

# IRE1 $\beta$ Inhibits Chylomicron Production by Selectively Degrading MTP mRNA

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## SUMMARY

Microsomal triglyceride transfer protein (MTP) is needed to assemble chylomicrons in the endoplasmic reticulum (ER) of enterocytes. We explored the role of an ER stress protein, inositol-requiring enzyme 1 $\beta$  (IRE1 $\beta$ ), in regulating this process. High-cholesterol and high-fat diets decreased intestinal IRE1 $\beta$  mRNA in wild-type mice. *Ire1b*<sup>-/-</sup> mice fed high-cholesterol and high-fat diets developed more pronounced hyperlipidemia because these mice secreted more chylomicrons and expressed more intestinal MTP, though not hepatic MTP, than wild-type mice did. Chylomicron secretion and MTP expression also were increased in primary enterocytes isolated from cholesterol-fed *Ire1b*<sup>-/-</sup> mice. There was no correlation between ER stress and MTP expression. Instead, cell culture studies revealed that IRE1 $\beta$ , but not its ubiquitous homolog IRE1 $\alpha$ , decreased MTP mRNA through increased posttranscriptional degradation. Conversely, knock-down of IRE1 $\beta$  enhanced MTP expression. These studies show that IRE1 $\beta$  plays a role in regulating MTP and in chylomicron production.

## INTRODUCTION

Chylomicrons are specialized vehicles synthesized by the intestine to transport large quantities of dietary fat and fat-soluble vitamins (Hussain et al., 1996; Hussain, 2000). Increased absorption of lipids is associated with metabolic disorders such as obesity, atherosclerosis, and diabetes. Chylomicron biosynthesis depends on apolipoprotein B (apoB), a structural protein, and microsomal triglyceride transfer protein (MTP), an endoplasmic reticulum (ER)-resident chaperone (Hussain et al., 2003a, 2003b). MTP transfers several lipids and helps form primordial apoB lipoproteins (Hussain et al., 2003a, 2003b). The phospholipid transfer activity of MTP is sufficient for lipoprotein assembly. Evolutionarily, it is the oldest activity (Sellers et al., 2003; Rava et al., 2006; Rava and Hussain, 2007).

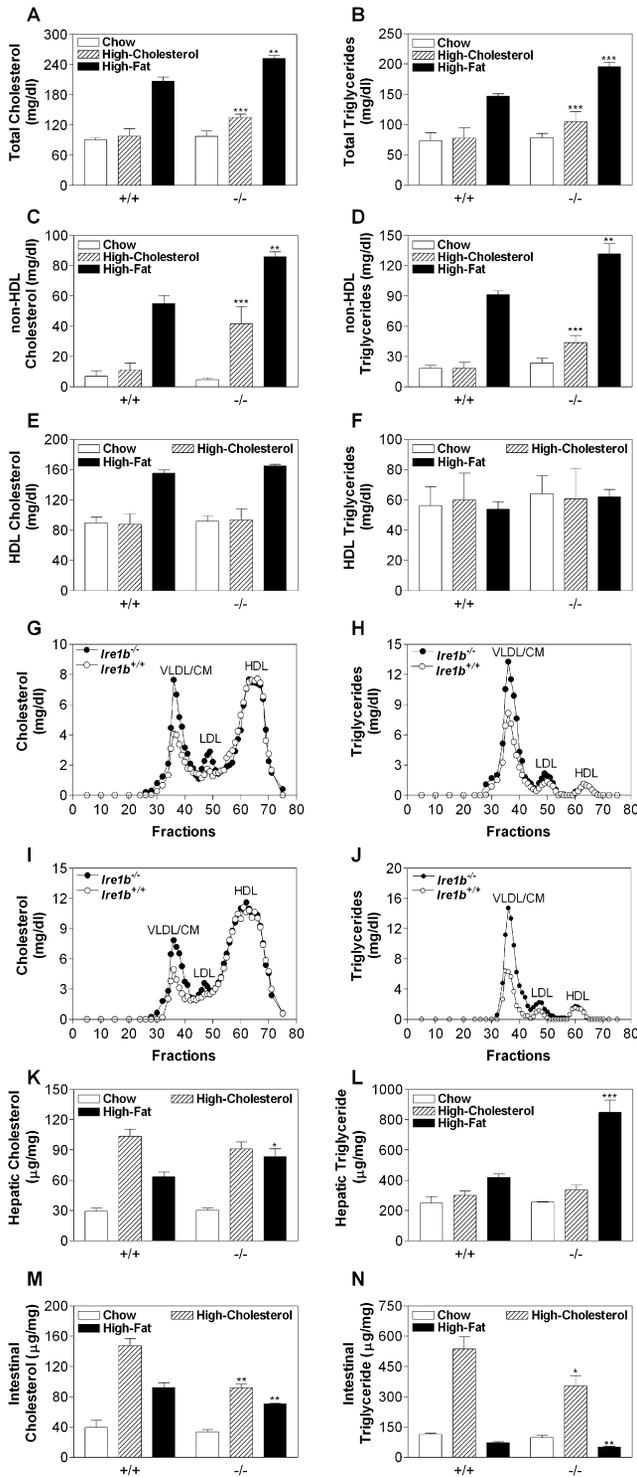
The central role of the ER in chylomicron assembly prompted us to examine the inositol-requiring enzyme 1 $\beta$  (IRE1 $\beta$ ) in the intestinal epithelial cells of vertebrates (Bertolotti et al., 2000, 2001; Iwawaki et al., 2001). IRE1 $\beta$  is closely related to the ubiquitously expressed ER stress-response protein IRE1 $\alpha$  (Bernales et al., 2006). IRE1 proteins anchored in the ER membrane contain a sensory domain that is exposed to the lumen and an effector domain facing the cytosol. The conserved effector domain of IRE1 is homologous to the viral-induced RNA-degrading enzyme RNase L. IRE1 proteins mediate the splicing of the X-box-binding protein 1 (XBP-1) mRNA precursor (Zhang et al., 2005; Niwa et al., 1999; Calton et al., 2002). The spliced mRNA is translated more efficiently, and the synthesized protein acts as a transcription factor for several genes involved in the unfolded protein response (Yoshida et al., 2001). Although IRE1 $\alpha$  acts mainly via XBP-1 splicing, there are exceptions. Glucose enhances IRE1 $\alpha$  phosphorylation in pancreatic  $\beta$  cells and augments insulin biosynthesis without increasing XBP-1 splicing (Lipson et al., 2006). In *Drosophila*, IRE1 can degrade specific mRNAs undergoing translation at the ER membrane and halt protein synthesis (Hollien and Weissman, 2006).

*Ire1b*<sup>-/-</sup> mice develop normally and are fertile, but they are resistant to toxin-induced colitis (Bertolotti et al., 2001). In this study, we show that *Ire1b*<sup>-/-</sup> mice respond to cholesterol-rich and fat-rich diets with enhanced intestinal MTP expression and chylomicron production.

## RESULTS

### Plasma Non-HDL Lipids Are Elevated in *Ire1b*<sup>-/-</sup> Mice Fed High-Cholesterol and High-Fat Diets

To test whether IRE1 $\beta$  plays a role in lipid metabolism, *Ire1b*<sup>+/+</sup> (WT) and *Ire1b*<sup>-/-</sup> (KO) mice were placed on a high-cholesterol diet or a high-fat western diet for 2 weeks (Figure 1). Compared with standard chow, a western diet significantly increased plasma levels of cholesterol and triglyceride in WT mice (Figures 1A and 1B, +/+), similar to the results of other studies (Napoli et al., 2003). A high-cholesterol diet did not increase plasma levels of cholesterol and triglyceride in WT SV129 mice (Figures 1A and 1B, +/+), which agrees with the results of Schwarz et al. (2001). In contrast, because of a 2- to 4-fold increase in non-high-density lipoprotein (non-HDL) apoB lipoproteins (Figures



**Figure 1. Plasma Non-HDL Lipids Are Elevated and Intestinal Lipids Are Decreased in *Ire1b*<sup>-/-</sup> Mice Fed High-Cholesterol and High-Fat Diets**

Wild-type *Ire1b*<sup>+/+</sup> (+/+) and *Ire1b*<sup>-/-</sup> (-/-) male mice were fed normal chow, chow supplemented with 2% w/w cholesterol (average of five experiments with n = 3–5 animals per group), or a high-fat diet (n = 3 per group) for 2 weeks. (A–F) Plasma samples were used to measure total, non-HDL, and HDL cholesterol as well as triglyceride.

1C and 1D), both of these diets significantly increased plasma levels of cholesterol (Figure 1A) and triglycerides (Figure 1B) in KO mice. HDL cholesterol and triglyceride levels were similar in WT and KO mice (Figures 1E and 1F). Gel filtration showed that lipid levels were increased in all apoB lipoproteins (very low-density lipoproteins/chylomicrons [VLDL/CM] and low-density lipoproteins [LDL]) but not in HDL lipids (Figures 1G–1J) of KO mice. Thus, on high-cholesterol or high-fat diets, *Ire1b*<sup>-/-</sup> mice develop more pronounced hyperlipidemia than WT mice do.

### Intestinal Lipid Levels Are Reduced in *Ire1b*<sup>-/-</sup> Mice Fed High-Cholesterol and High-Fat Diets

WT mice on high-fat and high-cholesterol diets accumulated significantly higher amounts of cholesterol in their livers compared with chow-fed animals (Figure 1K, +/+), as reported by Schwarz et al. (2001). Similar increases were seen in KO mice (Figure 1K, -/-). There were no significant differences in hepatic cholesterol and triglyceride levels in cholesterol-fed WT and KO mice (Figures 1K and 1L, hatched bars). However, hepatic cholesterol and triglyceride levels were higher in KO mice than they were in WT mice fed a high-fat diet (Figures 1K and 1L, black bars). KO mice accumulated less intestinal cholesterol and triglyceride on both diets compared with WT mice (Figures 1M and 1N). Thus, on high-cholesterol and high-fat diets, *Ire1b*<sup>-/-</sup> mice show lower intestinal lipid accumulation than WT mice do.

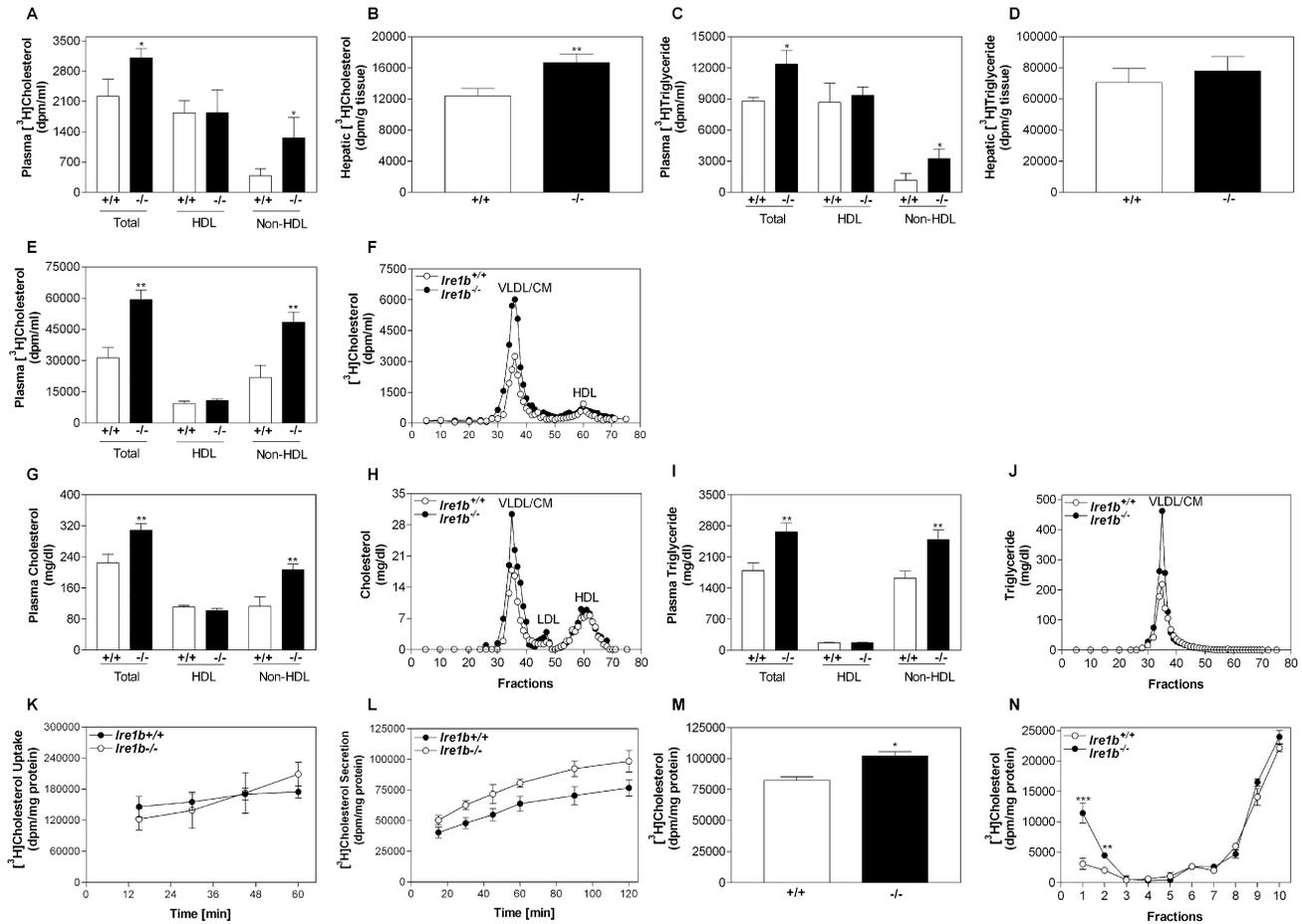
### Cholesterol-Fed *Ire1b*<sup>-/-</sup> Mice Absorb More Lipids

Increases in plasma apoB lipoprotein levels and decreases in intestinal lipid levels could be explained by similar uptake in WT and KO mice and enhanced secretion of intestinal lipoproteins in KO mice. Long-term cholesterol absorption studies using a dual-label fecal excretion method failed to show any significant differences (see Figure S1 available online), indicating similar uptake. Next, we performed short-term absorption studies. Radioactive cholesterol and triolein (Figures 2A–2D) were fed to cholesterol-fed WT and KO mice. After 2 hr, there was an approximately 50% increase in plasma [<sup>3</sup>H]cholesterol of KO mice compared with WT mice because of a 3.5-fold increase in non-HDL apoB lipoproteins, with no change in HDL levels (Figure 2A). The KO mice also showed an approximately 30% increase in hepatic [<sup>3</sup>H]cholesterol (Figure 2B). These studies show increased absorption of radiolabeled cholesterol by KO mice. Compared with WT mice, plasma [<sup>3</sup>H]triolein-derived counts were approximately 60% higher in KO mice because of a 2-fold increase in non-HDL apoB lipoproteins (Figure 2C), but hepatic [<sup>3</sup>H]triolein counts were similar in these mice (Figure 2D). These observations are consistent with the understanding that absorbed dietary cholesterol and fatty acids are primarily delivered to the liver and the peripheral tissues, respectively. These results suggest that IRE1 $\beta$  deficiency increases lipid absorption via apoB lipoproteins.

(G–J) Plasma samples from high-cholesterol (G and H) and high-fat (I and J) fed animals were separated by gel filtration to measure lipids in different lipoproteins.

(K–N) Liver and intestinal samples (n = 3) were used to measure cholesterol and triglyceride mass.

Values are mean  $\pm$  SD. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 versus *Ire1b*<sup>+/+</sup>.



**Figure 2. Intestines from *Ire1b*<sup>-/-</sup> Mice Secrete More Lipids with Chylomicrons**

*Ire1b*<sup>+/+</sup> (+/+; white bars) and *Ire1b*<sup>-/-</sup> (-/-; black bars) mice were fed a high-cholesterol diet for 2 weeks and fed 1  $\mu$ Ci of [<sup>3</sup>H]cholesterol or [<sup>3</sup>H]triolein as well as 0.1 mg of cholesterol in 15  $\mu$ l of olive oil.

(A and C) After 2 hr, plasma was used to measure radioactivity.

(B and D) Liver slices were digested with OptiSolv and used for radioactivity determinations.

(E–J) In another experiment, *Ire1b*<sup>+/+</sup> and *Ire1b*<sup>-/-</sup> mice fasted overnight were injected intraperitoneally with poloxamer 407 (30  $\mu$ g/mouse). After 1 hr, mice were fed 1  $\mu$ Ci [<sup>3</sup>H]cholesterol and 0.1 mg unlabeled cholesterol. Plasma was collected after 2 hr and used to measure radioactivity (E) as well as cholesterol (G) and triglyceride (I) mass in total plasma, HDL, and non-HDL lipoproteins. Plasma was separated by gel filtration to determine lipids in different lipoproteins (F, H, and J).

(K) Enterocytes were isolated from cholesterol-fed male *Ire1b*<sup>+/+</sup> and *Ire1b*<sup>-/-</sup> mice and incubated with 1  $\mu$ Ci/ml of [<sup>3</sup>H]cholesterol for different lengths of time to study uptake. At each time point, cellular lipids were extracted with isopropanol and counted.

(L) To study secretion, cells were labeled with [<sup>3</sup>H]cholesterol for 1 hr, washed, and chased for various lengths of time in the presence of 1.2 mM oleic acid. Radioactivity was measured in conditioned media.

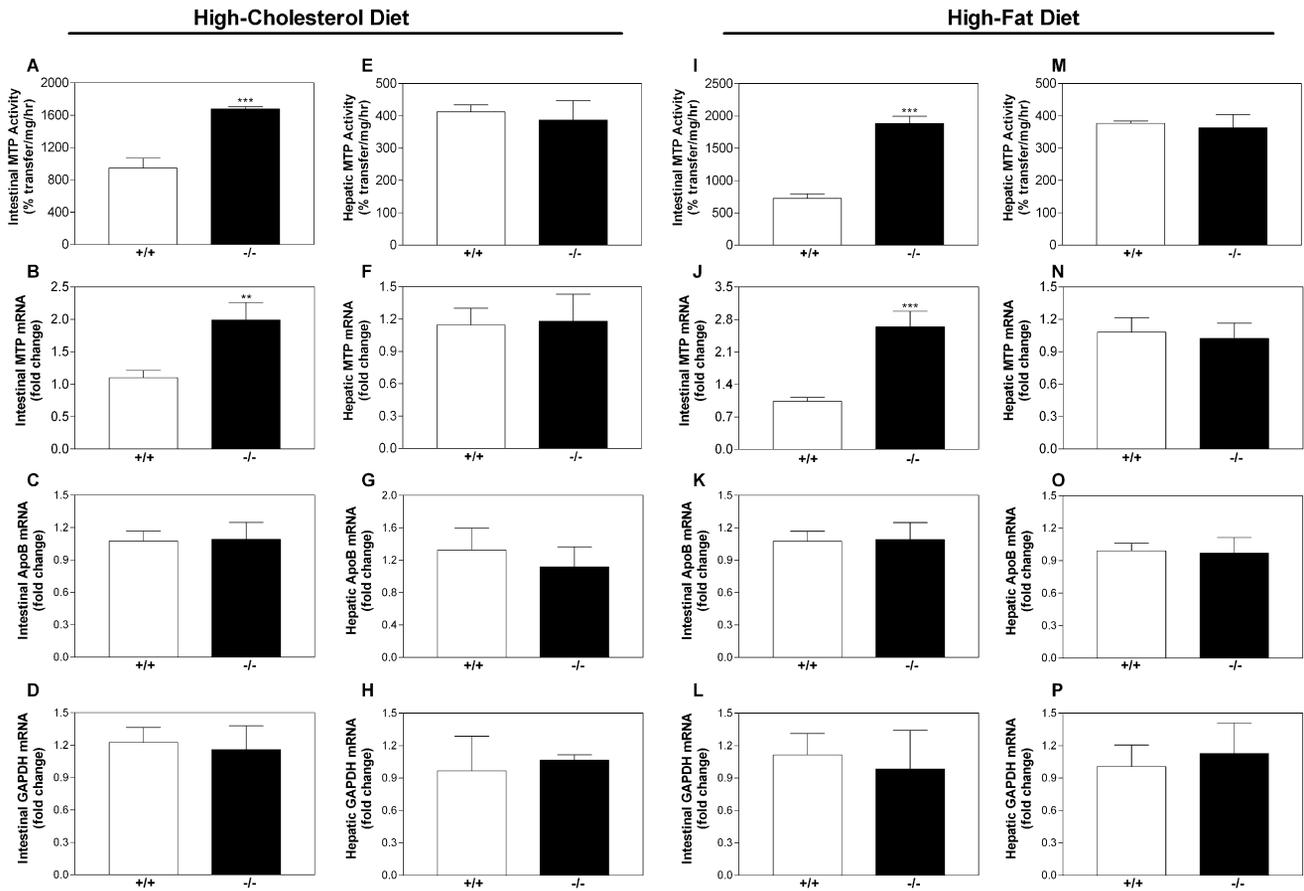
(M and N) To identify lipoproteins carrying cholesterol, enterocytes from cholesterol-fed male *Ire1b*<sup>+/+</sup> and *Ire1b*<sup>-/-</sup> mice were labeled for 1 hr with 1  $\mu$ Ci/ml of [<sup>3</sup>H]cholesterol, washed, and chased for 2 hr in the presence of 1.2 mM oleic acid-containing micelles. Conditioned media were used to measure radioactivity (M) or for density gradient ultracentrifugation. Fractions were collected from the top and assayed for radioactivity (N).

Each measurement was performed in triplicate with n = 3 mice per group. Values are mean  $\pm$  SD. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 versus *Ire1b*<sup>+/+</sup>.

To confirm increased lipid absorption in KO mice, mice fed high-cholesterol diets were injected intraperitoneally with the lipoprotein lipase inhibitor poloxamer 407 (Millar et al., 2005; Anwar et al., 2007) and fed 1 hr later with [<sup>3</sup>H]cholesterol in olive oil. There was a 90% increase in the appearance of [<sup>3</sup>H]cholesterol in the plasma of KO versus WT mice, which was associated with a 2.2-fold increase in non-HDL (Figure 2E). These counts were 20 times higher in poloxamer 407-injected animals than those seen in mice not injected with the lipase inhibitor (compare to Figure 2A), indicating significant inhibition of lipoprotein clear-

ance. Gel filtration analysis showed increased counts in the apoB lipoproteins (VLDL/CM) of KO mice (Figure 2F). Mass measurements showed that total plasma cholesterol and triglyceride levels were greater in apoB lipoproteins (Figures 2G–2J). These studies indicate increased lipid absorption via apoB lipoprotein production in *Ire1b*<sup>-/-</sup> mice.

Despite the decreased intestinal cholesterol level in KO mice (Figures 1M and 1N), isolated primary enterocytes showed no differences in the uptake of radiolabeled cholesterol (Figure 2K), and NPC1L1 mRNA levels were similar in WT and



**Figure 3. MTP mRNA, Protein, and Activity Are Increased in the Intestines of *Ire1b*<sup>-/-</sup> Mice**

Intestinal and liver samples from *Ire1b*<sup>+/+</sup> (+/+; white bars) and *Ire1b*<sup>-/-</sup> (-/-; black bars) mice (n = 3 per group) fed a cholesterol-enriched or fat-enriched diet were used for MTP activity assays (A, E, I, and M). RNA was used to measure MTP (B, F, J, and N), apoB (C, G, K, and O), GAPDH (D, H, L, and P), and ARPP0 mRNA in triplicate. The ratio between the gene of interest and ARPP0 in *Ire1b*<sup>+/+</sup> mice was used to normalize mRNA levels in all samples. Values are mean  $\pm$  SD. \*\*p < 0.01, \*\*\*p < 0.001 compared to *Ire1b*<sup>+/+</sup>. Data are representative of two experiments.

KO mice (Figures S2A and S2C). However, cholesterol secretion was higher in KO enterocytes (Figure 2L). Therefore, KO enterocytes take up cholesterol as efficiently as WT enterocytes but secrete more cholesterol than WT, explaining the lower levels of intestinal cholesterol in KO mice.

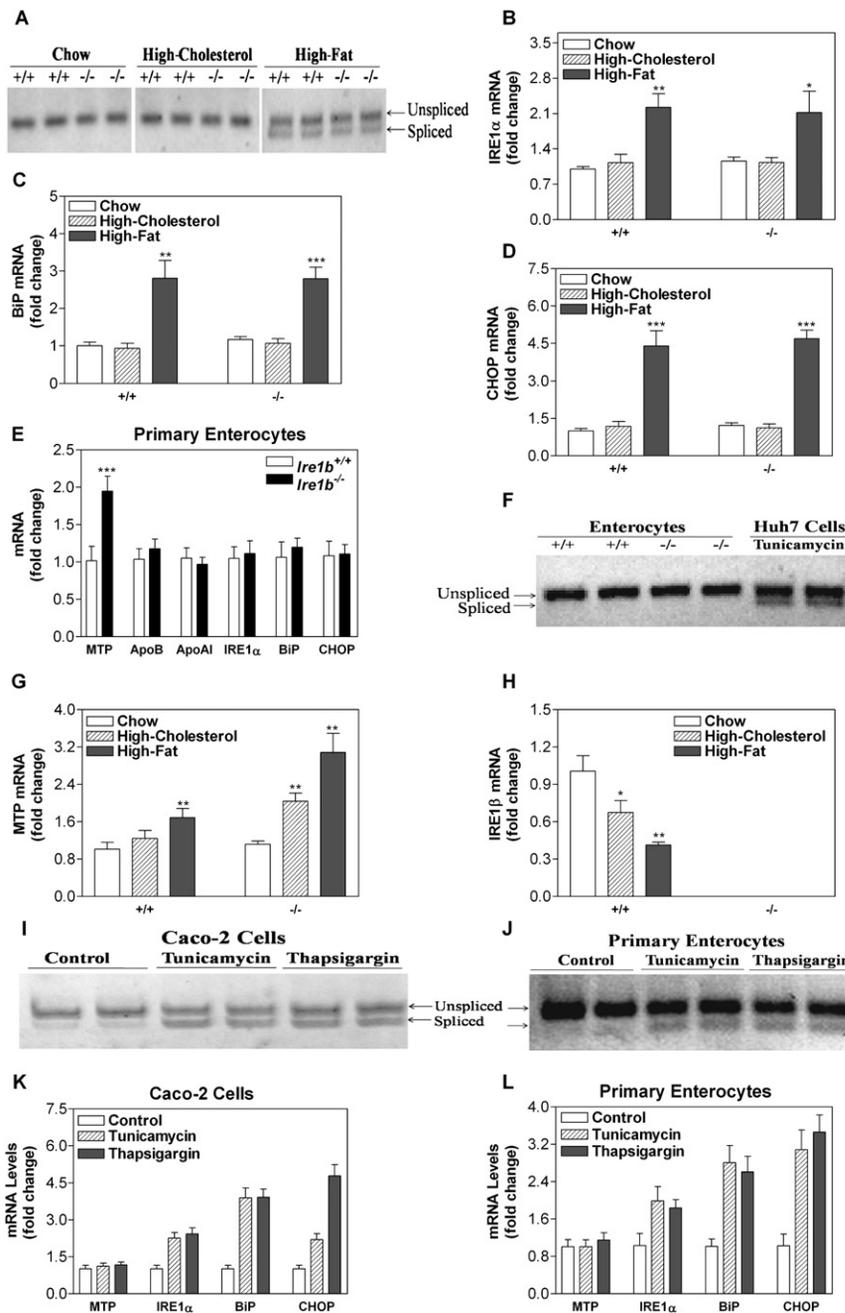
#### Primary Enterocytes from Cholesterol-Fed *Ire1b*<sup>-/-</sup> Mice Secrete More Chylomicrons

The gel filtration and lipoprotein precipitation methods used above (Figures 2A–2J) do not distinguish between different triglyceride-rich lipoproteins. To identify the types of intestinal lipoproteins increased in IRE1 $\beta$  deficiency, we studied lipoprotein production in primary enterocytes isolated from cholesterol-fed KO and WT mice. [<sup>3</sup>H]cholesterol secretion was approximately 25% greater in KO enterocytes than it was in WT enterocytes (Figure 2M), which could reflect a more substantial increase in chylomicrons with no increase in other lipoproteins. Therefore, we subjected the conditioned media to potassium bromide density gradient ultracentrifugation to separate chylomicrons from other lipoproteins. The WT enterocytes secreted [<sup>3</sup>H]cholesterol into chylomicrons at the top of the gradient (Luchoomun and

Hussain, 1999) and into HDL particles at the bottom of the gradient (Iqbal and Hussain, 2005), consistent with the secretion of cholesterol via both apoB-dependent and apoB-independent pathways (Iqbal and Hussain, 2005; Iqbal et al., 2003; Hussain et al., 2005). The secretion of [<sup>3</sup>H]cholesterol into chylomicron-like particles was approximately 3.7-fold greater in KO versus WT enterocytes (Figure 2N, fraction 1), while secretion into HDL lipoproteins was similar (Figure 2N, fractions 8–10). Thus, IRE1 $\beta$  deficiency selectively increases chylomicron secretion.

#### Intestinal MTP mRNA and Activity Are Elevated in Cholesterol- and Fat-Fed *Ire1b*<sup>-/-</sup> Mice

MTP is an essential chaperone and a rate-limiting factor for chylomicron assembly (Hussain et al., 2003a, 2003b). In fat-fed animals, the intestines (Figures S3A and S3C), but not the livers (Figures S3B and S3D), of KO mice had 2- to 3-fold higher amounts of MTP protein. The triglyceride transfer activity of MTP was 2- to 2.5-fold higher in KO intestines (Figures 3A and 3I), but hepatic MTP activity was not different (Figures 3E and 3M). Levels of MTP mRNA were 2- to 2.7-fold higher in the intestines of cholesterol-fed and fat-fed *Ire1b*<sup>-/-</sup> mice (Figures 3B



**Figure 4. XBP-1 Splicing Does Not Regulate MTP**

RNA was isolated from the intestinal tissues of *Ire1b*<sup>+/+</sup> and *Ire1b*<sup>-/-</sup> mice (n = 3) fed with or without high-cholesterol or high-fat diet for 2 weeks. (A) Spliced and unspliced XBP-1 mRNA levels. (B–D, G, and H) Intestinal RNA was used to determine in triplicate the levels of IRE1 $\alpha$  (B), BiP (C), CHOP (D), MTP (G), and IRE1 $\beta$  (H) mRNAs. Values are mean  $\pm$  SD. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 versus chow-fed animals. (E and F) RNA also was isolated from the primary enterocytes of *Ire1b*<sup>+/+</sup> and *Ire1b*<sup>-/-</sup> mice fed a high-cholesterol diet and used to quantify mRNA levels of ER stress and lipoprotein genes (E) and XBP-1 splicing (F). RNA from Huh7 cells treated with tunicamycin (5  $\mu$ g/ml) was used as a control (F). Values are mean  $\pm$  SD. \*\*\*p < 0.001 versus *Ire1b*<sup>+/+</sup>. (I and J) Differentiated Caco-2 cells were treated with tunicamycin (5  $\mu$ g/ml) or thapsigargin (1  $\mu$ M) for 17 hr and used to quantify spliced as well as unspliced XBP-1 mRNA (I) and to measure MTP, IRE1 $\alpha$ , BiP, CHOP, and ARPP0 mRNA (J). (K and L) Enterocytes isolated from *Ire1b*<sup>+/+</sup> mice were treated with tunicamycin (10  $\mu$ g/ml) or thapsigargin (5  $\mu$ M) for 3 hr and used to measure XBP-1 splicing (J) and MTP, IRE1 $\alpha$ , BiP, CHOP, and ARPP0 mRNA (L).

express apoB or MTP, with an apoB48 expression plasmid and different amounts of a human MTP-FLAG expression plasmid (Figure S4). No apoB was secreted in the absence of MTP, but apoB secretion increased as MTP expression increased, consistent with other studies (Gordon et al., 1994; Leiper et al., 1994).

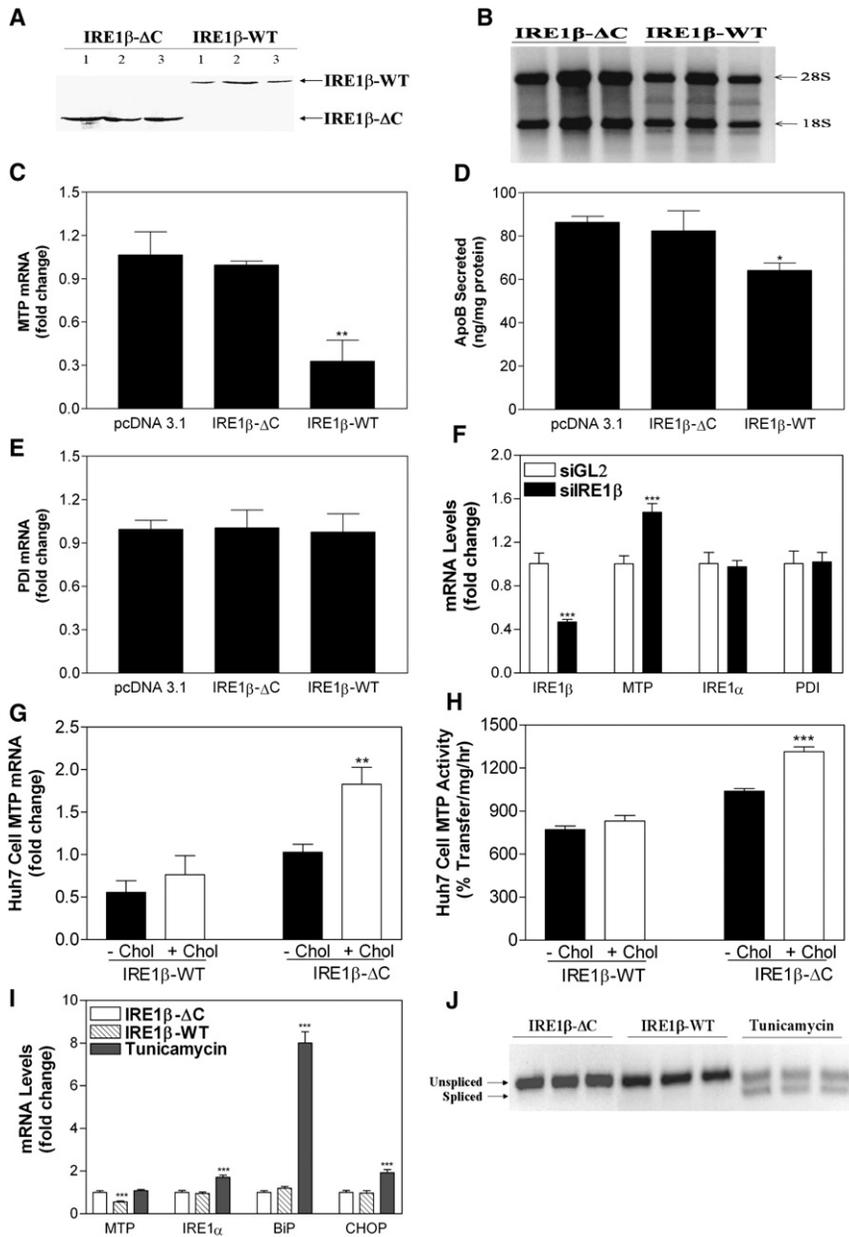
**Changes in MTP Expression Are Not Correlated with ER Stress**

We next sought to identify how IRE1 $\beta$  deficiency leads to increased MTP. Although IRE1 $\beta$ -mediated XBP-1 mRNA splicing in cholesterol-fed WT mice could theoretically suppress MTP mRNA, intestinal samples from chow-fed and cholesterol-fed animals did not induce XBP-1 mRNA splicing (Figure 4A) or IRE1 $\alpha$ , BiP, or CHOP mRNA levels, which increase during ER stress (Figures 4B–4D).

To increase sensitivity, we used enterocytes isolated from cholesterol-fed WT and KO mice and found an increase in MTP mRNA levels but no changes in IRE1 $\alpha$ , BiP, or CHOP (Figure 4E) or in XBP-1 mRNA splicing (Figure 4F). As a control, XBP-1 mRNA splicing was evident in tunicamycin-exposed human hepatoma Huh7 cells (Figure 4F). These data indicate that a high-cholesterol diet does not induce intestinal ER stress. In contrast, the high-fat diet induced similar levels of ER stress in the intestines of both WT and KO mice, as evidenced by XBP-1 mRNA splicing (Figure 4A), and increases in IRE1 $\alpha$ , BiP, and

CHOP mRNA levels, which increase during ER stress (Figures 4B–4D). To increase sensitivity, we used enterocytes isolated from cholesterol-fed WT and KO mice and found an increase in MTP mRNA levels but no changes in IRE1 $\alpha$ , BiP, or CHOP (Figure 4E) or in XBP-1 mRNA splicing (Figure 4F). As a control, XBP-1 mRNA splicing was evident in tunicamycin-exposed human hepatoma Huh7 cells (Figure 4F). These data indicate that a high-cholesterol diet does not induce intestinal ER stress. In contrast, the high-fat diet induced similar levels of ER stress in the intestines of both WT and KO mice, as evidenced by XBP-1 mRNA splicing (Figure 4A), and increases in IRE1 $\alpha$ , BiP, and

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**Figure 5. Overexpression of IRE1 $\beta$  Decreases MTP mRNA Levels**

(A) Huh7 cells transfected with different c-Myc-tagged IRE1 $\beta$  expression plasmids were used to determine protein expression using c-Myc antibodies.

(B) Total RNA from IRE1 $\beta$ - $\Delta$ C- and IRE1 $\beta$ -WT-transfected Huh7 cells was loaded on 1% agarose gels to visualize changes in 18S and 28S rRNA.

(C–E) Cells from three wells were used to measure MTP (C) and PDI (E) mRNA in triplicate. Media were used to measure apoB secretion (D). \* $p < 0.05$ , \*\* $p < 0.01$  compared to pcDNA3.1-treated cells.

(F) RNA interference was used to knock down IRE1 $\beta$  in undifferentiated Caco-2 cells. siGL2 served as a control. Cells were treated with siRNA targeting IRE1 $\beta$  and used to measure IRE1 $\beta$ , MTP, IRE1 $\alpha$ , and PDI mRNAs. \*\*\* $p < 0.001$  compared to siGL2-treated cells.

(G and H) To study the effect of cholesterol on MTP expression, Huh7 cells were transfected with either IRE1 $\beta$ -WT or IRE1 $\beta$ - $\Delta$ C expression plasmids. After 12 hr, cells were supplemented with or without cholesterol (40  $\mu$ g/ml), and MTP and ARPP0 mRNA were quantitated (G). Cells treated in parallel were used for MTP activity assays (H). \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to IRE1 $\beta$ - $\Delta$ C-overexpressing cells without cholesterol treatment.

(I and J) Huh7 cells were transfected with IRE1 $\beta$  expression plasmids or treated with 5  $\mu$ g/ml tunicamycin. Total RNA was isolated and used to measure MTP, IRE1 $\alpha$ , BiP, and CHOP mRNA (I). \*\*\* $p < 0.001$  compared to IRE1 $\beta$ - $\Delta$ C-overexpressing cells. In addition, RNA was used to determine XBP-1 splicing (J).

Data are representative of two experiments performed in triplicate. Values are mean  $\pm$  SD.

CHOP mRNA (Figures 4B–4D). Thus, induction of ER stress is independent of genotype and is a consequence of a high-fat diet.

In WT mice, a high-fat diet increased intestinal MTP mRNA by 68% (Figure 4G, +/+). High-cholesterol diet and high-fat diet increased intestinal MTP mRNA in KO mice 2-fold and 3-fold (Figure 4G, -/-), respectively, compared with chow-fed WT. The high-cholesterol and high-fat diets decreased IRE1 $\beta$  mRNA levels by 33% and 59%, respectively, in WT mice (Figure 4H, +/+). Thus, both diets reduce intestinal IRE1 $\beta$  in WT mice but increase MTP mRNA in *Ire1b*<sup>-/-</sup> mice. Direct cell stress and activation of XBP-1 splicing in Caco-2 human colon carcinoma cells (Figure 4I) and primary enterocytes (Figure 4J) by tunicamycin or thapsigargin did not affect MTP mRNA (Figures 4K and 4L) or activity (data not shown) but did increase IRE1 $\alpha$ , BiP, and CHOP mRNA levels.

ER stress and XBP-1 in intestinal cells does not affect MTP expression.

#### MTP mRNA Changes Proportionally to IRE1 $\beta$ Expression

To test whether MTP is directly suppressed by IRE1 $\beta$ , we transfected human hepatoma Huh7 cells, which express MTP but not IRE1 $\beta$ , with c-Myc-tagged plasmids expressing full-length IRE1 $\beta$  (IRE1 $\beta$ -WT) and C-terminally truncated IRE1 $\beta$  (IRE1 $\beta$ - $\Delta$ C) lacking the kinase and endoribonuclease domains (Figure 5A) (Urano et al., 2000; Wang et al., 1998). Expression of IRE1 $\beta$ - $\Delta$ C did not affect MTP mRNA (Figure 5C), apoB secretion (Figure 5D), or protein disulfide isomerase (PDI) mRNA (Figure 5E). IRE1 $\beta$ -WT expression reduced 28S rRNA (Figure 5B) consistent with Iwawaki et al. (2001), suppressed MTP mRNA (Figure 5C), and decreased apoB lipoprotein secretion (Figure 5D) but did not

In summary, a high-cholesterol diet does not induce ER stress but increases intestinal MTP mRNA in KO mice. A high-fat diet induces similar ER stress in both WT and KO mice but significantly induces MTP mRNA only in the intestines of KO mice. Furthermore, induction of

affect PDI (Figure 5E) or apoB, apoA1, ACAT1, ACAT2, MGAT2, MGAT3, DGAT1, DGAT2, or NPC1L1 mRNA (Figure S2E), suggesting no general effects on cell health.

We next determined the consequences of decreasing IRE1 $\beta$  on MTP expression in undifferentiated Caco-2 cells through RNA interference. IRE1 $\beta$  siRNA reduced its target mRNA by 60% with no effect on IRE1 $\alpha$  mRNA (Figure 5F). More importantly, IRE1 $\beta$  knockdown increased MTP mRNA by 50% (Figure 5F) and did not affect PDI mRNA. These data show that expression and suppression of IRE1 $\beta$  lead to specific changes in MTP mRNA through a mechanism that requires the kinase and ribonuclease domains.

To investigate why cholesterol feeding was necessary to observe differences in plasma lipid levels in WT and KO mice, Huh7 cells were transfected with IRE1 $\beta$ -WT or IRE1 $\beta$ - $\Delta$ C and cultured in the presence or absence of cholesterol. Cholesterol treatment of cells expressing IRE1 $\beta$ - $\Delta$ C significantly increased MTP mRNA (Figure 5G) and activity (Figure 5H), as occurred in KO mice fed a high-cholesterol diet (Figures 3A and 3B). In contrast, cholesterol supplementation of cells expressing IRE1 $\beta$ -WT did not affect MTP mRNA (Figure 5G) or activity (Figure 5H). Therefore, IRE1 $\beta$  counteracts cholesterol-induced increases in MTP expression. Similar studies were not performed with high fatty acid concentrations because they are toxic to cells.

Next, we asked whether overexpression of IRE1 $\beta$  in Huh7 cells increases ER stress and decreases MTP mRNA. Expression of IRE1 $\beta$ - $\Delta$ C and IRE1 $\beta$ -WT had no effect on IRE1 $\alpha$ , BiP, or CHOP mRNA (Figure 5I), and no splicing of XBP-1 occurred in these cells (Figure 5J). On the other hand, treatment of Huh7 cells with tunicamycin increased ER stress indicators, IRE1 $\alpha$ , BiP, CHOP, and XBP-1 splicing (Figures 5I and 5J). Thus, Huh7 cells respond to ER stress transducers but do not upregulate these genes when transfected with IRE1 $\beta$ . We conclude that IRE1 $\beta$  decreases MTP levels without inducing an ER stress response.

### IRE1 $\beta$ Decreases MTP mRNA by Promoting Posttranscriptional Degradation

To determine whether IRE1 $\beta$ -mediated suppression of MTP mRNA occurs at the transcriptional level, we determined the promoter activity of a 1.5 kb sequence upstream of the transcription initiation site. Luciferase activity expressed under the control of the MTP (*mtp*) promoter could be measured in Huh7 and Caco-2 cells but not HEK293 cells (Figure 6A), indicating that the promoter is sufficient for expression in the intestine and liver; this is consistent with other studies showing that 204 bases are sufficient for MTP expression (Hagan et al., 1994; Hirokane et al., 2004; Kang et al., 2003). To evaluate whether IRE1 $\beta$  affects *mtp* promoter activity, we cotransfected Huh7 cells with a plasmid expressing luciferase under the control of the 1.5 kb *mtp* promoter as well as pcDNA3.1, IRE1 $\beta$ -WT, or IRE1 $\beta$ - $\Delta$ C expression plasmids. IRE1 $\beta$ -WT did not suppress *mtp* promoter activity compared with vector control or IRE1 $\beta$ - $\Delta$ C (Figure 6B), indicating that IRE1 $\beta$  does not reduce MTP levels by altering *mtp* promoter activity.

To explore posttranscriptional mechanisms, we used mouse fibroblast L cells stably transfected with mouse MTP cDNA under the control of the cytomegalovirus promoter (Dogan et al., 2007). Expression of IRE1 $\beta$ -WT in these cells reduced

endogenous MTP mRNA (Figure 6C), activity (Figure 6D), and protein (Figure 6D inset). In contrast, IRE1 $\beta$ - $\Delta$ C and IRE1 $\alpha$  had no effect on MTP mRNA (Figure 6C), activity (Figure 6D), or protein (Figure 6D inset). To explore whether IRE1 $\beta$  decreases the stability of MTP mRNA, Huh7 cells expressing IRE1 $\beta$ -WT or IRE1 $\beta$ - $\Delta$ C were treated with actinomycin D to block new mRNA transcription. MTP mRNA, but not PDI mRNA, decayed at a greater rate in cells expressing IRE1 $\beta$ -WT than in cells expressing IRE1 $\beta$ - $\Delta$ C (Figure 6E), indicating that IRE1 $\beta$ -WT promotes MTP mRNA degradation.

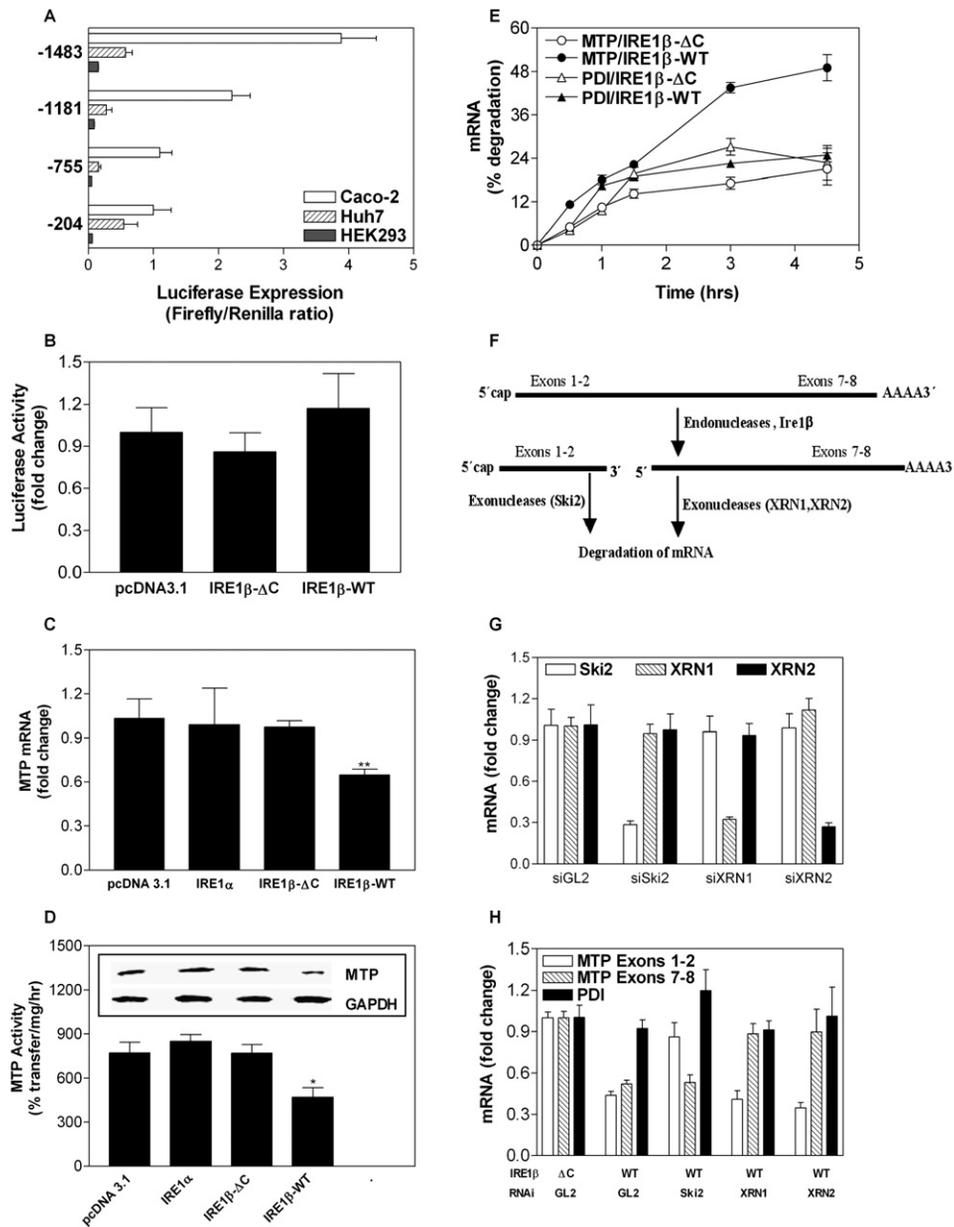
A plausible scenario for MTP mRNA degradation would involve its cleavage by IRE1 $\beta$  endonuclease activity (Figure 6F), followed by exonuclease digestion of the cleaved products. In this context, the exonucleases Ski2 and XRN1/2 may perform 3'  $\rightarrow$  5' and 5'  $\rightarrow$  3' exonuclease digestion, respectively, of endonuclease-cleaved mRNA (Newbury, 2006; Houseley et al., 2006; Meyer et al., 2004). Thus, knockdown of these exonucleases should preserve the putative 5' or 3' cleavage products of MTP mRNA. To test this, Huh7 cells were transfected with IRE1 $\beta$ -WT or IRE1 $\beta$ - $\Delta$ C and treated with Ski2, XRN1, XRN2, or control GL2 siRNA, which specifically reduced 70%–75% of the target mRNAs (Figure 6G). Expression of IRE1 $\beta$ -WT decreased MTP mRNA sequences corresponding to exons 1–2 (5') and exons 7–8 (3') when treated with control GL2 siRNA but did not affect PDI mRNA levels (Figure 6H, WT+GL2). Treatment with Ski2 siRNA prevented the loss of sequences corresponding to MTP exons 1–2 but not exons 7–8 (Figure 6H, WT+Ski2). Similarly, XRN1 and XRN2 siRNA selectively limited the loss of exons 7–8. These data are consistent with IRE1 $\beta$ -dependent endonuclease cleavage between exons 2 and 7, followed by 3'  $\rightarrow$  5' exonuclease cleavage of the 5' fragment(s) by Ski2 and 5'  $\rightarrow$  3' exonuclease cleavage of the 3' fragment(s) by XRN1 and XRN2.

### DISCUSSION

IRE1 $\beta$  deficiency in mice enhances intestinal MTP expression without affecting hepatic MTP and increases lipid absorption and chylomicron secretion in response to high-cholesterol and high-fat diets. IRE1 $\beta$  reduces MTP mRNA levels by augmenting posttranscriptional degradation. Thus, the present study identifies an intestine-specific mechanism controlling the assembly and secretion of apoB lipoproteins, wherein IRE1 $\beta$  regulates chylomicron production by degrading MTP mRNA.

Eating high-cholesterol and high-fat diets enhances MTP expression in the liver, and to a lesser extent in the intestine, of WT mice. In contrast, diet-induced increases in intestinal and hepatic MTP are similar in KO mice. These observations indicate that MTP expression is more responsive to dietary manipulations in the absence of IRE1 $\beta$  and that IRE1 $\beta$  blocks diet-induced upregulation of intestinal MTP in WT mice.

*Ire1b*<sup>-/-</sup> mice fed high-cholesterol or high-fat diets produce more intestinal apoB lipoproteins both in vivo (Figures 2A–2J) and in isolated enterocytes (Figures 2M and 2N), which eliminates generalized intestinal damage, permeability, and other pathological mechanisms as potential explanations for this increase. This increased apoB lipoprotein production is probably secondary to increased MTP activity because (1) IRE1 $\beta$  deficiency did not affect apoB mRNA, (2) changes in MTP



**Figure 6. IRE1 $\beta$  Enhances Posttranscriptional Degradation of MTP mRNA**

(A) Caco-2, Huh7, and HEK293 cells in 12-well plates were cotransfected with 40 ng of control plasmid (pCMV-RL) and 1  $\mu$ g of pGL2 plasmid DNAs with luciferase under the control of various lengths (–204 to –1483 bp) of 5' sequences upstream of the human *MTP* gene. Cells were collected 48 hr later and analyzed with a Dual-Luciferase Reporter kit (Promega).

(B) Promoter activity assays were performed using the dual-luciferase assay system. pCMV-Renilla luciferase served as a control for transfection efficiency. Huh7 cells were transfected with 20 ng pCMV-Renilla luciferase and 1  $\mu$ g pMTP-1483-luciferase (expressing luciferase activity under the control of a 1.5 kb *MTP* promoter) with pcDNA3.1, IRE1 $\beta$ - $\Delta$ C (100 ng), or IRE1 $\beta$ -WT (100 ng). After 48 hr, luciferase activity was measured.

(C and D) Mouse fibroblasts L cells stably expressing mouse MTP under the control of the cytomegalovirus promoter were transfected with different plasmids. After 48 hr, MTP mRNA (C) and activity (D) were measured in triplicate. Inset in (D) shows western blot of a representative sample. \* $p < 0.05$ , \*\* $p < 0.01$  versus pcDNA3.1-treated cells.

(E) Huh7 cells were transfected in triplicate with either IRE1 $\beta$ -WT or IRE1 $\beta$ - $\Delta$ C expression plasmid DNA. After overnight incubation, cells were cultured in the presence of actinomycin D (1  $\mu$ g/ml) for different lengths of time in triplicate. RNA was used to quantify MTP, PDI, and ARPP0 mRNA. The MTP/ARPP0 or PDI/ARPP0 ratio at time 0 was normalized to 100%, and loss of MTP was plotted versus time.

(F) Schematic representation of possible internal cleavage of MTP mRNA by IRE1 $\beta$  and its subsequent degradation by different exonucleases.

(G) Huh7 cells were transfected with IRE1 $\beta$ -WT. After 24 hr, cells were transfected with siRNAs against Ski2, XRN1, or XRN2. RNA was isolated after 48 hr and used to quantify Ski2, XRN1, or XRN2 mRNA in control (siGL2) and RNAi-treated cells to determine the efficacy and specificity of RNA interference.

(H) Huh7 cells were transfected in triplicate with IRE1 $\beta$ - $\Delta$ C or IRE1 $\beta$ -WT expression plasmids. After 24 hr, cells were transfected with indicated siRNAs. Total RNA from these cells was used to amplify different regions (exons 1–2 and exons 7–8) of MTP mRNA using primers listed in Table S1.

Data are representative of two experiments performed in triplicate. Values are mean  $\pm$  SD.

expression significantly altered the secretion of apoB lipoproteins (Figure S4), and (3) changes in MTP levels affect plasma lipid and apoB lipoprotein levels (Tietge et al., 1999; Ameen et al., 2005; Wolfrum and Stoffel, 2006; Lin et al., 1994). Conversely, repression of MTP was associated with decreased apoB secretion. Chenodeoxycholate reduces MTP expression and apoB secretion in HepG2 cells (Hirokane et al., 2004). MTP antagonists reduce plasma lipids (Cuchel et al., 2007; Wetterau et al., 1998). Thus, changes in MTP activity significantly affect plasma apoB lipoprotein levels. Therefore, increases in MTP due to IRE1 $\beta$  deficiency are sufficient to augment apoB lipoprotein production.

*mtp*<sup>-/-</sup> mice are not viable (Raabe et al., 1998). Chow-fed heterozygous *mtp*<sup>+/-</sup> mice have plasma lipid levels similar to WT mice but significantly lower intermediate-density lipoprotein (IDL)/LDL cholesterol. A high-fat diet significantly lowers plasma cholesterol in *mtp*<sup>+/-</sup> mice owing to a marked reduction in VLDL and LDL cholesterol, and hepatocytes isolated from *mtp*<sup>+/-</sup> mice secrete less apoB lipoprotein (Raabe et al., 1998). Half-normal MTP levels reduce apoB lipoprotein secretion to a similar extent in *apob*<sup>+/-</sup> and *apob* transgenic mice (Leung et al., 2000). Therefore, MTP deficiency reduces apoB lipoprotein production and decreases plasma cholesterol when challenged with a high-fat diet. In *Ire1b*<sup>-/-</sup> mice, increases in intestinal MTP increase apoB lipoprotein production and enhance plasma cholesterol levels.

In abetalipoproteinemia, MTP deficiency significantly reduces total and LDL cholesterol. In general, obligate *MTP* heterozygotes present with normal plasma cholesterol, but several parents of abetalipoproteinemia patients have been found to have low cholesterol (Raabe et al., 1998; Narcisi et al., 1995; Berriot-Varoqueaux et al., 2000; Di Leo et al., 2005). The variations in plasma cholesterol in heterozygotes might result from an adaptation involving increased synthesis of MTP by the normal allele (Berriot-Varoqueaux et al., 2000). Moreover, plasma apoB lipoproteins have not been characterized, and high-fat feeding studies have not been performed in heterozygotes. Therefore, there are insufficient data to draw conclusions about the effect of half-normal MTP levels on plasma apoB lipoproteins.

Although IRE1 functions primarily via splicing XBP-1 mRNA, it represses ER-targeted mRNA independently of the XBP-1 pathway (Hollien and Weissman, 2006). IRE1 $\beta$  may reduce protein synthesis by cleaving 28S ribosomal RNA (Iwawaki et al., 2001). IRE1 $\alpha$  regulates proinsulin synthesis without enhancing XBP-1 splicing (Lipson et al., 2006). We did not observe spliced XBP-1 mRNA in the intestines of cholesterol-fed WT mice, and induction of XBP-1 splicing by tunicamycin and thapsigargin did not alter MTP mRNA levels (Figure 4). Thus, intestinal IRE1 $\beta$  regulates MTP independently of the XBP-1 pathway.

Despite the sequence homology between IRE1 $\alpha$  and IRE1 $\beta$ , the effect on MTP mRNA regulation was specific to IRE1 $\beta$  (Figure 6C). The cytosolic domains that contain the kinase and endoribonuclease activities in mouse IRE1 $\beta$  and IRE1 $\alpha$  exhibit 62%–63% identity, but the identity of the luminal domains is only 41%. Therefore, IRE1 $\beta$  may have evolved to detect a specific luminal signal—for example, fat status.

The posttranscriptional degradation of MTP mRNA could involve direct endolytic cleavage. IRE1 $\beta$  and MTP mRNA translation are associated with the ER membrane, and overexpression

of MTP may therefore facilitate its degradation by IRE1 $\beta$ . Alternatively, increased chylomicron assembly may temporarily stall the protein translational machinery, leading to the recognition of the MTP mRNA by IRE1 $\beta$  directly or via recruitment/activation of another endonuclease. If so, all components are present in the liver, and IRE1 $\beta$  may be the limiting factor for MTP downregulation.

We can only speculate as to why the enterocytes of vertebrates have this regulatory pathway to control chylomicron assembly. One hypothesis is that enterocytes optimize fat absorption by activating the ER stress response and downregulating IRE1 $\beta$ . By upregulating the ER stress response, the ER is protected from the increased load of synthesizing and packaging chylomicrons. By downregulating IRE1 $\beta$ , chylomicron assembly is maximized by enhancing MTP. In the fasting state, it may be beneficial to limit chylomicron secretion by degrading MTP mRNA to conserve critical cellular lipids and fat-soluble vitamins and to avoid rapid fluctuations in membrane phospholipids, neutral lipids, and vitamin E. Alternatively, IRE1 $\beta$  may mediate downregulation of chylomicron secretion in response to satiety signals or respond to endocrine factors from the liver and adipose tissue under conditions of excess whole-body lipid accumulation. In the absence of IRE1 $\beta$ , this postprandial mechanism of optimizing fat absorption is derailed, leading to a sustained hyperlipidemia.

In summary, these studies highlight an intestine-specific mechanism controlling lipid absorption. In this mechanism, IRE1 $\beta$  regulates MTP mRNA levels involving posttranscriptional degradation. IRE1 $\beta$  restricts MTP induction in the intestine by high-fat and high-cholesterol diets. These findings raise the possibility that upregulation of IRE1 $\beta$  may be beneficial in avoiding diet-induced hyperlipidemia.

## EXPERIMENTAL PROCEDURES

### Materials

[1,2-<sup>3</sup>H]cholesterol and [<sup>3</sup>H]triolein were from NEN Life Science Products. Oleic acid, cholate, deoxycholate, taurocholate, and monoacylglycerol were from Sigma. Phosphatidylcholine was from Avanti Polar Lipids. Other chemicals and solvents were from Fisher Scientific.

### Animals and Diets

*Ire1b*<sup>-/-</sup> (KO) and *Ire1b*<sup>+/+</sup> (WT) mice on an SV129 background were used (Bertolotti et al., 2001). To study the effects of a high-cholesterol diet, 10- to 16-week-old male mice were fed a chow diet containing 2% w/w cholesterol for 14 days, with no significant effect on body weight. Histologic analyses revealed no overt differences in epithelial cell morphology or crypt cell mitotic figures. To study the effects of a high-fat diet, male WT and KO mice were fed a high-fat western diet containing 17%, 48.5%, 21.2%, and 0.2% by weight of protein, carbohydrate, fat, and cholesterol, respectively (TD88137, Harlan Teklad). Food was withdrawn the night before the experiments.

### Plasma Lipid Measurements

Total cholesterol and triglyceride levels were measured using kits (Thermo Trace Ltd.). HDL lipid levels were measured after precipitating apoB lipoproteins (Iqbal and Hussain, 2005). Lipid levels in apoB lipoproteins were determined by subtracting HDL lipid levels from total lipid levels. Plasma lipoproteins were separated by gel filtration (flow rate of 0.2 ml/min) using a Superose 6 column, and 200  $\mu$ l fractions were collected.

### Short-Term Lipid Absorption Studies

Age-matched male mice (n = 3 per group) fed a high-cholesterol diet for 2 weeks were fasted overnight and gavaged with 1  $\mu$ Ci of either [<sup>3</sup>H]triolein or [<sup>3</sup>H]cholesterol and 0.1 mg of unlabeled cholesterol in 15  $\mu$ l of olive oil (Iqbal

and Hussain, 2005). After 2 hr, plasma was used to measure radioactivity. Tissues (0.1 g) were rinsed and dissolved in 1 ml OptiSolv, and radioactivity was counted.

#### Secretion of [ $^3$ H]Cholesterol by Primary Enterocytes

Primary enterocytes from WT and KO mice (Iqbal et al., 2003; Iqbal and Hussain, 2005) were suspended in 4 ml of DMEM containing 1  $\mu$ Ci/ml of [ $^3$ H]cholesterol and incubated at 37°C. Enterocytes were incubated for 1 hr with micelles containing 1.2 mM oleic acid (Iqbal et al., 2003; Iqbal and Hussain, 2005). After 2 hr, enterocytes were centrifuged and supernatants were subjected to density gradient ultracentrifugation. Fractions were collected and radioactivity was counted. Cell pellets were incubated overnight at 4°C with 1 ml isopropanol to extract lipids. After extraction, proteins were dissolved in 1 ml of 0.1 N NaOH and quantified using Coomassie reagent (Pierce Chemical Company).

#### Determination of MTP Activity

Small pieces (0.1 g) of liver and proximal small intestine (~1 cm) were homogenized in low-salt buffer and centrifuged, and supernatants were used for protein determination and MTP assay (Athar et al., 2004; Rava et al., 2005) using a kit (Chylos Inc.).

#### RNA Isolation and Two-Step Quantitative RT-PCR

Total RNA from tissues and cells was isolated using TRIzol (Invitrogen). Purity and integrity were assessed by the  $A_{260}/A_{280}$  ratio and 1% agarose gel electrophoresis, respectively. RNAs with ratios greater than 1.7 were used for cDNA synthesis. The first-strand cDNA was synthesized with an Omniscript RT kit (QIAGEN) and used for quantitative RT-PCR (qPCR Core Kit for SYBR Green I, Eurogentec). Data were analyzed using the  $\Delta\Delta C_T$  method and are presented as arbitrary units. The primers used were designed using PrimerExpress 3.0 (Applied Biosystems) and are listed in Table S1. A two-step RT-PCR method was used to determine XBP-1 RNA splicing. The primers (Table S1) were designed to differentiate the two forms of XBP-1, which differ by 26 nucleotides. The PCR products were separated by 3% agarose or 12% polyacrylamide gels.

#### Plasmid Expression

The expression plasmids pIRE1 $\beta$ -WT and pIRE1 $\beta$ - $\Delta$ C have been described previously (Urano et al., 2000). Plasmid pMTP1483 was generated by cloning a 1.4 kb *mtp* promoter into a pGL2 basic vector. Huh7 cells with endogenous MTP and mouse fibroblast L cells expressing mouse MTP under the control of the cytomegalovirus promoter (Dougan et al., 2007) were plated in 100 mm dishes. ExGen 500 (Fermentas) and PolyFect (QIAGEN) were used to transfect cells. After 24–48 hr transfection, cells were used to measure MTP protein and activity (Athar et al., 2004; Rava et al., 2005) as well for RNA extraction.

#### Small RNA Interference

Huh7 cells transfected with IRE1 $\beta$ -WT or IRE1 $\beta$ - $\Delta$ C plasmids for 8 hr were transfected with siRNA against the exoribonucleases Ski2, XRN1, and XRN2 or control siGL2 by the siPORT NeoFX agent (Ambion). After 48 hr, RNA was extracted and used for quantitative RT-PCR.

#### Western Blot Analyses

Tissue and cell samples were homogenized in PBS containing 1% Triton X-100. Proteins (40  $\mu$ g) were resolved on 4%–20% gradient gels (Bio-Rad). A monoclonal MTP antibody (BD Biosciences), rabbit anti-PDI (Santa Cruz), and mouse anti-GAPDH (Santa Cruz) were used to detect endogenous proteins. Overexpressed tagged mouse IRE1 $\beta$  proteins were detected using anti-c-Myc (9E10, Santa Cruz) antibodies. Secreted apoB was immunoprecipitated with goat anti-human apoB100 antibody and immunoblotted with 1D1 monoclonal antibody (Academy Bio-Medical Company, Inc.). MTP-FLAG was detected using anti-FLAG (Sigma) antibody. The resulting blots were developed using an ECL kit (Amersham). To quantify mass, in some western blots, we used Alexa Fluor 633 (Invitrogen) anti-mouse or anti-rabbit secondary antibodies and evaluated the blots using Storm 860 (Amersham). Bands were quantified using Scion Image (Scion Corporation).

#### Statistics

Data are presented as mean  $\pm$  SD. Unless noted otherwise,  $n = 3$  for each group or condition. Statistical significance ( $p < 0.05$ ) was determined using Student's  $t$  test (GraphPad Prism).

#### SUPPLEMENTAL DATA

Supplemental Data include four figures, one table, and Supplemental References and can be found with this article online at <http://www.cellmetabolism.org/cgi/content/full/7/5/445/DC1/>.

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