bile salts, jaundice and pruritus in women with no previous history of liver or biliary abnormality, and can lead to fetal distress and death; it is hypothesized to be linked to elevated levels of female sex hormones (e.g. progesterone) in pregnancy. Cholestasis of pregnancy was also reported in an obligate heterozygous mother of PFIC1 infants (Clayton *et al* 1969, Linarelli *et al* 1972). A pattern emerges of heterozygous insufficiency of a protein required in bile secretion becoming symptomatic upon addition of a subsequent insult, in these cases the high hormone levels or other physiological imbalances of pregnancy.

The second example of an adult phenotype associated with *MDR3* mutations was suggested by the finding of gallstones in some children with PFIC3, and also in their heterozygous parents. Rosmorduc *et al* (2001) extended this finding by testing 6 adult patients with symptomatic intrahepatic sludge and gallbladder cholesterol stones for *MDR3* mutations. Three patients were found to have homozygous missense mutations, while the other three had a heterozygous mutation, one of which resulted in a frameshift and early protein truncation.

The detection of *MDR3* mutations in adult patients without PFIC disease provides significant validation for one of the tenets of genetic studies of rare inherited disease, which is that genes thus discovered play important physiological roles and may therefore also be defective in sporadic and common forms of disease. Accordingly, the authors of the *MDR3* reports speculate that further defects in this gene may be uncovered among patients with unexplained adult cirrhosis of the biliary type and also among patients with cholestasis secondary to drug sensitivity.

Other forms of PFIC

As evident from the discussion above, PFIC3 is clinically and mechanistically distinct enough from PFIC1 and PFIC2 to fall outside the main scope of this dissertation, though it nevertheless provides important parallel insights into biliary disease processes as well as into the interplay of clinical advances and of biological investigations in murine models. In addition, it is worth mentioning diseases of even more murky affiliation with the PFIC continuum. Specifically, the fair prominence of the PFIC nomenclature in the literature of the field has inspired efforts to name further described disease entities accordingly, and thus some forms of intrahepatic cholestasis arising from defects in enzymes of bile salt synthesis pathways have been shoehorned by some into the category of PFIC4 (Clayton *et al* 1987, Setchell *et al* 1988, Setchell *et al* 1998). Finally, many other rare familial syndromes of intrahepatic cholestasis of as yet unknown etiology exist, some with characteristic extrahepatic manifestations (Jansen *et al* 2001).

Objectives and overview of the dissertation

The goal of the research presented here was to elucidate the genetic defect in human patients with the BRIC and PFIC1 diseases, previously demonstrated to be allelic defects in the same gene (Bull *et al* 1998), and to create an in vivo model of human PFIC1 disease by a multi-pronged strategy of targeted mutagenesis of the mouse. We hypothesized that, since cholestasis represents dysfunction of the enterohepatic circulation of bile salts, affecting many organs and resulting in many systemic complications, only analysis of the effects of the mutation in a whole mammalian organism, i.e. the mouse model, would allow the recapitulation of the initiating events of the pathogenesis of cholestasis and the dissection of the site-specific aspects of the process. Furthermore, at the time the project was initiated, existing animal models of cholestasis utilized external surgical insult, i.e. bile duct ligation (Accatino 1993, 1995) or chemical insult: estrogen (Bossard *et al* 1993, Meier *et al* 2002), endotoxin (Mosely *et al* 1996), or ANIT (Chisholm *et al* 1996) to induce the pathophysiological process. As such, they were inadequate models for primary cholestasis due to intrinsic physiological defects, and did not separate pathology occurring in the hepatobiliary system from systemic and secondary complications very well. We hypothesized that a mouse strain replicating the genetic defect in PFIC1 patients could have utility as a general model of intrahepatic cholestasis or of defects in the enterohepatic circulation of bile salts.

This dissertation is organized chronologically and reflects the fairly linear narrative and scientific journey from the identification of *FIC1* as the gene mutated in BRIC and PFIC1 through generation and analysis of the mouse model of PFIC1. The chronological

organization means that certain aspects of the evolving scientific background of this work have been left out of the introductory chapter and are discussed in the relevant chapters in an effort to mirror the historical development of ideas in the field that occurred concurrently with the progress of the research presented here. As the work on this dissertation progressed and evolved, so did efforts elsewhere concerning the genetic basis of other forms of inherited cholestasis. The published results of functional studies of the *FIC1* and *BSEP* genes, and lessons from the *BSEP* knockout mouse inform the final chapters of this dissertation, but it must be appreciated that those insights were not yet available at the time *Fic1* was identified and the murine targeting strategy conceived.

Chapter 2, originally published in *Nature Genetics* 18, 219-224 (Bull *et al* 1998) and reprinted here by permission of the Nature Publishing Group, describes the positional cloning of the candidate gene for PFIC1 and BRIC by high-resolution genetic mapping, assembly of the full-length *FIC1* coding sequence by cDNA library screening, genomic sequencing and 5'RACE, the first homology sequence analyses of the novel gene and formulation of hypotheses as to its molecular function, and the identification of disease-causing mutations in *FIC1* in patients with both diseases. This work was led by Laura N. Bull; my contributions to it are outlined in the Dissertation Advisor's Statement.

Chapter 3, a manuscript in preparation (L. Klomp *et al*), describes the characterization of the human *FIC1* genomic locus including the delineation of intron-exon boundaries (a task now rendered almost obsolete by the advent of the Human Genome Project), and the identification of disease-causing mutations in many more PFIC1 and BRIC families. This

chapter also extends previous speculation on how different mutations in a single gene can cause a spectrum of cholestatic diseases spanning a wide range of severity. I performed a significant portion of the experimental work presented here (see Dissertation Advisor's Statement); the manuscript was written by Leo Klomp and edited/reviewed by the other authors including myself.

Chapter 4 sets the stage for the generation of mice carrying targeted mutations in *Fic1*. I describe the isolation of the murine orthologue of human *FIC1* and the design of a two-pronged targeting strategy aimed at both replicating a disease-causative homozygous point mutation in the hope of creating a mouse model of PFIC1, and at creating a null or knockout mouse to allow the unambiguous description of the systemic and tissue-specific effects of complete loss of FIC1. Chapter 4 concludes with a progress report on the generation of the mutant mice carrying null and conditional-null alleles of *Fic1*, which is continuing. The remainder of this dissertation describes the analysis of the PFIC1 (*Fic1*^{G308V/G308V}) mutant mice.

Chapter 5, a manuscript in preparation, describes the surprising phenotype and key experimental findings in mutant mice homozygous for the G308V substitution in *Fic1*. I discuss novel insights, gleaned from the mouse model, into the paramount role of the intestine in the etiology of *Fic1*-related liver disease and into the previously underestimated importance of the regulation of intestinal bile salt absorption for systemic bile salt pool homeostasis. I also discuss the advantages and frustrations associated with the differences in hepatobiliary physiology and between mice and humans, and the ways

in which these differences result in contrasting phenotypes in mice and humans with an identical point mutation in an evolutionarily highly conserved protein.

Chapter 6 describes further experimental findings in the $Fic1^{G308V/G308V}$ mutant mice, most of which are still at the pilot stage. Here I also discuss the substantial influence of mouse strain background on the phenotype of the $Fic1^{G308V/G308V}$ mutation, comparing and contrasting results obtained in the 129 and FVB/N strains as well as in mice of a mixed background composed of 50% C57Bl/6 and 50% 129/SvJae.

Finally, Chapter 7 provides overall conclusions for this body of work as well as some medical implications and future directions for experimental approaches utilizing the *Fic1* mutant mouse model system.

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CHAPTER 2

A gene encoding a P-type ATPase mutated in two forms of hereditary cholestasis.

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A gene encoding a P-type ATPase mutated in two forms of hereditary cholestasis

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Cholestasis, or impaired bile flow, is an important but poorly understood manifestation of liver disease. Two clinically distinct forms of inherited cholestasis, benign recurrent intrahepatic cholestasis (BRIC) and progressive familial intrahepatic cholestasis type 1 (PFIC1), were previously mapped to 18q21. Haplotype analysis narrowed the candidate region for both diseases to the same interval of less than 1 cM, in which we identified a gene mutated in BRIC and PFIC1 patients. This gene (called *FIC1*) is the first identified human member of a recently described subfamily of P-type ATPases; ATP-dependent aminophospholipid transport is the previously described function of members of this subfamily. *FIC1* is expressed in several epithelial tissues and, surprisingly, more strongly in small intestine than in liver. Its protein product is likely to play an essential role in enterohepatic circulation of bile acids; further characterization of *FIC1* will facilitate understanding of normal bile formation and cholestasis.

Bile formation and bile acid secretion are necessary for the intestinal absorption of dietary fats and fat-soluble vitamins, as well as for the elimination of cholesterol and various toxins. Cholestasis, or impaired bile flow, is one of the most common and devastating manifestations of hereditary and acquired liver disease¹. It presents clinically with itching, jaundice and fat malabsorption¹. The molecular mechanisms of bile formation and of intrahepatic cholestasis remain poorly understood, in part because of the inaccessibility of the biliary canaliculi and the intrahepatic biliary tree to direct physiological investigation. (The bile canaliculus is a small intercellular space that lies between hepatocytes, and through which bile is delivered from the hepatocytes to the bile ducts.) For this reason, identification and characterization of genes that are mutated in inherited forms of cholestasis may provide the best opportunity to elucidate these processes. Benign recurrent intrahepatic cholestasis (BRIC or Summerskill syndrome; refs 2–4; OMIM 243300) and progressive familial intrahepatic cholestasis type 1 (PFIC1 or Byler disease; refs 5–6; OMIM 211600) are of particular interest, because cholestasis is the predominant manifestation of these clinically distinct autosomal-recessive disorders. BRIC is characterized by recurrent episodes of intrahepatic cholestasis, lasting days to months, which can begin in childhood or adulthood and resolve spontaneously, causing no detectable lasting liver damage². In contrast, PFIC1 is manifest in early infancy as cholestasis that may be initially episodic but progresses to malnutrition, growth retardation and end-stage liver disease before adulthood⁵. (PFIC1 is so termed because a second PFIC locus, *PFIC2*, has recently been mapped to chromosome 2 (ref. 7). *PFIC1* and *PFIC2* are distinct clinically, histopathologically and ultrastructurally⁸.)

While BRIC and PFIC1 differ greatly in severity, they share certain clinical and biochemical features. Serum gamma glu-

tamyl transpeptidase activity is normal or slightly elevated in both BRIC and PFIC1 (refs 8,9), contrasting with the markedly elevated activity of this enzyme in most cholestatic disorders. Light microscopy of liver tissue from patients with BRIC or early stages of PFIC1 reveals only bland intracanalicular cholestasis^{2,8}. Substantially elevated concentrations of lithocholic acid, a strongly hepatotoxic bile acid, have been observed in BRIC and PFIC1 patients^{3,6,9}, but not in patients with PFIC of unspecified sub-type¹⁰. We now report that both BRIC and PFIC1 are caused by mutations in a single gene, which we term *FIC1* (for familial intrahepatic cholestasis 1).

High-resolution mapping of BRIC and PFIC1

The gene mutated in BRIC was mapped to 18q21–q22, using a search for shared genome segments in three distantly related affected Dutch individuals¹¹. Linkage analysis in a larger set of families confirmed this localization¹². The *PFIC1* locus was mapped to an overlapping interval through identification of a haplotype for which two distantly related Amish PFIC1 patients were homozygous; this result suggested that both diseases are caused by mutations in the same gene¹³. We refined the localization of both *BRIC* and *PFIC1* to the same interval of less than 1 cM (*D18S41–D18S49*) through examination of haplotype sharing in expanded sets of patients with each disorder. A microdeletion spanning the region containing markers *D18S381*, *D18S49* and *D18S1144* on a single chromosome in a PFIC1 patient S.K. further narrowed the candidate region to an interval of less than 300 kb, between *D18S977/D18S1401* and *D18S49* (Fig. 1a, and L.N.B. *et al.*, manuscript in preparation).

Use of CEPH YACs in PCR and hybridization experiments allowed us to determine the order of genetic markers in a 2 cM region containing the *BRIC* and *PFIC1* loci. Subsequently, we gen-

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Fig. 1 Positional cloning of the PFIC1/BRIC gene. **a**, Genetic map of the 1-cM region containing the PFIC1/BRIC gene, indicating the microsatellite markers used for genotyping studies. The location of the candidate region for this gene, based on haplotype and deletion analyses, is shown. Exact genetic and physical distances between the markers in this region are unknown. **b**, Physical map of the region (defined by markers D18S41 and D18S849) to which the PFIC1/BRIC gene was localized by haplotype analysis. **c**, The position of the candidate cDNA (*FIC1*) in relation to the physical map.

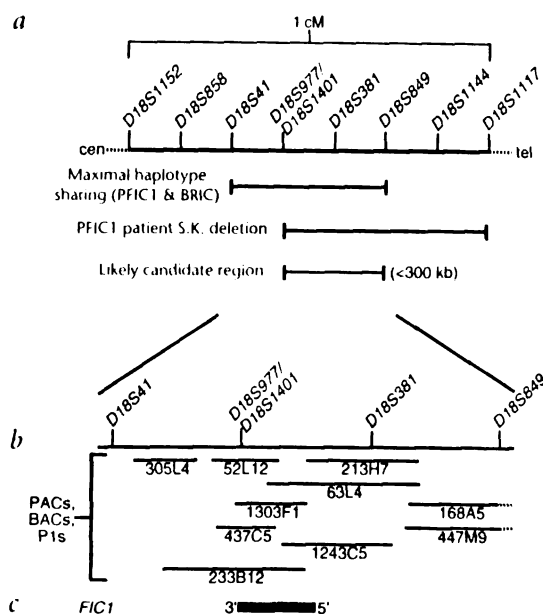
erated a contig spanning the candidate region between D18S977 and D18S849, consisting of P1, PAC and BAC clones (Fig. 1b).

Identification of the *FIC1* coding sequence

Initially, we hypothesized that the PFIC1/BRIC gene might be a member of the ATP-binding cassette (ABC) protein family. This hypothesis was based on the assumption that PFIC1 and BRIC were likely due to defective transport of bile acids across the canalicular membrane⁵ and on the fact that all previously identified proteins that conduct active (in-to-out) transport across this membrane are ABC-proteins¹⁴. An extensive screen, however, of DNA in the candidate region by Southern hybridization and PCR revealed no evidence of the presence of an ABC protein gene.

Therefore, we relied upon the following three approaches to identify genes in the candidate region: examination of previously mapped expressed sequence tags (ESTs), sequence scanning and hybridization of a PAC insert (from PAC 52L12, Fig. 1b) to a liver cDNA library. The only coding sequence identified that mapped to the narrow candidate region was a 1.6-kb partial cDNA obtained by hybridization using the PAC insert. A BLAST search showed that the protein sequence predicted for this partial cDNA exhibits strong similarity (56%) to that of the bovine ATPase II gene¹⁵. Hybridization of this cDNA to northern blots revealed a transcript of approximately 7 kb. Relative to the β -actin gene, this transcript was highly expressed in proximal small intestine and pancreas. It is also present in several other tissues, including liver (Fig. 2).

To identify additional coding sequence from this gene, we screened an intestinal cDNA library, continued sequence scanning, performed 5' RACE and screened the EST database with the additional sequence obtained. These approaches yielded a total of 5.9 kb of cDNA sequence. We named this candidate cDNA *FIC1* (Fig. 1c). The sequence included 61 bp of 5' UTR, followed by the putative translation start site and the complete



3753-bp coding sequence of *FIC1*. The stop codon is followed by 2.1 kb of 3' UTR and a polyA tail. (Both the 5' and 3' UTRs contain regions of Alu repeat sequence.)

The predicted protein sequence encoded by *FIC1* contains the 'signature' motifs that characterize P-type ATPases as a whole¹⁶. Other features, notably the predicted presence of ten transmembrane domains, identify the *FIC1* cDNA as that of a non-heavy metal-binding P-type ATPase¹⁷. Within the signature motifs in this cDNA, specific sequences are present that define a recently identified subfamily of P-type ATPase genes that encode putative aminophospholipid transporters (ref. 15; Fig. 3). These sequences differentiate members of this subfamily from genes for previously recognized P-type ATPases, which transport cations. When the predicted *FIC1* amino acid sequence was compared with publicly available sequences using BLAST, it exhibited strong similarity (52%) to that of bovine ATPase II, the first described member of this subfamily¹⁵, and to the mouse orthologue of the bovine gene (53%). The P-type ATPase subfamily of which *FIC1* is a member was previously known to contain four genes: the bovine ATPase II and one gene each from *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Plasmodium falciparum*¹⁵. Through screening of publicly available databases using BLAST, we identified several additional *S. cerevisiae*, *Schizosaccharomyces pombe* and *C. elegans* genes that exhibit sequences characteristic of this P-type ATPase subfamily. *FIC1* exhibited 89% similarity to an EST representing 89 amino acids of a cDNA from porcine small intestine. This similarity persisted in a region where the sequence of *FIC1* diverges substantially from that of the cDNA for bovine ATPase II. Furthermore, we subsequently identified a distinct partial cDNA from a human small-intestine library with a predicted amino acid sequence that is much more similar to that of bovine ATPase II than is the predicted *FIC1* amino-acid sequence. These observations indicate that *FIC1* is not the human counterpart of the bovine ATPase II gene.

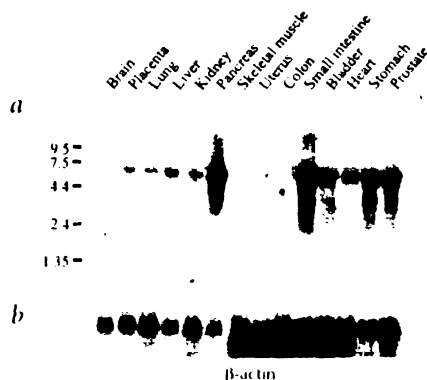


Fig. 2 Northern blot of a candidate (partial cDNA) for the PFIC1/BRIC gene. **a**, The 1.6 kb cDNA probe was hybridized to multiple-tissue northern blots (Clontech) containing 1–6 μ g of poly(A)⁺ RNA in each lane. Interpretation of the expression pattern is complicated by the fact that the pancreas lane of the blot was loaded with several-fold more RNA than the other lanes. **b**, Hybridization of these same blots with a β -actin probe.

Mutation identification

Samples of tissues in which *FIC1* is expressed were not available for most of the PFIC1 and BRIC patients in this study, and thus we searched for mutations in their genomic DNA. Exon-intron

Fig. 3 Predicted amino-acid sequence encoded by *FIC1*. Boxed sequences are P type ATPase signature domains; bold sequences within the boxes are characteristic of a recently defined subfamily of P-type ATPases¹⁵. Putative transmembrane domains, predicted with the aid of a Kyte-Doolittle hydrophobicity plot¹¹, are underlined.

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1  MSTERDSETT FDSDSQPNDE VVPYSDDDETE DELDDQGS AV EPEQNRVNRE AFEENREPFRK
61  ECTWQVKAND RKYHEQPHFM NTKFLCIKES KYANNAIKTY KYNATFTIPM NLFEQFKRAA
121 NLYFLALLIL QAVPQISTLA WYTTLVPLLV VLGVTAIKDL VDDVARHKMD KEINNRTCEV
181 IKDGRFKVAK WKEIQGVGDI RLKKNDFVPA DILLSSSEP NSLCYVTAE LDGETNLKFK
241 MSLEITDQYL QREDTLATFD GFIECEEPNN RLDKFTGTLF WRNTSFPLDA DKILLRGCVI
301 RNTDFCHGLV IFAGADTKIM KNSGKTRFKR TKIDYLMNYM VYTFIVVLIL LSAGLAIGHA
361 YWEAQVGNSS WYLYDGEDDT PSYRGFLIFW GYIIVLNTMV PISLYVSVEV IRLGQSHFIN
421 WDLQMYAAEK DTPAKARTTT LNEQLGQIHY IFSDKTGTLT QNINTFKKC INGQIYGDHR
481 DASQHNHNKI EQVDFSWNTY ADGKLAFYDH YLIEQIQSGK EPEVRQFFFL LAVCHTMVD
541 RTDQGLNYQA ASPDEGALVN AARNFGFAFL ARTQNTITIS ELGTERTYNV LAILDNFSDR
601 KRMSIIVRTV EGNIKLYCKG ADTVIYERLH RMNPTKQETQ DALDIPANET LRTLCLCYKE
661 IEEKEPTEWN KKFMAASVAS TNRDEALDKV YEEIEKDLIL LGATAIEDKL QDGV/PETISK
721 LAKADIKIYW LTGDKKETAE NIGFACELLT EDTTICYGED INSLHARME NQRNRGGVYA
781 KFAPPQESF FPPGNGRALI ITGSLWNEIL LEKKTNRKI LKLFKPTREE ERRMRTQSKR
841 RLEAKKEQRQ KNFVDLACEC SAVICCRVTP KQKAMVVDLV KRYKKAITLA IGDGANDVNM
901 XTAHIGVGI SGQEGMQAVM SSDYSFAQFR YLQRLLLVHG RWSYIRMCKF LRYFFYKNFA
961 FTLVHFWSYF ENGYSQAQY EDWFITLYNV LYTSLPVLML GILDQDVSDK LSLRFPGLYI
1021 VGQRDLLFNY KRFFVSLHLG VLTSMILFFI PLGAYLQTVG QDGEAPSDYQ SFAVTIASAL
1081 VITVNFQIGL DTSYWTFVNA FSIEGSIALLY FGIMFDFHSA GIHVLFPESAF QFTGTASNAL
1141 RQPYIWLTI LTVAVCLLPV VAIRFLSMTI WPSESDKIQK HKRKLKAEQ WQRQQVFRR
1201 GVSTRRSAYA FSHQRGYADL ISSGRSIRKK RSPDLAIVAD GTAERYRTGD S

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boundaries in *FIC1* were identified, and intronic primers were chosen to allow amplification of exons (L.W.J.K. *et al.*, manuscript in preparation). Because PFIC demonstrates locus heterogeneity and BRIC may as well^{7,12}, we screened for mutations in unrelated PFIC and BRIC patients who were either homozygous for many consecutive markers in the candidate region or carried at least one copy of an extended conserved haplotype identified in multiple patients. We also included patient S.K., who carried a deletion of part of the candidate region on one chromosome. We began screening for mutations in eight PFIC patients and three BRIC patients. As soon as we identified the first mutations (in four PFIC and two BRIC patients), we finished screening the gene in these six patients. Completion of mutation screening in the other five original patients and additional PFIC and BRIC patients is in progress.

To date, we have identified five PFIC mutations and two BRIC mutations in *FIC1*, indicating that mutations in this gene are responsible for both disorders (Fig. 4). In the patients in whom these mutations were identified, we sequenced exons spanning the 3.8-kb coding sequence of *FIC1*; no other sequence variants were found that were not also present in controls.

In PFIC patients, we identified three missense mutations, one deletion and one skipped exon (Fig. 4). One missense mutation, G308V (G923T), replaces a glycine that is highly conserved in P-type ATPases¹⁸. This mutation is found on the PFIC1 haplotype identified in the Amish; all nine Amish PFIC1 patients shown to be homozygous for the same haplotype are also homozygous for this mutation. It was not present on 107 control chromosomes. A second missense mutation, G892R (G2674A), present in homozygous form in a patient of European descent, replaces a highly conserved glycine present in a signature motif thought to be involved in ATP-binding in P-type ATPases¹⁶. This mutation was not present in a sample of 82 control chromosomes. The third missense mutation, L288S (T863C), was present in homozygous form in a Polish patient, and occurs at a position at which only conservative substitutions are generally seen in the subfamily of P-type ATPases to which *FIC1* belongs. It was not present on 107 control chromosomes.

We further defined the boundaries of the deletion present on one chromosome in PFIC1 patient S.K. through pulsed-field gel

electrophoretic (PFGE) analysis; this deletion includes the 5' 1.4 kb of the coding sequence of *FIC1* (Fig. 4). This result is consistent with the observed orientation of the gene. To identify the mutation on the second chromosome in this patient, we screened genomic DNA. This patient was heterozygous for a mutation, 2097+2 (T→C), in the splice site consensus sequence immediately 3' of the exon which encodes amino acid residues 645–699 (Fig. 4); this exon is predicted to encode part of the largest cytoplasmic domain. This mutation was not present on 77 control chromosomes. RT-PCR of this region of *FIC1* was performed using RNA from this patient's liver. The predominant PCR product was of smaller size than that seen in controls. Sequencing of this product from the non-deleted chromosome of patient S.K. showed that this exon was skipped; skipping of this exon does not change the reading frame of *FIC1*.

In BRIC patients, we identified a missense mutation and a small deletion. The missense mutation, I661T (T1982C), is a non-conservative mutation at a site at which all members of the subfamily of P-type ATPases to which *FIC1* belongs have a leucine or isoleucine residue (Fig. 4). This mutation is present on the most common conserved haplotype in BRIC patients of Western European descent. It is present in homozygous form in patients from 13 families, and in heterozygous form in patients from 6 additional families, and was not found on 84 control chromosomes. A Dutch patient was found to be homozygous for a deletion of 3 amino acids, 795delGNR (2384delGAAACCGTG; Fig. 4). This mutation occurs in a region of the gene that demonstrates little conservation, except between *FIC1* and its porcine homologue, in which the three amino acids are identical. This mutation was not found on 90 control chromosomes. The patient also displays a homozygous sequence alteration in the intronic sequence that precedes the exon containing the deletion, 2286–4 (CT→AA); this alteration was not present on 90 control chromosomes. Fibroblast RNA was available from this patient. When the portion of *FIC1* that contains this exon was amplified using RT-PCR, the predominant product was of expected size. However, a less abundant product of smaller size was also obtained (and was not observed in controls); sequencing of this smaller product showed that the exon containing the deletion was skipped. It is not certain whether this exon-skipping contributes to the phenotype or if the disease is due to the deletion alone.

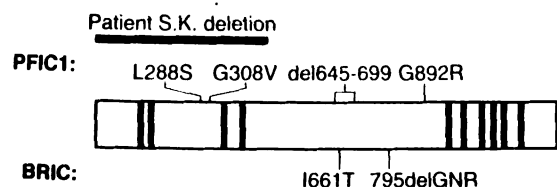


Fig. 4 Location of PFIC1 and BRIC mutations in the coding sequence of *FIC1*. Vertical black bars represent the putative transmembrane domains

Discussion

FIC1 was isolated through population genetic mapping; its initial localization and the subsequent refinement of this localization were based on identification of progressively smaller genome segments shared identical by descent in extremely distantly related single patients¹¹. The advantages of this approach (compared with mapping methods that depend on the availability of entire pedigrees) are striking for rare autosomal-recessive disorders such as BRIC and PFIC1 in which patients are from genetically isolated populations; in such cases it is possible to screen the genome and then map disease genes to small chromosomal regions by genotyping a small number of affected individuals.

The PFIC1 mutations that we identified are likely to destroy or severely compromise the function of *FIC1*. The BRIC mutations identified are in less highly conserved regions of the gene, suggesting that these changes may affect *FIC1* function less drastically than do PFIC1 mutations. These observations are consistent with the fact that PFIC1 is clinically more severe than BRIC. A more complete understanding of the relationship between particular *FIC1* genotypes and different phenotypes awaits elucidation of *FIC1*'s function.

Clues to the function of *FIC1* are provided by the known biology of the hepatobiliary system, the probable function of other members of the protein subfamily to which *FIC1* belongs and by clinical and biochemical features of PFIC1 and BRIC. *FIC1* presumably plays a role in the enterohepatic circulation of bile acids, which consists of several steps. First, bile acids are synthesized in hepatocytes. They are then secreted across the canalicular membrane into the biliary tract and from there into the small intestine. Finally, in the intestine they are absorbed and returned, through the portal circulation, to the liver where they are re-secreted into bile¹⁹. As bile acid synthesis is not defective in BRIC or PFIC1 (refs 3,8,9), *FIC1* probably plays a role in transport of bile acids into the canaliculus, uptake of bile acids from intestinal contents into intestinal mucosa or both.

Defective canalicular transport would presumably result in liver injury through intrahepatic accumulation of bile acids²⁰. Clinical and biochemical evidence suggests such a defect in PFIC1 and BRIC. (The clinical and biochemical findings that we describe were obtained using patients known to carry mutations in *FIC1*, or other patients in the same families.) Concentrations of bile acids are elevated in serum but diminished in bile of PFIC1 patients^{8,21}. Preliminary data from a small number of BRIC patients sampled during cholestatic episodes show similar results (K.D.R. Setchell, unpublished data). Additionally, PFIC1 patients experience dramatic amelioration of cholestasis following liver transplantation (A.S.K., unpublished observations).

FIC1 may directly transport bile acids into bile; however, previously identified molecules that mediate canalicular secretion of organic solutes are members of the ATP-binding cassette (ABC) protein family¹⁴, and a rodent ABC protein has been shown to mediate ATP-dependent canalicular bile acid transport (Gerloff, T. *et al. Hepatology* 26, 358A). *FIC1* could also play an indirect role in canalicular bile acid transport, for example, by maintaining normal function of the canalicular membrane. As in other membranes, phospholipids in this membrane are likely to be asymmetrically distributed, with phosphatidylcholine concentrated in the outer leaflet and aminophospholipids concentrated in the inner leaflet^{22,23}. Transport of phosphatidylcholine from the hepatocyte across the canalicular membrane, a process essential for normal bile formation, is apparently mediated by a previously identified ABC protein, MDR3 (refs 24–27). *FIC1* is the first identified human member of a recently reported subfamily of P-type ATPases whose other members are thought to function in the transport of aminophospholipids from the outer to the

inner leaflet of various membranes^{15,23,24}. It is thus possible that *FIC1*, together with MDR3, maintains an asymmetric distribution of phospholipids in the canalicular membrane.

FIC1's expression at particularly high levels in small intestine is consistent with evidence that intestinal processing of bile acids is abnormal in PFIC1 and BRIC. Defective transport of bile acids or phospholipids, impairing intestinal bile acid reabsorption, could account for the striking malabsorption and diarrhoea in PFIC1 patients^{3,6}. This diarrhoea does not resolve in PFIC1 patients following liver transplant, a finding that distinguishes these patients from individuals with other forms of PHC (A.S.K., unpublished observations; ref. 28). Intestinal bile-acid malabsorption could also account for the excessive faecal bile-acid loss described in some BRIC patients^{3,9} as well as for increased conversion by enteric flora of chenodeoxycholic acid to lithocholic acid. (Lithocholic acid causes experimental cholestasis and is present at abnormally high levels in the blood and bile of BRIC and PFIC1 patients, but not of PHC patients of indeterminate type^{3,6,9,10}.) The consequences of such intestinal bile acid malabsorption would likely vary in relation to factors including diet, infection, gastrointestinal motility and the composition of enteric flora. The effects of such factors could (for example, through alteration in the level of lithocholate production) cause the strikingly variable and episodic course of BRIC and also explain the observation that cholestasis in BRIC frequently follows viral illnesses^{3,9}.

While the data discussed above support the hypothesis that *FIC1* is important in both the intestinal and hepatic phases of bile acid circulation, other evidence suggests wider roles for this protein. For example, the fact that sweat electrolyte concentrations are frequently abnormal in PFIC1 patients^{28–30}, together with *FIC1*'s expression in a wide variety of epithelial tissues (including sites not known to be involved in bile acid handling), suggests that *FIC1* may act globally at sites of secretion and absorption.

The findings reported here suggest that *FIC1*'s function differs fundamentally from that of the proteins responsible for in-to-out transport across the canalicular membrane and support the hypothesis that BRIC and PFIC1 are distinct from other forms of intrahepatic cholestasis at the molecular, as well as the clinical and biochemical, levels²⁷. Functional characterization of *FIC1* will further understanding of the normal enterohepatic circulation of bile acids, the pathophysiology of intrahepatic cholestasis and the role of a novel subfamily of P-type ATPases in human biology and disease. It may also lead to new approaches for treating patients with PFIC1 and BRIC and to the improved management of cholestasis in patients with liver disease of all types.

Methods

Physical mapping. Preliminary mapping information on CEPH YAC clones was obtained from the Whitehead Institute for Biomedical Research/MIT Center for Genome Research web site and the clones obtained from Research Genetics. P1 clones (130311, 43705 and 124305) were identified by C. Collins. The human PAC library³¹ (Genome Systems) and BAC library (Research Genetics) were screened according to the protocols provided (PAC: 30514, 52112, 213117, 6314, 168A5, 233B12; BAC: 447M9). To fill in gaps in the contig, we used 2 methods to obtain PAC ends: direct cycle sequencing of PAC ends and a modification of DOP-PCR³². The latter protocol was modified as follows: Ampliqaq and its buffer (Perkin Elmer) were used for the initial primer extension. Second-round PCR products were gel purified, reamplified, and purified prior to cloning. STSs were designed from PAC end sequences, and used to rescreen the large insert libraries.

Screen for ATP binding cassette (ABC) protein genes. YACs and PACs from the candidate region were screened for the presence of an ABC protein through Southern hybridization at reduced stringency, using as probes STSs that are members of the ABC protein family and oligonucleotides cor-

responding to regions highly conserved in ABC proteins. These primers were also used in PCR-based screening of DNA from the region.

Screening of cDNA library with PAC insert. PAC 521.12 DNA was purified on a CsCl gradient, digested with *NorI* to excise the insert, and the insert was purified by pulsed-field gel electrophoresis (PFGE). 250,000 plaques from an amplified oligo dT primed human liver cDNA library (ATCC# 77402) in lambda gt11 were plated, and duplicate filter lifts prepared. Filters were prehybridized (2 137-mM filters/bottle, in 8 ml solution) for 24 h at 42 °C in 48% formamide, 4.8×SSC, 20 mM Tris pH 7.5, 1×Denhardt's solution, 0.1% SDS and 250 µg/ml denatured human placental DNA (Sigma). For each bottle, PAC insert (approximately 25 ng) was double labelled with ³²P-dCTP and ³²P-dATP. The probe was purified and prehybridized for 1 h at 65 °C with COT-1 DNA (50 µg, Gibco BRL) and linearized PAC vector DNA (1 µg). Hybridization was performed for 24 h at 42 °C in fresh hybridization solution (the same as prehybridization solution, except 1×Denhardt's was replaced by 10% dextran sulfate), with the probe at a concentration of approximately 2×10⁶ cpm/ml. Membranes were washed 2×5 min at RT in 2×SSC, 0.1% SDS, 3×20 min at 65 °C in 0.2×SSC, 0.1% SDS, and autoradiography was performed at -70 °C with intensifying screens. Inserts were isolated from seven plaque-purified positive clones, five of which mapped back to PAC 521.12. Three of these five clones contained an identical 1.6-kb partial cDNA sequence.

Subsequent cDNA library screening. We screened 1,350,000 phage from an amplified oligo dT- and random-primed human proximal small intestine cDNA library (Clontech), using the 1.6-kb partial cDNA sequence or its most 5' end as a probe. Nineteen cDNAs were identified and characterized. Six of these clones extended the cDNA sequence 3' (yielding 1.15 kb of new sequence) and two of them 5' (yielding 0.46 kb of new sequence). The additional 3' sequence aligned with another partial cDNA identified in the original PAC screen.

EST database screening. Iterative screening of the EST database identified nine ESTs that contain sequence from the gene. Three of these ESTs proved to be genomic DNA contaminants, while the other six contain sequence from the 3' UTR of the gene. Two of the latter clones were obtained (Research Genetics) and sequenced.

Sequence scanning. Sequence scanning was performed on PAC 631.4 which has an insert of approximately 175 kb, and was shown to contain the more 5' portion of the 1.6 kb-cDNA. A plasmid library with insert sizes from 1.5 to 3 kb was constructed from sheared 631.4 DNA, and clone inserts were sequenced from both ends. 245 clone ends were sequenced and identified as human, yielding 147 kb of raw human sequence which, assembled into contigs, resulted in 92 kb of non-redundant sequence. Sequence analysis was carried out using Sequencher (Gene Codes) and GCG (Wisconsin Package Version 9.1, Genetics Computer Group). The sequence was analysed with BLAST and, in most cases, XGRAIL. Seven 'excellent' candidate exons were identified by GRAIL 2. Two of these exons were present in the sequence obtained by cDNA library screening. One exon was novel and had strong similarity to a region of the bovine ATPase II, 50 amino acids from the 5' end of the coding sequence. The intervening 1 kb of cDNA sequence between this exon and the 5' most sequence obtained by cDNA library screening was obtained by PCR from human liver cDNA. Based on sequence analysis, four 'excellent' candidate exons identified did not appear to be a part of this candidate cDNA and did not demonstrate strong similarity to any known genes. One of these, however, later proved to be the exon containing the translation start site (boundaries predicted incorrectly by GRAIL).

RACE. To obtain the most 5' portion of the coding sequence, RACE was performed using the 5' RACE system (Gibco BRL). Total human liver RNA was reverse-transcribed with a gene-specific primer. Hemi-nested PCR

was then performed using two additional gene-specific primers, yielding 340 bp of additional sequence.

Screening for mutations. Exons of *FIC1* were individually amplified by PCR from genomic DNA using intronic primers. The resulting products were column-purified (columns from Qiagen or Edge Technologies) and sequenced using automated fluorescent sequencing techniques (Applied Biosystems), followed by sequence analysis using Sequencher (Gene Codes), or cycle-sequencing using ³²P-end-labelled primers and the Amplify Cycle Sequencing kit (Perkin-Elmer), followed by independent reading of the sequence by two people.

Mutation screening controls. Exons were sequenced in normal controls using automated fluorescent sequencing techniques (Applied Biosystems), followed by sequence analysis using Sequencher (Gene Codes). Sequence chromatograms of control and affected individuals were scored for the putative mutations independently by two people. Normal controls used to evaluate the possible *FIC1* and *BRIC* mutations were from the following sources: For *FIC1* mutations present in Amish and Polish patients: 29 Amish, 36 Polish, and 42 other European chromosomes; For the G892R *FIC1* mutation: 82 European chromosomes; For the 2097+2 (T→C) *FIC1* mutation: 77 European chromosomes; For the 1661T *BRIC* mutation: 84 European chromosomes; For the 795delG/R, 2286-4 (CT→AA) *BRIC* mutation: 90 European chromosomes.

Characterization of mutations in patient S.K. PFGE analysis of lymphoblastoid cell line DNA from patient S.K., her unaffected mother (also heterozygous for the deletion) and controls was performed with probes from different regions of *FIC1*. Characterization of fragments of altered size and detection of decreased dosage of fragments of normal size in the deletion carriers allowed localization of the deletion breakpoint. The PCR experiment that revealed exon-skipping in patient S.K. used a primer that is situated within the 1.4 kb deletion; this indicated that the skipped exon is on the patient's non-deleted chromosome.

GenBank accession numbers. *FIC1* coding sequence, AF038007; six ESTs that contain sequence from the 3' UTR of the gene (LMAG.E Consortium Clone IDs are indicated in parentheses): N56820/N32400 (258798) (sequenced), AA257072/AA257166 (682176) (sequenced), AA234928/AA234929 (669574), T10596, AA532514 (996303), D79900. Three ESTs that are genomic contaminants: T69680/T69606 (835631), AA283965/AA285159 (700728) and AA054063/AA047876 (380341) [apparently the same clone is also represented by W96264/W96174 (361642) and AA021355/AA021214 (364154)]. Porcine small intestine cDNA, F14675.

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CHAPTER 3

Characterization of mutations in *ATP8B1* associated with hereditary cholestasis.

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SUMMARY

Mutations in *ATP8B1* (previously termed *FIC1*) are found in some patients with benign recurrent intrahepatic cholestasis (BRIC) and progressive familial intrahepatic cholestasis (PFIC), two clinically distinct (though clinicopathologic intergrades exist) hereditary errors of bile acid metabolism. BRIC is characterized by intermittent attacks of cholestasis, which resolve spontaneously, whereas the cholestasis in PFIC1 patients is chronic, and ultimately progresses to liver fibrosis. Here we report the genomic organization of *ATP8B1* and describe novel mutations associated with PFIC1 and BRIC. The gene comprises 28 exons, spanning a minimum of 77 kb of genomic sequence on chromosome 18q21. Comprehensive analysis of the mutations underlying FIC1 disease, using single stranded conformation polymorphism analysis and direct sequencing of PCR-amplified *ATP8B1* genomic fragments, has to date detected 13 missense mutations, 1 nonsense mutation, 3 small deletions, 1 large genomic deletion, and 5 splice site mutations in *ATP8B1*. These mutations are distributed throughout the gene and are not present in at least 40 control individuals. The variety of mutations is consistent with the substantial clinical variability in FIC1 disease. Many patients are compound heterozygous for two mutations; some mutations are detected in BRIC as well as in PFIC1. The most common mutation associated with BRIC, I661T, displays reduced penetrance, suggesting that this mutation has a relatively mild effect on the function of the encoded protein. Finally, we present evidence for substantial locus heterogeneity underlying both BRIC and hereditary cholestasis that clinically resembles PFIC1.

INTRODUCTION

Familial cholestasis syndromes are a heterogeneous group of hereditary liver disorders. Progressive familial intrahepatic cholestasis (PFIC) is a severe chronic liver disorder that starts in early infancy and leads to hepatic fibrosis and end-stage liver disease. No congenital structural abnormalities are present in the hepatobiliary tree (Clayton *et al* 1969, Bourke *et al* 1996). Benign recurrent intrahepatic cholestasis (BRIC; MIM 243300) is characterized clinically by intermittent cholestasis without progression of the disease to liver failure. The initial episode in BRIC can occur at almost any age (usually in the first decade). Cholestatic attacks vary in severity and duration and resolve spontaneously (Summerskill, 1965). Both PFIC and BRIC are distinguished from most other cholestatic disorders by essentially normal serum concentrations of cholesterol and gamma-glutamyl transpeptidase (γ GT) activities ("low- γ GT cholestasis"). These disorders are thought to result from a defect in bile acid secretion and/or re-absorption, leading to accumulation of bile acids within hepatocytes with a concomitant reduction of bile acid availability in the intestine (Knisely, 2000, van Mil *et al* 2001, Oude Elferink and Groen, 2002).

Much work during the past decade has focused on identification and characterization of the gene defects underlying low γ GT cholestasis syndromes. Both BRIC and PFIC are inherited as autosomal recessive diseases (Clayton *et al* 1969, de Koning *et al* 1995) although an autosomal dominant form of BRIC has recently been described (Floreani *et al* 2000). Studies of PFIC revealed that locus heterogeneity exists in this disorder

(Strautnieks *et al* 1996). Therefore, low γ GT PFIC was further divided into at least two subtypes, PFIC1 (MIM 211600) and PFIC2 (MIM 601847), based on genetic criteria (Bull *et al* 1997, Strautnieks *et al* 1997). The loci for PFIC1 and autosomal recessive BRIC were mapped to a small region on chromosome 18q21 by searching for shared segments and shared haplotypes in patients from different populations (Houwen *et al* 1994, Carlton *et al* 1995, Sinke *et al* 1997, Bull *et al* 1997, 1998, 1999, Tygstrup *et al* 1999). Mapping of the PFIC1 locus was facilitated by the identification of a PFIC patient with a large genomic deletion in this region. Consistent with the hypothesis that these disorders are caused by a deficiency of the same protein, this region contained a single gene, *ATP8B1* (formerly called *FIC1* [familial intrahepatic cholestasis 1]). The protein that it encodes, a putative aminophospholipid translocase (Ujhazy *et al* 2000), is called FIC1. This gene was mutated in patients with PFIC1 and BRIC (Bull *et al* 1998). More recently, we established that Greenland familial cholestasis is also caused by a mutation in *ATP8B1* (Klomp *et al* 2000). Therefore, we now refer to these disorders in the collective as FIC1 disease.

The gene affected in PFIC2 patients was identified using a similar strategy. This gene, *ABCB11* (ATP-binding cassette, subfamily *B*), is located on chromosome 2q24 and encodes BSEP (Bile Salt Export Pump), an ATPase important in secretion of bile salts across the canalicular membrane of the hepatocyte (Strautnieks *et al* 1997, 1998, Gerloff *et al* 1998). By analogy with FIC1 disease, disorders caused by deficiency of BSEP can collectively be termed BSEP disease.

Some clinical and histopathological features may facilitate differentiation between FIC1 disease and BSEP disease (Bull *et al* 1997, Knisely 2000, Chen *et al* 2002). Genetic testing is at present required, however, to distinguish FIC1 disease from BSEP disease definitively. Therefore, a collaborative study, described here and in an accompanying paper, was designed to provide a genetic framework for discrimination between FIC1 disease and BSEP disease and to begin dissection of the molecular features underlying the differences in clinical presentation and disease progression between BRIC and PFIC. In this paper, we describe mutations in *ATP8B1* responsible for FIC1 disease. To avoid introducing biases in patient selection based on current hypotheses of how the two known genetically distinct forms of PFIC differ clinically and histologically, we did not attempt to differentiate PFIC1 from PFIC2 using clinical, biochemical, or histological criteria. Rather, genetic criteria were employed to enrich our patient sample for patients likely to carry mutations in *ATP8B1*.

SUBJECTS AND METHODS

Patients and control individuals

These studies were conducted under approved protocols in accordance with the guidelines of the Children's Study Committee of the University Medical Center Utrecht and the Committee on Human Research of the University of California, San Francisco. BRIC patients from 39 families and PFIC patients from 71 families, all of diverse ancestry, had been previously diagnosed on the basis of clinical symptoms in combination with routine laboratory investigations other than histological evaluation.

The clinical diagnosis of BRIC was made in patients with at least two bouts of cholestasis whose symptoms resolved completely between attacks and whose biliary tracts were normal on imaging studies. Growth and development were normal in patients with BRIC. Patients clinically diagnosed with PFIC had nonremitting pruritus first manifest in infancy. Patients failed to thrive, and (in all but rare instances) were icteric, with conjugated hyperbilirubinemia. All patients had normal or disproportionately low serum concentrations of γ GT activity. Primary disorders of bile acid synthesis were excluded by urine screening. None had findings on physical examination consistent with arthrogryposis - renal disease - cholestasis (ARC) syndrome (Eastham *et al* 2001) or clinical courses consistent with acute liver failure (Kajiwara *et al* 1991).

Genomic DNA was extracted from peripheral blood leukocytes by standard procedures. As reference control DNA was obtained from CEPH individuals 1331-1, 1331-2, or 1347-2 (Centre d'Étude du Polymorphisme Humain, Paris, France). DNA samples from 50 healthy Dutch female individuals were also included.

Determination of *ATP8B1* gene structure

Exon-intron boundaries of two exons were identified by sequence analysis of inserts in a previously generated genomic minilibrary made from PAC 63L4 (Bull *et al* 1998). We also constructed additional minilibraries by partial digestion of PAC inserts by *AluI* and *HaeIII*. The obtained fragments were ligated to adaptors containing *BstXI* restriction overhangs and subsequently ligated into *BstXI*-digested pcDNA I/Amp vector (Invitrogen, Carlsbad, CA). The resulting plasmids were electroporated into DH10 β

bacteria and plated at a density of 800 colonies per plate. Replicates were prepared on Hybond N-filters (Amersham Pharmacia Biotech, Little Chalfond, UK) and bacteria were lysed by soaking the filters in 10% SDS. Filters were hybridized with ^{32}P -labeled FIC1 cDNA probes. Plasmids were isolated from positive colonies and sequenced. To identify additional exons, portions of PAC clones 233B12 and 63L4 were sequenced directly using *ATP8B1* cDNA-derived primers and a [^{32}P] dATP-based adaptation of the cycle sequencing protocol (Roche Molecular Systems, Branchburg, NJ) (Vredendaal *et al* 1998).

Determination of intron sizes

Intron-specific primers were designed and used to amplify individual introns. For each intron, two independent primer pairs were used and both CEPH 1331-2 genomic DNA and PAC clones 233B122 and 63L4 were used as templates. PCR was performed using TaKaRa LA long PCR kit (Takara Shuzo, Otsu, Japan) according to the manufacturer's instructions. Intron sizes were estimated after separation of the PCR products on 0.8% agarose gels. Exon-intron boundaries were verified by sequencing.

Genotyping

Chromosome 18q21 markers were genotyped as described (Bull *et al* 1999). D18S41, D18S977, D18S1401, D18S381, and D18S849 (listed in order from centromere to telomere, D18S977 and D18S1401 lie within *ATP8B1*) were typed in all families. Some families were typed for additional markers flanking *ATP8B1*. Marker haplotypes were

constructed for patients. Genotyping of the *ATP8B1* region in some of these families was previously reported (Bull *et al* 1997, 1999).

Polymerase chain reaction, single stranded conformation polymorphism (SSCP) analysis, sequencing, and restriction fragment length polymorphism (RFLP) analysis

PCR: oligonucleotide primers complementary to the *ATP8B1* genomic sequence were designed for the amplification of all exons containing coding sequence, and of the flanking exon-intron boundaries (Table 1). PCR products were generated using AmpliTaq DNA polymerase (Roche Molecular Systems, Branchburg, NJ) for 35 cycles.

SSCP: formamide was added to PCR products at a final concentration of 50%. The samples were denatured at 95 °C for 5 min, and quickly chilled on ice. Aliquots were applied to 12.5% polyacrylamide gels (Excel gel, Amersham Pharmacia Biotech) and electrophoresed using the FBE-3000 apparatus at 600 V and 15°C. Gels were then silver-stained (Amersham Pharmacia Biotech).

Sequencing: PCR products purified on Qiaquick spin columns (Qiagen, Valencia, CA) were analyzed by cycle sequencing using the BigDye Terminator Cycle Sequencing kit. Samples were run on an ABI prism 377 sequencer (Applied Biosystems, Foster City, CA). For most exons, both strands of the PCR product were sequenced.

RFLP: genomic DNA underwent PCR amplification with forward primer 5'-CTATTCTTTGCATTGGTGGATTT and reverse primer 5'-TTCTGTAAATTCTTTTTCTGCA to yield a 112-bp product. The mismatch in the reverse primer (underlined) introduces a *Pst* I restriction site in combination with a cytosine but not with the thymidine at position 1982. Following amplification, the PCR products were incubated with *Pst* I (Roche Molecular Systems) for 4 hours at 37°C and separated by electrophoresis on a 6% polyacrylamide gel. Bands were visualized by UV light transillumination after staining with ethidium bromide.

RESULTS

Organization of *ATP8B1*

To determine the exon-intron junctions in *ATP8B1* we used the overlapping PAC clones 233B12 and 63L4 that together contain *ATP8B1* (Bull *et al* 1998). Fragments of these PAC clones were subcloned and identified by library screening as described above. In a complementary approach, PAC DNA was sequenced directly using *ATP8B1* cDNA-derived primers. We thus initially identified 27 exons ranging in size from 43 bp to 2.3 kb. These exons comprised the complete coding sequence of *ATP8B1*, the complete 3' UTR and 22 bp of 5' UTR. To investigate the possibility that additional upstream exons existed, we used the 5' UTR sequence to search the expressed sequence tag (EST) database with the BLAST algorithm and identified a corresponding 538-bp clone (accession number AW997038). RT-PCR experiments on human intestinal RNA using primers derived from this sequence and from previously known *ATP8B1* cDNA sequence

revealed that the EST contained 472 bp of novel sequence information contiguous with the 5' end of *ATP8B1* cDNA. This EST encompassed two exons (exons 1 and 2) and was located on PAC 63L4. The genomic sequence directly upstream of the novel *ATP8B1* cDNA sequence was determined and novel PCR primers were designed based on this sequence. Subsequent RT-PCR experiments revealed that exon 1 contains 437 additional bp at the 5'-end of the sequence contained within EST AW997038. The exact transcription start site has not been experimentally determined and this first exon may be even larger. We cannot exclude the unlikely possibility that more exons lie upstream of this first exon.

These data reveal that *ATP8B1* comprises 28 exons (Figure 1). The translation initiation codon is located in exon 2 and the stop codon in exon 28. The complete *ATP8B1* cDNA is at least 6805 bp long, in good agreement with size of the *ATP8B1* transcript as estimated by northern blot analysis (7 kb; Bull *et al* 1998). All splice junctions adhere to the ag-gt rule. The complete sequence of some small introns (introns 5, 7, 9, 25) was determined experimentally. The length of all other introns was estimated by long PCR (Table 2). The largest intron (intron 15) is almost 10 kb in size. *ATP8B1* spans at least 77 kb (Figure 1).

Strategy for the detection of disease-associated mutations

Elucidation of the genomic structure of *ATP8B1* allowed development of primer sets for individual PCR amplification of the exons, including intron-exon boundaries (Table 1). In this study we analyzed all exons containing protein-encoding sequence; only the

protein-encoding region of exon 28 was analyzed. PCR products were examined for nucleotide aberrations from the reference sequence by two methods. SSCP analyses were developed for most exons (Table 1). Eleven exons, including those that did not allow sufficiently specific and reproducible SSCP analysis, were sequenced directly (Table 1). In order to discriminate disease-associated mutations from common polymorphisms, we tested 50 healthy individuals for sequence variants detected in patients. When genomic DNA from family members was available, the segregation of disease-associated mutations in the affected families was investigated. None of the mutations described below was detected in our control samples. When segregation analysis was performed, the data were always consistent with autosomal recessive inheritance.

Mutation analysis in PFIC patients

Given known locus heterogeneity for PFIC, genetic criteria were used to enrich our sample set for patients likely to carry mutations in *ATP8B1*. Haplotype data generated using microsatellite markers from the *ATP8B1* region were evaluated in 71 families in which no disease mutation had yet been identified. We specifically excluded Amish families known to harbor the G308V mutation in *ATP8B1* associated with a conserved haplotype (Bull *et al* 1998, 1999). Four Amish families not carrying this mutation were included. Recently obtained data indicate that the patients in three of these Amish families have a distinct form of liver disease, familial hypercholanemia (Morton *et al* 2000; Carlton VE *et al*, manuscript in preparation). The remaining 67 families are of diverse ethnic backgrounds. Some of these families were also genotyped for markers in

the region containing *ABCB11*, the gene mutated in PFIC2; a subset of the DNA samples was screened for mutations in *ABCB11* (see accompanying paper).

Fifteen of these 71 PFIC patients were chosen for *ATP8B1* mutation screening using the following criteria, and assuming complete penetrance of the disease; 12 families in which the data were inconsistent with linkage to *ATP8B1* were excluded from screening.

Results of genetic studies of four of these families were presented previously (Bull *et al* 1997, 1999). At the time this screening was initiated, 3 of these families were known to harbor mutations in *ABCB11* (Strautnieks *et al* 1998, accompanying paper). Five additional families in which *ABCB11* mutations had been identified at the time this screen was initiated were also excluded (Strautnieks *et al* 1998). Among the remaining 54 families, we prioritized for *ATP8B1* mutation screening those families in which patients were homozygous for 4 or more consecutive markers, including D18S1401, D18S977 and D18S381. Patients from 6 families fulfilled this criterion. In one of these families, patient DNA was unavailable, but the parents were heterozygous for a shared haplotype; screening of DNA from one of the parents did not identify any mutations. Homozygous mutations were identified in patients from the remaining 5 families (families 43, 45, 50, 78, 81) (Table 3).

We chose patients from 9 other families for mutation screening, using less stringent genetic criteria, including homozygosity for fewer consecutive markers, or evidence suggestive of haplotype sharing between patients in different families. We also included families with 2 or more affected children, in which the genetic data were consistent with

linkage to *ATP8B1*. Of these 9 additional families, mutations in *ATP8B1* were identified in only one. The patient in that family was a compound heterozygote for 2 distinct mutations, despite having been chosen based on evidence suggestive of homozygosity in the region (family 60; Table 3).

Many of the 71 families included in this study have also been screened for *ABCB11* mutations. Such mutations have been found in 24 of them (Strautnieks *et al* 1998, accompanying paper). Of the 15 families screened for *ATP8B1* mutations, 6 indeed have defects in *ATP8B1*, 4 have defects in *ABCB11* (families 69, 85, 87, and 88), and one (family 39) has a distinct disease, familial hypercholanemia (Morton *et al* 2000, VE Carlton *et al*, manuscript in preparation). In 5 of the families screened for *ATP8B1* mutations, the genetic etiology of disease remains undetermined.

Analysis of the 6 families with mutations in *ATP8B1* revealed the previously published G892R mutation (Bull *et al* 1998) and 6 novel mutations: 2 splice site mutations, one 7-bp deletion leading to a frameshift, and 3 novel missense mutations (Table 3; Figures 1 and 2). The 279 G>A mutation in family 81 does not change the alanine amino acid residue encoded by codon 93; the affected guanine is the last base of exon 3, resulting in a predicted change in mRNA splicing. The other putative splice-site mutation (IVS3+1 *del gtg* in family 50) also affects the splice donor site of exon 3. RNA from these patients was not available and the effects of these putative mutations on the structure of the *ATP8B1* transcript could not be determined.

Detection of the common BRIC mutation

Thirty-nine families, each with at least one classical BRIC patient, were included in this study. These families were recruited from diverse ethnic backgrounds, but most families were of Western European descent. We had shown that the I661T mutation is common in Western European BRIC patients and in patients from the Faroe Islands (Bull *et al* 1998, Tygstrup *et al* 1999). Therefore, all probands in the 39 families were initially screened for this mutation by restriction enzyme analysis using a mutation-specific oligonucleotide. In 8 families, patients were heterozygous for I661T, suggesting possible compound heterozygosity. Affected individuals in 4 families were homozygous for this mutation. We have now detected clinically affected patients with the I661T mutation on both alleles from 14 families in all (Bull *et al* 1998, Tygstrup *et al* 1999). To confirm the recessive inheritance of this mutation, we analyzed its segregation in the affected families. In a large Italian pedigree, we detected 4 homozygous I661T individuals (one male and three females), who had remained symptom-free lifelong (Figure 3). One of these individuals was the father of three affected children. This observation suggested that I661T homozygosity could be associated with reduced penetrance of FIC1 disease. Data to support the hypothesis of reduced penetrance were provided by detection of another unaffected individual (from a Belgian family) who was homozygous for the T allele at position 661. This I661T homozygote, clinically unaffected to date, is an older brother of the proband. These results suggest gender-independent reduced penetrance of FIC1 disease caused by I661T homozygosity.

Mutation analysis in BRIC patients

DNA samples from the 8 patients heterozygous for the I661T mutation were screened for novel sequence variations in *ATP8B1*. A second heterozygous mutation was detected in 6 cases (families 2, 6, B3, B7, B20, B54; Table 4). No additional mutation could be found in 2 patients (families 13 and B18; Table 4).

In most of the remaining 27 families, genotyping experiments were not informative because DNA from the parents was not available, or the proband was an only child. We detected only two affected patients (families 1 and US1) who were homozygous for 4 or more consecutive markers, including D18S1401, D18S977 and D18S381. Evidence of locus heterogeneity in BRIC (Sinke *et al* 1997, Floreani *et al* 2000) has grown since this study began. When we screened DNA for *ATP8B1* mutations in probands from all 27 remaining families affected with BRIC, we were surprised not to detect any sequence variations that were not also detected in control individuals in 21 families. Of the 6 probands in whom *ATP8B1* mutations were detected, 2 were homozygous (families 1 and US1; Table 4) and 3 were compound heterozygous (families B6, B19, US4; Table 4). One additional patient was probably also compound heterozygous, but we identified a mutation at only one allele (family 21; Table 4).

Thirteen novel mutations associated with BRIC were detected overall (Table 4, Figure 1 and Figure 2). These mutations included 2 putative splice site mutations, 2 nonsense mutations, 1 single-bp deletion and 8 missense mutations. Some of these mutations were also detected in PFIC patients. The 279 G>A mutation (family B54) was found in PFIC

family 81. Both these families were from Belgium. The BRIC patient was compound heterozygous for this mutation, whereas 279 G>A was detected in homozygous form in the PFIC patient. The BRIC patient from family B3 was heterozygous for the G892R mutation. This was also detected previously in PFIC1 patients, either in homozygous form (Bull *et al* 1998) or as a compound heterozygous mutation (this study; family 60).

The IVS23-3 c>a mutation was detected in homozygous form in all 4 affected individuals of family 1; in this family from a small Dutch village the BRIC locus was initially mapped to chromosome 18q21 (Houwen *et al* 1994). During this work, we identified two more affected family members, a newborn and a young adult. These two patients were also homozygous for the IVS23-3 c>a mutation. Analysis of fibroblast RNA isolated from one revealed that the mutation leads to complete skipping of exon 24 (results not shown). The transcript containing this in-frame deletion is predicted to encode a protein lacking the complete sixth transmembrane domain.

The G308D mutation found in heterozygous form in family B20 alters the same amino acid residue mutated in PFIC1 patients of Amish descent. These PFIC1 patients are homozygous for the G308V mutation (Bull *et al* 1998). The proband from family B6 has an older, clinically unaffected brother, who carries the same mutations as the proband. This confirms our conclusion that BRIC may be characterized by reduced penetrance. Our data show that some mutations are shared between BRIC and PFIC patients and suggest that a considerable number of patients presenting with BRIC may not carry mutations in *ATP8B1*.

Single nucleotide polymorphisms

Several sequence variations in *ATP8B1* that did not seem associated with FIC1 disease were encountered. Some of these variations are located in the coding region of *ATP8B1* and are predicted to lead to changes in FIC1 amino acid sequence. Most notable was the non-conservative substitution of R952Q (G2855A) in heterozygous form in BRIC patients 13, B18 and B20. The same mutation was detected in our control group with an allele frequency of 11 %. It is thus a common polymorphism. This excludes the possibility that R952Q by itself can cause FIC1 disease. We cannot rule out the possibility that this polymorphism, in combination with another mutation, leads to clinical symptoms. Such a possibility would explain the detection of only one mutation in patients 13 and B18. Alternatively, this polymorphism may represent a genetic modifier of FIC1 disease or of other liver disorders.

DISCUSSION

Our description of the structure of *ATP8B1* and comprehensive analysis of gene mutations associated with BRIC and PFIC provide strong support for our previous conclusion that mutations in *ATP8B1* cause these disorders (Bull *et al* 1998, Klomp *et al* 2000). The unexpectedly high number of PFIC and BRIC patients screened who apparently have no *ATP8B1* mutations also provides strong indirect evidence for locus heterogeneity in BRIC and PFIC.

Genes encoding P-type ATPases often spread over a large genomic region and are divided into many exons (Petrukhin *et al* 1994, Thomas *et al* 1995, Dierick *et al* 1995). *ATP8B1* is now characterized as containing 28 exons and spanning at least 77 kb. No evidence was found for the existence of additional exons or alternatively spliced transcripts. Two previously characterized 98-bp and 74-bp deletions in *ATP8B1* mRNA, which are associated with PFIC (Chen *et al* 2002) can now be attributed to deletions of exons 3 and 7 respectively and the putative intronic mutations leading to these splicing artifacts can now be determined. The transcription start-site of *ATP8B1* must be established in experiments to define the upstream elements that govern regulation of *ATP8B1* gene expression. The results of these experiments will direct research aimed at pharmacologically increasing *ATP8B1* transcript abundance to ameliorate disease symptoms, especially in BRIC patients.

Preselection of patients based on marker haplotypes has assisted mutation screening in hereditary disorders such as Wilson disease (Shah *et al* 1997). PFIC patients were also preselected for mutation analysis on the basis of genetic data to increase the likelihood that they would carry mutations in *ATP8B1*. Since locus heterogeneity in BRIC was well-established only after this study began (Floreani *et al* 2000), we included BRIC patients without genetic selection. Strong evidence of marker homozygosity in the region containing *ATP8B1* was in retrospect the only effective genetic criterion for selection of patients with *ATP8B1* mutations. Of 8 families that fulfilled this criterion (6 PFIC; 2 BRIC), we detected the mutations in 7 (87.5%). In contrast, in only 5 (1 PFIC; 4 BRIC) of the 34 patients (9 PFIC; 25 BRIC) who were included based on less stringent genetic

criteria or despite uninformative haplotype analysis, could mutations be detected (14.7%). Some mutations may have remained undetected due to limitations of the detection methods used or because they reside within regions not analyzed (e.g. untranslated regions, upstream regulatory sequences and introns). It is unlikely that these technical limitations fully explain the low rate of mutation detection in patients who were not homozygous in the *ATP8B1* region, given the high frequency of mutation detection in the group of patients who were homozygous in the *ATP8B1* region. We therefore conclude that many patients with low γ GT cholestasis are not affected by mutations in *ATP8B1*. This is an unexpected finding, especially in BRIC, and it raises the interesting possibility that yet undiscovered loci associate with the disorder in some of these patients. Consistent with this possibility, the existence of a third locus for low γ GT PFIC was previously suggested (Strautnieks *et al* 2001).

With *ATP8B1* gene mutations published earlier (Bull *et al* 1998, Klomp *et al* 2000, Chen *et al* 2002), the current work provides a spectrum of mutations leading to FIC1 disease. In total, 18 different missense mutations, 3 nonsense mutations, 7 mutations associated with, or predicted to lead to aberrant splicing, 3 small deletions, and 1 large genomic deletion have been identified. These data indicate that disease-associated mutations in *ATP8B1* are diverse in nature and are not clustered in a specific region. Three mutations, each associated with a distinct haplotype, are encountered relatively frequently, at least within specific populations (G308V, I661T, D554N). Most mutations were detected in only one, or at most, a few. The variety of mutations and the large proportion of

compound heterozygotes hamper the identification of clear genotype-phenotype correlations in FIC1 disease.

This work also shows that several mutations can be associated with BRIC as well as PFIC. Mutations homozygous in PFIC1 can be seen in heterozygous form in BRIC, in association with a second mutation that has presumably milder effect. The diversity in mutations responsible for PFIC and BRIC is consistent with the marked clinical heterogeneity observed in these disorders. As an example, BRIC varies considerably in age of onset, frequency and duration of cholestatic episodes, and response to pharmaceutical intervention. Several individuals with homozygous mutations in *ATP8B1* identified in this study are indeed to date free of symptoms of FIC1 disease.

Representing an intermediate phenotype between BRIC and PFIC, other patients have been described who present early in life with recurrent episodes of cholestasis, which progress to permanent cholestasis later in life (van Ooteghem *et al* 2002). This pattern was seen in this study in family 1. The most severe form of FIC1 disease is found in the many PFIC patients with rapidly progressive, potentially fatal liver dysfunction that in the presence of documented *ATP8B1* mutations is defined as PFIC1. Severity of FIC1 disease thus ranges along a continuum, rather than being easily divided into mild (BRIC) and severe (PFIC1).

Distinct mutations may have variable effects on the expression and function of FIC1.

The S453Y, D454Y, and T456M missense mutations affect a region of FIC1 that is highly conserved in all P-type ATPases. This region encompasses the phosphorylation

domain and contains an aspartate residue (D454) that is transiently phosphorylated as a prerequisite for catalytic activity (Lutsenko and Kaplan 1995). The G892R mutation lies in a functionally conserved hinge region. D554N is located in a sequence motif that is not present in all P-type ATPases, but is highly conserved in the P4 subfamily (Palmgren and Axelsen 1998, Klomp *et al* 2000). These mutations are therefore predicted to abrogate catalytic activity of FIC1 entirely. In contrast, one nonsense mutation and two small deletions were observed in the last two exons, which encode a part of FIC1 that does not contain consensus domains of known function. Both small deletions lead to a frameshift. The resultant transcript encodes an elongated protein due to an out-of-frame stop codon. In PFIC patients with the 7-bp deletion (family 43 [Bourke *et al* 1996]), this probably leads to rapid degradation of the encoded protein. This was illustrated by the undetectable FIC1 immunoreactivity in liver samples of this patient using specific antisera raised against the large cytoplasmic loop of the FIC1 protein (Eppens *et al* 2001).

The mechanisms whereby mutations in *ATP8B1* lead to recurrent cholestasis are unknown. FIC1 is a 1251-amino acid protein with ten potential membrane-spanning domains that belongs to the P4 subfamily of P-type ATPases (Bull *et al* 1998, Palmgren and Axelsen 1998). FIC1 is located at the brush border of enterocytes, at the canalicular membranes of hepatocytes, and in cholangiocytes, where it may function as an aminophospholipid translocase (Ujhazy *et al* 2001, Eppens *et al* 2001). These data are consistent with an indirect role of FIC1 in bile salt secretion, as well as in enterohepatic and cholehepatic recirculation of bile salts. Mice homozygous for an engineered FIC1 G308V mutation displayed no decrease in biliary bile salt secretion, but had dramatically

increased serum bile salt concentrations when fed cholate. These findings suggested that dysfunction of FIC1 resulted in an inability to downregulate intestinal resorption of bile acids (Pawlikowska *et al* 2001, Chapter 5).

These data lay the foundation for analysis of the pathogenesis of FIC1 disease. Studies of expression and function will address the effects of the mutations described here on the localization and function of FIC1. Our work has direct implications for carrier detection, genetic counseling and prenatal diagnosis in affected families.

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Electronic-database information

The accession number and URLs for data in this article are:

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.gov/OMIM/> (for PFIC1 [MIM 211600], for PFIC2 [MIM601847] and for BRIC [243300]).

Basic Local Alignment Search Tool (BLAST), <http://www.ncbi.nlm.nih.gov/BLAST/> (accession number AW997038).

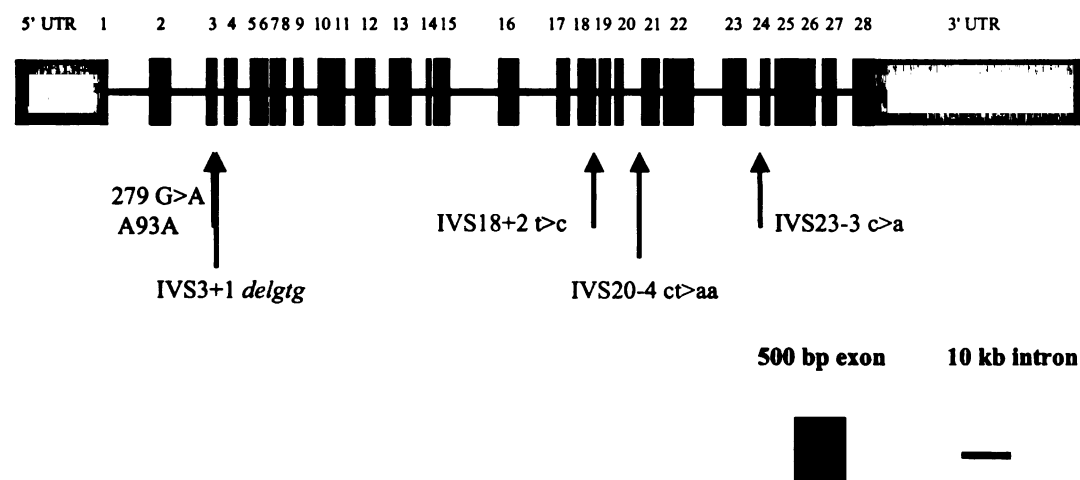


Figure 1

Structural organization of *ATP8B1* and location of splice site mutations.

A schematic representation of the exon/intron structure of *ATP8B1*; exons are numbered 1-28. Exons and introns are depicted at different scales as indicated. Arrows indicate splice site mutations.

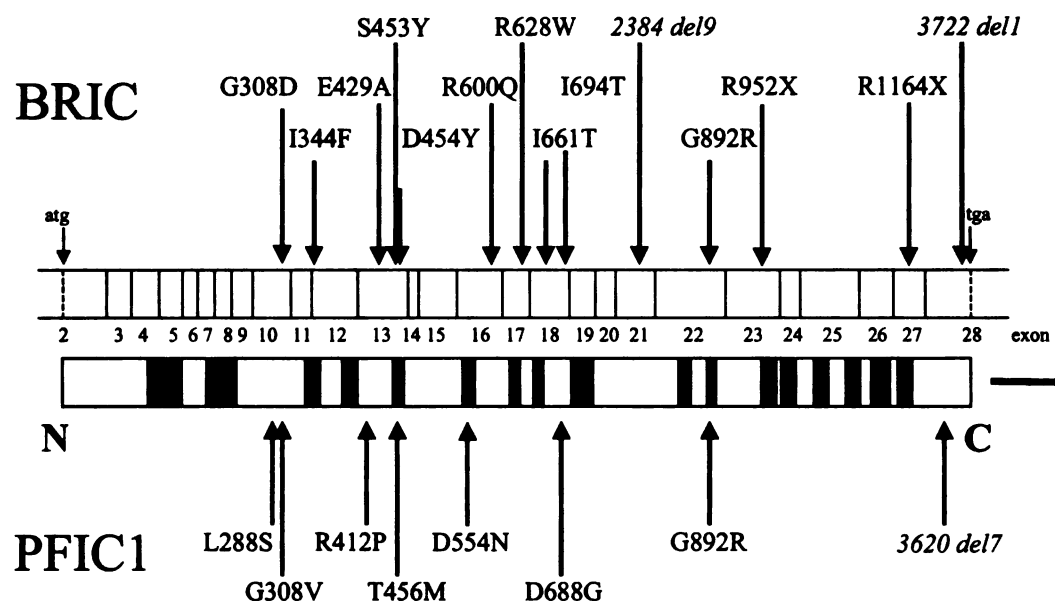


Figure 2

Location of mutations in the coding region of *ATP8B1*.

All mutations detected in this and our previous work are shown (Bull *et al* 1998, Klomp *et al* 2000). Exons, drawn to scale, are shown as numbered boxes; the dashed lines indicate the start (atg) codon and stop (tga) codon. Below the *ATP8B1* mRNA is a schematic representation of the FIC1 protein; N and C indicate amino- and carboxy-termini, respectively. Consensus sequences are depicted as: Grey: transmembrane regions; hatched, functional P-type ATPase consensus domains; black, P4 P-type ATPase specific consensus domains. Arrows indicate mutations detected in BRIC patients (top) and PFIC1 patients (bottom). Missense and nonsense mutations are represented as the predicted amino acid substitutions. The scale bar represents 100 codons or amino acid residues.

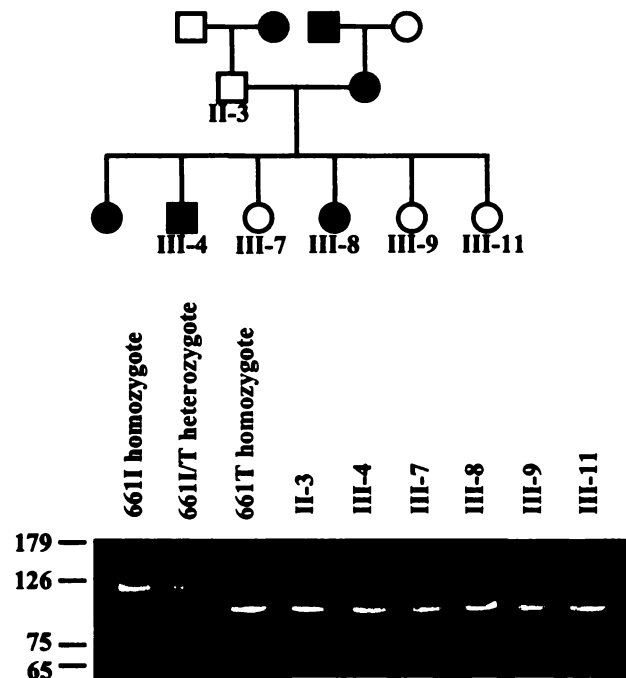


Figure 3

Segregation analysis of the I661T substitution in an Italian pedigree.

Exon 18-specific *ATP8B1* fragments were amplified by semi-nested PCR using a mismatch primer from genomic DNA of the individuals indicated in the pedigree and from indicated control individuals (C). After incubation with *Pst*I, samples were analyzed by polyacrylamide gel electrophoresis and stained with ethidium bromide. In the absence of the mutation, the *Pst*I restriction site introduced by the mismatch primer allows cleavage of the PCR-product to a large and a smaller fragment; the latter is not resolved on the gel. Molecular size markers are indicated in bp. Control samples are as follows: I/I: I661 homozygous; I/T: I661, T661 heterozygous, +/+ : T661 homozygous. Note that all unaffected individuals are homozygous for the T allele.

Table 1**Sequence of PCR oligonucleotides.**

Primers used to amplify *ATP8B1* genomic fragments are listed relative to the exons. Exons 5+6 and exons 7+8 were amplified in a single PCR reaction. Large exons were split in two overlapping fragments (a and b); ^aannealing temperature (°C); ^bmethod of analysis is indicated. SSCP: single-strand conformation polymorphism; seq: direct automated sequencing.

exon	forward primer 5'-3'	reverse primer 5'-3'	temp ^a	method ^b
2a	ccatgcaggcagttattcaac	ctgcttccctgttgactcgg	55	sscp
2b	tcagcctaattgacgaagtgg	atcatctttggcacgtgtgg	55	sscp
3	gggatggaggttttaggactt	aggcaggtgtagcatgaagg	55	seq
4	ttgtcactgtaagctgtggg	ctacagcttaaatgttatcgag	58	seq
5+6	tggacacacacataagccct	gaatgtgtttctaggcagag	55	seq
7+8	gcattttctctgtgacttgc	agccgtctggtccacaatgc	55	seq
9	gcttcatgtccaggatggc	caataggaattgaaccaagc	55	seq
10	gttgcttggttcaattccta	aggacagaaaagcaatcccc	55	seq
11	tggtgagctagtgaattgat	atgagagatctactgagatg	52	sscp
12a	ggttggaatccatcttcag	tgtatcgtcttctccatca	55	sscp
12b	ttattgggaagcacagggtgg	atggtgggagaagggtacaac	55	sscp
13	tatcgagctctctacggaaa	aggagacaggctatagtcca	55	seq
14	tgctcaggccgtatatggtc	ggcaacgaaaagagtcttccc	55	sscp
15a	accttgcccttgaagaaaagt	tgctgacttcttggtctt	55	sscp
15b	gctgatgggaagcttgcat	aacctggtctctaatagcctg	55	sscp
16a	agcagaggcctcaacaatga	gcaagaacattgttaagtcc	52	sscp
16b	gaccagaaacaccatcac	gggcacaagcaacatctaaa	55	sscp
17	tgagccgatactgaactgctgc	cagacagcatcagagagtaag	55	sscp
18a	ctattctttgcattggtggatt	tttatccagagcttcgtccc	50	sscp
18b	ccctatgcctttgctacaag	taaccttcttccattgtgcc	55	sscp
19	acatggcagccttccctggt	gcaaaggaaacggcagctat	55	seq
20	cagcaggaatgacatttgac	cccaagtgcacatcgtaac	55	sscp
21	tctcagagtcaagggcctat	ggcagttaacatgtgaaagc	55	sscp
22a	atcttggaatggtactcctg	gatgactgcgctgcactc	55	sscp
22b	agcagcggcagaaaaactttg	agcacgttgggcctcactg	55	sscp
23a	gttttgccctcaacaatcggg	tatcgtaggaacttgcacatc	55	sscp
23b	agaggctactgctggtgcat	gtaaggagacacagcccaaa	55	sscp
24	ctttgttggtgacatggagc	acgtttgcttgggacttctc	58	sscp
25	tatctctgactgctttgacc	gacactgaatacggccaatg	55	seq
25b	gagacttactattcaactataaga	gacactgaatacggccaatg	55	sscp
26	ctccccaaaatgagtgtctc	cactgtgcccagccattcca	52	sscp
27	ccacacctggcgaaatatta	acgctttggtttctgtgagg	55	sscp
28a	atcctccctggtgtggatct	cgatggcatcaagcggcgag	55	sscp
28b	ggctacgcggacctcatctc	cacacaaagtcctgagagtc	55	sscp

Table 2**Genomic organization of *ATP8B1*.**

^aExon sequences correspond to the published cDNA sequence (Bull et al 1998) and are depicted in uppercase, whereas intron sequences are depicted in lowercase.

N/A not applicable.

Exon	Exon length (bp)	Splice acceptor ^a	Splice donor ^a	Intron length(bp)
1	> 909	N/A	ACGAAAAGgtggg	8600
2	203	agcagGTAGTTCC	CAGAAAAGgtaac	7300
3	98	taaagAATGTACA	AATATGCGgtgag	1700
4	114	aacagAATAATGC	TCTTACAGgtaat	2850
5	99	tttagGCAGTTCC	ACGATGTGgtgag	113
6	62	tcaagGCTCGCCA	GATGGCAGgtact	500
7	73	cttagGTTCAAAG	TTGTTCCAgtaag	110
8	71	gacagGCTGACAT	CTGGACGGgtaag	2000
9	83	tttagAGAAACCA	ATTTGATGgtttg	120
10	159	aaaagGTTTTATT	TTTTGCAGgtacg	275
11	89	ttcagGTGCTGAC	TTTACACGgtatt	2200
12	191	tctagATCTTTGT	TATGTCAGgtaag	3000
13	209	cctagCGTGGAAG	GATATATGgtaag	3000
14	44	ctcagGGGACCAT	AAATAGAGgtaag	800
15	157	ttcagCAAGTTGA	GACTGATGgtgag	9750
16	189	gttagGTCAGCTC	TATCATTGgtaag	7800
17	113	accagTAAGAACC	CCCTGGATgtaag	1900
18	165	aacagATCTTTGC	ACTTAATTgtgag	810
19	112	tctagCTCCTGGG	CAAAAAGGgtaat	1000
20	76	tgtagAAACTGCT	GATATTAAgtgag	3800
21	133	cctagTTCTCTTC	CTTGGTTGgtacg	1000
22	289	cacagAATGAAAT	GATCAAAAgtgag	5800
23	224	catagCTGCCCAC	CTGCGCAGgttaa	1100
24	84	cacagACTGCATA	TCGACCAGgtagg	1150
25	246	ttaagGATGTGAG	ATTTCCAGgtacg	230
26	139	aacagATTGGCTT	ATTTACAGgttgg	1500
27	151	tacagGCACAGCT	GTGATAAGgtata	1500
28	2323	tgcagATCCAGAA	N/A	

Table 3**Mutations in PFIC1 patients.**

^aPatient is compound heterozygous for the described mutation; all other patients are homozygous. ^bMutation detected previously in another patient (Bull *et al* 1998).

Patient	ethnicity	mutations	exon/intron	(predicted) effect
43	Irish	del 3620-3627	exon 27	frameshift
45	Japanese	1235 G>A	exon 13	R412P
50	Bolivian	IVS3+1 <i>del gtg</i>	intron 3	disrupts canonical splice site
60^a	unknown	2063 A>G	exon 18	D688G
		2674 G>A	exon 22	G892R ^b
78	Italian	1367 C>T	exon 13	T456M
81	Belgian	279 G>A	exon 3	A93A; disrupts splice site

Table 4**Mutations in BRIC patients.**

^aDetermined experimentally. ^bMutation detected previously in other patients (Bull *et al* 1998). ^cPatients are homozygous for the described mutation; all other patients are compound heterozygotes. ^dMutation on second allele not detected.

Family	ethnicity	mutations	exon/intron	(predicted) effect
1^c	Dutch	IVS23-3 c>a	intron 23	skipping exon 24 ^a
2	Dutch	1982 T>C	exon 18	I661T ^b
		1799 G>A	exon 16	R600Q
6	Dutch	1982 T>C	exon 18	I661T ^b
		1799 G>A	exon 16	R600Q
13^d	Dutch	1982 T>C	exon 18	I661T ^b
20^c	Dutch	IVS23-3 c>a	intron 23	skipping exon 24
21	Dutch	1286 A>C	exon 13	E429A
B3	Italian	1982 T>C	exon 18	I661T ^b
		2674 G>A	exon 22	G892R
B6	French	1361 A>G	exon 13	D454Y
		2081 C>T	exon 18	I694T
B7	Italian	1982 T>C	exon 18	I661T ^b
		3490 C>T	exon 27	R1164X
B18^d	German	1982 T>C	exon 18	I661T ^b
B19	German	1802 C>T	exon 17	R628W
		2854 C>T	exon 23	R952X
B20	German	923 G>A	exon 10	G308D
		1982 T>C	exon 18	I661T ^b
B54	Belgian	279 G>A	exon 3	A93A; disrupts splice site
		1982 T>C	exon 18	I661T ^b
US1^c	Pakistani	3722 del G	exon 28	frameshift; elongated protein
US4	unknown	1030 A>T	exon 12	I344F
		1358 C>A	exon 13	S453Y

CHAPTER 4

**Identification of the murine orthologue of *FIC1* and design of a targeting strategy
for creation of a mouse model of PFIC1 disease.**

INTRODUCTION

Generation of a mutant mouse with a targeted mutation in the gene implicated in the BRIC and PFIC1 diseases had been formulated as a specific research objective long before *FIC1* was identified and characterized, and also before the distinction between PFIC1 and PFIC2 was made. Our rationale for pursuing a mouse model was multifold.

First, the identification of mutations in patients with a disease suggests that these mutations represent a genetic defect responsible for the disease, but does not prove it. In order to formally demonstrate disease causation, it is necessary to retrace the reasoning path backwards, insert the candidate mutation into the orthologous gene in a model system, and demonstrate that the mutation has functional or physiological consequences. If the molecular function of the protein in question is already characterized, this may be done in an *in vitro* system or in a cell-based assay. If the molecular function is unclear, phenotypic observations in a model organism can provide evidence of pathology. In the case of PFIC1, the candidate gene was novel, and its function could only be surmised (Bull *et al* 1998). Furthermore, the genetic defect was predicted to lead to a complex, systemic phenotype resulting from malfunction of a multi-organ system, the enterohepatic circulation of bile acids. This kind of higher physiological function can only be recapitulated in a mammalian model system, the simplest and most genetically tractable of which is the mouse.

The goals and overall scope of the project had always encompassed more than just the identification and verification of the genetic defect in PFIC1 and BRIC. We hoped to gain insight into both the normal function of the enterohepatic circulation of bile salts, as well as into the etiology of pathology that arises from a genetic defect. Experimental investigation of cholestasis had been hampered for several reasons. First, the bile canaliculus, a small space between two hepatocytes, is relatively inaccessible to experimental manipulation and cannot be modeled very well in cell culture. Second, bile secretion operates in the context of the enterohepatic circulation, and the intact mammalian organism including the liver, the circulation and the digestive tract is required to recapitulate it. Third, bile salt synthesis, secretion and re-absorption in the ileum are linked by many subtle regulatory mechanisms, including feedback loops, and affected by many environmental influences. Thus, although we hoped that *in vitro* and cell culture experiments conducted by our collaborators would provide insight into the molecular functions of FIC1, particularly the identity of its substrates, if it was indeed a transporter or translocase, we undertook the generation of FIC1 mutant mice in order to investigate the physiological functions of FIC1 in health and in disease in the only system that could provide physiologically accurate observations and still be amenable to experimental manipulation.

A mouse model that can be manipulated to recapitulate salient features of a human disease is an invaluable tool for the design and testing of therapeutic interventions. Few therapies other than liver transplantation produce significant improvement of primary disease manifestations in BRIC and PFIC1 patients, and the difficulty, trauma, risks

(several patients have died of surgical complications) and complications of transplantation as well as the dearth of suitable donors make it a measure of last resort. Furthermore, extrahepatic manifestations of disease, such as intractable diarrhea, often persist in transplanted PFIC1 patients, indicating transplantation is not fully curative (Chen *et al* 2001, Bhagat *et al* 1991, Knisely 2000 and personal communications). We therefore hoped that new treatment ideas could eventually be developed and tested with a good mouse model. Also, at the time *FIC1* was identified, all available rodent models of cholestasis relied on external pharmacological, chemical or surgical insult to produce cholestasis (Accatino *et al* 1993, 1995, Bossard *et al* 1993, Bosely *et al* 1996, Moseley *et al* 1996). The resulting phenotype did not recapitulate the molecular etiology of the inherited cholestasis diseases, and was complicated by nonspecific secondary manifestations. We hoped that a model of cholestasis due to a molecular defect would illuminate early stages of disease progression, and allow investigation of environmental influences that may trigger BRIC episodes or modulate severity of PFIC disease progression.

The identification of *FIC1* as the gene defective in BRIC and PFIC1 (Bull *et al* 1998) posed many unanticipated questions that could be addressed by analysis of a *Fic1* mutant mouse. The function of the novel gene was unknown; the homology *FIC1* exhibited to genes predicted to translocate aminophospholipids in the plasma membrane (Tang *et al* 1996) raised the question of how such a molecular function could be reconciled with development of cholestasis similar to that seen in PFIC2, which is associated with mutations in the bile salt export pump (BSEP) (Strautnieks *et al* 1998), and can be

explained with a clear (and long-predicted) etiological mechanism. Furthermore, the wide tissue distribution of FIC1, also a surprising discovery, shed new light on the extrahepatic manifestations of PFIC1 that had been underappreciated and thus not studied in any depth, and raised the hypothesis that intrahepatic cholestasis could be a consequence of defective bile salt absorption in the ileum (Bull *et al* 1998). Generation of mutant mice thus held great promise for the examination of the extrahepatic functions of FIC1, and of the impact of *FIC1* mutation in the many different tissues in which it was expressed, particularly the small intestine.

The generation and analysis of the FIC1 mutant mice forms the core of this dissertation, and is described in three chapters. This chapter describes isolation of the mouse orthologue of *FIC1* and the design and execution of two parallel mouse targeting strategies. Chapter 5 reports key findings in the *Fic1*^{G308V/G308V} mutant mice in the form of a manuscript soon to be submitted for publication. Chapter 6 describes additional experimental findings in the *Fic1* mutant mice, most of which are still preliminary, with particular emphasis on the phenotypic variation found among different mouse strains in which the *Fic1*^{G308V/G308V} mutation was studied.

RESULTS AND DISCUSSION

This phase of the project began in earnest as soon as mutations in *FIC1* were identified and the full length cDNA sequence obtained, with a search for the unknown mouse orthologue of *FIC1*.

Mouse *Fic1* shares very high sequence homology with human *FIC1*.

BLAST searches of the EST database performed with the complete coding sequence of *FIC1* identified an EST from a mouse liver cDNA library (AA242626) that showed 85% DNA sequence identity with the corresponding region (bp3220-3691) of the *FIC1* coding sequence, and when translated in the appropriate frame, would yield 98% amino acid identity and 100% similarity with *FIC1*. This sequence corresponded to the 3' end of *FIC1*, which is poorly conserved in other genes in the P-type ATPase family (Fagan & Saier 1994), or in the closest relatives of *FIC1* in the aminophospholipid-transporting subfamily (Tang *et al* 1996). It appeared highly probable that a sequence with such high degree of similarity in such a poorly conserved region represented the mouse orthologue of *FIC1*, now referred to as *Fic1*.

Since preliminary attempts to obtain *Fic1* sequence by PCR screening of several mouse cDNA libraries had been unsuccessful, I based my strategy for cloning the mouse orthologue of *FIC1* on this mouse EST. Sequencing, iterated primer walking and 5' RACE performed with a variety of EST-derived and human *FIC1* primers (used based on the assumption that the rest of the *Fic1* sequence would share the same high level of sequence conservation with *FIC1*) yielded the full length sequence of *Fic1*, which was contiguous with the mouse EST and aligned with the human *FIC1* coding sequence while maintaining 85% DNA identity and 98% amino acid similarity. There were no indications at any stage of this iterative process, that multiple different but homologous sequences were being amplified, suggesting that the obtained sequence represented a unique gene in the mouse genome (this was confirmed when Southern blotting of mouse

genomic DNA with *FIC1* probes identified a single band). This suggested that *FIC1* had a single murine orthologue, and not a corresponding family of genes, as is the case with some other rodent equivalents of human genes (e.g. the multiple hepatic *OATP* genes in rat and human [St. Pierre *et al* 2001]).

The mouse *Fic1* coding sequence had the same length as the human *FIC1* coding sequence; both the start codon and stop codon were conserved. The 98% amino acid similarity, maintained throughout the gene (Figure 1), represented an extremely high degree of sequence conservation between human and mouse, which suggested that the function of the protein was conserved in evolution and essential. (Even now, 4 years after these experiments were originally performed, when the mouse and human genome sequencing projects have identified many related genes in the P-type ATPase subfamily to which *FIC1* belongs, no other mouse gene found by BLAST homology searching of the non-redundant sequence database approaches the level of homology shared by human and mouse *FIC1* orthologues). A similar level of conservation is also seen with the rat orthologue of *FIC1* (Mutero *et al* 1998), and is reflected in the finding that antibodies raised against human *FIC1* also recognize the mouse and rat proteins (Eppens *et al* 2001).

Designing the targeting approach

Many considerations influenced the design of the targeting strategy to create *Fic1* mutant mice. First, we decided to aim for a mouse model of PFIC1, the more severe of the two *FIC1*-linked diseases, and the one hypothesized to be due to a complete loss of *FIC1*

protein function. We hypothesized that the more severe phenotype would be more likely to be reproduced in the mouse, overcoming differences in physiology.

The standard strategy in targeted mutagenesis in the mouse is to create a null allele, or a “knockout” with unequivocally compromised expression and function of the gene of interest. This is achieved by replacing exons essential for function, most often the initial several exons including the translation start site, with a selectable cassette (e.g. the *Bsep* knockout mouse, Wang *et al* 2001). This aborts transcription and/or translation of the gene, preventing the protein from being synthesized, or resulting in incomplete polypeptides that are degraded or nonfunctional (e.g. the *MDR2* knockout mouse, Smit *et al* 1993). However, since our objective was to reproduce a human disease-causing genetic defect (a strategy analogous to the Cfr delta F508 mouse, Colledge *et al* 1995), and not just to create a knockout mouse, we wanted to replicate one of the actual mutations identified in human PFIC1 patients.

None of the FIC1 disease mutations known at the time were unequivocal nonsense mutations, homozygous deletions or frameshifts (Chapter 2). Some concern thus existed that a profound “knockout” deletion in the mouse would result in a different, possibly more severe phenotype (such as embryonic lethality) than that seen in human disease, and thus be of limited utility. Furthermore, historically, there was particular interest in the mutation found in the PFIC1 patients belonging to the large Amish kindred in which the disease was first identified (Clayton *et al* 1969, Bull *et al* 1998). This mutation, Gly308Val (G923T), a conservative homozygous substitution in a residue located outside

the known conserved signature domains of P-type ATPases (Figure 3), was difficult to interpret at first glance. However, since the phenotype in Amish patients with this mutation was not appreciably less severe than that of patients with other mutations, including patients with the one identified mutation that most resembled an unequivocal functional null (the G892R substitution, in an invariant glycine residue in the conserved ATP-binding domain), we hypothesized that the G308V point mutation caused profound functional impairment of FIC1 function, and was thus a good candidate for reproduction in the mouse.

Homology analyses lent further support for this hypothesis. The G308 residue was found to be highly conserved in related proteins. The initial sequence alignments included the 13 (not counting the rat and mouse orthologues of *FIC1*) predicted members of subfamily IV of P-type ATPases; G308 was conserved in 10 of these 13 genes. We now know that Gly308 is conserved in 30 out of the 33 most closely related proteins (Figure 2).

Furthermore, a second PFIC1 mutation, L288S (T863C) was located nearby, suggesting that this segment of the protein was an important one. This segment comprised part of the small cytoplasmic loop, neighboring a conserved functional domain (thought to be involved in phosphorylation) and possibly, in the three-dimensional conformation of the protein, facing the conserved phosphorylation domain on the large cytoplasmic loop (Figure 3). Also, glycine residues are thought to be important in protein structures as their small size permits a tight turn that cannot accommodate any other residue. Thus any substitution in a glycine residue may compromise a protein's structural integrity. Finally, two interesting postscripts that support our theory about the importance of the G308

residue and the region surrounding it have come recently. Another, independent mutation in the same amino acid, G308D, was found in compound heterozygous form in a BRIC patient (Klomp *et al*, manuscript in preparation and Chapter 3, Figure 2) and, a third distinct PFIC1 mutation, R296C (C886T) was identified in the same area of the FIC1 protein (Chen *et al* 2002) (Figure 3).

We therefore decided to insert the G923T mutation, resulting in the G308V substitution, into mouse *Fic1*. In order to create an allele with no gene disruptions other than the desired point mutation, we utilized the Cre-loxP recombination system (Sauer & Henderson 1988). We used a “floxed” (flanked by the loxP sites recognized by the Cre recombinase) neomycin selection cassette (Gu *et al* 1993), which would provide the necessary selectability for screening of electroporated embryonic stem cells, but would be deleted by Cre-loxP recombination, so as not to obscure the phenotypic effects of the point mutation with any deleterious effects of the expression of the neomycin resistance gene within the *Fic1* locus (Figure 4).

We also decided to pursue a second targeting strategy that would lead to unambiguous disruption of the FIC1 transcript and protein, and thus safeguard against the possibility that the importance of the Gly308 residue would not be conserved in the mouse protein, and the *Fic1*G308V mice would have no phenotype. For experimental simplicity, this second targeting strategy was also designed within the genomic region that included the G308V mutation. Deleting the three exons 9, 10 and 11 (amino acids 232-342) would not only remove part of the “energy transduction” conserved domain, half the small

cytoplasmic loop, and part of a transmembrane domain, but would also result in a frameshift and premature transcript termination, thus preventing a greater part of the protein from being made, and resulting in a null allele (Figure 5).

Generation of this second, “*Fic1* null” mouse strain was intended as a safeguard against the possibility of the *Fic1*G308V mutation having no phenotype in mice. However, the converse problem also had to be considered: that the impact of both the G308V and the deletion mutations would be more severe in the mouse than in the human, resulting, for example, in embryonic lethality or developmental complications. To safeguard against such an outcome, and also to provide an interesting opportunity to study the effects of *Fic1* mutation in a tissue-specific manner, we decided to design the second targeting construct with the capacity to make either a simple null, or a conditional null allele. Thus, the three exons to be deleted were flanked by loxP sites, while the neomycin selection cassette was flanked by *frt* sites. Thus, Cre-mediated recombination (in embryonic stem cells or by mating to a transgenic, Cre expressing mice) would delete the “floxed” exons, creating the null allele, while, alternatively, FLP mediated recombination would remove the *frt*-flanked selection cassette (Meyers *et al* 1998), resulting in an allele competent for subsequent conditional targeting. Such conditional targeting could then be pursued through use of, for example, transgenic mouse lines expressing Cre recombinase in a tissue-specific (e.g. only in liver or intestine) pattern (Orban *et al* 1992), or under control of an inducible promoter (Rajewsky *et al* 1996).

Thus, the two targeting protocols would potentially produce several strains of mice carrying different *Fic1* mutant alleles (Figures 4 and 5). We anticipated that, although the minimal objectives of the research project would be accomplished with just one mouse strain with phenotypic abnormalities related to loss of Fic1 function, further interesting discoveries might arise from comparisons between the effects of the different mutations.

Gene targeting with the Fic1G308Vneo construct

In order to construct both the Fic1G308Vneo and the Fic1Null targeting constructs, the genomic segment encompassing exons 8-13 of the mouse protein was cloned and fully sequenced. Intron-exon boundaries and intron lengths were found to be conserved between mouse and human protein in this 9kb region. The Fic1G308Vneo targeting construct was assembled and targeted recombination in embryonic stem cells followed by blastocyst injection resulted in the birth of chimeric mice, which transmitted the mutation to their offspring. Mice heterozygous for the G308V (G923T) point mutation were then intercrossed to produce the $Fic1^{G308V/G308V}$ mutant mice. Cre-loxP recombination in germline transmission resulted in a mutant allele with the deleted neo cassette and only a single loxP site remaining in addition to the point mutation. The PC3 ES cells used carry a *Cre* transgene under the control of a protamine promoter, and the recombinase is expressed in the male germline but not in the embryonic stem cells. Thus the neo selection cassette was present during screening but deleted in germline transmission from chimeric fathers to heterozygous mutant pups (O’Gorman *et al* 1997). The analysis of these mice is described and discussed in the remainder of this dissertation.

Gene targeting with the *Fic1* null targeting construct

The assembly of the *Fic1* null targeting construct was delayed by cloning difficulties encountered in joining large pieces of mouse genomic DNA together in bacterial vectors. Generation of the second line of *Fic1* mutant mice lags approximately two years after the generation of *Fic1*^{G308V/G308V} mutant mice, and is still in progress. At the time of the writing of this dissertation, the targeting construct has been completed and successfully electroporated into mouse ES cells. 470 independent targeted clones await screening for homologously recombined mutants.

Once correctly targeted clones are identified, mice carrying the targeted allele will be generated and bred first to transgenic mice with pleiotropically expressed Cre recombinase to create the null mutants with the three-exon deletion, frameshift and premature transcription termination in *Fic1*. In parallel, mice with the targeted allele will be bred to Flp-expressing mice (Meyers *et al* 1998), so as to create the functionally wild-type allele competent for conditional targeting. The discovery of the paramount role of the intestine in the etiology of FIC1 disease in the *Fic1*^{G308V/G308V} mutant mice (Chapter 5) makes generation of mice carrying liver-specific or intestine-specific *Fic1* null alleles of particular interest.

METHODS

Identification of mouse orthologue of *FIC1* and assembly of full-length mouse cDNA

BLAST searches of the EST database performed with the complete coding sequence of *FIC1* produced a 472 bp EST from a mouse liver cDNA library (AA242626, 5' end of Soares mouse NML *Mus musculus* cDNA clone 680806) with 85% DNA sequence identity, 89% amino acid identity and 100% amino acid similarity with the corresponding region (bp 3220-3691, residues 1075-1231) of the *FIC1* coding sequence. Full length sequencing of the mouse EST (obtained from the IMAGE Consortium/Research Genetics) confirmed the 98% amino acid similarity with *FIC1* extended from the 5' end of the EST through to the 3' end of the *FIC1* coding sequence, including the conserved stop codon. Nested reverse PCR primers were designed from the part of the EST corresponding to *FIC1* coding sequence. Total RNA was extracted from fresh mouse liver (Trizol reagent, Gibco BRL), and cDNA reverse-transcribed from the RNA, priming with oligo-dT, random hexamers or the mouse *FIC-1* orthologue gene-specific reverse primers (Superscript RT-PCR kit, Gibco BRL). Two rounds of PCR using nested primers, with EST- derived reverse primers and several human *FIC1*-specific forward primers, were performed, yielding 2kb products, which were cloned into vector PGM-T (Promega) and sequenced. This strategy was repeated two more times, walking in the 5' direction from the EST. 5'RACE (Rapid Amplification of cDNA Ends, Gibco BRL) was performed to obtain the 5' end of the mouse cDNA.

Characterization of *Fic1* genomic sequence in region to be targeted

PCR primers were designed to amplify a fragment encompassing base-pairs 779-973, spanning exons 10 and 11, from mouse embryonic stem cell genomic DNA. The genomic fragment amplified was 340 bp long, indicating the presence of a short intron between two exons, as expected from the known intron-exon structure of human *FIC1* (Chapter 3, Figure 1). Screening of a mouse strain 129/SvJ embryonic stem cell genomic P1 library (Genome Systems) produced two genomic clones positive for the screening amplicon. P1 DNA was prepared and clone identity verified by Southern hybridization of the 340 bp product. The P1 DNA was digested with 12 different restriction enzymes and Southern blots of the digests hybridized with the screening amplicon to identify genomic fragments containing the region of interest. A 7 kb Bam HI fragment and a 12 kb Eco RI fragment were identified, subcloned into vector PGEM-3zf (Promega) and completely sequenced, using mouse *Fic1* PCR primers generated from the cDNA sequence and iterated sequence walking to determine the genomic organization and restriction map of the locus. The Bam HI fragment was found to encompass exons 9-12 (bp 735-1481), the Eco RI fragment - exons 9-13 (bp 735-1682) (exon numbering based on genomic structure of the human gene, which is conserved in the mouse in the above region sequenced; bp numbering for the coding sequence of mouse *Fic1*).

Assembly of targeting constructs

For the *Fic1*G308V targeting construct, the G923T point mutation was inserted into exon 10 by a PCR-based site-directed mutagenesis protocol. A loxP-flanked neomycin selection cassette derived from plasmid pSP2L2neo (Gu *et al* 1993) was inserted into the

unique intronic Bcl I site downstream of exon 11. This selection cassette introduces an Eco RI site that was subsequently used in screening of targeted embryonic stem cell clones (Figure 4).

For the *Fic1* null/conditional null targeting construct, a neomycin selection cassette flanked by *frt* sites and containing a loxP site 3' of the 3' *frt* site (from plasmid pM30, kind gift of Gail Martin, Meyers *et al* 1998) was inserted, in reverse orientation, into the unique intronic BclI site. Additionally, an oligonucleotide containing the second required loxP site (and an EcoRI restriction site to be used in targeted clone screening) was inserted into the DraIII site 5 of exon 9, in the reverse orientation. Thus, exons 9-11 were flanked by loxP sites to enable their excision by Cre-mediated recombination to produce a null allele; while the neomycin selection cassette was flanked by *frt* sites, to allow Flp-mediated recombination to produce a functionally wild-type allele competent for subsequent conditional targeting approaches (Figure 5).

Homologous recombination in embryonic stem cells

Targeting with the *Fic1*G308Vneo construct was performed in strain 129/SvJae PC3 mouse embryonic stem cells (O'Gorman *et al* 1997) according to standard techniques (Bradley *et al* 1987, Thomas & Capecchi 1987, Mansour *et al* 1988). Briefly, the targeting construct was linearized and electroporated into the PC3 ES cells, and recombinant clones selected on neomycin (G418). 650 independent recombinant clones were isolated; 300 were digested with Eco RI and screened by Southern blot with a 5' genomic probe positioned outside the targeting construct (Eco RI-Bam HI 229 bp

fragment, Figure 4). 27/300 of the screened clones had recombined at the targeted locus. Further screening with a 3' genomic probe, an internal neo probe and testing for the presence of the G923T point mutation by PCR with mutation-specific forward and reverse primers resulted in 8 independently derived but genetically indistinguishable clones that appeared correctly targeted and carried the point mutation heterozygously. Metaphase spread karyotyping verified the correct chromosome count of the ES cells.

Three of the ES cell clones were used in blastocyst injections (performed by Killeen laboratory technicians) and 2 injected clones resulted in the birth of chimeric mice, as assessed by coat color. Five of eight chimeric males derived from the two clones fathered agouti-colored pups that had the ES cell-derived coat color, when bred to C57/BL6 females (Jackson laboratory, Bar Harbor, Maine), in order to test for germline transmission of the targeted mutation. DNA was extracted from mouse tails and PCR genotyping and sequencing revealed that approximately 50% of these pups were heterozygous for the G923T point mutation. Correct deletion of the neo selection cassette during germline transmission of the mutant allele was verified by PCR with neo-specific and flanking genomic primers.

All animal experiments were performed by trained staff in the UCSF animal care facility under approved protocols of the UCSF Committee for Animal Research.

Targeting with the FicNull construct was performed in strain 129/SvJae RF8 embryonic stem cells (the kind gift of Robert Farese) (Bradley 1987). The linearized targeting

construct was electroporated into the ES cells and 470 recombinant clones isolated by neomycin (G418, Sigma) selection. Screening for homologous recombination with the 5' genomic probe (see above) is currently underway.

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FIC1	1	MSTERDSETT	FDEDFQPNDE	VVPYSDDDETE	DEADDQGSAAV	EPEQNRVNRE	AEENREPPRK	ECTWQVKAND	RKYHEQPHFM	NTKFLCIKES	KYANNAIKTY	100
F1c1		MSTERDSETT	FDEESQPNDE	VVPYSDDDETE	DELEDQGSTV	EPEQNRVNRE	AEKRETFRK	DCTWQVKAND	RKFHEQPHFM	NTKFFCIKES	KYASNAIKTY	200
FIC1	101	KYNAFTFIPM	NLFEQFKRAA	NLYFLALLIL	QAVPOISTLA	WYTLVPLLV	VLGVTAIKDL	VDDVARHKMD	KEINNETCEV	IKDGRFKVAK	WKBIQVGDVI	200
F1c1		KYNAFTFIPM	NLFEQFKRAA	NFYFLAILIL	QAIPQISTLA	WYTLVPLLL	VLGITAIDKL	VDDVARHKMD	KEISNMTXXX	XXXXRFXIHK	WKDIQVGDVI	300
FIC1	201	RLKKNDFVPA	DILLSSSEP	NSLCYVETAE	LDGETNLKFK	MSLEITDQYL	QREDTLATFD	GFIECEEPNN	RLDKFTGTFL	WRNTSPFLDA	DKILLRGCVI	300
F1c1		RLKKNDFIPA	DILLSSSEP	NSLCYVETAE	LDGETNLKFK	MALEITDQYL	QIEDNLATFD	GFIECEEPNN	RLDKFTGTFL	WKNQSPFLDA	DKILLRGCVI	400
FIC1	301	RNTDFCHGLV	IFAGADTKIM	KNSGKTRPKR	TKIDYLMNYM	VYTFIVLIL	LSAGLAIGHA	YWEAQVGNSS	WYLYDGEDDT	PSYRGFLIFW	GYIIVLNTMV	400
F1c1		RNTDVCHGLV	IFAGADTKIM	KNSGKTRPKR	TKIDYLMNYM	VYTFIVLIL	VSAGLAIGHA	YWEAQVGNYS	WYLYDGENAT	PSYRGFLNFW	GYIIVLNTMV	500
FIC1	401	PISLVSVSEV	IRLGQSHFIN	WDLQMYAEB	DTPAKARTTT	LNEQLGQIHY	IFSDKTGTLT	QNTWTFKKCC	INGQIYGDHR	DASQHHNHI	EQVDFSWNTY	500
F1c1		PISLVSVSEV	IRLGQSHFIN	WDLQMYAEB	DTPAKARTTT	LNEQLGQIHY	IFSDKTGTLT	QNTWTFKKCC	INGTIYGDHR	DASQHSISKI	ELVDFSWNTY	600
FIC1	501	ADGKLAFYDH	YLIBQIQSGK	EPEVRQPFPL	LAVCHTMVD	RTDQQLNYQA	ASPDGALVN	AARNFGFAFL	ARTONTITIS	ELGTERTYNV	LAILDFNSDR	600
F1c1		ADGKLAFYDH	YLIBQIQSGK	EPEVRQPFPL	LSICHTVMVD	RIDGQINYYQA	ASPDGALVN	AARNFGFAFL	ARTONTITVS	ELGSERTYNV	LAILDFNSDR	700
FIC1	601	KRMSIIVRTP	EGMIKLYCKG	ADTVIYERLH	RMNPTKQETQ	DALDIFANET	LRTLCLCYKE	IEEKEFTWNN	KKFMAASVAS	TNRDEALDKV	YEEIEKDLIL	700
F1c1		KRMSIIVRTP	EGSIRLYCKG	ADTVIYERLH	RMNPTKQETQ	DALDIFASET	LRTLCLCYKE	IEEKEFTWNN	MKFMAASVAS	SNRDEALDKV	YEEIEKDLIL	800
FIC1	701	LGATAIEDKL	QDGVPTISK	LAKADIKIWW	LTGDKKETAE	NIGFACELLT	EDTTICYGED	INSLLHARME	NORNRGVYA	KFAPPVQESF	FPPGGRNALI	800
F1c1		LGATAIEDKL	QDGVPTISK	LAKADIKIWW	LTGDKKETAE	NIGFACELLT	EDTTICYGED	INSLLHTRME	NORNRGVSA	KFAPPVYEPF	FPPGGRNALI	900
FIC1	801	ITGSWLNEIL	LEKTKRINKI	LKLKFPRTTE	ERRMRQSKR	RLEAKKEQRQ	KNFVDLACEC	SAVICCRVTP	KQKAMVVDLV	KRYKKAITLA	IGDGANDVNM	900
F1c1		ITGSWLNEIL	LEKTKRSKI	LKLKFPRTTE	ERRMRQSRR	RLEEKKEQRQ	KNFVDLACEC	SAVICCRVTP	KQKAMVVDLV	KRYKKAITLA	IGDGANDVNM	1000
FIC1	901	IKTAHIGVGI	SGQEGMQAVM	SSDYSFAQFR	YLQRLLLVHG	RMSYIRMKCF	LYRFFYKNFA	FTLVHFWYSF	FNGYSAQATAY	EDWFTILYNV	LYTSLPVLMM	1000
F1c1		IKTAHIGVGI	SGQEGMQAVM	SSDYSFAQFR	YLQRLLLVHG	RMSYIRMKCF	LYRFFYKNFA	FTLVHFWYSF	FNGYSAQATAY	EDWFTILYNV	LYTSLPVLMM	1100
FIC1	1001	GLLDQDVSDK	LSLRFPPGLYI	VGQRDLLFNY	KRFFVSLLHG	VLTSMLFFI	PLGAYLQTVG	QDGEAPSDYQ	SPAVTASAL	VITVNFQIGL	DTSYMTFVNA	1100
F1c1		GLLDQDVSDK	LSLRFPPGLYV	VGQRDLLFNY	KRFFVSLLHG	VLTSMLFFI	PLGAYLQTVG	QDGEAPSDYQ	SPAVTVASAL	VITVNFQIGL	DTSYMTFVNA	1200
FIC1	1101	FSIFGSIALY	FGIMFDFHSA	GIHVLFPFSAF	QFTGTASNAL	RQPYIWLTI	LTVAVCLLPV	VAIRFLSMTI	WPESEDKIQK	HRKRLKAEQ	WQRRQVFR	1200
F1c1		FSIFGSIALY	FGIMFDFHSA	GIHVLFPFSAF	QFTGTASNAL	RQPYIWLTI	LTVAVCLLPV	VAIRFLSMTI	WPESEDKIQK	HRKRLKAEQ	WQRRQVFR	1201
FIC1	1201	GVSTRRSAYA	FSHQRGYADL	ISSGRSIRKK	RSPLDAIVAD	GTAEYRRTGD	S					1251
F1c1		GVSTRRSAYA	FSHQRGYADL	ISSGRSIRKK	RSPLDAIAD	GTAEYRRTVH	S					1251

Figure 1

Protein sequence conservation between human FIC1 and mouse F1c1.

Divergent residues are highlighted. Known functional domains characteristic of P-type ATPases are underlined.

Organism	Gene name	
C.elegans	F36H2.1	slnvenlvwantvvas.gtavGivVvyGretsrsmNttl.peskvglldievnnltklfcfvvlsvsmvmkgl
C.elegans	F02C9.3	sldevlvwantvvas.gtaiGvVvyGcetrsmNttv.peykvglldieinnltklfcfvvlsvsmvmkglpt...
ATP9A		slsientlwagtvlvas.gtvvGvVlytGcklrsmNtsd.prskgifdlievncldklfgalvvvalmvvalqhf...
ATP9A		slsientlwagtvlvas.gtvvGvVlytGrelrsmNtsn.prskgifdlievncldklfgalvvvalmvvalqhf...
Homo sapiens		slnventawantlvadsemcalviytg.....ndtrlartrkqgnvkrktedevnfskifcslmgsilmimpdl
Entamoeba h.		plsvdntlwantvlavsgfciaevvvtGrtqamNttt.akvktggleleinsisKlcaacvafalsilvafagfnh...
S.cerevisiae	Yil1048w	plsvdntlwantvlavsg.vyGvVvytGktrgmNssk.aktKvgilekeinfysklctfvvlvsiagltshgik...
S.pombe	SPAC6C3.05c	asrsrwmpsgrrwrtgrrwllpsvtrvrgtraalNtsn.prnKlgllelelnliavlcfaallilslflvaqqfr...
Leishmania d.	APL21	viikemlllrgcrlrntfveGivVvyaGhdtkvnmqNngr.aphtKstgiektlnKfiacifmlllmvlygaitsavvvgq
C.elegans	T24H7.5	ilakenvllrgcvvktndfveGivVlyGkdkkmlNnng.pryKrslektmndvdiKsvctllalcvgavlsqvlrt
C.elegans	W09D10.2	glykenlllrgctlrntdavuGivViyGhetkallNnsg.pryKrsqlerqmcndvKvcvlllvcmslfsavghglwiwr
ATP10C		ghkenlllrgctlrntdavuGivViyGhetkallNnsg.pryKrsqlerqmcndvKvcvlllvcmslfsavghglwiwr
ATP10C		pfdekgtlrgcklkrntdwiGivViyGretkqimNssk.sikktskleitnktmliiwiqmicliciaayynailvss
PfATPase2		qitidnlvrgtsilvntkwilGivVintGnrktjmkNastksrqrKwrlfeyvghvliicqvvisliavaiaqvlwmrr
MAL3P6.27		plgpdnvlyrgtsilvntqnvGmVifGseetkirmNalKmptrKaplqrktkinmliivmfvfvatisfslgylghvhhkk
ymr162c		plgadqillrgaqlrntqvhGivVytGhdtkImqNsts.pplKlsnveritnvqililfcilliamslvesvgsaiwnrr
ATPaseII		plgadqillrgaqlrntqvhGivVytGhdtkImqNsts.pplKlsnveritnvqililfcilliamslvesvgsaiwnrr
ATPaseII		plgadqillrgaqlrntqvhGivVytGhdtkImqNsts.pplKlsnveritnvqililfcilliamslvesvgsaiwnrr
ATP8A1/ATPaseII		plgadqillrgaqlrntqvhGivVytGhdtkImqNsts.pplKlsnveritnvqililfcilliamslvesvgsaiwnrr
Y49E10.11		hfgidqillrgaqlrntawifGivViyGhdsckllmNskr.aplKstgidevqnyriiflffvvalalzatgseilwrgn
DRS2/yal026c		plspdqillrgaqlrntawifGivViyGhdsckllmNskr.aplKstgidevqnyriiflffvvalalzatgseilwrgn
ATP8B1/FIC1		pldadkllrgcrlrntdfchGivVifagadtKjmkNsgk.trfKrtkidyImnyvvtifvvlilllsaglaighayweaq
ATP8B1/Fic1		pldadkllrgcrlrntdfchGivVifagadtKjmkNsgk.trfKrtkidyImnyvvtifvvlilllsaglaighayweaq
H06H21.10		pldadkllrgcrlrntdchGivVifagadtKjmkNsgk.trfKrtkidyImnyvvtifvvlilllsaglaighayweaq
gwnndnillrgcrlrntwcyGvVvfaGkdtKjmmNsgk.tkfrtsldtrfnililgvlflfiamclictilcavweyq		
ATP8B3		slidgnillrgcrlrntdcyGivVifagadtKjmkNsgk.ihlKrtklldlmmklvwifisvvlvclvafgfgfsvke
SPBC887.12		plspdqillrgaqlrntpwvyGivVvftGheskImkNkte.pikrtKsvekvnsqililfcifvclfaslglalhrs
F24J5.6		plspdqillrgaqlrntdfiGvVifGhdtkvqNstd.ppsKrsmeiekkmdkiylmfmfvitmafisvifgvttrd
F20D21.10		plspdqillrgaqlrntayvyGvVvftGhdtkvqNstK.spsKrsrieekmdkiylmfmfvitmafisvifgvttrd
F3F19.24		plspitqillrgaqlrnteyiyGvVvftGhdtkvqNstd.ppsKrsrieekmdkiylmfmfvitmafisvifgvttrd
SPAC24B11.12c		plsidmlllrgcrlrntkwivGvVvftGddtkjmlNsga.pplKrsritrnlmwnvylnfllfsmctfcavvegiawrg
SPAC4F10.16c		pfslsnnllrgcrlrntkwivGvVvftGddtkjmlNsga.pplKrsritrnlmwnvylnfllfsmctfcavvegiawrg
ydr093w		pvninnlllrgcrlrntkwamGmVifGddtkjmiNagv.tptKksrstrlnfsvlnfvlflfclftagivngvykq
yer166w		pitinnvllrgcrlrntkwamGmVifGddtkjmlNsgi.tptKksrstrlnfsvlnfvlflfclftagivngvykq
MUG13.22		slqpsnillrgcrlrntawalgvVvyaGgetkamlNnsg.apskrsrletrmnleillslflvlctiaataavwlrt

IDENTICAL IN 28/33 SEQUENCES

CONSERVED SEGMENTS IN ALL P-TYPE ATPASES

-----G-V--G-----N-----K-----

CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC

Figure 2

Phylogenetic conservation of the FIC1 Gly308 residue among P-type ATPases of subfamily IV.

Adapted from Axelsen & Palmgren 1998 and from the website "P-type ATPase database" <http://biobase.dk/~axe/Patbase.html>.

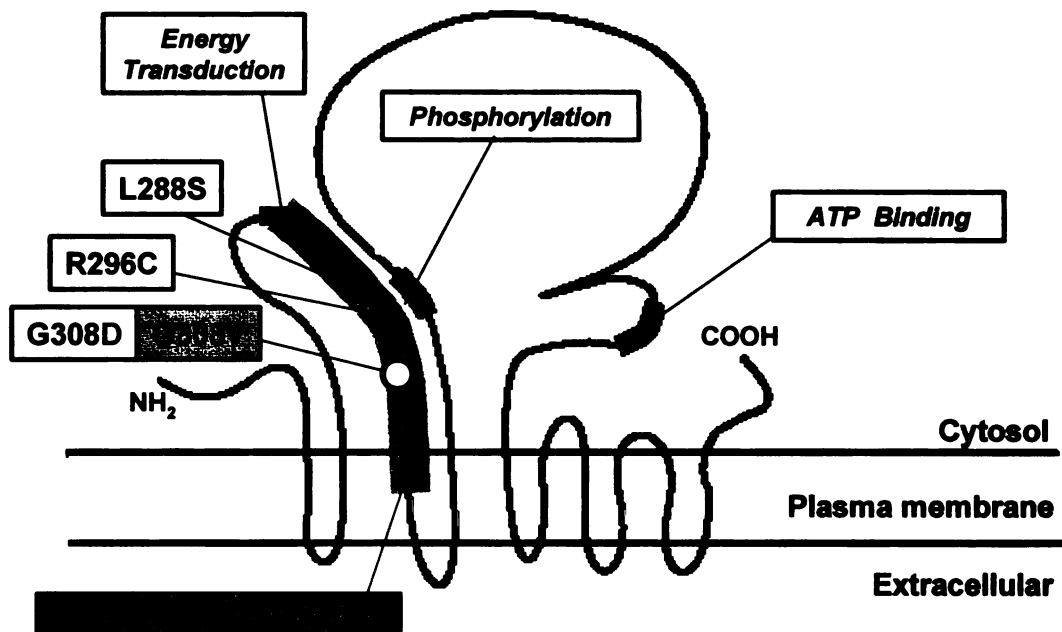


Figure 3

Location of targeted mutations in Fc11.

Conserved P-type ATPase functional domains are shown as black boxes and labeled in *italics*. The three exons targeted for deletion in the Fc1Null mouse are highlighted with a gray bar. The G308V Amish PFIC1 mutation, which is reproduced in the Fc1^{G308V/G308V} mutant mouse, is shown as a white circle; other PFIC1 and BRIC disease mutations located in the same region of the protein are shown as black circles.

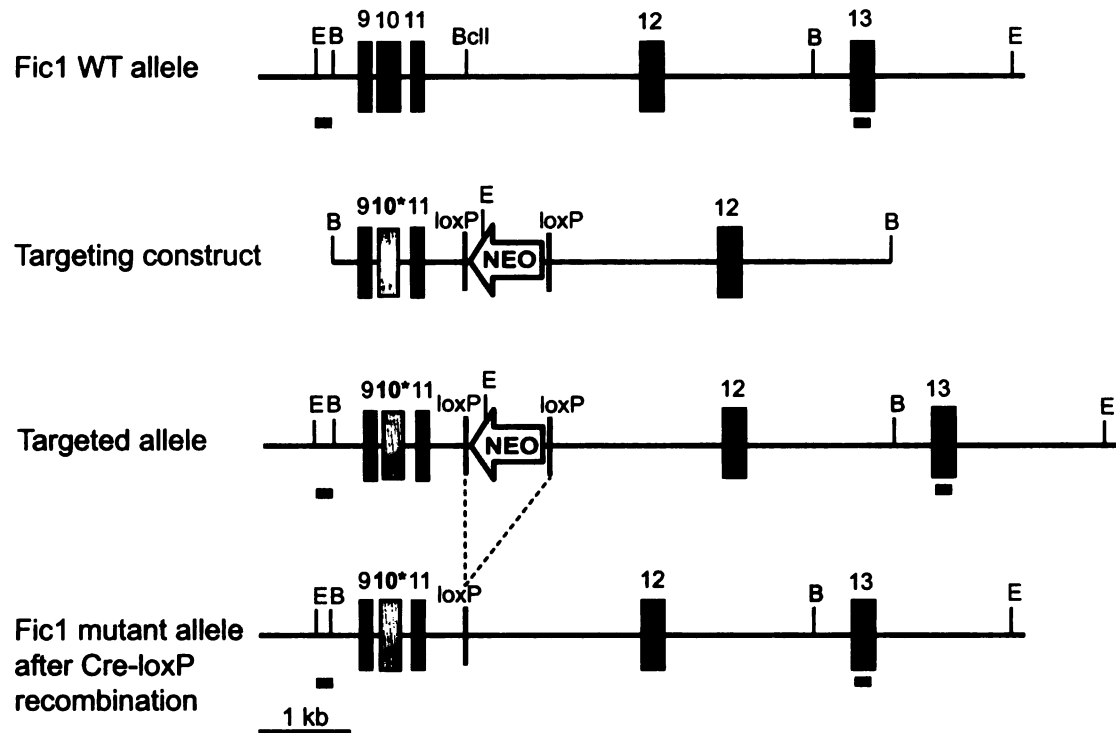


Figure 4

Targeting strategy for the *Fic1*^{G308V/G308V} mutant mouse line.

Exons are depicted as black, numbered boxes, the mutagenized exon 10* containing the G923T point mutation introduced by site-directed mutagenesis is shown as a gray box. “Neo” represents the neomycin resistance cassette cloned into the unique intronic Bcl I restriction site. The location of Eco RI (E), Bam HI (B) and Bcl I restriction sites is shown. Small horizontal boxes depict the locations of the 5’ and 3’ genomic probes used in ES cell clone screening. Cre-loxP recombination in germline transmission results in a mutant allele that differs from the wildtype only in the G923T point mutation and in a single remaining intronic loxP site.

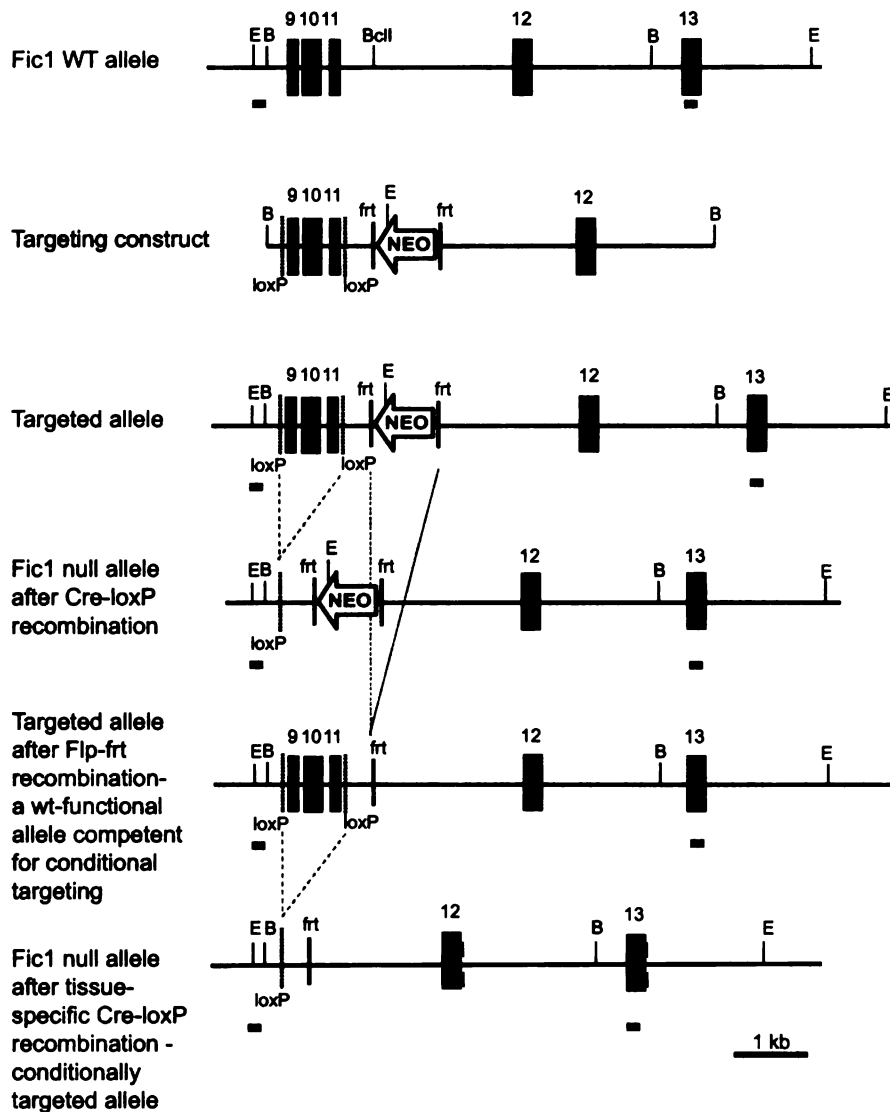


Figure 5

Targeting strategy for the *Fic1* null and conditional-null mutant mouse lines.

Exons are shown as black, numbered boxes. "NEO" represents the neomycin resistance cassette cloned into the unique intronic Bcl I restriction site. The location of Eco RI (E), Bam HI (B) and Bcl I restriction sites is shown. Small horizontal boxes denote locations of the 5' and 3' genomic probes used in ES cell clone screening. Cre-loxP recombination results in deletion of 3 exons to create the *Fic1* null allele. Alternatively, Flp-FRT recombination deletes the neo cassette, resulting in a wildtype-functional allele competent for conditional targeting. Subsequent Cre-loxP recombination with a Cre recombinase expressed under the control of a tissue-specific promoter deletes 3 exons, producing a tissue-specific, conditionally targeted *Fic1* null allele.

CHAPTER 5

***Fic1* mutant mice have a defect in the regulation of intestinal bile salt absorption.**

The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I found it!) but 'That's funny...'

Isaac Asimov

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***Fic1* MUTANT MICE HAVE A DEFECT IN THE REGULATION OF INTESTINAL BILE SALT ABSORPTION**

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ABSTRACT

Mutations in *FIC1* (*ATP8B1*), a broadly expressed P-type ATPase, result in impaired bile secretion and liver damage, but the etiological role of *FIC1* in liver disease is unclear. We investigated the systemic impact of FIC1 loss by generating *Fic1*^{G308V/G308V} mutant mice. In contrast to human patients with the same mutation, *Fic1*^{G308V/G308V} mice had unimpaired bile secretion and no liver disease. However, *Fic1*^{G308V/G308V} mice fed a bile salt-supplemented diet showed enhanced biliary secretion, but accumulated high levels of serum bile salt caused by excessive intestinal bile salt absorption. Our results show that *Fic1* deficiency disables regulation of intestinal bile salt absorption, but the low toxicity of the murine bile salt pool probably protects *Fic1*^{G308V/G308V} mice from severe liver damage. The *Fic1* mutant mice reveal the importance of regulation of ileal bile salt absorption for bile salt homeostasis and suggest that such defective regulation may be a primary insult in the etiology of human FIC1 liver disease.

INTRODUCTION

Secretion of bile by the liver is required for the absorption of lipids and fat-soluble vitamins in the intestine, and for elimination of toxins, metabolic byproducts and cholesterol. Cholestasis, the impairment of bile production or flow, has serious pathological consequences, including irreversible liver damage and fat and vitamin malabsorption. The discovery of the genetic defects responsible for several inherited cholestasis syndromes has informed our understanding of both the etiology of cholestasis and the molecular physiology of bile metabolism (Bull *et al* 1998, Strautnieks *et al* 1998, de Vree *et al* 1998).

The principal components of bile are bile salts (BS), phospholipids and cholesterol. BS are secreted at the canalicular (apical) membrane of the hepatocyte by the ABC transporter BSEP (*ABCB11*) and pass with bile into the small intestine (Gerloff *et al* 1998, Green *et al* 2000). Over 95% of BS are reabsorbed in the ileum, mainly via the apical sodium-dependent bile salt transporter (ASBT) (Wong *et al* 1994, Shneider *et al* 1995, Craddock *et al* 1998), and return via the portal circulation to the liver; the remainder is excreted in the feces (Shneider 2001).

Recent studies have identified the molecular basis of several inherited forms of cholestasis. MDR3 (*ABCB4*) is a translocator responsible for phospholipid secretion into bile; in the absence of phospholipids, liver cells are damaged by the toxic action of the detergent BS (Smit 1993). This condition is now classified as progressive familial

intrahepatic cholestasis type 3 (PFIC3) (Deleuze *et al* 1996, de Vree *et al* 1998, Jacquemin *et al* 2001). Defects in *BSEP* (*ABCB11*), the canalicular bile salt exporter, also result in PFIC (PFIC2) (Strautnieks *et al* 1998). A third set of cholestatic disorders results from mutations in *FIC1* (*ATP8B1*), a gene of unclear function (Bull 1998). *FIC1* disorders range from an episodic, non-progressive condition (benign recurrent intrahepatic cholestasis, BRIC) (Summerskill *et al* 1965) to the progressive and potentially fatal PFIC1 (Clayton *et al* 1969, Linarelli *et al* 1972). Although PFIC1 has much in common with PFIC2, including low biliary BS, high serum BS, elevated serum bilirubin and liver enzyme activities, but low cholesterol and gamma glutamyl transferase (Bull *et al* 1997), it differs from the latter condition in that the patients suffer from extrahepatic complications, such as watery diarrhea, that are not ameliorated by liver transplantation (Bull *et al* 1997, ASK personal communication, Chen *et al* 2001). Consistent with this observation, *FIC1* is broadly expressed in epithelial tissues, with particularly high levels in the ileum and pancreas (Bull *et al* 1998). In contrast, *MDR3* and *BSEP* are present predominantly in the hepatocyte canalicular membrane (Smit *et al* 1993, 1994, Strautnieks *et al* 1998). Additionally, *FIC1* does not share sequence homology with any known BS transporter genes. Instead, it appears most closely related to a subfamily of P-type ATPases thought to flip aminophospholipids from the outer to the inner leaflet of the plasma membrane (Bull *et al* 1998, Tang *et al* 1996), and has recently been reported to demonstrate such translocase activity (Ujhazy *et al* 2001). How such function relates to the development of cholestasis is still unclear.

We hypothesized that evaluation of the effects of *Fic1* mutation in a mouse model would allow dissection of the physiological roles of FIC1 and of the etiology of FIC1 disease. We have therefore constructed a mutant mouse line that reproduces a human PFIC1 disease mutation.

RESULTS

Generation of *Fic1*^{G308V/G308V} mutant mice

We isolated the mouse orthologue of *FIC1*, and found that murine *Fic1* shares 86% DNA identity and 98% amino acid similarity with human *FIC1*. We designed a targeting vector, which would introduce into mouse *Fic1* the G308V (G923T) substitution (in a glycine residue conserved between human and mouse) found in multiple patients in the Amish kindred in which PFIC1 disease was first described (Fig. 1a) (Clayton *et al* 1969, Bull *et al* 1998). This mutation results in absence of FIC1 protein in fibroblasts from a human PFIC1 patient (EFE, unpublished observations). Gene targeting in mouse embryonic stem cells resulted in germ-line transmission of the *Fic1*G308Vneo allele (Fig. 1b and c). Deletion of the neo selection cassette was confirmed, and *Fic1*^{G308V/+} heterozygous mice were intercrossed to produce *Fic1*^{G308V/G308V} homozygous mutants (Fig. 1d). Approximately 25% of pups born to heterozygous parents were *Fic1*^{G308V/G308V}, indicating that *Fic1* is not required for embryonic development. *Fic1* mutant mice were fertile and did not have a shortened lifespan, compared to wild-type mice of the same strain.

Western blotting revealed that *Fic1* mutant mice had no Fic1 protein in the liver, and only traces in the small intestine (Fig. 1e). The gene targeting approach was not expected to affect *Fic1* RNA transcription, and normal levels of *Fic1* RNA were seen in the liver and intestine of *Fic1* mutant mice (RT-PCR, data not shown). Thus, the G308V mutation resulted in the loss of functional Fic1 protein in mice, reproducing the defect in PFIC1 patients with the same mutation.

Fic1^{G308V/G308V} mice are healthy but have mild perturbations in bile salt homeostasis

Fic1 loss had an impact on pup growth in the nursing period, as Fic1^{G308V/G308V} pups (n=34) gained weight more slowly than their wild-type and heterozygous littermates (n=73), displaying 4% lower weight at weaning (Table 1). The weight difference resolved with age and adult Fic1^{G308V/G308V} mice appeared normal. Unexpectedly and unlike human patients with the corresponding mutation (Clayton *et al* 1969, Linarelli *et al* 1972, Bull *et al* 1998), the mutant mice did not suffer from jaundice or diarrhea and had normal serum levels of bilirubin and of diagnostic liver enzyme activities, except for mildly elevated ASAT (Table 1). *Fic1*^{G308V/G308V} mice had slightly enlarged livers (Table 1), but histopathological examination of the liver, duodenum, ileum, colon and pancreas revealed no abnormalities and no evidence of cholestasis. However, *Fic1*^{G308V/G308V} mice had elevated total serum BS levels as compared to wild-type controls (Table 1), confirming Fic1 involvement in BS homeostasis.

In order to investigate bile secretion in *Fic1* mutant mice, we cannulated the gallbladders and collected bile (Fig. 2). Contrary to expectation, bile flow and output of principal bile

components were not reduced in *Fic1* mutant mice (Table 1). *Fic1* mutant mice secreted significantly higher amounts of BS than wild-type animals throughout 150 minutes of bile depletion (Fig. 2), indicating that *Fic1* loss did not impair canalicular BS secretion, but instead resulted in an enlarged BS pool. Next, we investigated the maximal BS secretion capacity of *Fic1* mutant mice by intravenous infusion of gradually increasing amounts of BS (tauroursodeoxycholate (TUDC), Fig 2; also taurocholate and taurodeoxycholate, data not shown), but observed no reduction in BS output levels reached by *Fic1*^{G308V/G308V} mice. This showed that maximal BS secretion capacity was not impaired in *Fic1*^{G308V/G308V} mice. We also analyzed the BS composition of mouse bile and found that *Fic1* mutants had a somewhat more hydrophilic composition, with more muricholate and less cholate than wild-types (Table 1).

Hepatic expression of Bsep, the principal canalicular BS transporter, was equivalent in wild-type and mutant animals (Western Blot, data not shown).

***Fic1* mutant mice accumulate serum bile salt on a cholate diet**

We subsequently investigated BS absorption in the intestine and the systemic effects of excess BS by feeding the mice a diet containing 0.5% cholate. In wild-type mice, cholate feeding did not have any deleterious effects, but strongly up-regulated *Fic1* expression in the ileum and mildly up-regulated *Fic1* expression in liver (Fig. 3a). *Fic1* mutant mice fed the cholate diet lost weight (Fig. 3b). Their serum bilirubin and liver enzyme levels rose, as compared to wild-type controls (Fig. 3c and 3d), and their livers became enlarged ($7.5 \pm 1.1\%$ vs. $3.6 \pm 0.3\%$ body weight, $n=8$, $p<0.001$), indicating liver damage.

Histopathological evaluation showed enhanced hepatocyte proliferation, but no cholestasis (data not shown). Serum BS levels rose dramatically in *Fic1* mutant mice on the cholate diet while remaining constant in controls (Fig. 3e). Both endogenous and post-depletion BS output was enhanced in the mutant mice, revealing a markedly enlarged BS pool; nevertheless, maximal BS secretion capacity as measured by TUDC infusion was comparable between the two groups (Fig. 3f). These results suggested that excessive or inadequately controlled intestinal absorption caused the BS accumulation in the serum.

Since ASBT is considered the primary transporter responsible for BS absorption in the ileum (Shneider 2001), we examined the effects of *Fic1* abrogation and BS feeding on ASBT expression. Surprisingly, no difference in ASBT expression was seen between mutant and wild-type mice on the cholate diet (Fig. 3g). Similarly, ileal expression of IBABP (Intestinal Bile Acid Binding Protein, Gong *et al* 1994) was comparable in *Fic1* mutant and wild-type animals on cholate diet, though down-regulated from control diet levels (data not shown).

Analysis of the serum BS composition revealed that cholate conjugation by the liver was unimpaired, as taurocholate was the predominant BS species accumulating in *Fic1*^{G308V/G308V} mice (Fig. 3h).

***Fic1* mutant mice accumulate high levels of ^3H -labelled TC after oral administration**

In order to confirm enhanced absorption of BS in the intestine of *Fic1* mutant animals, we administered an oral dose of ^3H -labelled taurocholate to animals equilibrated to control or cholate diets, and determined residual total-body ^3H levels after 4 days. ^3H levels were similar in wildtype and mutant animals on a control diet. However, on cholate diet, total residual ^3H was 16 times higher in *Fic1* mutants than in controls ($n=6$, $p<0.0001$; Fig. 4a), confirming excessive intestinal BS absorption in *Fic1* mutant mice on the cholate diet. Cholate feeding significantly decreased ^3H retention in controls ($p<0.0001$), demonstrating that wild-type mice down-regulated their intestinal BS absorption upon dietary overload. This downregulation was completely absent in *Fic1*^{G308V/G308V} mice.

We also compared the proportional distribution of the retained ^3H label among the organs of the enterohepatic circulation in wild-type and mutant mice on the cholate diet (Fig. 4b and 4c). In the case of defective BS secretion, a proportional accumulation of label in the liver of mutant mice would have been expected. However, in mutant mice, as compared to wild-types, a much larger proportion of the label was found in the bile, and a relatively small proportion in the liver, confirming efficient hepatic secretion. In addition, a relatively small proportion of the label was observed in the colon of mutant mice on cholate diet, as compared to wild-types on the diet, suggesting enhanced absorption of the label upstream of the colon, in the ileum. To determine whether *Fic1*^{G308V/G308V} animals had a defect in hepatic uptake of BS, we examined the amount of label in the peripheral serum. The mutant mice did not display an uptake defect, as their proportional serum ^3H

levels were lower than in wild-types ($8.0 \pm 6.3\%$ vs. $21 \pm 5.4\%$ of ^3H retained in body, $n=6$, $p<0.005$, data not shown).

DISCUSSION

We have created mice homozygous for the *Fic1* G308V point mutation, which results in loss of *Fic1* from the liver and intestine, probably due to degradation of a structurally unstable protein. *FIC1* is also absent from fibroblasts from a PFIC1 patient with the analogous mutation (EFE unpublished data), suggesting that the G308V substitution acts as a functional null mutation in both mice and humans.

In contrast to human patients with the analogous mutation, *Fic1*^{G308V/G308V} mice had unimpaired biliary secretion and no evidence of cholestasis, even upon cholate feeding. Nonetheless, these animals displayed indications of a disturbed enterohepatic circulation of BS, most strikingly a dramatic increase in serum BS levels on cholate diet, suggesting that BS absorption in the intestine, rather than secretion by the liver, was affected by the loss of *Fic1*. The high total-body retention of orally administered ^3H -TC in cholate-fed *Fic1* mutant mice confirmed this conclusion. Importantly, wild type mice effectively maintained a low BS pool during identical BS overloading; this indicated that the dramatic increase of the bile salt pool in *Fic1*^{G308V/G308V} mice was due to impaired downregulation of intestinal uptake during BS overload. These results are the first to reveal the importance of the regulation of intestinal uptake for BS homeostasis.

Early hypotheses ascribed FIC1 disease to a primary defect in canalicular BS secretion (Clayton *et al* 1969, Bull *et al* 1998). However, our discovery that the primary defect in *Fic1* mutant mice resides in the intestine suggests that cholestasis and liver damage seen in human FIC1 disease could be secondary to an intestinal defect. The broad expression pattern of FIC1 (Bull *et al* 1998) is consistent with extrahepatic roles for this protein, as are clinical observations about PFIC1 patients. For example, the onset of diarrhea precedes the development of jaundice in PFIC1 infants; this is not usually seen in other forms of cholestasis (Clayton *et al* 1969, Linarelli *et al* 1972, Bourke *et al* 1986). Furthermore, intractable diarrhea often persists in transplanted PFIC1 patients, indicating that restoration of hepatic FIC1 expression cannot compensate for ileal FIC1 loss (Chen *et al* 2001, ASK, personal communication). Finally, reports of fatty liver change in transplant recipients suggest that the transplanted normal liver is susceptible to damage when placed downstream of deranged BS absorption in the *Fic1*-deficient ileum (ASK, in preparation). Nonetheless, the sustained therapeutic effect of liver transplantation and the presence of *Fic1* in the canaliculus and in cholangiocytes imply that, in humans, both intestinal and hepatic expression of FIC1 are required.

Intestinal events may also play a role in the substantial phenotypic variability observed in *Fic1*^{G308V/G308V} mice as well as in PFIC1 patients, which contrasts with the more predictable phenotype observed in other forms of inherited cholestasis, for example PFIC2. This variability may reflect an environmental component, and fluctuation in the composition of intestinal flora is a likely culprit. Our finding of defective regulation of intestinal BS uptake in *Fic1* mutant mice suggests that PFIC1 may be caused by

enhanced intestinal uptake of cholestatic agents, such as BS metabolites produced by intestinal bacteria, which secondarily cause cholestasis in the liver.

The paradox of an identical mutation producing contrasting phenotypes - cholestasis in humans, but enhanced BS secretion in mice - can be resolved by considering the impact of a primary intestinal insult in FIC1 disease in the context of the much higher relative hydrophobicity and hepatotoxicity of the human BS pool. The major human primary BS species are cholate and chenodeoxycholate; mice have cholate and the more hydrophilic muricholate (Coleman *et al* 1979). Consequently, human secondary BS species are also more hydrophobic than the murine species. Significantly, FIC1 is expressed in the colon, where resident bacteria produce much larger amounts of cholestatic lithocholate in humans than in mice (L. Klomp, personal communication). Disregulated intestinal BS re-absorption in humans may account for the observed increased levels of hydrophobic, hepatotoxic BS, including lithocholate, in the bile and/or serum of PFIC1 and BRIC patients (Linarelli *et al* 1972, Kuipers *et al* 1985, Bijleveld *et al* 1989).

Mice are probably protected on two levels from cholestasis and resultant liver injury due to absence of Fic1. First, the murine liver does not encounter significant amounts of any BS species more hydrophobic than cholate. Second, the endogenous BS pool of *Fic1*^{G308V/G308V} mice shifts towards even greater hydrophilicity. This shift is probably accomplished through mobilization of BS hydroxylation and export pathways unique to mice. The importance of such pathways was highlighted by *Bsep* knockout mice, which compensated for disabled cholate secretion by increased hydroxylation and secretion of

tetrahydroxylated BS species, probably via alternative canalicular transporters specific for hydrophilic BS (Wang *et al* 2001). Although the BS output of *Fic1* mutant mice is enhanced, not diminished, and the relative proportion of cholate only slightly reduced, in both mouse models the net result is an even more hydrophilic biliary BS pool, which explains in part the absence of hepatic damage and the conservation of bile secretion that sets both mouse phenotypes apart from the human diseases. The murine system responds to insult with adaptations in BS pool composition that may parallel the therapeutic principle behind the benefits of choleretic BS such as UDCA in some human cholestasis syndromes (Jacquemin 1997, Ferrenchak 2001).

Differences in BS pool composition between mouse and human explain why the *Fic1* mutants (as well as *Bsep* and *Mdr2* mutants) (Wang *et al* 2001, Smit *et al* 1993) are less severely affected than their human counterparts. Nonetheless, these mutant mice show how valuable mouse models can be even when the mouse phenotypes do not replicate the human phenotypes. For *Fic1* mutants, the mildness of the mouse phenotype facilitated analysis of the physiologic role of the protein and of the pathogenic process of FIC1 disease, revealing the primary importance of intestinal regulation of BS homeostasis. In contrast, in humans, the severity and early onset of PFIC1 conflate primary and secondary manifestations of BS mishandling and liver disease and therefore preclude elucidation of these processes.

Both sequence analysis and recent functional studies have suggested that FIC1 is an aminophospholipid flippase involved in maintenance of the asymmetrical distribution of

lipids in plasma membranes (Bull *et al* 1998, Ujhazy *et al* 2001). How could such a molecular function be reconciled with our physiological observations? Transporter activity and vesicular trafficking of membrane components are sensitive to membrane lipid distribution (Zachowski *et al* 1989, Kipp & Arias 2002). Thus, lipid imbalance caused by FIC1 malfunction could disrupt regulation of the activity of ASBT, the principal ileal BS re-uptake protein (Wong *et al* 1994, Craddock *et al* 1998). Since ASBT expression in the ileum appeared equivalent in wild-type and *Fic1* mutant mice on the cholate diet, such regulation is likely to happen at the level of ASBT activity or localization. Interestingly, both ASBT (Lazaridis *et al* 1997) and FIC1 (Eppens *et al* 2001) are also expressed in cholangiocytes: cells lining the bile ducts; dysregulation of cholangiocyte ASBT function might contribute to the hepatic phenotype in PFIC1 disease by permitting excessive shunting of BS from bile back to the hepatocytes. A general role in membrane maintenance and vesicular trafficking is also consistent with the extrahepatic manifestations of PFIC1, particularly in epithelial secretory cells whose apical domains may be particularly sensitive to membrane dysfunction due to the high volume of trafficking events.

The finding that dysregulation of BS absorption in the intestine plays the primary role in the pathogenesis of FIC1 disease highlights the importance of identifying the molecular mechanisms for this previously unappreciated regulatory process. This shift in our understanding of the initiation of cholestatic disease may eventually inspire new approaches to ameliorating the symptoms of PFIC1 and BRIC patients. Definitive elucidation of the functions of FIC1 will be necessary to complete our understanding of

feedback regulation of the enterohepatic circulation. Such understanding is of broad medical importance, as this feedback regulation is an important target for pharmacological approaches to modulate cholesterol excretion from the body by controlling BS re-absorption.

METHODS

Isolation of the mouse orthologue of *FIC1*

BLAST homology searching identified a mouse EST (GenBank accession number AA242626), with 98% protein sequence identity to bp 3229-3694 of human *FIC1*. The full-length mouse *Fic1* cDNA sequence was assembled using primer walking and RACE on mouse liver cDNA, and deposited in Genbank.

Generation of *Fic1*^{G308V/G308V} mutant mice

We constructed the targeting vector from a 7.1 kb BamHI genomic DNA fragment derived from a P1 clone (mouse 129/Sv embryonic stem cell DNA library, Genome Systems, St. Louis, Missouri), encompassing exons 9-12 of *Fic1*. The G923T point mutation was introduced into exon 10 by site-directed mutagenesis. A loxP-neo-loxP resistance cassette (Gu *et al* 1993) was inserted into an intronic BclI restriction site. Linearized vector was electroporated into strain 129/SvJae PC-3 ES cells carrying a male-germ-line specific Cre transgene (O’Gorman *et al* 1997). G418-resistant colonies were screened for homologous recombination by Southern blot using 5’ EcoRI-BamHI and 3’ BamHI-EcoRI genomic probes. Recombinant clones were re-screened with an internal neo probe and by PCR using primers specific for the G923T point mutation. 2

independent heterozygous mutant ES cell clones injected into strain C57Bl/6 blastocysts yielded chimeric male mice, which transmitted the targeted allele when bred to C57Bl/6 females. The deletion of the loxP-flanked neo selection cassette from the targeted allele during germline transmission was confirmed by PCR. Chimeric founders were bred to strain 129/SvImJ female mice (JAX, Bar Harbor, Maine) to produce mutant heterozygotes, which were intercrossed to produce $Fic1^{G308V/G308V}$ mutants.

***In vivo* experiments**

Age-matched male mice aged 2-5 months were used in all experiments. Mice were fed standard rodent chow or, for diet experiments, a commercial purified diet (K4068.02, Hope Farms, Woerden, The Netherlands) with or without 0.5% w/w sodium cholate (Merck, Darmstadt, Germany). Food and water were supplied *ad libitum*. For bile collection and organ isolation, mice were anesthetized with an intraperitoneal injection of Hypnorm/Diazepam. Gallbladder cannulation, bile collection for up to 240min, and intravenous infusion of TUDC (Calbiochem, La Jolla, California) in PBS pH 7.4 were performed as previously described (Oude Elferink *et al* 1995). Isoflurane anesthesia was used for blood collection by orbital puncture. For intestinal absorption analysis, 1 μ Ci of ^{3} H-taurocholate (PerkinElmer, Boston, Massachusetts) in 100 μ L PBS was administered by gavage to animals that had been fed control or cholate diets for 10 days and then fasted for 12h. Animals were maintained on their respective diets and sacrificed 4 days after ^{3} H-taurocholate administration. Organs were excised, weighed aliquots treated with Soluene (Packard, Meriden, Connecticut) and ^{3} H levels determined by scintillation

counting. All animal experiments were performed under approved protocols of the UCSF and/or AMC Committees on Animal Research.

Assays

Phospholipid, cholesterol and BS concentration in bile were determined enzymatically as previously described (Oude Elferink 1995). Serum bilirubin levels and liver enzyme activities were measured by routine clinical chemistry. Serum and biliary BS composition was determined by HPLC/MS.

Western Blotting

Preparation of protein homogenates from mouse tissues, SDS-PAGE and Western blotting were performed as previously described. L. Klomp provided anti-FIC1 polyclonal antibody (Eppens *et al* 2001). Anti-ASBT and anti-IBABP antibodies were a gift from L. Agellon. Anti-CPS (carbamoylphosphate synthetase) antibody, used as a reference, was a gift from Wout Lamers.

Statistical analysis

Values are reported as mean $\pm$ s.d. Comparisons between two groups were evaluated with the Mann-Whitney nonparametric test. Data in Figures 2, 3b-f, 4b and Table 1 was analysed by repeated measures two-way ANOVA with Tukey's correction for multiple testing, after $\log_{10}(x+1)$ transformation. Data in Figure 4a was analysed by two-way ANOVA.

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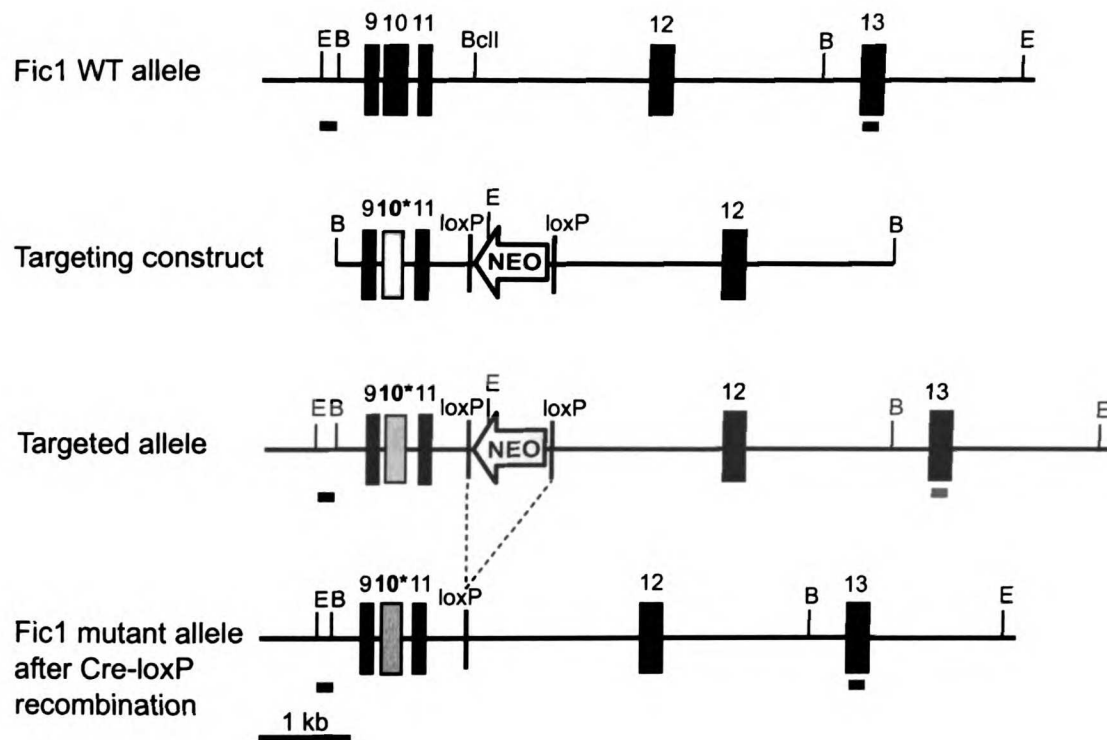


Figure 1a

Generation of *Fic1*^{G308V/G308V} mice.

Schematic representation of the targeted mutagenesis of the *Fic1* genomic locus (WT allele) with the *Fic1*G923Tneo targeting construct, followed by Cre-loxP recombination in germline transmission to produce the *Fic1*G308V mutant allele. Black boxes represent exons; exon 10* after insertion of the G923T mutation is shown as a light box. NEO indicates the loxP-flanked neomycin selection cassette inserted into a Bcl I site. BamHI (B), EcoRI (E), and Bcl I restriction sites and the 5' and 3' genomic probes used for screening targeted ES cell clones are shown.

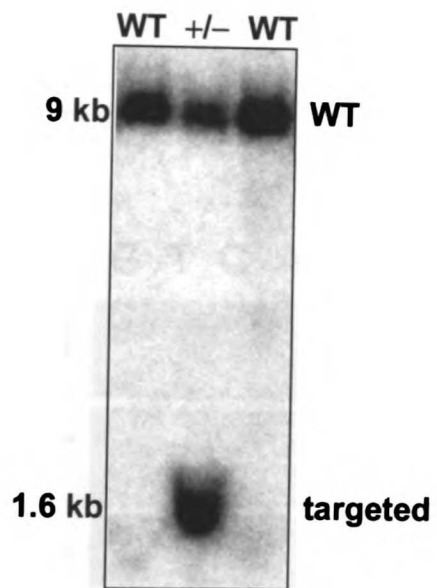


Figure 1b

Southern blot analysis of targeted ES cell clones.

The 229 bp genomic 5' probe shown in panel a was used. A correctly targeted heterozygous clone (+/-, *Fic1*^{+/*G923T*}) is shown flanked by two wildtype (WT) clones.

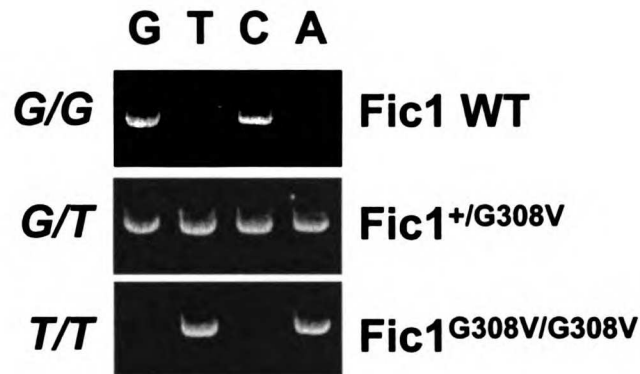


Figure 1c

PCR genotyping of mutant mice.

DNA from wildtype (WT), heterozygous mutant ($Fic1^{+/G308V}$), and homozygous ($Fic1^{G308V/G308V}$) mutant mice was amplified using PCR primers specific for the G923T point-mutation. Products amplified using mutation-specific primers: G: forward wildtype, T: forward mutant, C: reverse wildtype, A: reverse mutant. DNA sequence genotype for the G923T point mutation (G/G, G/T, T/T) is shown on the left.



Figure 1d

Loss of Fic1 protein in liver and ileum of Fic1^{G308V/G308V} mutant mice.

Western blot of total protein extracts (15 µg protein/lane) from liver and ileum of wildtype (WT) and Fic1^{G308V/G308V} mice probed with antibodies against FIC1 and CPS (carbamoylphosphate synthetase, used as a reference).

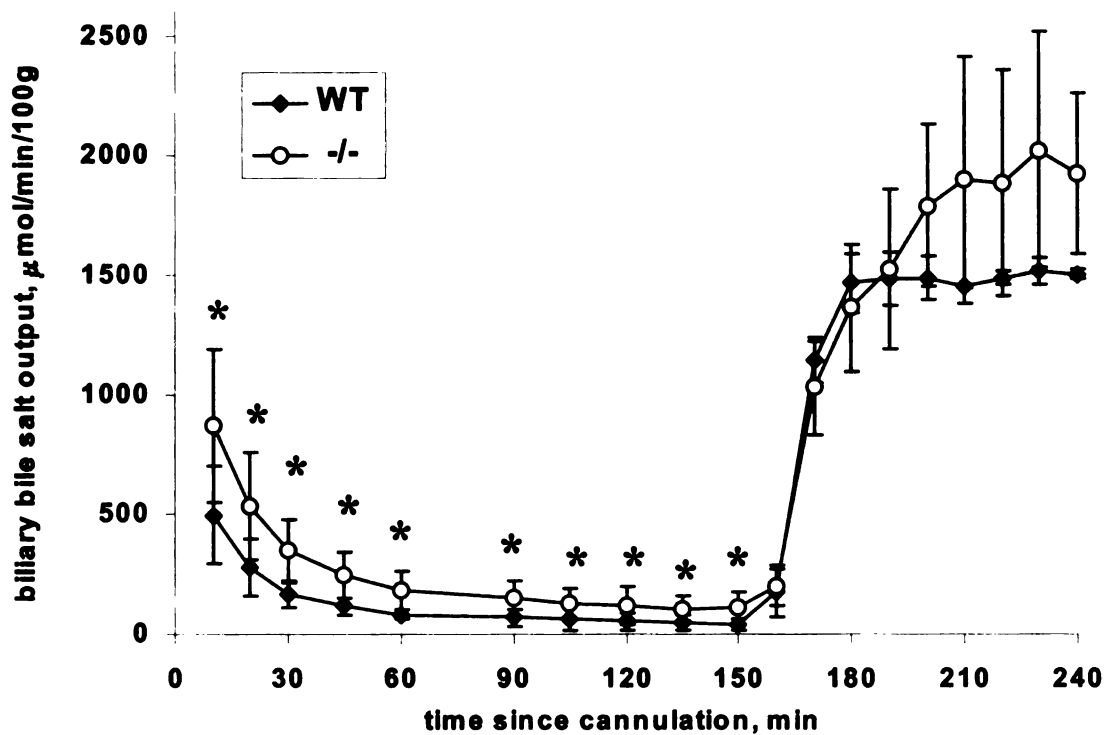


Figure 2

Biliary bile salt output in *Fic1* WT and *Fic1*^{G308V/G308V} mice.

Gallbladders were cannulated at t=0 and bile collected continuously for 240 minutes. T=0-150 bile depletion period; t=150-240min stepwise increasing intravenous infusion of TUDC. n=3-8. The overall difference between bile salt output in *Fic1* WT and *Fic1*^{G308V/G308V} mice for the 150min bile depletion period was significant, *p=0.0003 (two-way repeated measures ANOVA with Tukey's correction for multiple testing).

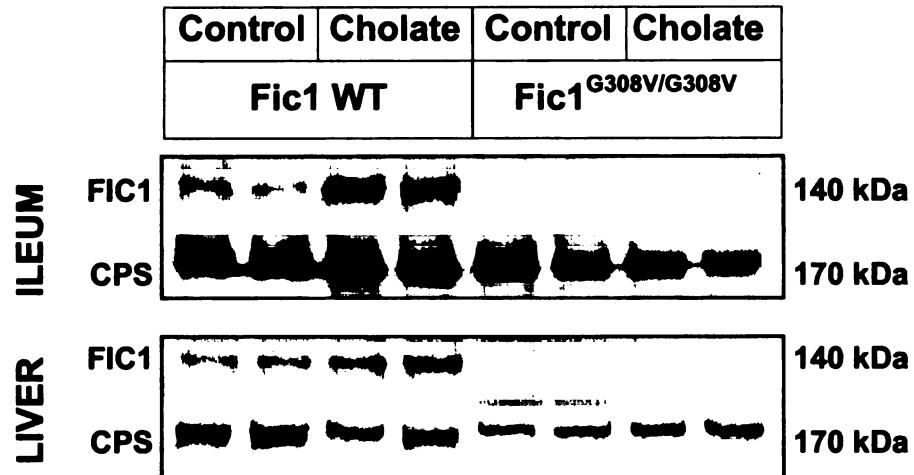


Figure 3a

Bile salt feeding upregulates Fic1 expression in wildtype mice.

Western Blot analysis of protein expression levels of Fic1 and CPS (carbamoylphosphate synthetase, used as a reference) in total protein extracts (15µg protein/lane) from the ileum and liver of wildtype and Fic1^{G308V/G308V} mice on control and 0.5% cholate diet (n=2).

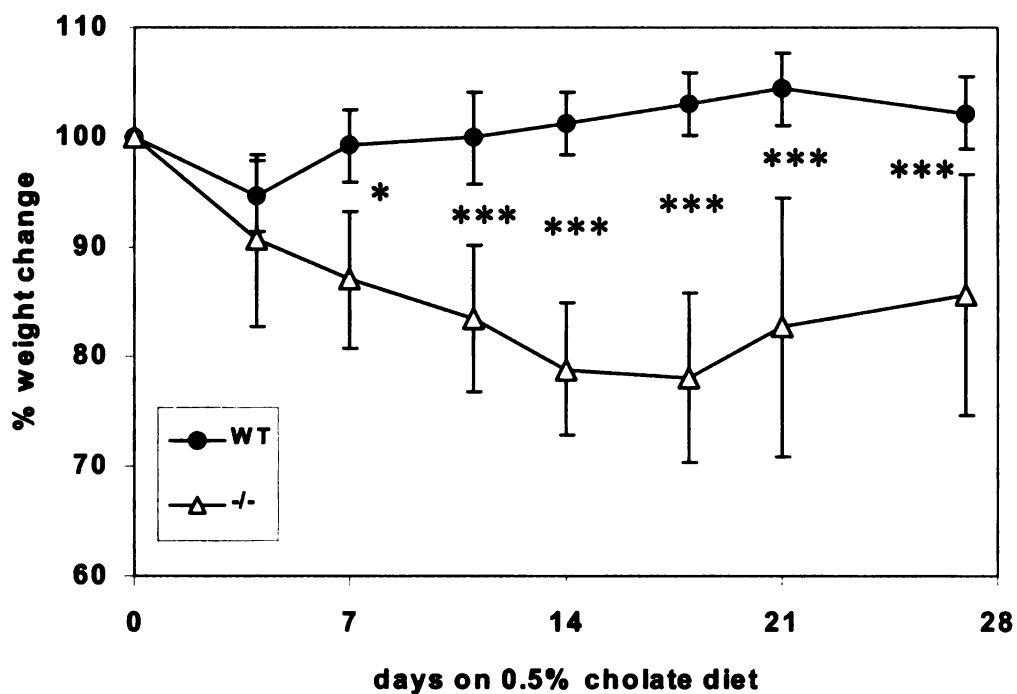


Figure 3b

Fic1^{G308V/G308V} mice lose weight on 0.5% cholate diet.

n=8, mouse weights normalized to weight on day 0. *p<0.0005, ***p<0.0001 (two-way repeated measures ANOVA).

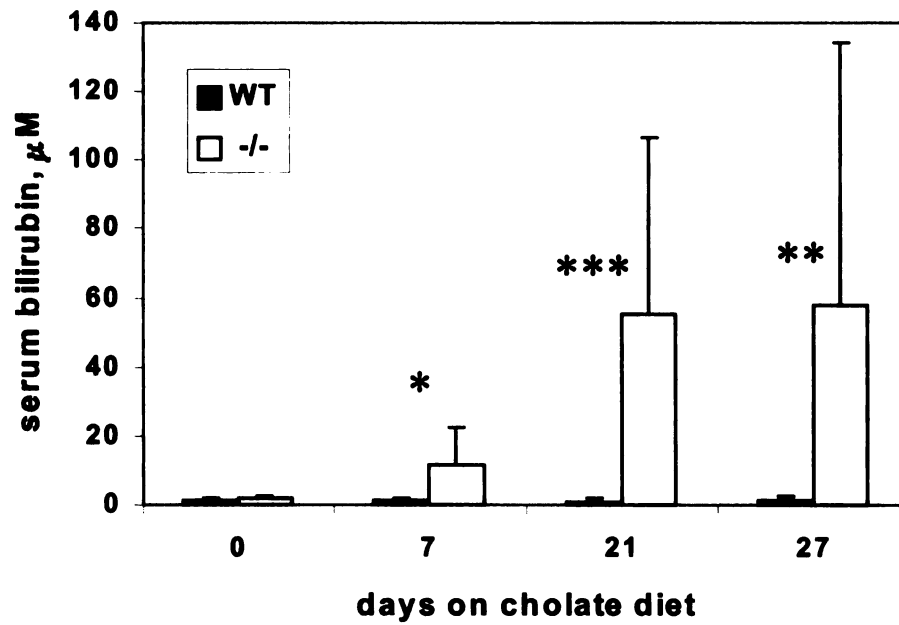


Figure 3c

Serum total bilirubin levels (μM) in wildtype (WT) and $\text{Fic1}^{\text{G308V/G308V}}$ ($-/-$) mice on 0.5% cholate diet.

$n=3-8$, * $p<0.05$, ** $p<0.005$, * $p<0.0001$ (two-way repeated measures ANOVA).**

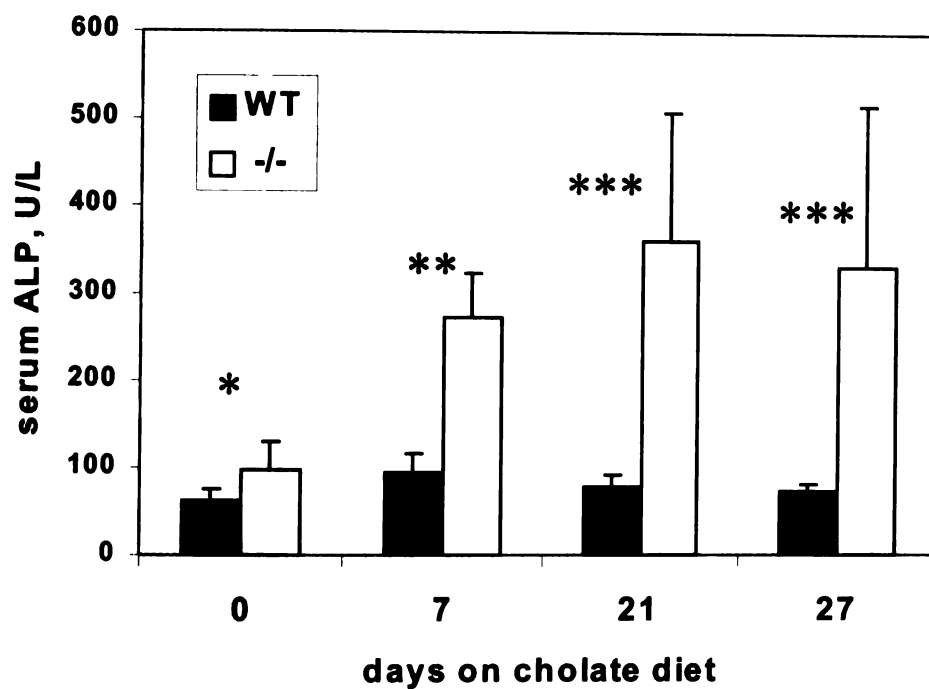


Figure 3d

Serum alkaline phosphatase activity (ALP, U/L) in wildtype and *Fic1*^{G308V/G308V} (-/-) mice on 0.5% cholate diet.
 n=3-8; *p<0.01, **p<0.0005, ***p<0.0001 (two-way repeated measures ANOVA).

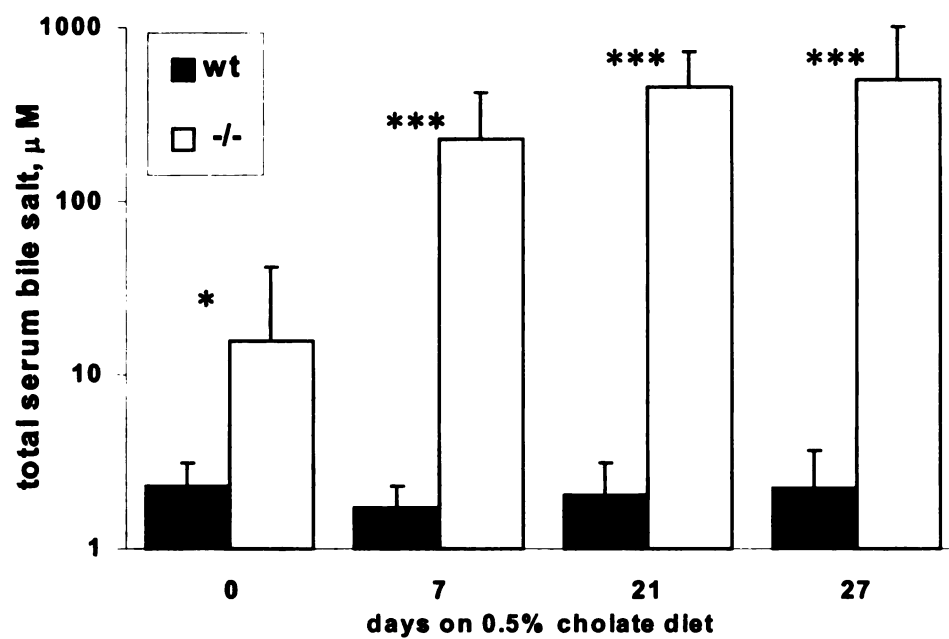


Figure 3e

Total serum bile salt levels (μM) in wildtype and *Fic1*^{G308V/G308V} mice (-/-) on 0.5% cholate diet.

n=6-8, *p<0.05, ***p<0.0001 (two-way repeated measures ANOVA).

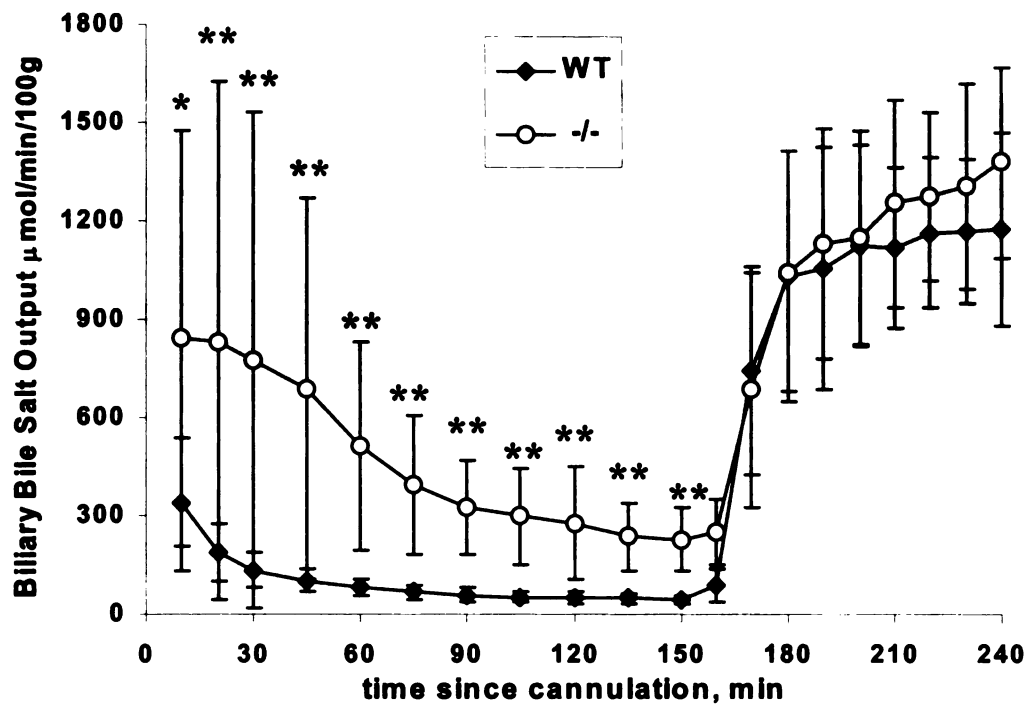


Figure 3f

Biliary bile salt output of wildtype (WT) and $Fic1^{G308V/G308V}$ mice (-/-) after 4 weeks on 0.5% cholate diet.

Gallbladders were cannulated at t=0 and bile collected continuously for 240 minutes.

Stepwise intravenous infusion of TUDC was started at t=150min.

N=6-8, *p<0.0005, **p<0.0001 (two-way repeated measures ANOVA).

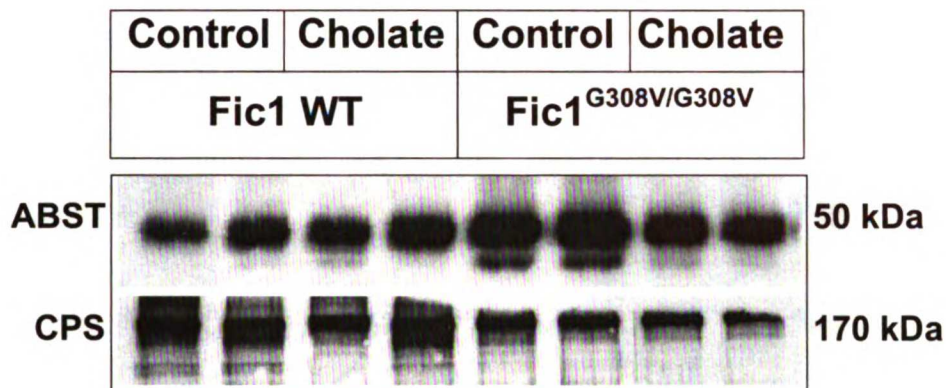
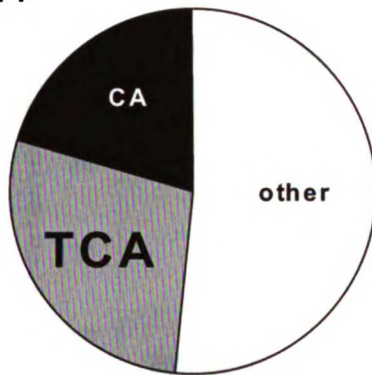


Figure 3g

Western Blot analysis of protein expression levels of ASBT and CPS.

Total protein extracts (15μg) from the ileum of wildtype and Fic1^{G308V/G308V} mice on control and 0.5% cholate diet were analyzed.

Fic1 WT



Fic1^{G308V/G308V}

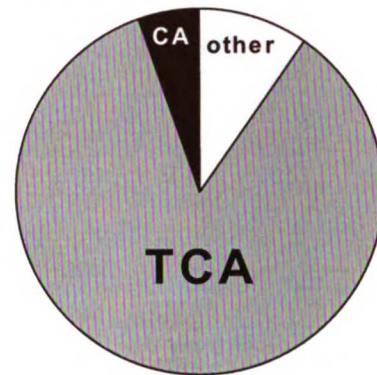


Figure 3h

Serum bile salt composition of wildtype (WT) and Fic1^{G308V/G308V} mice on 0.5% cholate diet.

% TCA: WT 28% ± 14%; Fic1^{G308V/G308V} 86% ± 11%; n=8; p<0.0003 (Mann-Whitney nonparametric test).

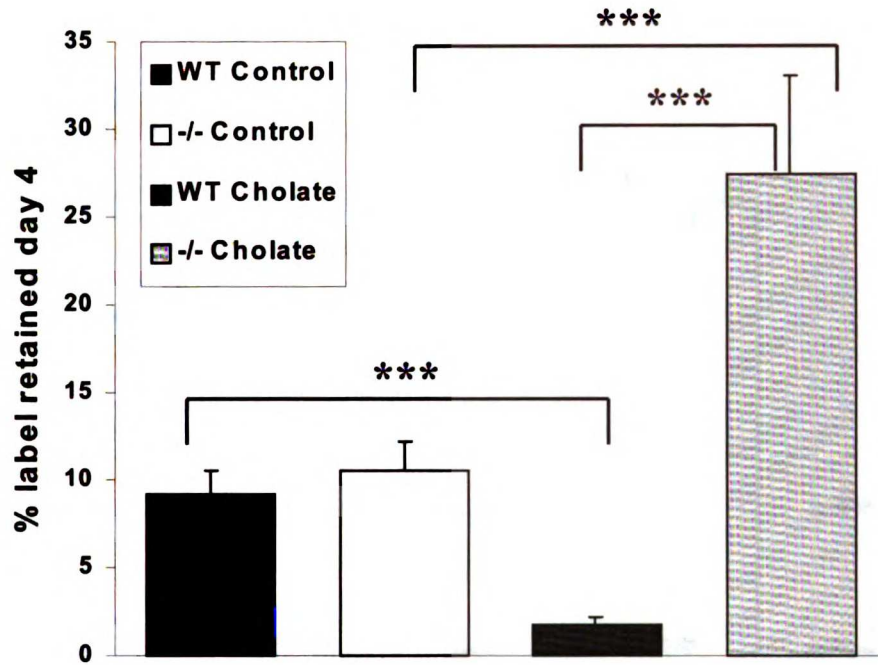


Figure 4a

Excess label retention in cholate-fed *Fic1*^{G308V/G308V} mice after oral administration of ³H-taurocholate.

Total ³H levels (bile, liver, intestines) in *Fic1* wildtype and *Fic1*^{G308V/G308V} mice on control and 0.5% cholate diet, 4 days after oral administration of $\approx 1 \mu\text{Ci}$ ³H-taurocholate; n=6, ***p<0.0001 (two-way ANOVA). Ileum = ileum with jejunum, large intestine = colon with cecum.

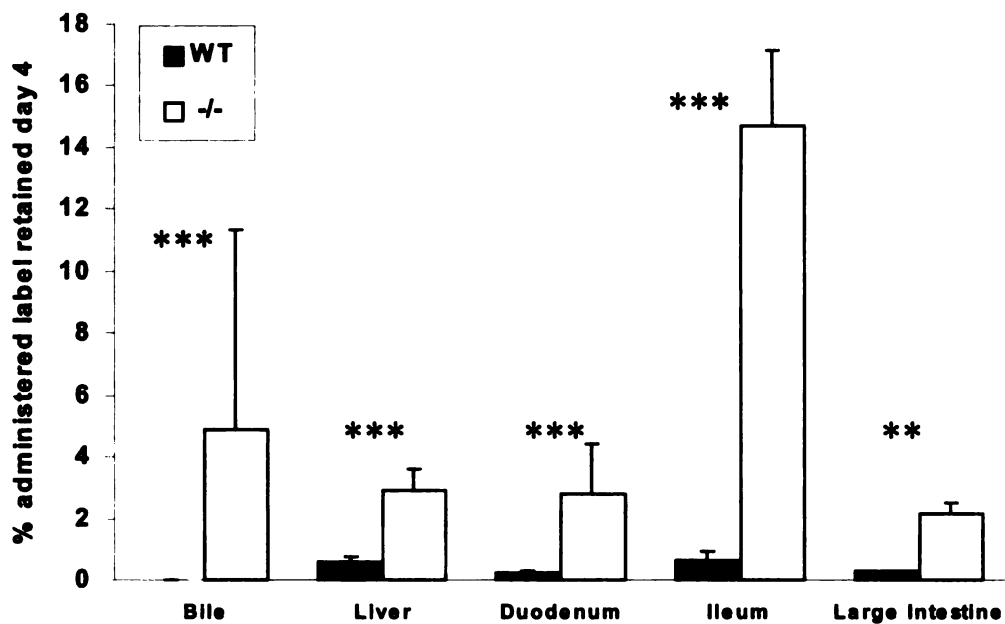


Figure 4b

Distribution of retained ^3H label in organs of the enterohepatic circulation in *Fic1* wildtype and *Fic1*^{G308V/G308V} mice on 0.5% cholate diet.

****p=0.0001, ***p<0.0001 (two-way repeated measures ANOVA).**

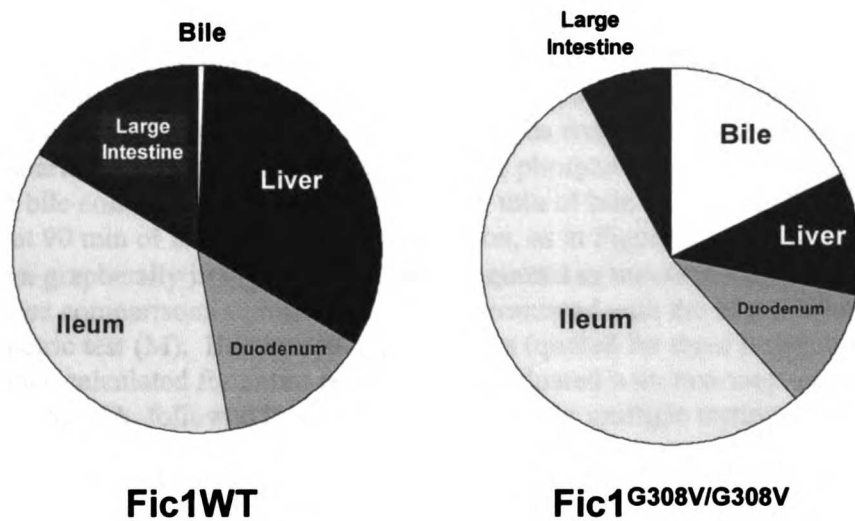


Figure 4c

Proportional distribution of retained ^3H in the organs of the enterohepatic circulation in *Fic1* wildtype and *Fic1*^{G308V/G308V} mice on 0.5% cholate diet. The same data as in Figure 4b is shown.

Table 1

Phenotypic characteristics of *Fic1*^{G308V/G308V} mice.

All data except for pup weights are from male mice of strain 129/SvJ, aged 3-7 months. Weight at weaning age was determined in pups (males and females combined) from mixed litters (heterozygote intercrosses) and normalized to average pup weight in litter to account for litter size variation. As there was no weight difference between wild-type and heterozygous pups ($p=0.3$), ^athese two genotypes were combined for the comparison. ASAT: aspartate aminotransferase; ALP: alkaline phosphatase; endog: endogenous, after 10 min of bile collection; dep: depleted, after 90 min of bile collection; TUDC: after a subsequent 90 min of intravenous TUDC infusion, as in Figure 2. Bile salt output data is also shown graphically in Figure 2. Values are quoted as means $\pm$ standard deviation. For pairwise comparisons significance (P) was evaluated with the Mann-Whitney nonparametric test (M). Bile secretion parameters (quoted for three timepoints, but ^bsignificance calculated for entire course) were evaluated with two-way repeated measures ANOVA, followed by Tukey's correction for multiple testing. Statistically significant P-values are shown in bold.

Parameter	Unit	WT (n)	<i>Fic1</i> ^{G308V/G308V} (n)	P
Weight at weaning	g/g mean pup weight in litter	1.01 $\pm$ 0.079 (73) ^a	0.97 $\pm$ 0.098 (34)	0.0001(M)
Hepatic function parameters				
Liver weight	g/100g body weight	3.8 $\pm$ 0.4 (14)	4.5 $\pm$ 0.5 (17)	0.008 (M)
Serum total bilirubin	μ M	0.6 $\pm$ 0.8 (12)	0.7 $\pm$ 1.1 (15)	0.8 (M)
Serum ASAT	U/L	92 $\pm$ 8 (12)	225 $\pm$ 179 (15)	0.006 (M)
Serum ALP	U/L	52 $\pm$ 16 (12)	69 $\pm$ 36 (15)	0.3 (M)
Total serum bile salt	μ M	2.0 $\pm$ 0.92 (10)	18.5 $\pm$ 29.8 (11)	0.0006(M)
Bile secretion parameters^b				
Biliary bile salt, endog.	mM	60.4 $\pm$ 15.3 (11)	74.7 $\pm$ 25.7 (11)	0.08
Biliary bile salt, 90 min dep	mM	23.1 $\pm$ 8.5 (8)	34.9 $\pm$ 8.9	0.08
Biliary bile salt, after TUDC	mM	147 $\pm$ 14.5 (3)	161 $\pm$ 8.9 (5)	0.08
Bile flow, endog.	μ L/min/100g	8.0 $\pm$ 1.6 (11)	12.4 $\pm$ 3.6 (11)	0.001
Bile flow, 90 min dep.	μ L/min/100g	3.0 $\pm$ 0.7 (8)	4.3 $\pm$ 1.3 (8)	0.001
Bile flow, after TUDC	μ L/min/100g	10.3 $\pm$ 1.0 (3)	12.8 $\pm$ 1.8 (3)	0.001
Bile salt output, endog.	μ mol/min/100g	498 $\pm$ 278 (11)	868 $\pm$ 320 (11)	0.0003
Bile salt output, 90 min dep.	μ mol/min/100g	69.5 $\pm$ 35.2 (8)	148 $\pm$ 75.6	0.0003
Bile salt output, after TUDC	μ mol/min/100g	1500 $\pm$ 22.3 (3)	1920 $\pm$ 338 (5)	0.0003
Phospholipid output, endog.	nmol/min/100g	58.5 $\pm$ 17.4 (8)	66.4 $\pm$ 27 (8)	0.03
Phospholipid output, 90 min dep.	nmol/min/100g	17.8 $\pm$ 6.7 (8)	31.3 $\pm$ 13.2 (8)	0.03
Phospholipid output, after TUDC	nmol/min/100g	53.4 $\pm$ 3.9 (3)	56.2 $\pm$ 8.4 (3)	0.03
Cholesterol output, endog.	nmol/min/100g	4.2 $\pm$ 1.0 (8)	5.9 $\pm$ 3.7 (8)	0.002
Cholesterol output, 90min dep.	nmol/min/100g	0.7 $\pm$ 0.4 (8)	1.8 $\pm$ 1.1 (8)	0.002
Cholesterol output, after TUDC	nmol/min/100g	2.6 $\pm$ 0.8 (3)	4.6 $\pm$ 0.9 (3)	0.002
Biliary bile salt composition				
% tauromuricholate	% total bile salt	39 $\pm$ 0.6% (4)	50 $\pm$ 4.2% (4)	0.03 (M)
% taurocholate	% total bile salt	59 $\pm$ 0.7% (4)	49 $\pm$ 4.9% (4)	0.03 (M)
% other	% total bile salt	2.6 $\pm$ 0.5% (4)	0.9 $\pm$ 0.7% (4)	0.03 (M)

CHAPTER 6

Further phenotypic studies of the *Fic1* mutant mice.

Two paradoxes are better than one; they may even suggest a solution.

Edward Teller

INTRODUCTION

Chapter 5 of this dissertation presents the key phenotypic findings in *Fic1*^{G308V/G308V} mutant mice. However, several interesting findings were left out of that manuscript for reasons of space, and also because they represent further investigations into the physiology of the mutant mice, that are at the time of writing still in pilot or preliminary stage. Some of these results are presented here as a prelude to discussion of the implications and future directions of this work, since they offer hints and suggestions for follow-up experiments.

A key issue that emerged from these experiments was the substantial impact of strain background on the *Fic1*^{G308V/G308V} mouse phenotype. It is well-known and much discussed in the literature that genetic differences between different strains of inbred laboratory mice can have large effects on physiological phenotypes of targeted mutant mice (e.g. Humphries *et al* 2001, Le Roy *et al* 2001, Fleming *et al* 2000). In addition, many such findings remain anecdotal and unreported, being seen as confounding factors, and if such issues are encountered, researchers try to standardize the strain in which each mutation is studied. This often requires backcrossing to preferred strains, as most mice with homologously targeted mutations originate in one of the several 129 substrains from which the most widely used embryonic stem cell lines were made. Mice of most 129 substrains breed poorly, which is another reason why mutations are moved off that background. In the field of enterohepatic physiology, cholesterol metabolism in particular has been found to differ substantially among many studied inbred mouse

strains (Wang *et al* 2001), C57B and, to a lesser degree, C57Bl/6 mice have been noted for their susceptibility to gallstone disease on high-fat lithogenic diet (Khanuja *et al* 1995, Paigen *et al* 2000), and C57Bl/6 mice appear to differ in their intestinal bile salt absorption from other strains (Bruno Steiger, unpublished observations). On the other hand, FVB/N mice, long used in transgenic mouse generation and thus well-characterized (Taketo *et al* 1991), are becoming one of the preferred strains for study of targeted mutations, in part for their excellent breeding performance. For example, the laboratory where this work was done maintains a colony of *Mdr2*^{-/-} mice in the FVB/N background (Oude Elferink *et al* 1995). The interaction of *Fic1* and *Mdr2* (phosphatidylcholine translocase) is of interest, since both are mutated in distinct syndromes of inherited cholestasis, PFIC1 and PFIC3 (Chapter 1). Therefore, the decision to move the *Fic1* mutation into the FVB/N background was made early on. However, even a partial backcross takes over a year, so preliminary experiments were performed when these mice reached 93.75% FVB/N.

Phenotype characterization experiments were initially performed in the very first *Fic1* homozygous mutant mice bred. These were of mixed background composed of 50% 129SvJae and 50% C57Bl/6, the descendants of the first matings in the breeding scheme, designed to test germline transmission of ES-cell derived genetic material by coat color. Subsequently, to examine the effects of the mutation in a genetically uniform, inbred background, and hopefully to minimize variation related to genetic modifier loci, homozygous mutant mice of a 129SvJ background were generated, and these mice were used for all of the experiments presented in Chapter 5 and in most other work. Thus,

some comparisons of the *Fic1* mutant phenotype can be made between the FVB/N and 129SvJ strains, and some hypotheses proposed as to the possible phenotypic contribution of the C57Bl/6 strain from the analysis of the mixed background mice.

A second area of investigation concerned the effect of bile duct ligation (BDL) on the *Fic1*^{G308V/G308V} mutant mouse phenotype. BDL is a surgical manipulation used since the 1970s as an experimental model of obstructive cholestasis in rats and mice; the flow of bile out of the liver and into the intestine is prevented. Concomitant with jaundice and serum bile salt buildup, bile duct-ligated rats (Williams *et al* 1981), and rats with drug induced cholestasis (Chisholm and Dolphin, 1996), accumulate high serum lipid levels in the form of an abnormal low density lipoprotein termed LpX (lipoprotein X), composed almost exclusively of phosphatidylcholine and cholesterol. This mirrors the appearance of LpX in human obstructive jaundice (Seidel *et al* 1969) and in some forms of both extrahepatic and intrahepatic cholestasis (e.g. in primary biliary cirrhosis [Kunkel & Ahrens 1949] and in Alagille syndrome [Davit-Spraul *et al* 1996]), but, interestingly, not in BRIC (Summerskill 1965) and PFIC1 (Clayton *et al* 1969), PFIC2 (Whittington *et al* 1994) and PFIC3 (Jacquemin *et al* 2001), where serum cholesterol levels tend to be low to normal. The accumulation of LpX in the plasma is hypothesized to result from regurgitation of biliary lipid vesicles at the basolateral (sinusoidal) surface of the cholestatic hepatocyte, though the exact mechanism of its formation remains unclear. Analysis of plasma lipoproteins in bile duct-ligated *Mdr2*^{-/-} mice showed that formation of LpX was prevented by loss of the phosphatidylcholine translocase (*Mdr2*), the protein required for secretion of lipid vesicle into bile (Oude Elferink *et al* 1998). In wild-type

mice after BDL, immunofluorescence localization revealed Mdr2 protein still present at the canalicular and not at the basolateral membrane, but more diffusely localized, suggesting its presence in subapical vesicles and not just in the canalicular membrane itself. These results indicated that the canalicular bile secretion machinery is implicated in LpX formation, and suggested a possible reason why all forms of PFIC share the characteristic low cholesterol and absence of LpX. All PFIC subtypes have defects in the canalicular bile secretion machinery that prevent correct lipid vesicle secretion into bile (whether by loss of phospholipid translocase or loss of bile salt secretion which facilitates vesicle formation) and may also prevent the intracellular formation of such vesicles and their subsequent release on the basolateral side of the hepatocyte, into the sinusoidal plasma, seen in obstructive forms of cholestasis (Oude Elferink & Groen 2002).

We decided to follow up these observations in Mdr2 mutant mice (a model of PFIC3) in the $\text{Fic1}^{\text{G308V/G308V}}$ mice (a model of PFIC1), to see if a similar inability to leach lipid into the plasma even upon severe cholestatic insult could be demonstrated. Since, unlike human patients, the $\text{Fic1}^{\text{G308V/G308V}}$ mice were not intrinsically cholestatic to any clinical degree, though did have elevated serum bile salt levels (Chapter 5), we also wanted to test whether they would be more or less severely affected than wild-types by the surgical imposition of extrahepatic cholestasis.

RESULTS AND DISCUSSION

1. The impact of strain background on $Fic1^{G308V/G308V}$ mouse phenotype

$Fic1^{G308V/G308V}$ mice of mixed 129SvJae/C57Bl/6 strain background have more pronounced depression of weight at weaning than $Fic1^{G308V/G308V}$ 129SvJ mice. The first $Fic1^{G308V/G308V}$ homozygous mutant mice we generated were of a mixed genetic background composed of 50% strain 129/SvJae (the strain from which the ES cells used to create the mice were derived) and 50% strain C57Bl/6 (this mixed background is abbreviated here as 129/B6). When the first $Fic1^{G308V/G308V}$ homozygous mutants were born in mixed-genotype litters from heterozygote intercrosses, we noticed that, around 3 weeks of age, *Fic1* homozygous mutant pups tended to be smaller and runted in appearance compared to their wildtype and heterozygous littermates. Weaning weight data was therefore collected from all mice born in mixed litters, and normalized to average pup weight in litter to account for differences in litter size (pups from smaller litters tend to be significantly bigger than pups from large litters) and genotype distribution. This analysis revealed that $Fic1^{G308V/G308V}$ pups tended to have 8% reduced weight at weaning ($p < 0.0005$), compared to their wildtype and heterozygous littermates, which were indistinguishable ($p > 0.5$, all comparisons: Mann-Whitney nonparametric test) (Figure 1A). The weight depression resolved with age in most (though not in all) *Fic1* mutant animals and was no longer significant in adult animals (10 weeks old).

Subsequently, mice of more uniform 129 background were generated (a 50/50 mixture of the ES cell-derived 129SvJae and the closest commercially available match, 129SvImJ; referred to as 129SvJ). Depression of weight at weaning (4%, $p < 0.001$) was also observed in *Fic1* mutant mice of this background, but it was less pronounced than in the mice that had the C57Bl/6 strain contribution (Figure 1B). The difference between *Fic1*^{G308V/G308V} mutant mice of the two different backgrounds approached statistical significance ($P = 0.06$).

Overall, the slight weight depression in mutant mice suggested a growth defect, possibly one associated with the change from milk diet to solid food. It is known that expression of several molecules important in the enterohepatic circulation of bile salts, including ASBT, the ileal bile acid transporter (Hwang & Henning 2001), and cholesterol 7 α -hydroxylase, the rate limiting enzyme in bile salt synthesis pathway (Schwartz *et al* 1996), changes around the age of weaning in rodents. It is possible that *Fic1* mutation results in partial dysregulation of this developmental program of expression.

Alternatively, the period of adaptation to a different diet with a dramatically different fat content, and the subsequent metabolic shifts, may stress an enterohepatic circulation compromised by the loss of *Fic1*.

The difference in mutant pup phenotypes between the two backgrounds suggested that the C57Bl/6 genetic component may aggravate the *Fic1*^{G308V/G308V} phenotype. Data from several other pilot experiments performed with the 129/B6 mice were also suggestive of such differences, but high enough numbers for statistical comparisons were not obtained.

Preliminary comparisons of very old (11-18 months of age) mutant and wildtype mice of the two backgrounds also revealed that the old $Fic1^{G308V/G308V}$ mice of the mixed background had a higher incidence of liver steatosis and liver neoplasia than both wildtype mice and mutant mice of the 129SvJ background. These data are still under analysis. Finally, in an anecdotal, but interesting observation, one *Fic1* mutant mouse (out of 6 examined) of the mixed 129/B6 background had pathological changes in the liver consistent with mild cholestasis, including yellow biliary pigment deposits, significant inflammation and fibrosis and bile duct proliferation. Although a sporadic liver problem or environmental insult unrelated to *Fic1* genotype cannot be ruled out in this single mouse, it is also possible that, since this mouse came from an F2 generation of the two-strain intercross, it could be homozygous for some C57Bl/6 derived modifier alleles, and thus represent the form the *Fic1* mutant phenotype might take on uniform C57Bl/6 background. These hints, as well as the published reports of differences in lipid metabolism in C57Bl/6 mice (compared to 129 and FVB/N strains), have inspired the decision to move the $Fic1^{G308V/G308V}$ mutation into the inbred C57Bl/6 background by performing a backcross, now underway (generation F3).

Pup mortality

No outward indications of disease, other than the mild and transient runting in weaning-age pups discussed above, were observed in the first generation nor in any subsequent generations of $Fic1^{G308V/G308V}$ mice of either 129/B6 or 129SvJ background born to heterozygous or homozygous mutant parents in the UCSF animal facility. A breeding colony of the 129SvJ mutant mice and control mice was subsequently established at the

animal facility at the Academic Medical Center, Amsterdam, where physiological characterization of the *Fic1* mutant mice was performed. Initial breeding in heterozygous parent couples yielded no unusual phenomena. Since the *Fic1*^{G308V/G308V} mice were found to be fertile, F3 generation wildtype and *Fic1* homozygous mutant breeding couples were subsequently established, in order to maximize production of animals for experiments. Much to our surprise, over the next few months, i.e. during the reproductive span of this generation of breeders, a high incidence of *Fic1* mutant pup mortality was observed – out of 271 *Fic1*^{G308V/G308V} pups born to *Fic1*^{G308V/G308V} mothers, 31% died before weaning, most late in the third week of life. These pups appeared normal at birth, but showed signs of sickness, diarrhea, ruffled fur, and dehydration at around 14 days of age and almost invariably died within 1 week of developing problems (Figure 2). Most litters had both severely affected and completely unaffected pups. The diarrhea and mortality was not seen in wildtype pups bred in the same room under identical conditions.

Serum collected from four of these extremely sick *Fic1*^{G308V/G308V} pups was clear, indicating that they did not have jaundice; measurement of serum bile salt also did not reveal any consistent elevation over levels seen in 2 healthy wildtype pups (Sick mutants: 25.2; 22.4; 6.8 μ M; healthy controls: 13.2; 5.7 μ M). Gross liver appearance and histopathology were normal. The stomach was full of milk, indicating that the mice had been feeding and had still been nursing; it was not clear whether they had started eating solid food yet. The intestines were empty of chyme and filled with mucus, consistent with diarrhea. Histopathological examination of the ileum of three sick *Fic1*^{G308V/G308V}

pups revealed abnormal hyperproliferation and cellular disorganization of the enteric mucosa, compared with the normal appearance of the ileum of healthy wildtype pups. The high pup mortality was perplexing given that no similar problems had been observed in previous breeding, and especially since there was such variability within litters composed of pups of the same genotype. However, the occurrence of the problem near weaning age and the presence of diarrhea and intestinal abnormalities appeared consistent with other features of the $Fic1^{G308V/G308V}$ phenotype, notably the weight depression at weaning and the intestinal defect demonstrated by cholate feeding (Chapter 5). We hypothesized that the problem was related to the changes in the expression program of bile salt transporters and metabolic enzymes that occur near weaning age in mice (Schwartz *et al* 1996, Hwang & Henning 2001). The pup mortality was probably also related to a maternal influence, since it had not been seen in $Fic1^{G308V/G308V}$ pups born to heterozygous mothers, and probably linked either to an environmental factor, such as composition of intestinal flora, or a genetic modifier effect (since these pups had background contribution from the 129SvJae and from the 129SvImJ strains), since the intra-litter variability was so high.

Ultimately, it was the last hypothesis that appeared most likely, since when the breeding couples were replaced, the effect disappeared, and all $Fic1^{G308V/G308V}$ pups survived to adulthood without developing diarrhea. The explanation for the transient effect may still be due to a temporary environmental influence such as an impurity in water, an ingredient in food or an undetected pathogen. However, the phenotype was most probably due to a genetic variant that was subsequently bred out of the population, since

almost all pups who developed the problem died, while only formerly healthy pups were used as the next generation of breeders. This variation may therefore be related to a genetic divergence between the SvJae and SvJmsJ substrains of 129, and probably represents one of four independent genetic modifiers of the $Fic1^{G308V/G308V}$ phenotype that we have observed to date. The others are the possible aggravation of phenotype in C57Bl/6 *Fic1* mutants, the resistance to TDC infusion in 129SvJ mutants, and the protection from cholate diet effects in FVB/N mutants, see below. These results suggest that genetic investigation of these putative modifier loci may be of great interest. Finally, whether the mortality/diarrhea phenotype in $Fic1^{G308V/G308V}$ pups was caused by an environmental insult or a genetic modifier, the fact that it produced such a strong intestinal phenotype lends further support to the primary role of the intestine in the etiology of FIC1 disease.

$Fic1^{G308V/G308V}$ mice of 129SvJ background are resistant to taurodeoxycholate-induced cholestasis

Hydrophobic bile salts such as lithocholate and deoxycholate are known to be cholestatic in humans and in rodents (Delzenne *et al* 1992). Intravenous infusion of deoxycholate results in an initial rise, and then a concentration-dependent decrease in bile flow and bile salt output in FVB/N and 129/Ola wild-type mice (Oude Elferink *et al*, unpublished observations). On the basis of our surprising findings of increased bile salt secretion and bile flow in *Fic1* mutant mice (Chapter 5), we speculated that, since the endogenous bile salt pool of mice is significantly more hydrophilic than the human pool, any bile salts that accumulate in $Fic1^{G308V/G308V}$ mice as a consequence of dysregulated intestinal absorption

are not sufficiently cholestatic to impair bile salt secretion. In order to investigate the response of the *Fic1*-deficient liver to an excess of hydrophobic bile salt, we cannulated the gallbladders of wild-type and *Fic1*^{G308V/G308V} mice, collected bile for 90-150 min as described previously (Chapter 5) in order to deplete the endogenous bile salt pool, and then infused taurodeoxycholate (TDC, 15mM in PBS) intravenously into the tail vein in stepwise rate increments, with a top infusion rate of 200nmol/min/25g.

This experiment was initially performed in the mixed background 129/B6 mice, in both males and in females (n=2 for each). Two variations of the infusion protocol with a 90 min or 150 min depletion phase, and a 2-step or 4-step increase in TDC infusion rate were used; both protocols arrive at the same final TDC infusion rate (see Methods).

Female mice of the FVB/N strain are known to have higher bile salt secretion rates than males (Van Nieuwerk *et al* 1997); we saw this pattern in our 129/B6 strain background as well (Figure 3a compared to 3b). In both sexes, comparison of wildtype and *Fic1*^{G308V/G308V} mutant animals indicated that the ability of *Fic1* mutants to secrete TDC was not impaired. In TDC-infused females, a clear plateau of bile salt output, followed by the onset of TDC-induced cholestasis and gradual reduction of bile salt secretion was observed in both genotypes; although endogenous and depleted bile salt secretion was higher in the *Fic1*^{G308V/G308V} mice, there was no clear difference between genotypes during the TDC-infusion phase (Figure 3a). The results in males were more difficult to interpret, but again no indication of any defect in TDC secretion was seen in the *Fic1* mutant animals (Figure 3b).

When this experiment was repeated in strain 129/SvJ animals, a striking difference between wild-type and *Fic1*^{G308V/G308V} animals manifested itself, though this trend took the opposite direction from that predicted by the hypothesis that increasing the hydrophobicity of the bile salt pool would impair biliary secretion in *Fic1* mutant mice. We found that wild-type mice of the 129/SvJ background were extremely susceptible to TDC-induced cholestasis (Figure 3c & d). Bile flow and bile salt output dropped precipitously and ultimately ceased entirely upon increasing TDC infusion rates, resulting in some cases in the death of these mice before the end of the experimental protocol, probably as a result of bile salt induced hemolysis (the serum was red). Such severe TDC-induced cholestasis had not been previously seen in the laboratory, although a strong reduction in bile salt secretion had also been noted in TDC-infused 129/Ola wild-type mice; this indicates that the 129/SvJ substrain is particularly sensitive to TDC.

Most strikingly, while the wild-type mice ceased bile secretion, the *Fic1*^{G308V/G308V} mice continued to secrete high amounts of bile salt even at the maximal TDC infusion rate, and appeared to be almost entirely resistant to the cholestatic effects of TDC (Figure 3c and d). Thus, we concluded that in the 129/SvJ background, *Fic1* loss is protective against TDC-induced cholestasis. Since this effect was not observed in the mixed 129/B6 background, this suggests that the responsible genetic variant acts in a recessive manner, as the 129/B6 mice were presumably heterozygous for a 129-derived allele and a C57Bl/6 derived allele of this unknown modifier locus. One heterozygous mouse was tested in the experiment (Figure 3c); its level of bile salt secretion upon TDC infusion

was intermediate between that seen in the wildtypes and homozygous mutants, suggesting that the protective effect of *Fic1* loss was gene-dosage dependent.

That *Fic1* mutant mice should not only have elevated bile salt secretion (instead of the cholestasis expected from the human PFIC1 phenotype), but also a higher resistance to the cholestatic effects of infused hydrophobic bile salt is the double paradox hinted at by the quotation that prefaces this chapter. The solution of this conundrum is not yet at hand, but some explanation may be attempted. First, since the bile salt composition of *Fic1* mutant mice appears to be slightly more hydrophilic (Chapter5, Table1), and the bile salt pool slightly enlarged, we hypothesized that the protection against TDC-induced cholestasis is due to a dilution effect, i.e. that the impact of the same amount of TDC as infused into wild-types is lessened in *Fic1*^{G308V/G308V} mutants, due to the protective effect of higher amounts of hydrophilic endogenous bile salts still present in the mutant mice even after 90 or 150min of bile salt depletion. To test this hypothesis, we analyzed biliary bile salt composition of bile aliquots collected prior to and during the TDC infusion, and compared both the absolute TDC concentration and % contribution of TDC in the bile of *Fic1*^{G308V/G308V} and control mice (n=2). Although the maximal level of TDC reached, both in concentration terms and in percentage contribution to the bile salt pool, was higher in the wildtype mice that had become cholestatic, than in the *Fic1* mutants which had not, the *Fic1* mutants did eventually reach TDC levels at which wildtype mice had already become profoundly cholestatic, without any signs of cholestasis (Figure 4). It therefore appeared that *Fic1* mutant mice were able to tolerate equivalent amounts of TDC in their bile without becoming cholestatic. The mutant mice did retain higher

amounts of endogenous hydrophilic bile acids at the start of the TDC infusion, but those amounts were very low, compared to the amount of TDC infused, so it seems unlikely that such a minor contribution of hydrophilic bile salt could have provided sufficient dilution and protection to prevent TDC-induced cholestasis. Therefore, other mechanisms whereby loss of *Fic1* is protective in the TDC-challenged mouse liver must be considered.

One other significant difference between the wildtype and mutant mice became apparent from the bile composition analysis. TDC-infusion appeared to stimulate secretion of biliary taurocholate (TC), in *Fic1*^{G308V/G308V} mice, but not in wildtype mice (Figure 4e). Since this effect was specific to taurocholate and not taumuricholate (Figure 4c), it may represent the greater ability of *Fic1* mutant mice to immediately hydroxylate the TDC (producing TC) in the liver. One possibility, first suggested by the enhanced hydrophilicity of the bile salt pool in *Fic1*^{G308V/G308V} mutant mice, is that *Fic1* loss results in an upregulation of hydroxylation pathways in the liver, as a protective mechanism for coping with deranged bile salt influx from the ileum (Chapter 5). Intravenously infused TDC could therefore become a substrate for upregulated hydroxylation, leading to the observed increase in taurocholate output.

Bile salt depletion-infusion experiments such as those done with TUDC in Chapter 5, and with TDC as reported here, examine only the uptake of bile salts into the liver and subsequent secretion of bile salts into bile in the context of an artificially interrupted enterohepatic circulation. Thus, these experiments, while invaluable for assessment of

hepatic function, do not account for the interplay of intestinal absorption and biliary secretion that is at the center of bile salt homeostasis. Furthermore, IV infusion for a maximum duration of 2½ hours allows only the measurement of acute response to bile salt loads, and of the hepatic bile salt transport capacity, but not of the chronic effects of bile salt challenge and accumulation such as those seen with feeding experiments. The TDC infusion experiment demonstrates that no intrinsic bile salt uptake or secretion defect, whether general or specific for hydrophobic bile salts, exists in the *Fic1*-deficient mouse liver. However, this does not rule out the possibility that buildup of cholestatic bile salts over time and the resulting chronic hepatotoxic insult would lead to liver damage and secondary cholestasis. This latter question is of great interest and merits investigation by oral or dietary administration of TDC to animals over a period of several days. Finally, the striking difference between *Fic1*^{G308V/G308V} and control mice in their response to infused TDC, which occurred when intestinal mechanisms were excluded from consideration, supports the notion that *Fic1* mutation affects bile salt handling not only in the *Fic1*-deficient intestine, but also in the liver. The cholate diet experiments (Chapter 5) demonstrated convincingly that the intestinal defect is primary in *Fic1* deficient mice, nonetheless, it is important to keep in mind this evidence of *Fic1* loss altering hepatic bile salt handling as well, albeit only in a strain-specific manner.

***Fic1*^{G308V/G308V} mice of the FVB/N genetic background are less sensitive to the effects of cholate diet feeding than mutant mice of the 129/SvJ background**

Mice of the various 129 substrains do not breed very well, and while the breeding performance of the *Fic1*^{G308V/G308V} strain 129/SvJ mice was not as bad as in e.g. 129/Ola

(once the pup mortality problem resolved spontaneously, see above), we did have some difficulty assembling sufficient cohorts of mice for experiments. Furthermore, we had an interest in investigating the impact of *Fic1* mutation on the *Mdr2*^{-/-} phenotype, and the latter mice were readily available in the FVB/N background, the biliary physiology of which had been well-characterized in the laboratory where these physiological experiments were performed (Oude Elferink *et al* 1995, van Nieuwerk *et al* 1997). We knew from comparing our results with published and unpublished data from strain FVB/N mice, that differences in bile secretion existed between these two strains, and thus intercrossing the mutations in different backgrounds was likely to produce too many confounding independently segregating genetic differences (which it did – see below). Furthermore, the extremely high experimental variability in the *Fic1* mutant mice (but not the wildtype mice) of the 129SvJ strain led us to suspect that there was a significant genetic difference even between the two very closely related substrains (129SvJae and 129SvImJ) that contributed to the genetic background of our mice, which had not been formally backcrossed onto the recipient commercially available 129SvImJ strain. It was therefore important to generate *Fic1*^{G308V/G308V} mice of a truly inbred strain, without any independently segregating modifier loci, in order to verify whether the variability was an intrinsic feature of the *Fic1*^{G308V/G308V} phenotype, or whether it was simply the unfortunate result of background strain variation. All these considerations contributed to our decision to backcross the *Fic1* mutation onto the FVB/N background.

A complete backcross takes around 18 months; so in order to test the impact of FVB/N strain background on *Fic1*^{G308V/G308V} phenotype sooner, we intercrossed heterozygous

mice at the F4 generation to produce $Fic1^{G308V/G308V}$ mutants with, on average, 93.75% FVB/N strain contribution. Although these mice still carried an average 6.25% contribution from the donor 129SvJ background, they had been through 4 generations of backcrossing to the recipient FVB/N strain, followed by a single intercross, so their 129 contribution was dispersed and unlikely to be homozygous.

The FVB/N $Fic1^{G308V/G308V}$ mutant mice were outwardly normal, like mice of all previous backgrounds examined. In order to investigate the effect of the FVB/N background on phenotype, we fed 0.5% cholate diet to these animals, replicating the experiment that had produced the most striking evidence of the impact of $Fic1$ loss on the enterohepatic circulation of bile salts (Chapter 5).

The disappointing result of the experiment was that in the FVB/N background the $Fic1^{G308V/G308V}$ mutant phenotype was considerably milder than in the 129/SvJ background. Neither weight loss (Figure 5a) nor serum bile salt accumulation were very dramatic in the FVB/N $Fic1^{G308V/G308V}$ mice (Figure 5b), although the expected trends could nevertheless be perceived. Most confoundingly, the experimental variability was still extremely high; the backcrossing had not reduced it. Interestingly, FVB/N wildtype mice seemed slightly more sensitive to cholate feeding than 129SvJ wildtype mice had been, which further obscured the findings in the mutant mice.

That the strain background had an impact on the severity of the phenotype was less surprising than the persistence of such extreme variability in a nearly inbred genetic

background. We concluded that variability is an intrinsic aspect of the phenotype caused by Fic1 loss in mice. This may be explained by the fact that Fic1 loss probably impacts regulation of intestinal absorption and biliary secretion indirectly. Regulatory effects within the enterohepatic circulation are likely to be subtle, cumulative and modulated by multiple feedback loops; such iterative processes are thus inherently more subject to variability than direct transport processes. Furthermore, if Fic1 acts as an aminophospholipid flippase and has a role in maintenance of the lipid homeostasis of the plasma membrane (Ujhazy *et al* 2001), it can be anticipated that Fic1 loss leads to lipid imbalances in the membrane that would have a certain random component, as plasma lipids are at least in part able to move freely, and thus the effect of these lipid imbalances on transporter activation and localization within the plasma membrane (Asamoto *et al* 2001) might be quite variable. Finally, environmental influences may also play a role in the severity of the consequences of Fic1 loss, and effects such as individual variations in food intake and enteric bacterial flora are not easily controlled for in experimental investigation.

Generation of Fic1Mdr2 double-mutant mice

Since backcrossing of the Fic1^{G308V/G308V} mutation onto the FVB/N background took a long time, we tried a “quick and dirty” test of any interaction between the Fic1^{G308V/G308V} and Mdr2 (phosphatidylcholine translocase) knockout phenotypes by intercrossing the two mutations in the available strain backgrounds. This resulted in the creation of Fic1^{G308V/G308V} Mdr2^{-/-} double-knockout mice in an independently segregating mixture of three backgrounds: 50% FVB/N, 25% 129SvJae and 25% C57Bl/6.

We compared serum bilirubin and liver enzyme levels between double-knockout and *Mdr2*^{-/-} animals to see if the *Fic1* mutation had any impact on the severity of the *Mdr2* knockout phenotype (which includes mild jaundice and elevation of liver serum enzymes [Smit *et al* 1993]), but no clear pattern emerged. We also investigated biliary secretion by cannulating the gallbladder, collecting bile and analyzing the bile salt, phospholipid and cholesterol output of two *Fic1*^{-/-}*Mdr2*^{-/-} and *Fic1*wt*Mdr2*^{-/-} mice (Figure 6).

Unfortunately, one of these mice was misgenotyped and an *Mdr2*^{+/-} heterozygote (and also appeared to have the 129SvJ wild-type sensitivity to TDC), so no real conclusions could be drawn from of this experiment, since we could only compare the two tested double knockout mice to one *Fic1*wt*Mdr2*^{-/-}. This comparison suggested that some cholesterol and phospholipid secretion was possible upon TDC infusion (but not endogenously) in *Mdr2*^{-/-} mice that were also homozygous for the *Fic1* mutation, while only traces of phospholipid could be eluted into bile in *Mdr2*^{-/-} mice wildtype for *Fic1* (Ronald Oude Elferink, personal communication). This investigation bears repeating (once double knockouts in the FVB/N background are available) since substantiated evidence of phospholipid secretion in the absence of *Mdr2* would suggest that *Fic1* loss alters the characteristics of the plasma membrane making it possible for TDC secretion into bile to elute some phospholipid and cholesterol into bile without any phospholipid translocase activity. This would indirectly support the hypothesis of *Fic1* involvement in maintenance of membrane lipid composition (Ujhazy *et al* 2001).

2. Variable levels of LpX in plasma of bile-duct ligated Fic1^{G308V/G308V} mice

The final experimental investigation undertaken in Fic1^{G308V/G308V} mutant mice aimed to examine the influence of Fic1 loss on the physiological consequences of bile duct ligation, i.e. surgically induced cholestasis. As discussed above, patients with obstructive jaundice, and bile duct ligated mice accumulate high levels of lipid in the plasma, in the form of the abnormal lipoprotein LpX, but formation of LpX is entirely prevented in Mdr2^{-/-} mice, implicating the canalicular bile secretion machinery, particularly the phosphatidylcholine translocase in LpX formation.

We performed bile duct ligation, collected plasma and organs for analysis at day 5 post surgery (or earlier if mouse condition was very bad) and assayed total plasma cholesterol levels in Fic1^{G308V/G308V} and wildtype mice of the 129SvJ background. Plasma was fractionated on an FPLC column and free and total cholesterol and phospholipid concentrations determined in fractions to create a lipoprotein density profile for bile duct ligated and control mice, as previously described for Mdr2^{-/-} mice (Oude Elferink *et al* 1998).

Total plasma bilirubin levels mirrored the substantial jaundice (yellow plasma and peritoneal membranes, dark urine and pale feces) observed in all wildtype and Fic1^{G308V/G308V} mice following bile duct ligation, and confirmed the induction of cholestasis (Figure 7a).

Cholesterol levels were low in control animals of both genotypes, confirming that *Fic1* mutant mice, like PFIC1 patients, do not have elevated serum cholesterol levels (Figure 7b).

However, while cholesterol levels in wildtype BDL animals were high, as expected,

Fic1^{G308V/G308V} animals could be divided into two groups depending on whether or not their plasma cholesterol was elevated following BDL: 6 animals had cholesterol levels indistinguishable from those of the control group, while 3 animals had elevated levels close to those seen in wildtype BDL animals (Figure 7b). Furthermore, the overall condition of these latter 3 animals appeared worse than that of their counterparts which had normal cholesterol levels.

Lipoprotein fractionation showed that control wildtype (n=4) and Fic1^{G308V/G308V} (n=5) mice had similar lipoprotein profiles featuring a small HDL peak around fraction 30 and traces of LDL around fraction 23. This indicated that there are no significant differences in endogenous lipoprotein profiles between wildtype and Fic1^{G308V/G308V} mice (Figure 8a, all 4 wildtype and all 5 Fic1^{G308V/G308V} control mice are shown; lipoproteins are represented by phospholipid concentration in FPLC fractions; similar profiles were obtained by measurement of free and total cholesterol in the fractions).

As expected, 5/5 of the wildtype bile-duct ligated mice tested had large amounts of very low density lipoprotein in their plasma, previously demonstrated to represent the LpX fraction (Oude Elferink *et al* 1998). This was shown by the very high cholesterol and phospholipid concentrations in fractions 14-25 (Figure 8c, three representative WT-BDL mice are shown out of 5 tested.)

The results from the bile duct ligated Fic1^{G308V/G308V} mice correlated with the perplexing disparity in total plasma cholesterol levels. Plasma samples from 7 jaundiced, cholestatic

Fic1^{G308V/G308V} mice that underwent successful BDL treatment, were tested. 4/7 of these Fic1^{G308V/G308V}-BDL mice did not form significant LpX in the plasma, which appeared to support the hypothesis, suggested by low cholesterol levels in PFIC1 patients, that Fic1^{G308V/G308V} mutant mice, like Mdr2^{-/-} mice, do not form LpX during cholestasis (Figure 8c). However, the other 3/7 Fic1^{G308V/G308V} BDL mice tested did have high levels of LpX in their plasma, as suggested by the prior total cholesterol measurements. These mice had done poorly after the BDL surgery; one of the mice had to be sacrificed 2 days after BDL instead of 5.

Furthermore, close examination of the lipid profiles of the 4 Fic1^{G308V/G308V} mice which did not form LpX revealed that small but discernible amounts of lipid were eluting in the LpX fraction range in at least 2 of these mice (Figure 8b), suggesting that these mice were showing early signs of future LpX formation as well.

Finally, histopathological analysis revealed severe liver damage in the Fic1^{G308V/G308V} BDL mice that had become sick (and generated LpX) with areas of focal necrosis. Liver damage this extensive was not observed in the wildtype BDL mice nor in the Fic1^{G308V/G308V} BDL mice that had tolerated BDL well and not formed LpX .

These observations suggested the hypothesis that the apparent disparity between the two kinds of response to BDL seen in Fic1^{G308V/G308V} mice was due to the time-dependent nature of the physiological insult, further influenced by the general variability seen in the Fic1^{G308V/G308V} phenotype. We hypothesized that in the initial phases of surgically induced

cholestasis, $Fic1^{G308V/G308V}$ mice resist formation of LpX, but as the duration of the insult increases, they succumb to massive liver damage concomitant with release of large quantities of LpX into the plasma. The finding that at days 2-5 some of the mice had deteriorated and were already in this postulated second phase, while most were still resistant, suggests individual variation in the time it takes for each mouse to develop the degree of cholestasis that causes the transition. This variation might be directly linked to the endogenous bile salt output rate and bile flow of the $Fic1^{G308V/G308V}$ mice, which varied widely (as shown by the large error bars in the early time-points in Figure 2, Chapter 5).

We intend to test this hypothesis by performing bile duct ligation and analyzing plasma lipid either within 36 hours of surgery (which is long enough to induce cholestasis and generate LpX in wildtype mice, Oude Elferink *et al* 1998) or after 7 days. We expect that at the early time point, none of the $Fic1^{G308V/G308V}$ mutant mice will be secreting LpX, and at 7 days (or at onset of severe illness, whichever is sooner) all of them will.

The mechanism of this time-dependent response to surgical cholestasis in *Fic1*-deficient mice is unclear. Bile analysis (Chapter 5, Table 1) showed that $Fic1^{G308V/G308V}$ mice generally manifest slightly enhanced biliary phospholipid and cholesterol levels, which correlate with their enhanced bile salt output. They therefore have no intrinsic defect in biliary lipid secretion. However, if *Fic1* has a lipid translocase activity that is specific for aminophospholipids (Ujhazy *et al* 2001, Harris *et al* 2001), loss of such activity may not manifest itself in altered biliary lipid composition, as bile contains mostly phosphatidylcholine and very little aminophospholipid. Since the function of *Fic1* is

most likely to be regulatory with regard to the activity of canalicular biliary transporters such as Mdr2^{-/-}, it may be that upon bile duct ligation, loss of Fic1 function could have contrasting effects depending on the degree of overall cholestasis and membrane damage. One possible mechanistic scenario is as follows. In the initial, LpX-free phase in *Fic1* mutant mice with surgical cholestasis, Mdr2 protein remains in the canalicular membrane, as loss of Fic1 interferes with membrane function including vesicle fusion and internalization events, and thus prevents the withdrawal of Mdr2-bearing lipid vesicles to the cytoplasm that occurs in wildtype BDL mice (Oude Elferink *et al* 1998). However, upon increasing cholestasis, the plasma membrane may become so compromised that either lipid vesicles enter into the sinusoidal blood as a result of membrane failure and cell death, or withdrawal of lipid vesicles into the cytoplasm happens independently of normal mechanisms, perhaps as a physical consequence of high lipid burden. Thus the first phase of the response could be seen as failure of Mdr2 regulation; but the second phase more as a nonspecific severe insult to hepatocyte cell and plasma membrane integrity, as evidenced by the necrosis seen on histopathological examination.

Overall, both the TDC infusion experiments and the bile duct ligation experiment demonstrate that Fic1 loss does have an impact on the murine liver, but this impact is difficult to dissect, due to its most likely indirect and regulatory nature.

METHODS

Mouse strains

The mixed background $\text{Fic1}^{\text{G308V/G308V}}$ mice used in some experiments were 50% 129SvJae (ES-cell derived genetic material passed through chimeric males to offspring by germline transmission) and 50% C57Bl/6, and represented the F2 generation obtained after intercrossing F1 mutant heterozygotes born to C57Bl6 mothers mated to chimeric founders.

The animals referred to as of 129SvJ background were in a 50/50 mixture of the original strain 129SvJae ES cell genetic contribution and the closest commercially available strain, 129SvImJ (Jackson Laboratories, Bar Harbor, Maine), and represented F-2 and F-3 generations, descended from the F1 heterozygotes born to 129SvImJ females mated to chimeric founders. Since, in the interest of time, a backcross to the commercial 129 substrain was not performed, the experimental animals were the product of one or two intercrosses (F-1 or F-1 and F-2) and therefore were randomly segregating the loci that are different between these two 129Sv substrains. This probably contributed to the phenotypic variability demonstrated by the animals.

To move the $\text{Fic1}^{\text{G308V/G308V}}$ mutation into the FVB/N background, animals were backcrossed for 5 generations to achieve over 96% FVB/N contribution. The preliminary experiments presented here were performed with an intermediate stage of the backcross, with animals that were on average 93.75% FVB/N and 6.25% 129/SvJ.

Mdr2^{-/-} animals in the FVB/N strain background were obtained from the breeding colony at the AMC Amsterdam (from Ronald Oude Elferink) and intercrossed with Fic1^{G308V/G308V} a mutant of mixed 129/B6 background to produce double heterozygous animals, which were further intercrossed to produce double knockout animals.

Animal experiments

Age- and background strain-matched Fic1^{G308V/G308V} and wild-type male mice were used for all experiments except the bile duct ligations, where age matched female mice were used (these were pilot experiments and not enough males were available at the time); one TDC infusion experiment was also done with females. 0.5% cholate diet feeding was performed as described in Chapter 5. All animal experiments were performed by trained staff under approved protocols of the Academic Medical Center, Amsterdam Committee for Animal Research.

TDC infusion

Gallbladder cannulation, bile collection and intravenous bile salt infusion were performed as previously described (Chapter 5).

Taurodeoxycholate (TDC) intravenous infusion experiments were performed according to two slightly different protocols. In the first, gallbladders were cannulated, bile collected in aliquots for 150 min (depletion phase) and TDC (15mM TDC [Sigma] in PBS pH 7.4) infused intravenously in the tail vein for the remainder of the experiment, in two infusion rate increments: t=150-180, 150 nmol/min TDC; t=180-240min: 200

nmol/min TDC. These infusion rates represent the top two rates of previously tested 4-step infusion protocols (ref). In the second protocol, to better understand the relationship between TDC concentration and bile secretion, a 4-step TDC infusion protocol was followed, after a shorter 90 min depletion period: t=90-120min: 50 nmol/min TDC; t=120-150 min: 100 nmol/min TDC; t=150-180 min: 150 nmol/min TDC; t=180-240 min: 200 nmol/min (all quoted for a 25g mouse and adjusted to individual mouse weight). The highest infusion rate was the same in both protocols; a total of 1.1 and 1.4 mL of 15mM TDC was infused respectively. Total time of experiment (time since gallbladder cannulation) was the same in both protocols (4 hours). In both experiments the full infusion protocol was administered to Fc1^{G308V/G308V} mice; with the two-step TDC infusion protocol, some wild-type mice stopped secreting bile and died late in the infusion phase, and had to be removed from the experiment.

Serum and biliary bile salts were determined by HPLC-MS.

Bile duct ligation (BDL)

Bile duct ligation was performed as previously described (Oude Elferink *et al* 1998).

Briefly, animals were anesthetized by intraperitoneal injection of 1mL/kg Hypnorm (fentanyl/fluanisone) and 10mg/kg diazepam. The abdomen was opened, the common bile duct ligated in two places, the gallbladder removed and a section of the bile duct between two ligatures excised to prevent re-attachment, and the abdomen reclosed.

Animals were monitored daily, sacrificed 5 days after the BDL surgery and EDTA blood

and liver collected for analysis. In a few cases the deteriorating condition of the mice necessitated earlier euthanasia.

Plasma lipid analysis

EDTA blood was obtained from anesthetized animals by heart puncture, and immediately centrifuged to obtain plasma. Plasma samples were stored at 4°C and used within 24 hours. Fast performance liquid chromatography (FPLC) on a Superose 6B column (Pharmacia, Uppsala, Sweden) was performed on 200µL plasma samples as previously described (Oude Elferink *et al* 1998) to fractionate the lipoproteins by density. 500µL fractions were collected and assayed for total cholesterol (free + esterified) and phospholipid (phosphatidyl choline) content enzymatically.

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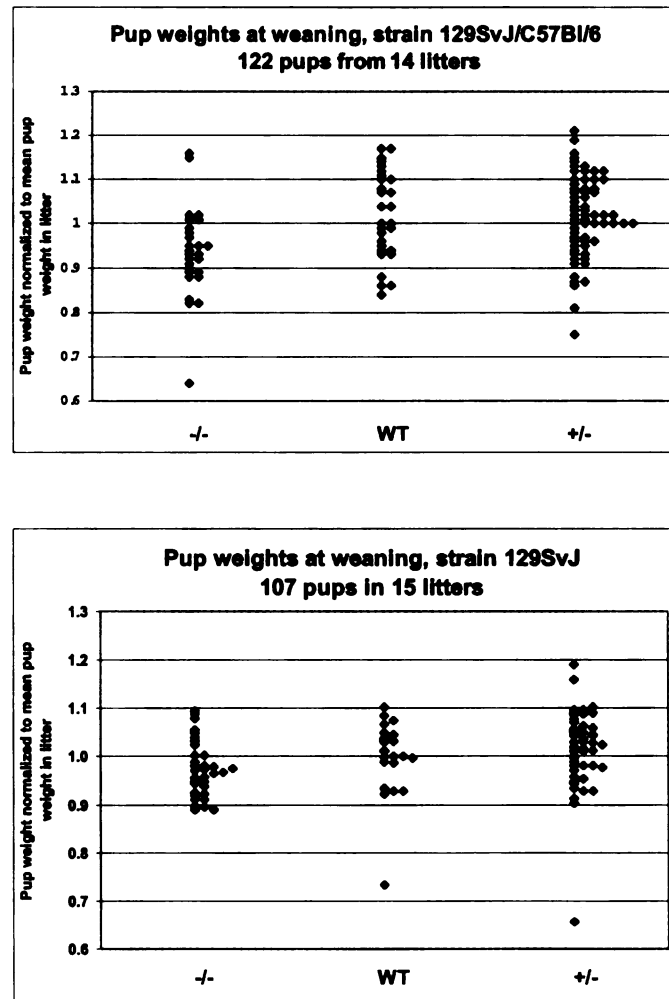


Figure 1

Effect of *Fic1* genotype and strain background on pup weight at weaning.

Pup weights at weaning age in *Fic1*^{G308V/G308V} (-/-), wildtype (WT) and heterozygous (+/-) mice in two strain backgrounds: mixed (50% 129/SvJ & 50% C57Bl/6, upper panel) and 129/SvJ (lower panel). Pup weights were normalized to average pup weight in litter. All pups came from mixed litters from (+/-) parents. **p<0.0005, *p<0.001 (-/- vs. WT & +/- - combined). There was no difference between WT and +/- pups in either strain (p>0.5). p<0.06 between -/- animals of the two strains. All comparisons evaluated by Mann-Whitney nonparametric test.

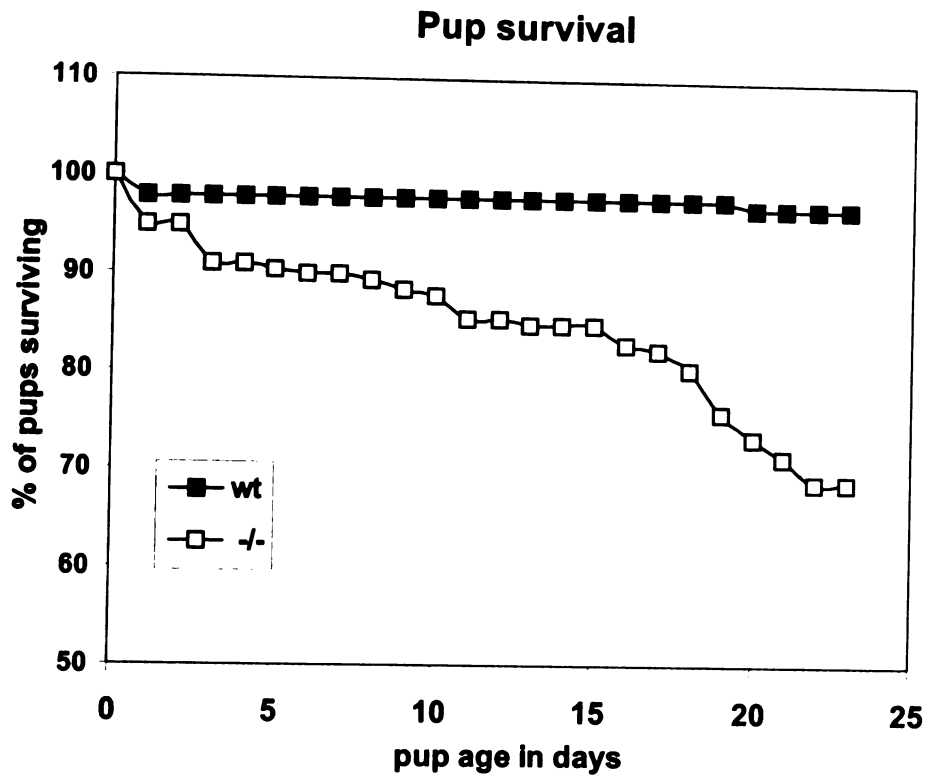


Figure 2

Increased mortality in $Fic1^{G308V/G308V}$ pups born to $Fic1^{G308V/G308V}$ mothers.
 Data includes 271 wildtype (wt) pups born to 7 wildtype breeding couples and 199 mutant (-/-) pups born to 10 $Fic1^{G308V/G308V}$ breeding couples of the 129SvJ strain. When the breeding couples were replaced, this phenomenon disappeared and pup survival was comparable between the two genotypes.

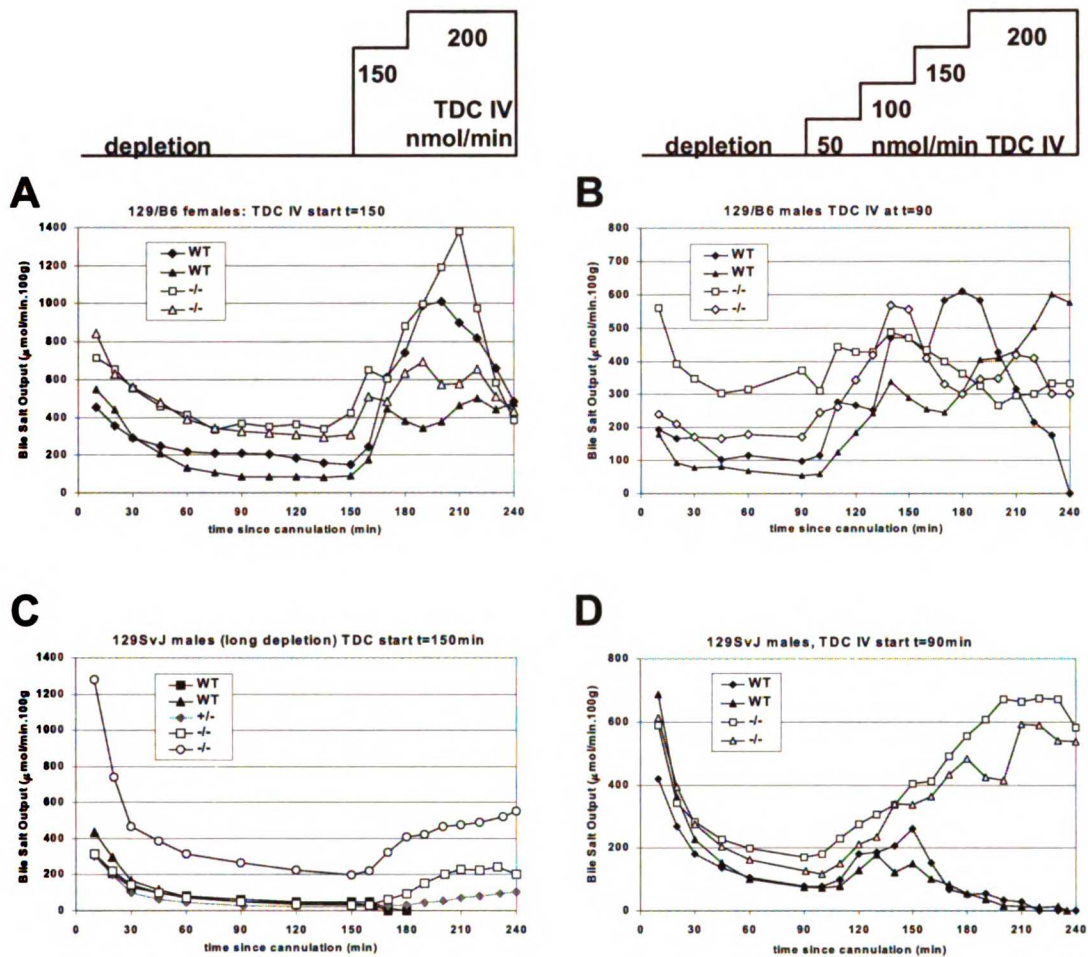


Figure 3

Resistance to TDC-induced cholestasis in 129SvJ *Fic1*^{G308V/G308V} mice.

Bile depletion followed by stepwise TDC intravenous infusion in *Fic1*^{G308V/G308V} (–/–) and wildtype (WT) mice of two strain backgrounds: mixed 129SvJae/C57Bl/6 (A & B) and 129SvJ (C & D). Two different variations of the TDC infusion protocol were used and are represented graphically above the panels: in all cases the maximal TDC infusion rate was 200nmol/min/25g. The animals in panel A were female; the rest male. One heterozygous (+/–) animal was included in the experiment shown in panel C.

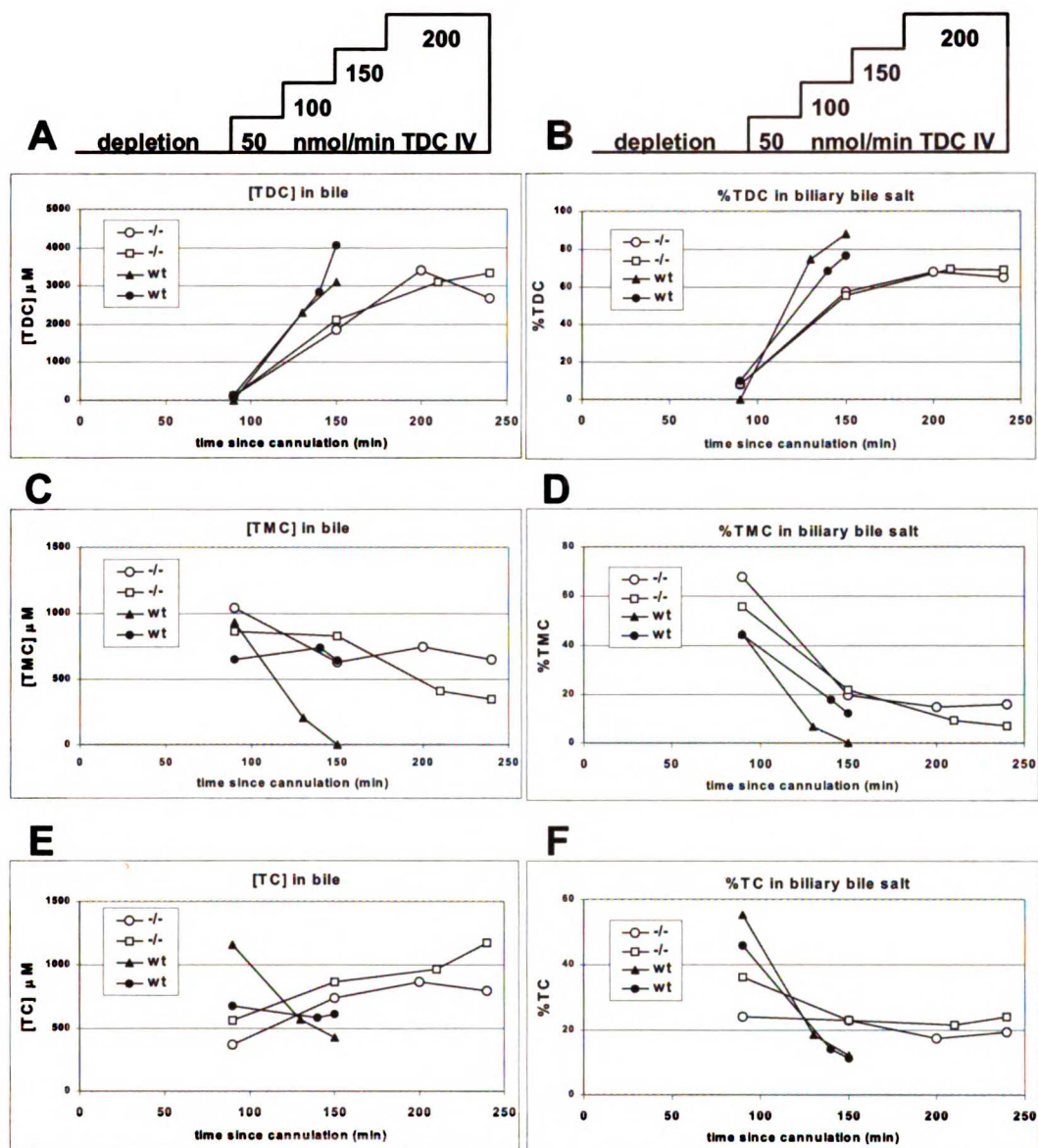


Figure 4

Biliary bile salt composition of TDC-infused mice of strain 129/SvJ.

Bile aliquots from the animals shown in Figure 4D (n=2, start of TDC IV at t=90min) were analyzed for concentration and percentage contribution of infused taurodeoxycholate (TDC) and of endogenous tauromuricholate (TMC) and taurocholate (TC). Absolute concentrations of bile salt species in bile (μM) are shown in panels A, C, E; % contribution of each bile salt species to total biliary bile salt in panels B, D, F.

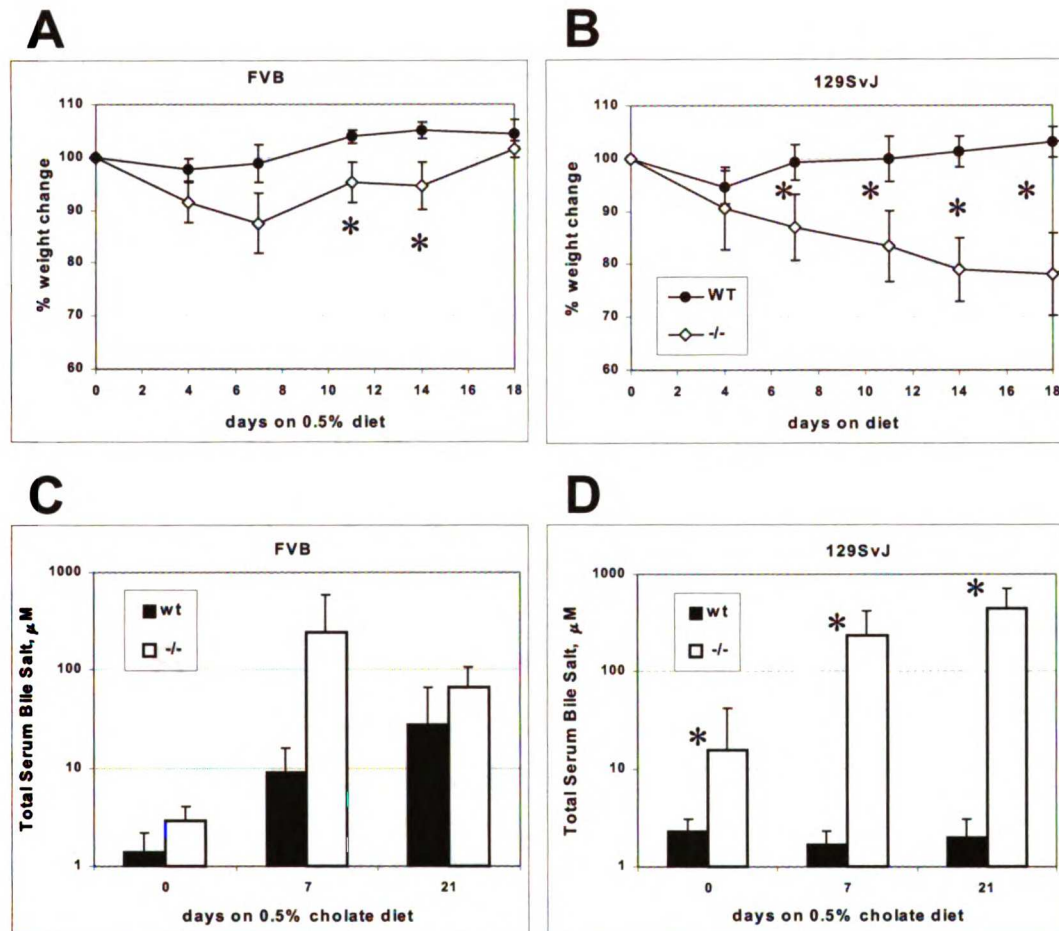


Figure 5

Impact of strain background on the response of *Fic1* mutant mice to cholate diet.

The difference in phenotypic response to 0.5% cholate diet in wildtype (WT) and *Fic1*^{G308V/G308V} mice of the FVB (n=4) and 129/SvJ (n=8; same data as Chapter 5, Figure 3) strain backgrounds. Panels A & B show weight variation normalized to starting weight, panels C & D show total serum bile salt levels. *p<0.05; all comparisons evaluated by Mann-Whitney nonparametric test.

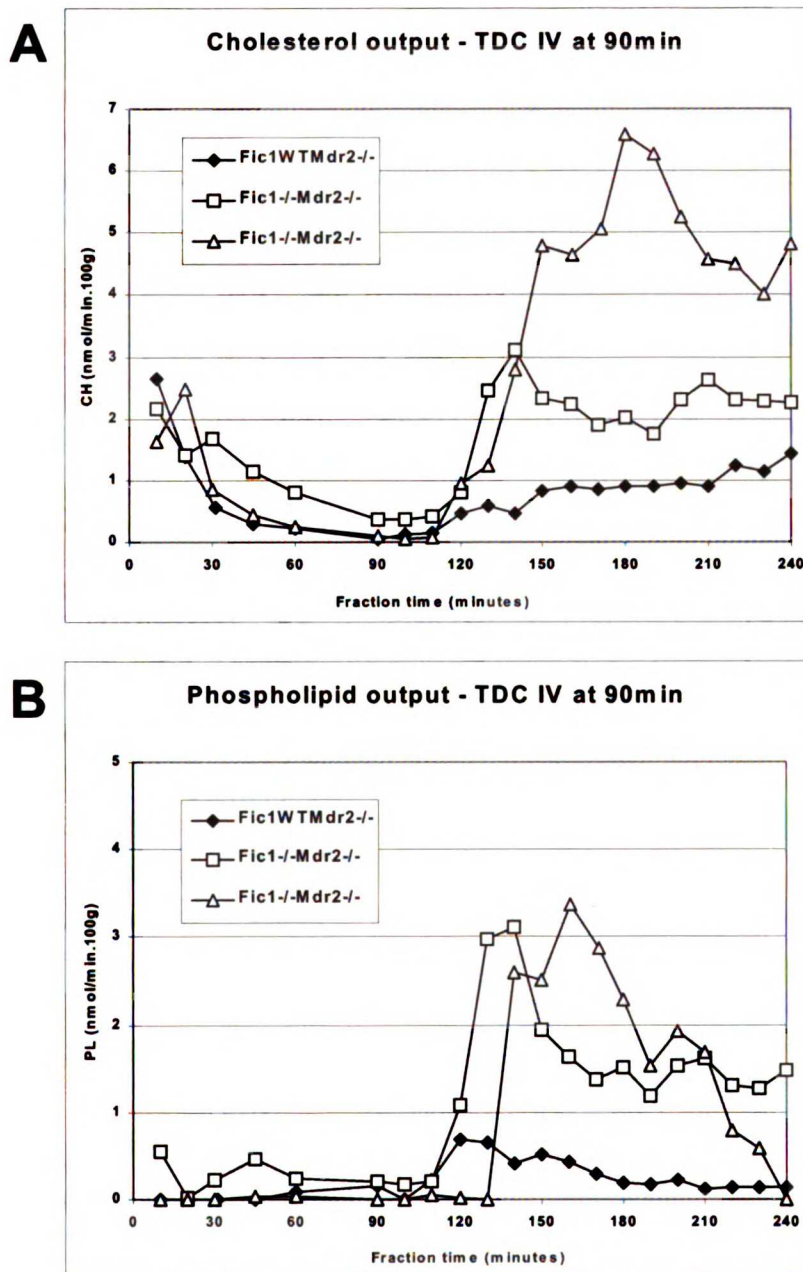


Figure 6

Influence of *Fic1* genotype on lipid secretion in *Mdr2*^{-/-} mutant mice.
 Cholesterol and phospholipid output in bile of one *Fic1*^{WT}*Mdr2*^{-/-} and two *Fic1*^{G308V/G308V}*Mdr2*^{-/-} double-knockout mice during 90 min of bile salt depletion followed by 150 min of TDC infusion (as in Figure 3B,D).

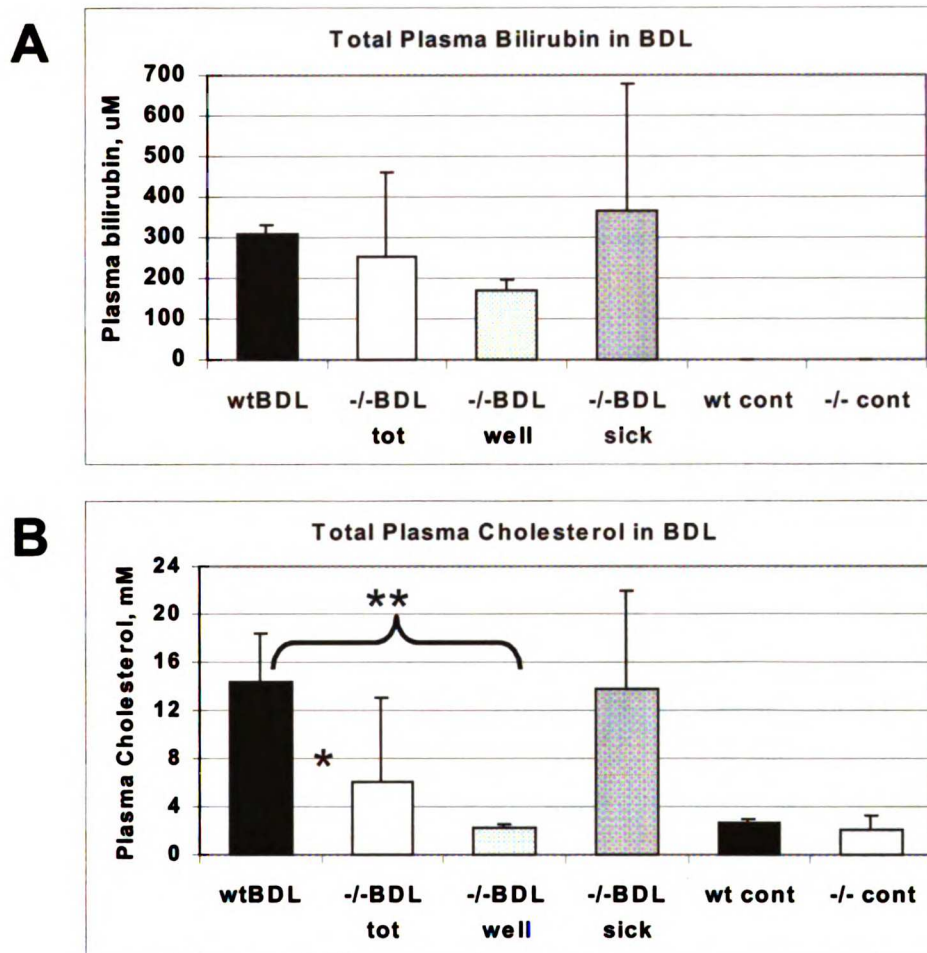


Figure 7

Cholestasis induced by bile duct ligation increases plasma cholesterol in some but not all *Fic1*^{G308V/G308V} mutant mice.

Total plasma cholesterol (A) and bilirubin (B) levels are shown in wildtype (n=5) and *Fic1*^{G308V/G308V} (n=9) mice sacrificed 5 days after bile duct ligation (BDL) and in controls (cont). All BDL mice were very cholestatic, as shown by highly elevated plasma bilirubin. All wildtype mice developed high plasma cholesterol after BDL. 3/9 *Fic1* mutant mice had elevated plasma cholesterol (-/-BDL sick); 6/9 did not (-/-BDL well). (-/-BDL tot) represents sick and well -/-BDL animals combined.

**p<0.0001, *p<0.01 by Student's t-test (two-tailed, unequal variance).

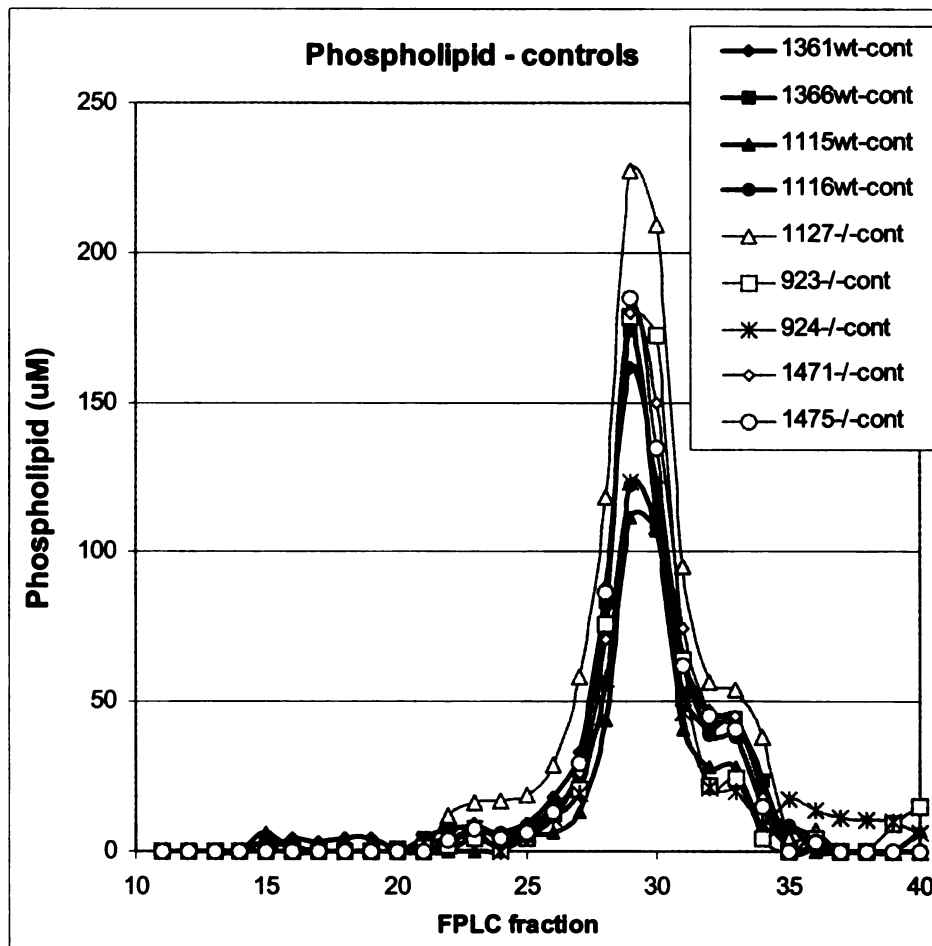


Figure 8a

Endogenous plasma lipid profiles in wildtype and *Fic1*^{G308V/G308V} mice.

Plasma lipoprotein profiles of control wildtype and *Fic1*^{G308V/G308V} mice are shown. Phospholipid concentrations in plasma fractions separated by FPLC represent lipid amounts in FPLC fractions; similar profiles were obtained from analysis of cholesterol concentrations in the same FPLC fractions. Lipoprotein X elutes in fractions 13-17, LDL in fractions 23-26 and HDL in fractions 28-35.

Figure 8b and 8c show the plasma lipid profiles of bile duct ligated mice (overleaf).

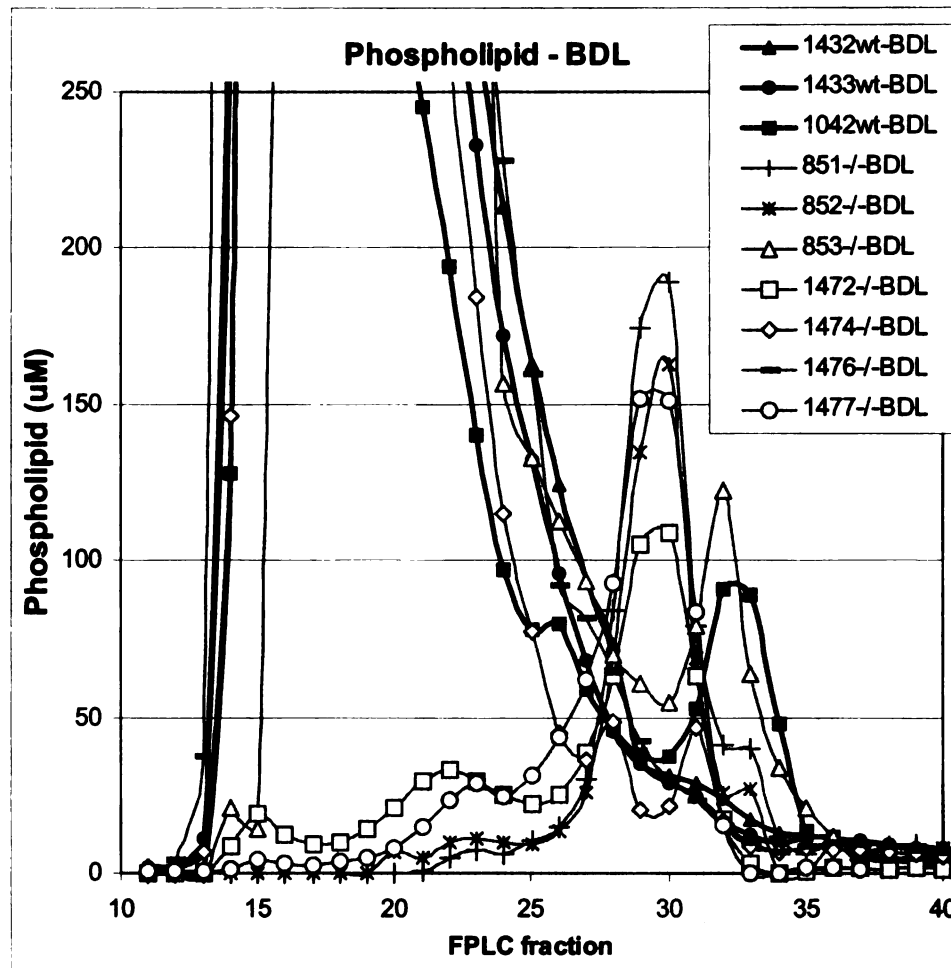


Figure 8b

Effect of bile duct ligation on plasma lipid profiles in wildtype and *Fic1*^{G308V/G308V} mice.

Plasma lipoprotein profiles of bile-duct-ligated (BDL) wildtype (3 representative of 5 tested) and *Fic1*^{G308V/G308V} mice (7/7 tested) are shown. Phospholipid concentrations in plasma fractions separated by FPLC represent lipid amounts in FPLC fractions; similar profiles were obtained from analysis of cholesterol concentrations in the same FPLC fractions. Lipoprotein X elutes in fractions 13-17, LDL in fractions 23-26 and HDL in fractions 28-35. Figure 8b (above) and 8c (overleaf) show the same data, but on a different vertical scale to illustrate trace amounts of LpX lipid in 4/7 BDL *Fic1* mutant mice (Figure 8b, animals 851, 852, 1472 and 1477) and very high amounts of LpX in 3/7 BDL *Fic1* mutant mice (Figure 8c, animals 853, 1474, 1476). These LpX levels correlate with the total cholesterol levels shown in Figure 7.

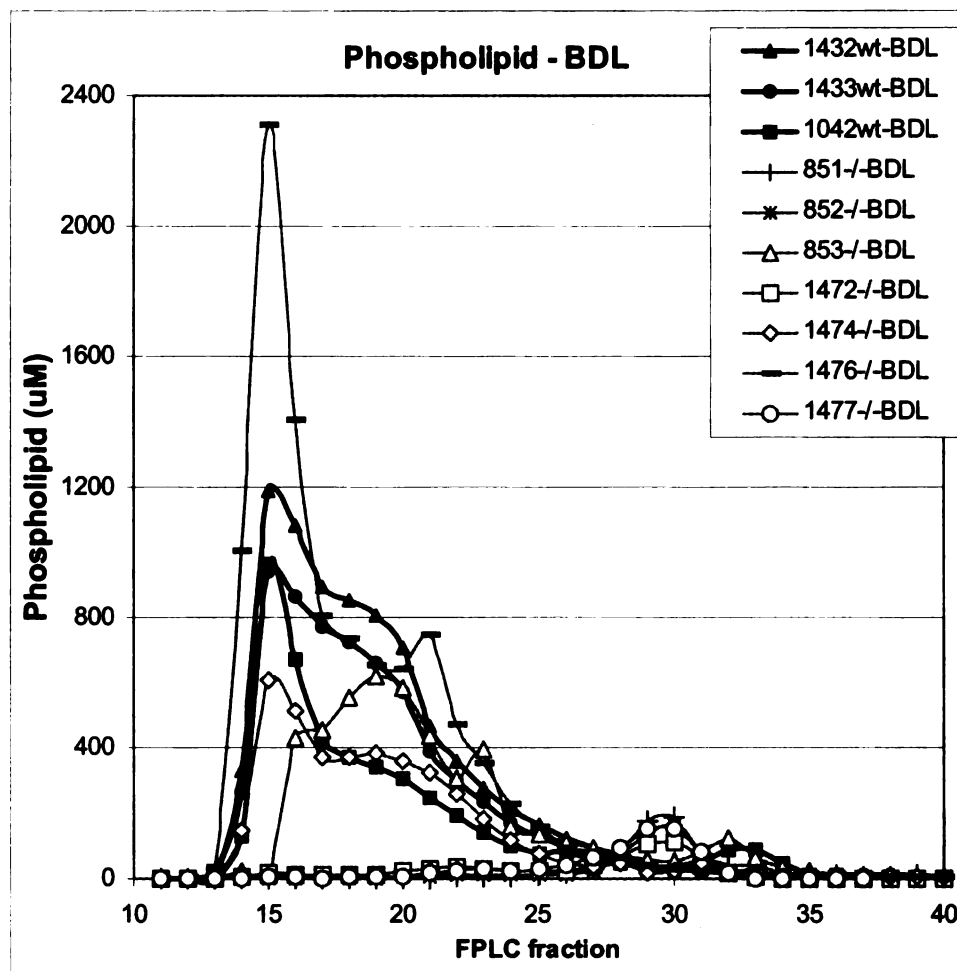


Figure 8c

Effect of bile duct ligation on plasma lipid profiles in wildtype and *Fic1*^{G308V/G308V} mice.

Plasma lipoprotein profiles of bile-duct-ligated (BDL) wildtype (3 representative of 5 tested) and *Fic1*^{G308V/G308V} mice (7/7 tested) are shown, as in Figure 8b, except for the different vertical scale to illustrate very high amounts of LpX in BDL wildtype mice and 3/7 BDL *Fic1* mutant mice (animals 853, 1474, 1476). These LpX levels correlate with the total cholesterol levels shown in Figure 7.

CHAPTER 7

Conclusions and future directions

There is something fascinating about science. One gets such wholesale returns of conjecture out of such a trifling investment of fact.

Mark Twain

The overall objectives of the research presented in this dissertation included elucidation of the genetic defect in human patients with BRIC and PFIC1 diseases, and creation of an *in vivo* model of human PFIC1 disease by targeted mutagenesis of the mouse, so as to recapitulate the initiating events of the pathogenesis of cholestasis and dissect the site-specific aspects of the process. We had also envisioned that a mouse strain replicating the genetic defect in PFIC1 patients could ultimately serve as a general model of intrahepatic cholestasis, or of defects in the enterohepatic circulation of bile salts.

The results of these investigations fulfilled many of these goals, though sometimes in surprising ways. Although some of the original research objectives were not directly met, the many unanticipated insights gained into the physiology of the enterohepatic circulation of bile salts in humans and in mice compensated for the necessary changes of focus made along the way.

One of the key hypotheses driving this research, that BRIC and PFIC1, two inherited disorders of the bile secretion, were allelic, was proved correct by the positional cloning of FIC1 and identification of mutations associated with both diseases (Bull *et al* 1998). As expected, homozygous mutations in evolutionarily highly conserved residues tended to be associated with PFIC1, the more severe disease previously hypothesized to result from complete loss of FIC1 function. This was subsequently confirmed by the absence of FIC1 protein in tissue from patients with two different PFIC1 mutations (liver from a patient with a small deletion near the C-terminus, and fibroblasts from a patient with the G308V point mutation [Eppens *et al* 2001, Chapter 5]) and in the targeted mice with the

G308V point mutation. BRIC patients were more likely to have milder mutations, such as conservative substitutions in less conserved residues, or one milder and one more severe mutation together in compound heterozygous form, which was consistent with the idea that some FIC1 function is retained in BRIC (Bull *et al* 1998, Chapter 5).

The surprising finding that *FIC1* was a P-type ATPase with homology to plasma membrane aminophospholipid translocases disproved another original hypothesis: that the gene mutated in these two diseases would be a canalicular bile salt transporter of the ABC transporter family. That postulated gene, BSEP, was indeed found by genetic mapping and positional cloning, but it was associated with a different subtype of PFIC, PFIC2 (Strautnieks *et al* 1998).

The characterization of the novel *FIC1* gene disproved several assumptions that had been made about BRIC and PFIC1 and raised key questions about the etiology of cholestasis in these two diseases. Unlike BSEP (and MDR3, the gene mutated in PFIC3), which is essentially specific to the hepatocyte canalicular membrane (Strautnieks *et al* 1998), FIC1 was broadly expressed in mostly epithelial tissues, with fairly low levels in the liver, and very high expression in the ileum and pancreas (Bull *et al* 1998). This immediately suggested that FIC1 disease could be related to a defect in intestinal absorption of bile salts, a key step in the enterohepatic circulation of bile acids, or indeed to a defect in bile salt handling along the entire loop of the enterohepatic circulation, as FIC1 expression was ultimately found at all the sites of bile salt passage: the hepatocyte canalculus, the cholangiocytes lining the bile ducts (Eppens *et al* 2001), throughout the

small and large intestine (Leo Klomp, unpublished observations), and in the kidney (Bull *et al* 1998). The broad expression also served to refocus attention on certain extrahepatic manifestations of PFIC1 disease: the discovery of FIC1 expression in the pancreas, reproductive system and fibroblasts suggested that symptoms such as pancreatic insufficiency (Knisely *et al* 1995), failure to pubesce (Knisely 2000) and elevated sweat chlorides (Bourke *et al* 1996) could be primary results of FIC1 loss, and not just secondary complications of malabsorption and vitamin deficiency, as had once been thought.

The postulated function of FIC1 as an aminophospholipid flippase was puzzling in the context of the original hypothesis that a specific defect in canalicular bile salt secretion was the cause of disease. A new hypothesis, supported by the broad expression pattern, was formulated, that FIC1 was involved in maintenance of the asymmetric lipid composition required for correct plasma membrane function, and that it indirectly affected function of bile salt transporters by modulating their lipid environment. Subsequent investigations elsewhere into the associations between lipid composition and transporter activity and localization support this hypothesis (Kipp & Arias 2002). However, since some doubt was shortly thrown on the specificity of the postulated aminophospholipid flippase activity of proteins related to FIC1 (Siegmund *et al* 1998), we also kept in mind the alternative possibility, that Fic1 was indeed a bile salt transporter itself. However, this hypothesis did not envision FIC1 as a primary canalicular transporter responsible for bile salt secretion (analogous to BSEP), but instead as a broadly distributed one with a gatekeeper role in exclusion of passively entering

hydrophobic bile salts from tissues throughout the body. Such a role would be particularly crucial at the epithelial sites lining the route of bile passage; these sites encounter the highest concentrations of bile salts and are thus most vulnerable to the membrane damaging, cytotoxic effects of these detergent molecules.

The quest for physiological clues that would support these hypotheses of FIC1 function thus became another key objective in the generation of *Fic1* mutant mice. Of the multi-pronged targeting strategy we envisioned, the first mouse line generated was that replicating a PFIC1 disease mutation, Gly308Val (G923T). Reproduction of a specific human disease-causing mutation is still a fairly uncommon approach to construction of mutant mice, and in this case it served to confirm the dramatic effect of a fairly modest sequence alteration on protein function. Abrogation of FIC1 function by the Gly308Val substitution was confirmed when we found FIC1 expression in mice (liver and intestine) and in human patients homozygous for this mutation (fibroblasts) to be profoundly decreased. This suggested that the mutation resulted in degradation of the protein and represented the hypothesized functional null.

The mutant mice accordingly provided the sought genetic proof of principle. However, on the phenotypic level, findings in the mice could not have been more unexpected. At first glance, it appears that in mice, the G308V mutation produces an opposite phenotype from that seen in human patients. PFIC1 patients have profound cholestasis and progressive liver damage, which can be fatal if untreated. The *Fic1*^{G308V/G308V} mice showed no clinical abnormalities, no liver damage, and most strikingly, enhanced rather

than reduced bile salt secretion. The only phenotypic manifestations that mice and humans had in common were elevation of serum bile salt levels, and growth retardation early in life, but these manifestations were much milder in mice than in humans.

However, the dramatic response of the *Fic1*^{G308V/G308V} mice to bile salt feeding revealed that they did indeed have a defect in the regulation of the enterohepatic circulation of bile salts. The striking accumulation of serum bile salt on cholate diet, associated weight loss, hepatomegaly and signs of liver damage, and retention of orally administered labeled bile salt in the body, which occurred regardless of a further enhancement of bile secretion, demonstrated that the primary defect in the *Fic1*^{G308V/G308V} mice lay in their inability to downregulate ileal bile salt absorption upon bile salt overload.

Thus the high ileal expression of *Fic1* was correlated with physiological function, and the utility of the mouse model in dissecting events in the etiology of FIC1-related disease was demonstrated. Furthermore, these results for the first time highlighted the importance of the regulation of bile salt absorption in the intestine for bile salt homeostasis and particularly for prevention of bile salt accumulation and damage to the liver. However, as with most scientific ventures, more questions were raised than answered.

The striking disparity between mouse and human phenotypes arising from an identical genetic defect and identical loss of protein expression remained to be elucidated. Clues from analysis of the composition of the bile salt pool of *Fic1* mutant and wildtype mice,

and observations made in Bsep knockout mice, the model of PFIC2 disease, provided a possible explanation.

The murine bile salt pool is much less hydrophobic and thus intrinsically less cholestatic and hepatotoxic than the human (Coleman *et al* 1979, Alvaro *et al* 1986). This in itself partly explains why the consequences of FIC1 and BSEP loss are much milder in mice than in humans. Furthermore, mice have a much higher intrinsic capacity for hydroxylation and detoxification of hydrophobic bile salts, and can probably further upregulate this metabolic capacity in response to insult, as demonstrated by the appearance of unusual tetrahydroxylated bile salt species in *Bsep* mutant mice (Wang *et al* 2001), and the shift to a more hydrophilic bile salt pool composition, and the ability to instantly metabolize infused taurodeoxycholate into cholate in *Fic1* mutant mice.

These differences may be particularly significant in light of the hypothesis that the primary insult in FIC1 disease lies in defective regulation of intestinal bile salt absorption. The etiology and severity of disease in mice and in humans may thus depend, to a large extent, upon exactly which specific bile salt species may be excessively absorbed in the intestine under different physiological conditions. In mice, as mentioned above, endogenous intestinal bile salt species are hydrophilic and of low toxicity. Even upon cholate diet administration, the resulting high levels of serum bile salt are primarily due to accumulating taurocholate. The mice maintain high bile salt secretion under these conditions, and, as taurocholate is not very cholestatic, mild liver stress ensues, but no cholestasis. In humans, however, any excessive absorption in the intestine and especially

in the colon (where FIC1 is also expressed) probably results in entry of very toxic and hydrophobic bile salt species produced by intestinal flora, such as lithocholate (the substrates and the bacterial agents that produce lithocholate are not found in mice). This is indeed seen in human FIC1 disease, where abnormally high levels of lithocholate and other hydrophobic bile salt species have been detected in serum and bile (Linarelli *et al* 1972, Kuipers *et al* 1985, Bijleveld *et al* 1989).

Admission of these toxic species from the intestine in human patients may result in their accumulation in the liver and consequent hepatic damage and secondary cholestasis. Furthermore, the iterative nature of bile salt circulation in the body probably compounds the insult, as bile salt species that do not stagnate in the liver, causing damage to the hepatocytes at first pass, will be secreted at the canaliculus and exert their cytotoxic effects on the plasma membranes of the bile ducts. Furthermore, cholehepatic recirculation of bile salts within the liver may compound the insult, as loss of FIC1 expression in the cholangiocytes may cause excessive bile salt absorption from the bile duct lumen, whether by loss of ability to control bile salt uptake activity mediated by ASBT, or by loss of ability to re-excrete passively entering hydrophobic species.

The results from the mutant mice thus suggest a plausible mechanism for how, in human patients as well as in mice, cholestasis may be secondary to deranged absorption of toxic bile salt species in the enterocyte and the cholangiocyte. However, both the therapeutic effects of liver transplantation in PFIC1 patients (Knisely 2000, Chen *et al* 2001), and the demonstrated presence of FIC1 in the biliary canaliculus (Eppens *et al* 2001) indicate that

FIC1 has an essential function in the hepatocyte as well. Indications of this function are also seen in the response of *Fic1* mutant mice to bile duct ligation.

Since evidence that Fic1 is indeed an aminophospholipid flippase has been reported, the overall role of FIC1 in the enterohepatic circulation of bile salts is best considered in light of this proposed molecular function. The broad expression of Fic1 suggests that it may be required for maintenance of membrane lipid homeostasis in many tissues. It may therefore impact the correct function and regulation of many different membrane transporters and proteins. However, it may be particularly critical in tissues that see high concentrations of bile salts, since the detergent properties of these molecules render them so damaging to cellular membranes. This would explain why loss of a pleiotropically expressed protein produces particularly severe hepatic manifestations in humans.

Alternatively, plasma membrane bile salt transporters may have particularly stringent environmental requirements for lipid composition of the plasma membrane leaflets, since they handle molecules that are lipophilic and amphipathic, and may present a particular challenge in translocation between aqueous and lipid environments. Furthermore, the activation of bile salt transporters may be largely regulated at the level of localization, which occurs through membrane fusion events in vesicle trafficking that are highly sensitive to lipid composition. Finally, the presence of FIC1 in secretory and/or epithelial tissues (e.g. prostate, pancreas) may be related to the fact that, in such tissues, the plasma membrane is under particular stress due to a high volume of vesicle fusion events in vesicle mediated secretion and in delivery of transporters to the plasma membrane, and in maintenance of transmembrane solute gradients.

Most of these molecular and functional hypotheses await verification in cellular transport assays that will utilize tissues, e.g. enterocytes from the *Fic1* mutant mice in parallel with cell lines stably transfected with wildtype and mutant forms of FIC1.

The *Fic1* mutant mice provided other unanticipated insights which suggest avenues for further investigation. The considerable effects of genetic background on the severity and individual manifestations of the *Fic1*^{G308V/G308V} phenotype suggest that several modifier effects may influence *Fic1* function. We are presently performing a backcross of the *Fic1*^{G308V/G308V} mutation onto the C57Bl/6 genetic background in the hope of obtaining a more severe version of the murine phenotype, which could serve as a better model of FIC1 disease in humans, and may also allow subsequent genetic mapping of modifier loci which are distinct between the C57Bl6 and 129SvJ strains. Such modifier loci are of profound interest as they may represent proteins implicated in the regulation of bile salt homeostasis. Finally, detailed analysis of the strain-specific resistance of *Fic1* mutant mice to TDC-induced cholestasis may further illuminate metabolic distinctions between murine and human hepatic physiology, which have been underscored by our research.

We hope to follow up the discovery that the intestinal defect may be primary in *Fic1*^{G308V/G308V} mice by analysis of mouse lines with conditional, tissue specific abrogation of *Fic1*. The availability of mice with *Fic1* defects confined to the intestine or to the liver (or, more specifically, to the enterocyte or the hepatocyte) will allow further deconstruction of the effects of the mutation in different tissues.

The findings presented here also illuminate the larger themes in human genetic investigations discussed in the Introduction. During the course of our journey from the characterization of two rare human inherited diseases to analysis of a mutant mouse, we have demonstrated the importance of genetic diagnosis for clinical phenotyping and vice versa. It was only once the genetic defects underlying PFIC1, 2 and 3 were delineated that the true significance of the subtle distinctions in the phenotypes of these diseases was appreciated. Thus, human genetics approaches have served to dissect the molecular etiology of a group of diseases which were previously poorly understood. The converse observation is of course that, while fascinating to the scientist, these genetic advances offer few concrete hopes to patients with these diseases, at least at this early stage. Some advances in rational therapy design have been made. For example, evaluation whether partial biliary diversion helps PFIC2 patients more than PFIC1 patients is currently underway; also, with careful management of secondary complications, PFIC1 patients can now be offered a somewhat better prognosis, at least with respect to liver disease, than PFIC2 patients (Bull et al 1997, Chen *et al* 2001), though, conversely, they are probably at a higher risk of developing post-transplant extrahepatic complications. However, making the correct differential diagnosis between these two disease subtypes is still not possible based on clinical findings alone, and identification of disease-causing mutations, even in patients with haplotype sharing consistent with genetic linkage to one of the PFIC disease loci, is still time-consuming, expensive and usually not clinically available. Ongoing studies of phenotype-genotype correlations in PFIC1 and 2 hope to ameliorate this situation.

As for the *Fic1* mutant mice, their immediate utility for modeling therapeutic interventions in PFIC1 disease may be limited by their mild phenotype. However, we expect that either breeding the mutation onto the C57Bl/6 background, or oral or dietary administration of hydrophobic bile salts may aggravate the murine phenotype, thus making it a better model for human disease. Nonetheless, the advances in our understanding of the physiological roles of Fic1 gleaned from the *Fic1*^{G308V/G308V} mouse may still inspire new therapeutic approaches in human patients. For example, the utility of an ileal transplant in preventing bile salt accumulation on cholate diet could be investigated in the mouse model, and if found promising, extended to human disease. More realistically, lessons from the mouse's superior ability to accommodate and detoxify an abnormal influx of bile salts caused by deranged ileal bile salt absorption may inspire new ideas for design of pharmacological therapies. The murine metabolic response can be seen as a form of self-medication through enhancement of the hydrophilicity of the bile salt pool. A similar approach is already used in humans with some cholestatic diseases in the form of UDCA therapy, though this does not consistently improve the condition of human PFIC1 patients. However, if the hepatic insult in PFIC1 is indeed secondary and cumulative, as suggested by the mouse results, and also if the key insult happens early on in disease progression, while the infant liver is still immature, and bile salt homeostasis mechanisms in flux (Hwang & Henning 2001), one could hypothesize that early commencement of bile salt pool replacement by UDCA or by even more hydrophilic bile salt analogs, could delay disease progression in newborn infants predicted by prenatal testing to carry defective *FIC1* alleles. Alternatively, if loss of FIC1 activity results in loss of regulation and thus enhancement of intestinal bile salt

absorption, perhaps upregulation of FIC1 activity would be beneficial in BRIC patients who are hypothesized to retain some functional protein. Research is underway in the laboratories of our collaborators to find ways to modulate FIC1 expression, though currently only bile salt feeding in vivo appears to accomplish this predictably. Interestingly, unlike *BSEP*, *FIC1* may not be regulated via the recently characterized FXR/BAR pathway since the corresponding consensus binding domain has not been found in the *FIC1* promoter, E. Eppens unpublished observations). Since murine Fic1 expression does respond to bile salt levels, it is of great interest to elucidate the unknown mechanism of this regulation.

Moving outside the immediate context of FIC1 disease, modulation of intestinal bile acid absorption is of great therapeutic interest in cardiovascular disease, as bile acid excretion in the feces is the primary pathway for cholesterol elimination from the body. If upregulation of *FIC1* could be demonstrated to result in reduced bile salt absorption, and thus increased fecal excretion in humans (as seen in wildtype mice fed cholate in the diet and subsequently given an oral dose of labeled bile salt), and a safe pharmacological agent that accomplished this upregulation could be found, this would provide a novel way to increase cholesterol excretion and thus decrease cholesterol levels in hyperlipidemic patients.

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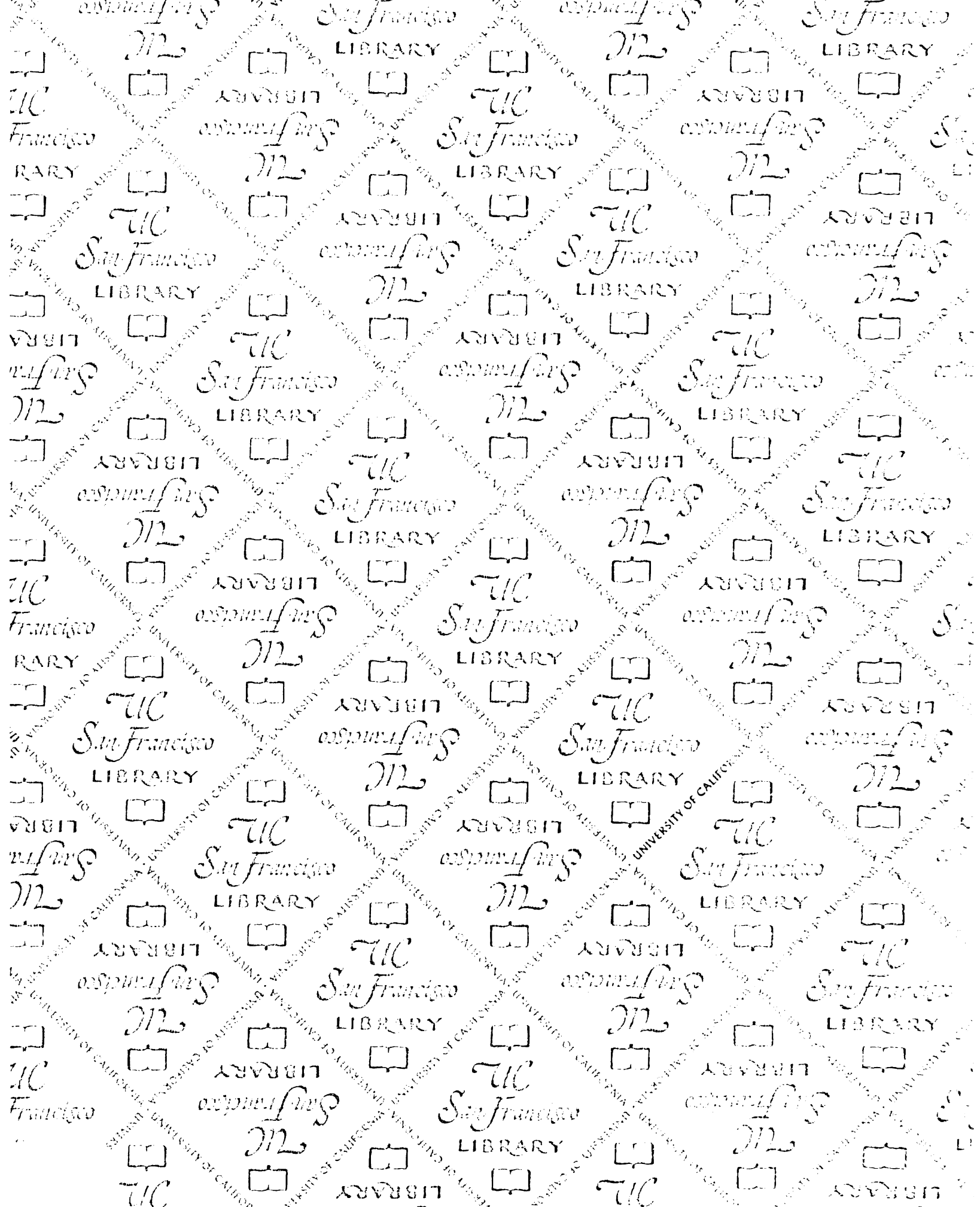
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