

# MOUSE HEPATITIS VIRUS INFECTION ACTIVATES THE IRE1/XBP1 PATHWAY OF THE UNFOLDED PROTEIN RESPONSE

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## 1. INTRODUCTION

The infection and replication of coronaviruses is likely to impose significant stress on the host cell exocytic pathway because of the extensive use of intracellular membranes for assembly of replication complexes and viral particles. MHV utilizes the ER to generate membrane-associated replication complexes, termed double membrane vesicles,<sup>1</sup> and to assemble progeny virus particles. We hypothesized that this extensive use of the ER induces the unfolded protein response. The UPR is a multifaceted signaling pathway that is triggered by perturbations in the normal ER environment (reviewed in Ref.2). The UPR emanates from the ER membrane and has the capacity to increase expression of ER resident chaperones and folding enzymes, to facilitate disposal of misfolded protein, to downregulate protein synthesis, to regulate production of membrane components necessary for expansion of the secretory pathway, and to regulate both cell cycle progression and cell death. Recently, investigators have reported that hepatitis C virus<sup>3,4</sup> and human cytomegalovirus<sup>5</sup> activate and regulate aspects of the UPR. One arm of the UPR, the Ire1-XBP1 pathway, leads to increased ER chaperone levels, upregulation of lipid biosynthesis, and alterations in protein degradation and synthesis, all of which might influence MHV replication. We found that MHV infection activates induces Ire1-mediated splicing of XBP1 mRNA, thereby resulting in synthesis of the active transcription factor, XBP1(S). To investigate the role of the Ire1-XBP1 pathway in MHV infection, we used RNA interference to generate XBP1-silenced cell lines (XBP1si). As expected, XBP1si cells exhibited reduced levels of XBP1 mRNA during MHV infection; however, XBP1(S) protein accumulated in the MHV-infected XBP1si cells. These data indicate that MHV infection is a potent activator of the Ire1/XBP1 pathway and suggest that XBP1(S) protein is stabilized in MHV-infected cells. Future studies will determine if MHV replication regulates the function of XBP1(S) as a transcriptional activator.

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## 2. MATERIALS AND METHODS

*Virus and cells:* MHV strain A59 was propagated as previously described.<sup>6</sup> DBT cells were cultured in MEM supplemented with 5% tryptose phosphate broth, 2% penicillin/streptomycin, 2% L-glutamine, and 5% fetal calf serum.

*RNA isolation and Northern blot analysis:* RNA was isolated from untreated or 2 ug/ml tunicamycin-treated DBT cells using Qiashredder and RNeasy columns according to the manufacturer's instructions (Qiagen). Total RNA (10 ug) was separated by electrophoresis on a formaldehyde agarose gel, transferred to a nitrocellulose membrane, and probed with radio-labeled DNA specific for XBP1, ERdj4 and ChoB as described in Ref. 7.

*XBP1si cell lines:* XBP1-specific oligonucleotide primers designed to generate shRNA (short hairpin RNA) that targets XBP1 for RNAi-mediated degradation were synthesized and cloned into the pU6 expression vector (Biomyx). Primer UPR10: 5'-TTTGAGTCAAACCTAACGTGGTAGTGACTTCCTGTCATCACTACCACGTTAGTTTGACTCTTTT-3' and primer UPR11: 5'-CTAGAAAAGAGTCAAACCTAACGTGGTAGTGATGACAGGAAGTCACTACCACGTTAGTTTGGACT were annealed and cloned into the BbsI and XbaI sites of the pU6 vector. The resulting plasmid DNA was designated pU6-XBP1si1. DBT cells were transfected with pU6-XBP1si1 DNA and stable cell lines were selected using 90 ug/ml G418 (Invitrogen). Clonal cell lines were isolated and tested to determine the level of XBP1 mRNA. A cell line with greater than 80% reduction in XBP1 mRNA was designated XBP1si, and a cell line with wild-type levels of XBP1 mRNA was designated as a control cell line (Con).

*Real-time RT-PCR:* Real-time RT-PCR was performed on RNA isolated from untreated, tunicamycin treated and MHV infected cells using Taqman reagents and probes according to the manufacturer's instructions (Applied Biosystems). For detection of XBP1: forward primer 5'-GCCATTGTCTGAGACCACCTT-3', reverse primer 5'-TCTGTACCAAGTGGAGAAGACATG-3', Taqman probe fam-TGCCTGCTGGACGCTCACAGTGAC-3'. For detection of MHV N: forward primer 5'-ATCCCGTGGGCCAAATAATCG-3', reverse primer 5'-TTAGCCAAAACAAGAGCAGCAATT-3', Taqman probe: fam-AAGCAGTTCCAACCAGCGCCAGCC-3'. The relative concentration of ribosomal RNA was determined using the ribosomal RNA detection system (Applied Biosystems).

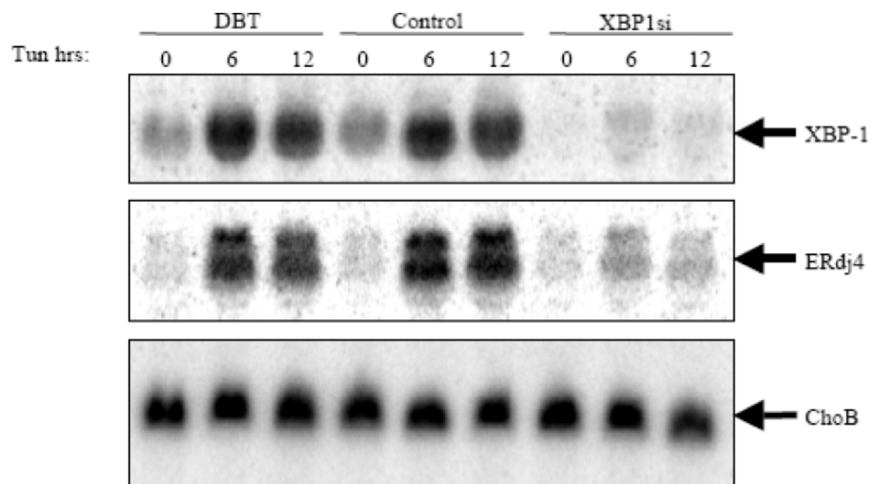
*Western blotting:* Whole cell lysates were prepared by scraping the cells in lysis buffer A (4% sodium dodecyl sulfate, 3% dithiothreitol, 40% glycerol, 0.065 M Tris, pH 6.8) and passing the lysate through a 25-gauge needle to shear the DNA released from the nucleus. Lysates were subjected to electrophoresis on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Optitran BA-S, Schleicher and Schuell). Proteins were detected by Western blotting (Western Lightning Chemiluminescence Reagent Plus, Perkin Elmer Life Sciences) using anti-calnexin (kindly provided by Linda Hendershot, St. Jude Children's Research Hospital, Memphis, TN), anti-D14 (detects p22 also termed nsp8<sup>1</sup>) and anti-XBP1 (Santa Cruz).

## 3. RESULTS AND DISCUSSION

Using RT-PCR analysis to investigate splicing of XBP1 mRNA and Western blotting to detect XBP1(S), we found that MHV infection activates the IRE1-XBP1 pathway

(Bechill et al., in preparation). MHV replication induces Ire1-mediated splicing of XBP1 mRNA, thereby resulting in synthesis of the transcription factor, XBP1(S). These results indicate that MHV replication is indeed a potent activator of the Ire1/XBP1 pathway of the UPR.

The Ire1-XBP1 pathway has previously been linked to lipid biosynthesis and expansion of the ER,<sup>8,9</sup> both of which might be critical to MHV replication. Therefore, we wanted to determine if knocking down the level of XBP1 mRNA would have an effect on MHV replication. To reduce the level of XBP1 mRNA in cells, we generated a stable cell line, designated XBP1si, that expresses a shRNA targeting XBP1 for RNAi-mediated degradation (as described in "Materials and Methods"). To test whether the XBP1si cells efficiently reduced steady-state levels of XBP1 mRNA, we compared the levels of XBP1 mRNA in untreated cells and cells treated with tunicamycin for 6 or 12 hours (Fig. 1). Northern blot analysis shows that XBP1 mRNA levels are induced by treatment with tunicamycin in DBT cells and control cells. In contrast, the XBP1si cell line has reduced amounts of XBP1 mRNA under all conditions tested. Furthermore, the mRNA level of the XBP1-responsive gene, ERdj4 is also reduced in the XBP1si cell line (Fig. 1, center panel). Overall, we conclude that the XBP1si cell line has reduced levels of XBP1 mRNA and exhibits reduced levels of mRNA of XBP1 responsive genes such as ERdj4 after tunicamycin treatment.



**Figure 1.** XBP1si cells have reduced levels of XBP1 mRNA and exhibit a reduced response to tunicamycin. Northern blot analysis of total RNA isolated from DBT cells, control and XBP1si cell lines. RNA (10  $\mu$ g) was subjected to electrophoresis on a formaldehyde-agarose gel, transferred to nitrocellulose and subjected to hybridization with radiolabeled probes to XBP1, ERdj4, and ChoB (a mitochondrial RNA used as a loading control). Hybridization was detected by phosphoimage analysis using a Typhoon Imager.

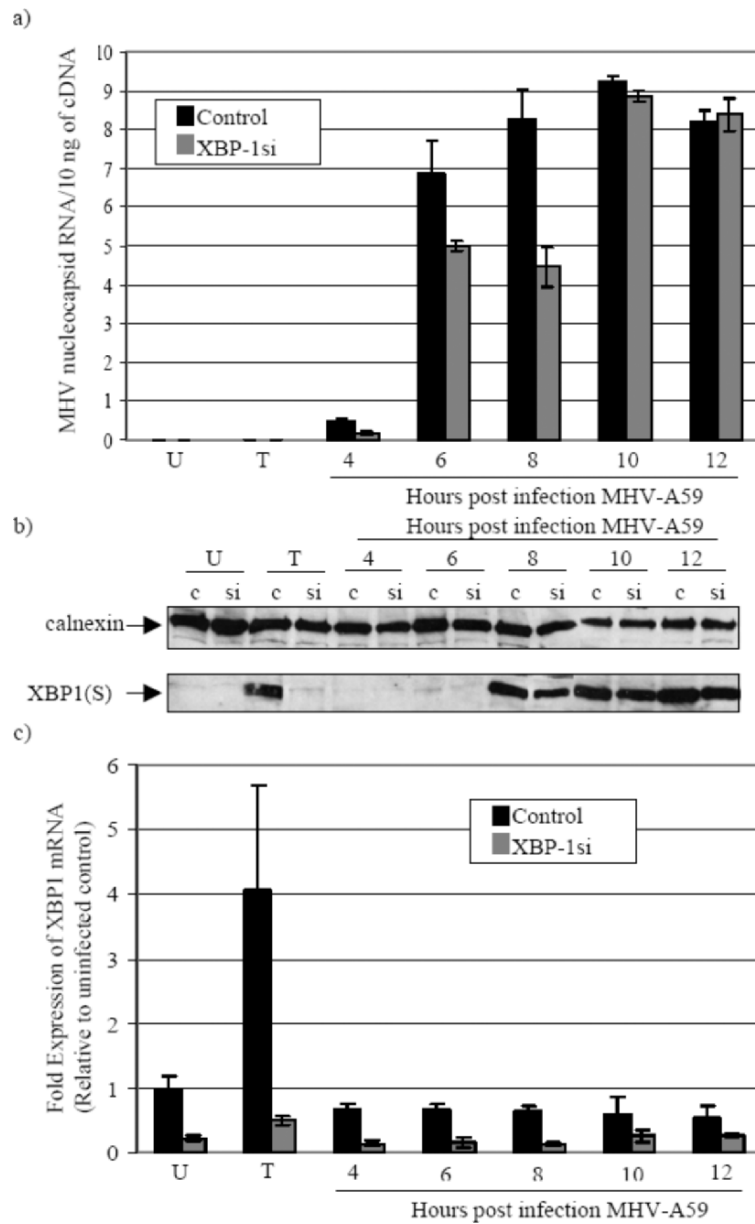
Next, we wanted to determine if the replication of MHV was altered in the XBP1si cells. We hypothesized that if MHV activation of the Ire1/XBP1 pathway was beneficial to replication, then reduced levels of XBP1 mRNA and protein may inhibit or delay MHV RNA synthesis. XBP1si and control cells were infected with MHV-A59 at MOI 1, RNA was isolated at hourly intervals after infection, and the level of MHV nucleocapsid mRNA was measured by real-time RT-PCR analysis (Fig. 2a). We found that the level of MHV replication (as monitored by accumulation of nucleocapsid RNA) was delayed in the XBP1si cells (6- and 8-hour time points), but ultimately rose to the same level as detected in the control cells (10- and 12-hour time points). Furthermore, the production of virus particles detected in the supernatant was essentially identical from the two cell lines (data not shown). To determine if the Ire1/XBP1 pathway was activated in the XBP1si cells, we generated whole cell lysates and performed Western blotting to examine the level of XBP1(S) protein. Surprisingly, we detected XBP1(S) in both MHV-infected control and MHV-infected XBP1si cells (Fig. 2b). One possible explanation for the presence of the XBP1(S) protein in the XBP1-silenced cells would be that silencing was diminished by MHV infection. To determine if MHV infection affected the level of silencing, we measured the level of XBP1 mRNA in control and XBP1si cell during the time course of MHV infection (Fig. 2c). We found that the silencing of XBP1 was maintained throughout the time course of MHV infection. Therefore, MHV infection does not impede the silencing of the XBP1 mRNA. Overall, we found that MHV infection is a potent inducer of the Ire1/XBP1 pathway of the UPR and that XBP1(S) protein is detected during MHV infection, even in XBP1si cell lines where the XBP1 mRNA concentration is reduced. These data suggest that XBP1(S), typically a short-lived protein, is stabilized in MHV-infected cells.

#### **4. CONCLUSIONS AND FUTURE DIRECTIONS**

We found that MHV infection is a potent activator of the Ire1/XBP1 pathway of the unfolded protein response. Indeed, MHV activation of Ire1/XBP1 pathway is detected even in cell lines with reduced expression of XBP1 mRNA (XBP1si cells). Future experiments will assess the turnover of XBP1(S) in MHV-infected cells and determine if XBP1(S) induces UPR responsive genes during MHV infection.

#### **5. ACKNOWLEDGMENTS**

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**Figure 2.** MHV replicates and induces XBP1(S) protein expression in the XBP1si cell line. (a) Real-time RT-PCR analysis of MHV nucleocapsid mRNA levels detected in MHV-infected control and XBP1si cells. (b) MHV infection activates the Ire1/XBP1 pathway in both control and XBPsi cells. Whole cell lysates were prepared at the time indicated and subjected to electrophoresis on a 10% polyacrylamide gel, transferred to nitrocellulose, and calnexin and XBP1(S) proteins detected by Western blotting. (c) RNA silencing is maintained during MHV infection. Real-time RT-PCR analysis of XBP1 mRNA levels during MHV infection of control and XBP1si cells.

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