Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis

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Summary

The liver and intestine play crucial roles in maintaining bile acid homeostasis. Here, we demonstrate that fibroblast growth factor 15 (FGF15) signals from intestine to liver to repress the gene encoding cholesterol 7α-hydroxylase (CYP7A1), which catalyzes the first and rate-limiting step in the classical bile acid synthetic pathway. FGF15 expression is stimulated in the small intestine by the nuclear bile acid receptor FXR and represses *Cyp7a1* in liver through a mechanism that involves FGF receptor 4 (FGFR4) and the orphan nuclear receptor SHP. Mice lacking FGF15 have increased hepatic CYP7A1 mRNA and protein levels and corresponding increases in CYP7A1 enzyme activity and fecal bile acid excretion. These studies define FGF15 and FGFR4 as components of a gut-liver signaling pathway that synergizes with SHP to regulate bile acid synthesis.

Introduction

Bile acids are amphipathic cholesterol metabolites that are synthesized in the liver, stored in the gall bladder, and released postprandially into the small intestine, where they are crucial for the absorption of lipophilic nutrients (Chiang, 2004; Russell, 2003). As part of their enterohepatic circulation, most bile acids (>90%) are reabsorbed in the ileum and returned via the portal vein to the liver. Because many bile acids are strong detergents, their concentrations are tightly regulated to prevent damage to enterohepatic tissues. Expression of the gene encoding cholesterol 7α-hydroxylase (CYP7A1), which catalyzes the rate limiting step in bile acid synthesis, is repressed in a feedback fashion by bile acids (Jelinek et al., 1990; Li et al., 1990). Expansion of the bile acid pool by administration of bile acids suppresses CYP7A1 expression and reduces bile acid synthesis (Jelinek et al., 1990; Li et al., 1990). Conversely, interruption of the enterohepatic circulation by biliary drainage increases CYP7A1 expression and activity (Pandak et al., 1991).

Although most studies addressing the regulation of bile acid synthesis have focused on liver, there is evidence that the intestine has an important role in this process beyond simply being the site for bile acid reabsorption. In rodents, blocking the flow of bile acids into the intestine by bile duct ligation increases CYP7A1 expression and activity in liver (Dueland et al., 1991; Gustafsson, 1978). Since hepatic concentrations of bile acids increase under these conditions, this unexpected result suggests a role for the intestine in feedback repression of bile acid synthesis. Subsequent studies in rats subjected to biliary diversion showed that intraduodenal administration of taurocholic acid inhibits CYP7A1 expression whereas intravenous or portal administration of taurocholic acid do not (Nagano et al., 2004; Pandak et al., 1991). One possible explanation for these results is that the intestine secretes a factor that is important for feedback regulation of bile acid synthesis in liver.

The nuclear bile acid receptor, FXR (NR1H4), a member of the steroid/thyroid hormone receptor family of ligand-activated transcription factors, plays an important role in the regulation of bile acid homeostasis (Kok et al., 2003; Sinal et al., 2000; reviewed by Chiang, 2004; Russell, 2003). FXR is expressed at high levels in liver and intestine, where it is activated by the binding of bile acids including cholic acid and chenodeoxycholic acid. FXR regulates transcription by binding as a heterodimer with retinoid X receptors (RXRs) to DNA response elements in the regulatory regions of target genes. Most FXR binding sites consist of two AGTTCA half sites organized as an inverted repeat with a single nucleotide spacer (IR-1). In liver, FXR plays a prominent role in the feedback regulation of bile acid synthesis (Edwards et al., 2002). Activation of FXR induces expression of the gene encoding the orphan nuclear receptor SHP (small heterodimer partner; NR0B2), which in turn binds to the orphan nuclear receptor LRH-1 (liver receptor homolog 1; NR5A2) and inhibits its activation of the *CYP7A1* promoter (Goodwin et al., 2000; Lu et al., 2000). The importance of SHP in the feedback regulation of bile acid synthesis was demonstrated in $SHP^{-/-}$ mice, which have increased CYP7A1 expression and activity and a corresponding increase in the

bile acid pool size (Kerr et al., 2002; Wang et al., 2002), and in SHP transgenic mice, which have reduced expression of CYP7A1 and a smaller hepatic bile acid pool size (Boulias et al., 2005).

A recent study showed that in primary cultures of human hepatocytes, FXR induces expression of fibroblast growth factor (FGF) 19, a secreted protein that represses *CYP7A1* through a c-Jun N-terminal kinase (JNK)-dependent pathway (Holt et al., 2003). FGF19 selectively binds to FGF receptor 4 (FGFR4), a transmembrane receptor with tyrosine kinase activity (Xie et al., 1999). Mice lacking FGFR4, have an increased bile acid pool size, reduced JNK activity and enhanced expression of CYP7A1 (Yu et al., 2000). Conversely, transgenic mice expressing a constitutively active form of FGFR4 have increased JNK activity, decreased CYP7A1 expression, and a reduced bile acid pool size (Yu et al., 2005). These studies implicate the FGF19/FGFR4 pathway in the regulation of bile acid homeostasis. However, it is puzzling that FGF19 mRNA is not detected in human liver samples by RT-PCR assays (Nishimura et al., 1999) whereas FGFR4 is expressed in liver (Kan et al., 1999; Nicholes et al., 2002; Stark et al., 1991).

In this report, we have evaluated the expression and function of FGF15, the mouse ortholog of FGF19. FGF15 is shown to be expressed and induced by FXR in small intestine but not liver. FGF15 represses hepatic bile acid synthesis through a mechanism that involves both FGFR4 and the orphan receptor SHP. These studies define FGF15 and FGFR4 as components of a gut-liver signaling pathway that cooperates with hepatic SHP to maintain bile acid homeostasis.

Results

FGF15 is induced by FXR in intestinal epithelium

FXR was shown previously to regulate *FGF19* transcription in primary cultures of human hepatocytes by binding to an IR1 motif in the second intron of the FGF19 gene (Holt et al., 2003). There is no FGF19 gene in the mouse genome; the most closely related FGF family member is FGF15, which shares 51% amino acid identity (McWhirter et al., 1997). Despite their sequence divergence, FGF15 and FGF19 appear to be orthologs based on synteny (Katoh, 2003; Wright et al., 2004). Moreover, the position and sequence of the IR1 motif is conserved in the FGF15 gene (Figure S1 in the Supplemental Data available with this article online). Through the use of electrophoretic mobility shift assays and cell-based reporter assays we showed that FXR binds to the *Fgf15* IR1 motif as an RXR heterodimer and directly regulates the *Fgf15* promoter (Figure S1). Similar results were recently reported by another group (Li et al., 2005).

To test whether FXR stimulates FGF15 expression in vivo, mice were treated for 14 hr with the potent, synthetic FXR agonist GW4064 (Goodwin et al., 2000; Maloney et al., 2000), the natural FXR agonist cholic acid, or vehicle. RNA was prepared from enterohepatic tissues including liver, small intestine, and colon. No FGF15 mRNA was detected in liver, even in mice treated with GW4064, as assessed by northern analysis (Figure 1A) and real-time quantitative PCR (RTQ-PCR) (data not shown). Similarly, the mRNA encoding FGF19, the human ortholog of FGF15, was not detected by RT-PCR in human liver samples (Nishimura et al., 1999). FGF15 mRNA was present in ileum, where its levels were increased by administration of

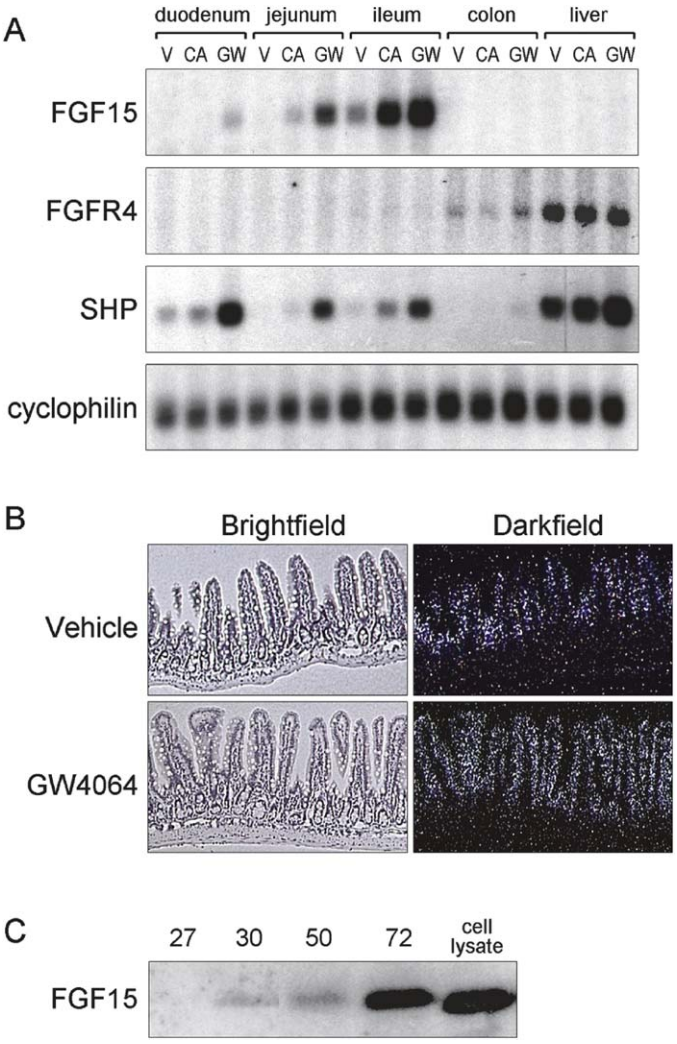

Figure 1. FGF15 expression is induced by FXR in intestinal epithelium

A) Multiple tissue Northern blot analysis was done using [^{32}P]-labeled probes for FGF15, SHP, FGFR4, and cyclophilin and pools of mRNA prepared from enterohepatic tissues of male mice (n = 5/group) treated for 14 hr with vehicle (V), cholic acid (CA; 200 mg/kg), or GW4064 (GW; 100 mg/kg).

B) In situ hybridization was done with transverse sections of distal ileum from a male mouse treated for 14 hr with vehicle or GW4064 using an FGF15 antisense riboprobe.

C) Caco-2 cells were infected with an FGF15-expressing adenovirus and media samples were collected at the indicated times (hr postinfection). There was no significant cell death throughout the course of the experiments as determined by light microscopy (data not shown). At the 72 hr time point, cell lysates were prepared in a volume equal to that in which the cells were cultured. Equal volumes of media and cell lysates were assayed for FGF15 protein by immunoblotting.

GW4064 or cholic acid (Figure 1A). FGF15 mRNA was also detected in the jejunum and duodenum of mice treated with FXR agonists. As expected, SHP mRNA was detected in liver, where expression was increased modestly by GW4064 (Figure 1A). SHP mRNA was also present and its expression enhanced by FXR agonists throughout the small intestine (Figure 1A). FGFR4 mRNA, which encodes a receptor for FGF15 (see below), was most highly expressed in liver and colon, with little or no expression detected in small intestine (Figure 1A).

To determine where FGF15 is expressed along the crypt-villus axis, in situ hybridization analysis was done using an FGF15 antisense riboprobe and transverse sections of distal ileum prepared from vehicle- and GW4064-treated mice. FGF15 mRNA was detected in the enterocytes of the villus epithelium in vehicle-treated mice with highest expression in the intervillus regions (Figure 1B). Little or no signal was detected in the crypts or the lamina propria. GW4064 treatment increased FGF15 mRNA hybridization and resulted in expression to the tips of the villi (Figure 1B). No signal was detected in control experiments performed with a sense probe (data not shown).

Like other FGFs, FGF15 has a hydrophobic N-terminal sequence that is presumed to be a signal sequence for secretion (McWhirter et al., 1997). To test whether FGF15 is secreted, Caco-2 intestinal epithelial cells, which are non-permissive for adenovirus replication, were infected with either a recombinant adenovirus that expresses FGF15 or a control virus, and immunoblotting was performed with an FGF15 antibody. FGF15 accumulated in the media of cells infected with the FGF15-expressing adenovirus in a time-dependent manner (Figure 1C). No FGF15 was detected in experiments done with media from control virus-infected cells at any time point (data not shown). Comparison of FGF15 concentrations in media and cell lysates at the 72 hr time point revealed that ~50% of the FGF15 protein was in each compartment (Figure 1C). These data show that FGF15 is a secreted protein and suggest that it may act in either a paracrine or endocrine fashion.

FGF15 represses *Cyp7a1* in liver

Since FGF19, the human ortholog of FGF15, regulates CYP7A1, we next examined the relationship between FGF15 and CYP7A1 using mice in which bile flow into the small intestine was blocked by ligation of the common bile duct. Bile duct ligation resulted in a ~3-fold increase in CYP7A1 mRNA in liver (Figure 2A) as previously described (Dueland et al., 1991; Gustafsson, 1978) without a corresponding decrease in SHP expression (Figure 2B), suggesting that an activity required for repression of *Cyp7a1* is absent in the bile duct ligated mice. Notably, FGF15 expression in ileum was extinguished in bile duct-ligated mice (Figure 2C). Treatment of bile duct-ligated mice with GW4064 resulted in a ~50-fold increase in FGF15 mRNA in ileum, a 3.5-fold increase in SHP mRNA in liver, and a >10-fold decrease in hepatic CYP7A1 mRNA (Figure 2). Thus, there is an inverse relationship between FGF15 expression in ileum and CYP7A1 expression in liver in a model of obstructive jaundice.

The reciprocal relationship between FGF15 and CYP7A1 expression raised the possibility that FGF15 regulates *Cyp7a1*. To test this hypothesis directly, wild-type mice were injected with either a recombinant adenovirus that expresses FGF15 or a control adenovirus. In wild-type mice, infection with the FGF15-expressing adenovirus decreased CYP7A1 mRNA levels ~5-fold (Figure 3A). Notably, SHP mRNA levels were not increased by infection with the FGF15-expressing adenovirus (Figure 3B), demonstrating that increased SHP expression is not essential for efficient FGF15-mediated repression of *Cyp7a1*.

In complementary studies, purified recombinant FGF15 (Supplementary Figure 2) was injected intravenously into wild-type mice. FGF15 resulted in a >100-fold reduction in CYP7A1 mRNA levels 6 hr after injection without causing a significant increase in SHP mRNA levels (Figure 3D). These data demonstrate that FGF15 can function in an endocrine fashion to suppress *Cyp7a1*.

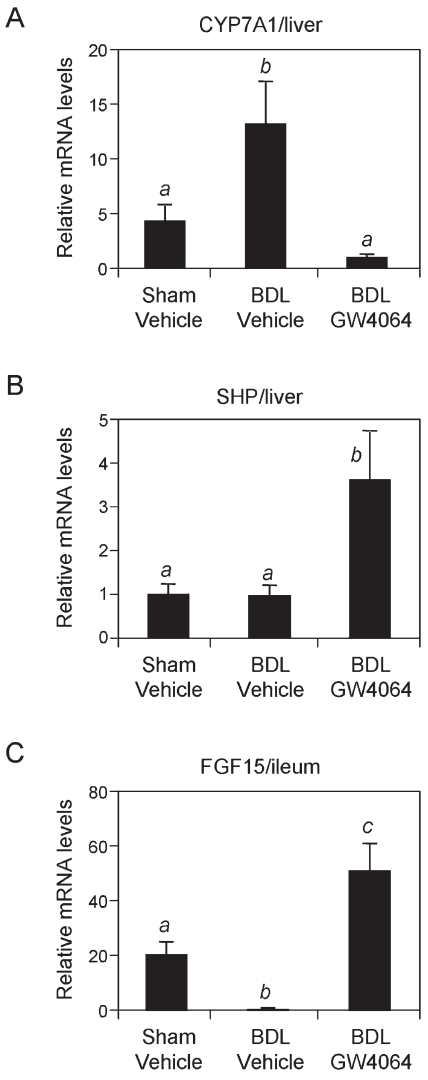

Figure 2. Inverse relationship between FGF15 mRNA level in ileum and CYP7A1 mRNA level in liver in bile duct ligated mice

Total RNA was prepared from ileum and liver of male mice (n = 5–9) subjected to sham operation and vehicle treatment, bile duct ligation (BDL) and vehicle treatment, or bile duct ligation and GW4064 treatment. GW4064 (100 mg/kg/day) and vehicle treatments were initiated two days prior to bile duct ligation or sham operation and continued for 4 days after surgery. Gene expression was measured by RTQ-PCR using cyclophilin as the internal control. Data represent the mean ± SEM and are plotted as fold change. The presence of different lowercase letters indicates statistical significance ($p < 0.05$).

FGFR4 and SHP contribute to FGF15-mediated repression of *Cyp7a1*

Whether FGF15-mediated repression of *Cyp7a1* requires FGFR4 was investigated because (1) FGF19 binds selectively to FGFR4 (Xie et al., 1999), (2) mice lacking FGFR4 have a bile acid phenotype that includes elevated CYP7A1 mRNA levels and a corresponding increase in the bile acid pool size and fecal bile acid excretion (Yu et al., 2000); and (3) FGFR4 is the

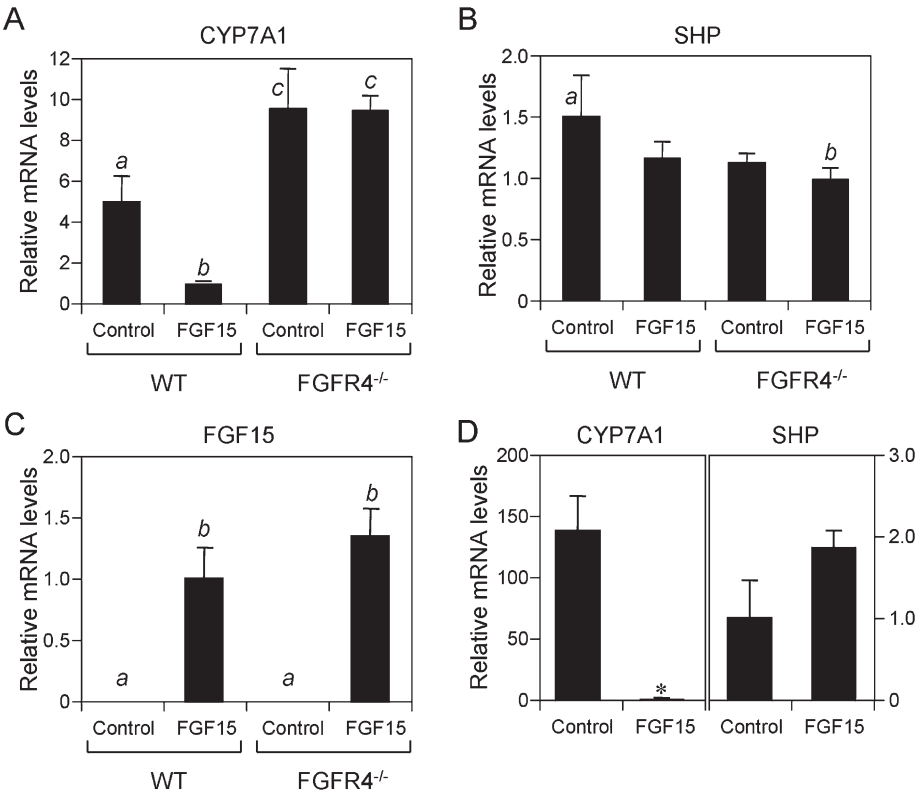

Figure 3. FGF15-mediated repression of *Cyp7a1* requires FGFR4

A–C) Wild-type (WT) and FGFR4$^{-/-}$ mice (n = 5/group, sex matched) were infected by intravenous injection with either a control adenovirus or an adenovirus that expresses FGF15. After 5 days, the mice were killed and CYP7A1 (**A**), SHP (**B**), and FGF15 (**C**) mRNA levels were measured by RTQ-PCR using total RNA prepared from liver. Data represent the mean ± SEM and are plotted as fold change. The presence of different lowercase letters indicates statistical significance ($p < 0.05$).

D) Wild-type male mice (n = 4/group) were injected with recombinant FGF15 (150 μg/kg) or control saline. Mice were killed 6 hr after injection and CYP7A1 and SHP mRNA levels were measured by RTQ-PCR using total RNA prepared from liver. Data represent the mean ± SEM and are plotted as fold change. *$p < 0.001$. SHP expression is not significantly different between the two groups.

predominant FGFR expressed in mature hepatocytes (Kan et al., 1999; Nicholes et al., 2002). To determine whether FGF15 represses *Cyp7a1* through FGFR4, the adenovirus experiments described above were performed in FGFR4$^{-/-}$ mice. As expected, CYP7A1 mRNA levels were elevated in FGFR4$^{-/-}$ mice compared to wild-type littermates (Figure 3A). In contrast to the findings in wild-type mice, infection of FGFR4$^{-/-}$ mice with the FGF15-expressing adenovirus did not repress *Cyp7a1* (Figure 3A). Comparable amounts of FGF15 mRNA were present in livers of wild-type and FGFR4$^{-/-}$ mice infected with the FGF15-expressing adenovirus (Figure 3C). Thus, FGF15-mediated repression of *Cyp7a1* requires FGFR4.

SHP is essential for FXR-mediated repression of bile acid synthesis (Kerr et al., 2002; Wang et al., 2002). Does repression of *Cyp7a1* by FGF15 involve SHP or do these two pathways function independently? To address this issue, wild-type and SHP$^{-/-}$ mice were treated with FGF15-expressing adenovirus or control virus, and hepatic CYP7A1 mRNA levels were measured by RTQ-PCR. Administration of FGF15-expressing adenovirus to wild-type mice resulted in a >10-fold reduction in CYP7A1 mRNA levels (Figure 4A). As expected, SHP$^{-/-}$ mice had increased CYP7A1 mRNA levels (Figure 4A) as previously reported (Kerr et al., 2002; Wang et al., 2002). However, infection of the SHP$^{-/-}$ mice with FGF15-expressing adenovirus resulted in only a 1.4-fold repression that was not statistically significant (Figure 4A). FGF15 mRNA levels were comparable in wild-type and SHP$^{-/-}$ mice infected with FGF15-expressing adenovirus (Figure 4B). These data reveal that SHP contributes to FGF15-mediated repression of CYP7A1 but do not rule out that SHP-independent mechanisms are also involved.

Bile acid homeostasis is dysregulated in FGF15$^{-/-}$ mice

FGF15$^{-/-}$ mice were recently described (Wright et al., 2004). Most of the FGF15$^{-/-}$ mice die during late embryonic development or shortly after birth, but the few FGF15$^{-/-}$ mice that survive appear normal. Through a large breeding program, we were able to obtain enough FGF15$^{-/-}$ mice to test for alterations in bile acid parameters. Notably, FGF15$^{-/-}$ mice had 3.5-fold higher levels of CYP7A1 mRNA than wild-type mice (Figure 5A) with no corresponding change in SHP mRNA levels (Figure 5B). Consistent with the mRNA data, FGF15$^{-/-}$ mice had increased levels of cholesterol 7α-hydroxylase protein (Figure 5C) and activity (Figure 5D) and a corresponding increase in fecal bile acid excretion (Figure 5E). Thus, FGF15 plays a crucial role in regulating bile acid synthesis.

Is FGF15 required for FXR-mediated repression of CYP7A1 expression? To address this question, wild-type and FGF15$^{-/-}$ mice were administered GW4064 or vehicle alone, and hepatic CYP7A1 and SHP mRNA levels were analyzed by RTQ-PCR. Administration of GW4064 to wild-type mice stimulated FGF15 expression in ileum (data not shown). Whereas GW4064 repressed CYP7A1 mRNA levels by 4-fold in wild-type mice, no repression was seen in FGF15$^{-/-}$ mice (Figure 5A). GW4064 administration resulted in a ~2-fold induction of SHP expression in both wild-type and FGF15$^{-/-}$ mice, although the data in the FGF15$^{-/-}$ mice did not achieve statistical significance (Figure 5B). Taken together, these data show that FGF15 is required for FXR-mediated repression of *Cyp7a1*.

A similar series of experiments were performed in FGFR4$^{-/-}$ mice, which were administered either GW4064 or the natural FXR agonist, cholic acid. As expected, GW4064 and cholic

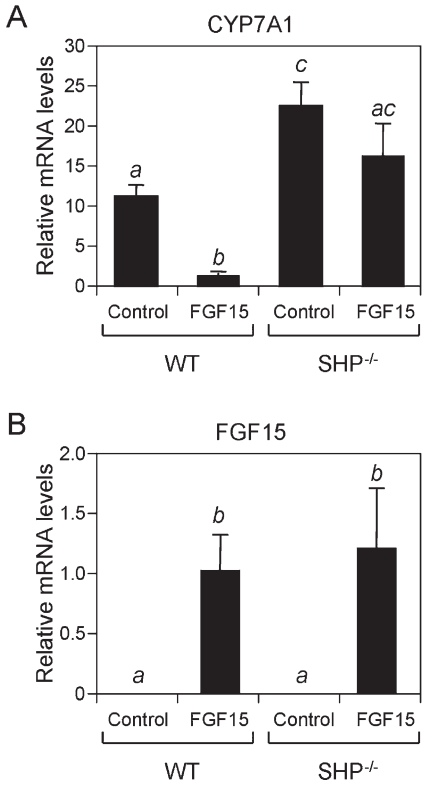

Figure 4. SHP contributes to FGF15-mediated repression of *Cyp7a1*

Wild-type (WT) and SHP$^{-/-}$ mice (n = 5-7/group, sex matched) fed a chow diet containing 0.2% cholesterol were injected with either a control adenovirus or an adenovirus that expresses FGF15. After 5 days, the mice were killed and CYP7A1 (**A**) and FGF15 (**B**) mRNA levels were measured by RTQ-PCR using total RNA prepared from liver. Data represent the mean ± SEM and are plotted as fold change. The presence of different lowercase letters indicates statistical significance ($p < 0.05$).

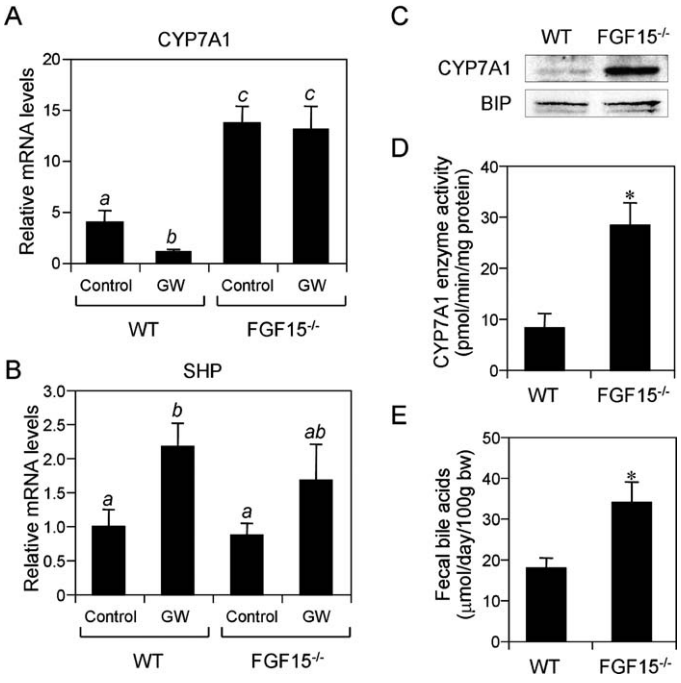

Figure 5. Bile acid homeostasis is dysregulated in FGF15$^{-/-}$ mice

A) Wild-type and FGF15$^{-/-}$ mice (n = 10/group; male) were treated with either vehicle or GW4064 (GW; 100 mg/kg/day) for 4 days. (**A and B**) CYP7A1 (**A**) and SHP (**B**) mRNA levels were measured by RTQ-PCR using total RNA prepared from liver. Data represent the mean ± SEM and are plotted as fold change. The presence of different lowercase letters indicates statistical significance ($p < 0.05$).
C) Immunoblotting was done with liver microsomal protein prepared from wild-type (WT) and FGF15$^{-/-}$ mice (n = 4; pooled samples) and antibodies against CYP7A1 and heavy chain binding protein (BIP), which serves as a loading control.
D) Cholesterol 7α-hydroxylase activity was measured by LC/MS/MS using hepatic microsomes isolated from wild-type (WT) and FGF15$^{-/-}$ mice (n = 4/group). Data represent the mean ± SEM. *p = 0.019.
E) Fecal bile acid concentrations were measured in male wild-type (WT) and FGF15$^{-/-}$ mice (n = 5/group). *p = 0.018.

acid repressed *Cyp7a1* in wild-type mice (Figure 6A). The effect of both the natural and synthetic FXR agonists on CYP7A1 expression was lost in the FGFR4$^{-/-}$ mice (Figure 6A) even though FGF15 mRNA was present at high levels in ileum (data not shown). Thus, FGF15 and FGFR4 are required in addition to SHP for FXR to repress *Cyp7a1*.

Discussion

Bile acids are powerful detergents whose concentrations must be tightly regulated. Most studies of the feedback mechanisms regulating bile acid homeostasis have focused on the liver with little attention paid to the intestine. Nevertheless, several reports reveal that the intestine is not simply a site for bile acid reclamation but that it also plays an active and integral role in bile acid-mediated suppression of bile acid synthesis in liver (Nagano et al., 2004; Pandak et al., 1991). Specifically, in rodents subjected to biliary diversion, intraduodenal administration of taurocholic acid inhibited CYP7A1 expression and activity whereas portal and intravenous administration of bile acids did not. Patients with obstructive jaundice have also been reported to have increased CYP7A1 mRNA and activity levels (Bertolotti et al., 2001). These data suggest that feedback regulation of bile acid synthesis requires that bile acids traverse the intestine. However, the mechanism whereby the intestine signals to liver was unknown.

In this report, we present several lines of evidence that FGF15 made in small intestine regulates bile acid synthesis in liver. First, *Fgf15* expression is strongly induced by the bile acid receptor FXR in the villus epithelium of ileum (Figures 1A and 1B). This effect is mediated by an FXR response element located in the second intron of the FGF15 gene (Figure S1). Second, FGF15 and CYP7A1 mRNA levels are regulated in a reciprocal fashion in bile duct ligated mice (Figures 2A and 2C). Third, in vivo administration of FGF15 by either infection with an FGF15-expressing adenovirus or injection of recombinant protein results in a marked repression of *Cyp7a1* (Figures 3A and 3D). Finally, mice lacking FGF15 have increased CYP7A1 mRNA, protein, and enzyme activity levels and a corresponding increase in fecal bile acid excretion (Figure 5). These data define a novel mechanism for the repression of bile acid synthesis that involves communication between the intestine and liver. Moreover, they reveal a new biological function for FGF15, which was previously shown to be highly expressed in the developing central nervous system where it is postulated to regu-

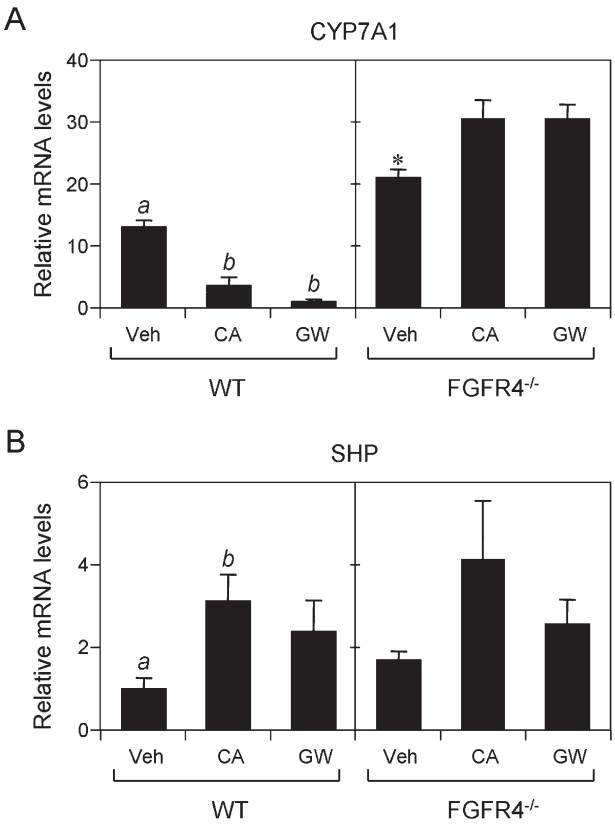

Figure 6. FXR-mediated repression of *Cyp7a1* requires FGFR4$^{-/-}$

Wild-type (WT) and FGFR4$^{-/-}$ mice (n = 3–4/group, sex matched) were treated with vehicle, cholic acid (CA; 1% administered in diet) or GW4064 (GW; 50 mg/kg/day administered by oral gavage) for 4 days. CYP7A1 (**A**) and SHP (**B**) mRNA levels were measured by RTQ-PCR using total RNA prepared from liver. Data represent the mean ± SEM and are plotted as fold change. The presence of different lowercase letters indicates statistical significance ($p < 0.05$). The asterisk indicates statistical significance ($p < 0.05$) between the vehicle-treated wild-type and FGFR4$^{-/-}$ groups.

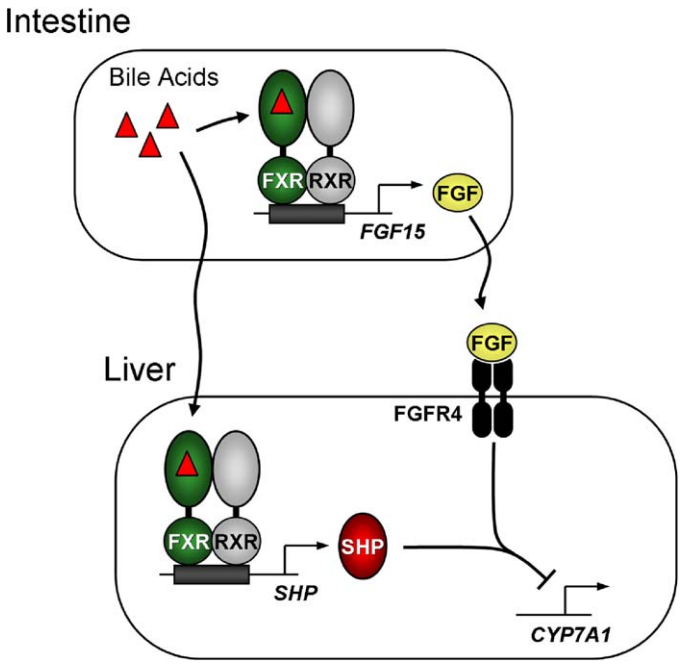

Figure 7. Model for FXR-mediated repression of bile acid synthesis

Bile acids activate FXR to induce FGF15 in small intestine and SHP in liver. FGF15 is secreted from intestine to activate FGFR4 in liver. The FGFR4 signaling pathway cooperates with SHP to repress *CYP7A1* in liver. Although SHP contributes to FGF15-mediated repression of *CYP7A1*, SHP-independent mechanisms cannot be ruled out.

late the growth and differentiation of neural precursor cells (Gimeno et al., 2003, 2002; Ishibashi and McMahon, 2002).

It was previously shown that the gene encoding FGF19, the human ortholog of mouse FGF15, is induced by FXR in primary cultures of human hepatocytes (Holt et al., 2003). However, we and others have not detected FGF19 mRNA in human liver samples using sensitive RT-PCR assays (Nishimura et al., 1999) (data not shown). These findings suggest that FGF19 may not be expressed in human liver. Although expression in adult intestine was not tested, *FGF19* is expressed in fetal intestine (Xie et al., 1999) and is induced in the Caco-2 intestinal cell line by FXR agonists (Li et al., 2005), suggesting that FGF19 may be regulated in human intestine in a manner analogous to FGF15.

How does FGF15 signal between intestine and liver? One possibility is that FGF15 affects CYP7A1 expression indirectly by modulating the activity of bile acids or other molecules that signal from intestine to liver. For example, FGF15 might enhance bile acid absorption in ileum or increase the intestinal synthesis of a bile acid metabolite that in turn inhibits bile acid synthesis in liver. However, it is hard to reconcile this model with our finding that induction of FGF15 expression in ileum of mice subjected to bile duct ligation, which blocks bile acid flow into the small intestine, correlates with a >10-fold reduction in CYP7A1 mRNA levels in liver (Figure 2).

A second, more intriguing possibility is that FGF15 acts as a hormone to signal between intestine and liver (Figure 7). The intestine produces many hormones that act in either paracrine or endocrine fashions to influence digestion. For example, secretin and cholecystokinin, which are released from enteroendocrine cells in the small intestine, are major regulators of exocrine pancreas activity. FGF15 could travel from intestine to liver through either the portal circulation or the lymph. The latter possibility is suggested by the finding that disruption of lymph flow increases cholesterol 7α-hydroxylase activity in rats (Bjorkhem et al., 1978). The hypothesis that FGF15 functions as an enterohepatic hormone is supported by data showing that FGF15 is a secreted protein (Figure 1C) and that intravenous injection of recombinant FGF15 represses CYP7A1 expression efficiently (Figure 3D). Moreover, FGFR4, which is required for FGF15-mediated repression of *Cyp7a1* (Figure 3A), is the predominant FGFR in mature hepatocytes (Kan et al., 1999; Nicholes et al., 2002; Stark et al., 1991). To date, we have not been able to detect FGF15 in serum, intestine, or liver from GW4064-treated mice using several different polyclonal antibodies. FGFs generally interact with their cognate receptors with K_d values in the picomolar range (Ornitz et al., 1996), so it is likely that our antibodies are not sufficiently sensitive to detect biologically active concentrations of FGF15.

There is precedent for FGFs acting in an endocrine manner.

FGF23, which is part of the same FGF subfamily as FGF15, functions as a phosphaturic hormone (Quarles, 2003). FGF23 is expressed in liver, lymph nodes, thymus, and heart and signals to bone and kidney to regulate phosphate homeostasis. Mutations in the FGF23 gene cause autosomal dominant hypophosphatemic rickets (Consortium, 2000). There is also evidence that FGF19, the human ortholog of mouse FGF15, can act in an endocrine fashion: ectopic expression of FGF19 in the skeletal muscle of transgenic mice enhanced proliferation of pericentral hepatocytes and caused hepatocellular carcinomas (Nicholes et al., 2002; Tomlinson et al., 2002), suggesting that FGF19 signaled from muscle to liver. Interestingly, the crystal structure of FGF19 revealed that it contains two disulfide bonds, which may stabilize the protein to permit it to act as a hormone (Harmer et al., 2004). Modeling studies predict that FGF15 is the only other FGF family member in which both disulfide bonds are conserved (Harmer et al., 2004). Five other FGFs, including FGF23, are predicted to have a single disulfide bond, which may increase their stability so that they too can act in an endocrine fashion (Harmer et al., 2004).

Previous gene knockout studies showed that SHP is required for FXR-mediated repression of *Cyp7a1* (Kerr et al., 2002; Wang et al., 2002). In this report we demonstrate that the FGF15/FGFR4 pathway synergizes with SHP in vivo to repress CYP7A1 expression (Figure 7). FGF15-mediated repression of *Cyp7a1* decreases from >10-fold in wild-type mice to <1.5-fold in SHP$^{-/-}$ mice (Figure 4A). These data demonstrate that SHP contributes to FGF15-mediated repression of *Cyp7a1* but do not rule out the possibility that SHP-independent mechanisms are also involved. Notably, administration of ectopic FGF15 represses CYP7A1 expression without increasing SHP mRNA levels (Figures 3A and 3B). Conversely, CYP7A1 mRNA levels are increased in FGF15$^{-/-}$ mice without a corresponding decrease in SHP mRNA (Figures 5A and 5B). The lack of a strict correlation between SHP and CYP7A1 mRNA levels suggests that modulation of SHP activity may be more important for *CYP7A1* repression than simply altering its expression levels. While the mechanism responsible for cooperation between the FGF15/FGFR4 and SHP pathways is not known, results from two recent studies raise the possibility that they are linked by a JNK-dependent pathway. First, FGF19 activates JNK in primary cultures of human hepatocytes, and FGF19-mediated repression of *CYP7A1* is blocked by inhibitors of the JNK pathway (Holt et al., 2003). Second, liver-specific expression of a constitutively active FGFR4 in transgenic mice increased the amount of activated JNK in a manner that correlated with decreased expression of CYP7A1 (Yu et al., 2005).

In summary, we have demonstrated that FGF15 is selectively induced by bile acids in small intestine and acts through FGFR4 to repress CYP7A1 expression in liver. This work integrates the FGF15/FGFR4 and SHP signaling pathways and provides a molecular explanation for the longstanding puzzle as to why intestinal administration of bile acids represses hepatic CYP7A1 expression and activity, whereas portal and intravenous administration do not.

Experimental procedures

Animal experiments

All experiments were performed on age- and sex-matched mice. The FGFR4$^{-/-}$ mice (a gift from Dr. Wallace McKeehan) are on a pure 129/Sv background (Yu et al., 2000); both the FGF15$^{-/-}$ mice (a gift from Dr. Thomas Reh) (Wright et al., 2004) and the SHP$^{-/-}$ mice (a gift from Dr. David Russell) (Kerr et al., 2002) are on mixed C57BL/6/129/Sv backgrounds and have been backcrossed >10 generations. Mice were housed in a temperature-controlled environment with 12 hr light/dark cycles and fed standard rodent chow *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center.

Bile duct ligation

Wild-type male mice were treated daily with GW4064 (100 mg/kg/day, in 1% Tween80, 1% methylcellulose) or vehicle by gavage for 2 days prior to surgery and 4 days after. Animals treated with GW4064 or vehicle for 2 days were randomly assigned to one of two groups: bile duct ligation or sham operated. The abdomen was shaved and the skin was sprayed with 70% ethanol and wiped. Surgery was performed under aseptic conditions and general anesthesia through an upper midline abdominal incision. The common bile duct was mobilized and ligated using 4/0 silk for the bile duct ligated group. Sham operated mice had the same incision followed by mobilization of the common bile duct without ligation. All abdominal incisions were closed in two layers using 3/0 catgut and Micron 9 mm autoclips (Becton Dickinson and Company, Sparks, MD).

FGF15 adenovirus and in vivo adenovirus infections

The FGF15 coding region was cloned into the pACCMVpLpA vector at unique *XbaI* and *HindIII* sites. A $(His)_6$ tag was included at the C terminus to facilitate purification. Virus was prepared by cre/loxP-mediated recombination with a *dl*309 adenoviral cosmid in 911 cells as described previously (Aoki et al., 1999). Viruses were propagated on 911 cells and purified using discontinuous CsCl gradients and gel filtration on Sepharose CL-4B columns as previously described (Gerard and Meidell, 1989). Mice were infected with adenovirus by injection into the jugular vein using a 3/10 cc syringe (Becton Dickinson and Company, Franklin Lakes, NJ). Each mouse received 7.5×10^9 particles/g body weight in 0.1 ml of saline. Mice were killed 5 days after injection and total RNA was prepared from the liver and ileum.

Expression of FGF15 in Caco-2 cells

Confluent Caco-2 cells were infected in D2 medium with an FGF15-expressing adenovirus or control β-galactosidase-expressing adenovirus (6×10^8 pfu/ml). After infection, the media was replaced with DMEM and media samples were collected at various times post-infection. At the final time point, the cells were rinsed and lysed in 2 ml of 0.2% NP40, 20 mM Tris-HCl, (pH 8.0) and the lysates centrifuged to remove debris. Immunoblotting was done using 30 μl of medium or cell lysate and a goat polyclonal FGF15 antibody (SC-16816, Santa Cruz, CA) followed by a donkey anti-goat horseradish peroxidase antibody (SC-2020, Santa Cruz, CA). No FGF15 was detected in medium or cell lysates from control infected cells (data not shown).

Recombinant FGF15 studies

911 cells were infected with His-tagged FGF15-expressing adenovirus (6×10^8 pfu/ml) for 16 hr. FGF15 was purified from the media using a 1 ml nickel-agarose column (Qiagen, Valencia, CA). The column was washed successively with 25 ml of 50 mM Tris-HCl, (pH 8.0) containing 0.25 M NaCl, 25 ml of 50 mM Tris-HCl, (pH 8.0), and 25 ml of 20 mM Tris-HCl, (pH 8.0) containing 10 mM imidazole before FGF15 was eluted with 0.5 ml of 20 mM Tris-HCl, (pH 8.0) containing 0.5 M imidazole. The protein was dialyzed overnight in Tris-buffered isotonic saline. The purified material was a single band as assayed by either SDS-PAGE followed by coomassie brilliant blue staining or immunoblotting with polyclonal FGF15 antibody (Figure S2). Purified FGF15 was injected into the jugular vein of BL6 mice at a dose of 150 μg/kg body weight. Six hours after injection, the mice were anesthetized and killed and total RNA prepared from their livers.

Fecal bile acid excretion

Wild-type and FGF15$^{-/-}$ male mice were housed individually in plastic cages containing wood shavings and fed *ad libitum*. Stools were collected from each animal over 72 hr, dried, weighed and ground in a mechanical blender. Aliquots of ground stool were treated with sodium borohydride and then subjected to alkaline hydrolysis at 120° for 12 hr. Fecal bile acid content was measured enzymatically as previously described (Turley et al.,

1997). The daily stool output (g/day/100 g body weight) and fecal bile acid content (mol/g) were used to calculate the rate of bile acid excretion (mol/day/100 g body weight).

RNA preparation

Mice were killed and liver samples were flash-frozen in liquid nitrogen and stored at –80°C until RNA was prepared. Small and large intestine was removed and flushed with ice cold phosphate-buffered saline. Small intestine was divided into three equal lengths designated duodenum (proximal), jejunum (medial), and ileum (distal). The three segments were cut open longitudinally, and the mucosa were gently scraped and flash frozen in liquid nitrogen. Total RNA was extracted using RNA STAT-60 (Tel-Test, Inc., Frindswood, TX).

RTQ-PCR analysis

The following primers were designed using Primer Express Software (Applied Biosystems, Foster City, CA) based on GenBank sequence data: CYP7A1: 5′-agcaactaaacaacctgccagtacta-3′; 5′-gtccggatattcaaggatgca-3′; FGF15: 5′-gaggaccaaaacgaacgaaatt-3′; 5′-acgtccttgatggcaatcg-3′; SHP: 5′-cgatcctcttcaacccagatg-3′; 5′-agggctccaagacttcacaca-3′; RTQ-PCR reactions contained 25 ng of cDNA, 150 nM of each primer and 5 μl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a total volume of 10 μl. All reactions were performed in triplicate on an Applied Biosystems Prism 7900HT Sequence Detection System, and relative mRNA levels were calculated by the comparative threshold cycle method using cyclophilin as the internal control.

Northern blot analysis

Equal amounts of total RNA from five mice/treatment group were pooled, and mRNA was prepared using oligo(dT)-cellulose columns (Amersham Pharmacia Biotech Inc., Piscataway NJ). mRNA (5 μg/lane) was size fractionated on 1% formaldehyde agarose gels and transferred to nylon membrane for hybridization with ^{32}P-labeled cDNA probes for FGF15, SHP, FGFR4, and cyclophilin.

In situ hybridization analysis

In situ hybridization experiments were performed with paraffin-embedded sections of ileum using ^{35}S-labeled FGF15 sense and antisense riboprobes (nucleotides 421–740, Genbank accession number AF007268). Slides were exposed at 4°C for 14 days. The sections were developed, counterstained with hematoxylin and examined using bright and darkfield optics. In all cases, no signal was detected in sections hybridized with the sense probe (data not shown).

CYP7A1 immunoblotting and activity

Hepatic microsomes were prepared from wild-type and FGF15$^{-/-}$ mice by differential centrifugation as previously described (Schwarz et al., 1997). CYP7A1 immunoblotting was done with a rabbit polyclonal antibody against amino acids 476–490 of the murine protein (a gift from Dr. David Russell) followed by an anti-rabbit IgG-conjugated horseradish peroxidase antibody (NEF812, Perkin Elmer, Boston MA). Cholesterol 7α-hydroxylase activity was measured in reactions performed with hepatic microsomal preparations as previously described (Chiang, 1991). Briefly, 7α-hydroxycholesterol was synthesized from endogenous cholesterol in hepatic microsomes incubated in the presence of NADPH. 7α-Hydroxycholesterol was then converted to 7α-hydroxy-4-cholesten-3-one by addition of cholesterol oxidase. β-Sitosterol, which is converted by cholesterol oxidase to β-sitostenone, was included in the reactions as an internal standard. LC/MS/MS was used to quantitate the amount of 7α-hydroxy-4-cholesten-3-one and β-sitostenone. All data were normalized to β-sitostenone.

Statistical analyses

All results are expressed as mean ± SEM. Statistical analyses were performed using Minitab Release 14 software (Minitab Inc, State College, PA). Multiple groups were tested by one-way ANOVA followed by Fisher's least significant difference test for unpaired data followed by Mann Whitney U test where appropriate. Comparisons of two groups was performed using a Student's t test. A p value < 0.05 was considered to be significant.

Supplemental data

Supplemental Data include two figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/2/4/217/DC1/.

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