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ENDOPLASMIC RETICULUM STRESS RESPONSE AND TRANSCRIPTIONAL REPROGRAMMING

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Kezhong Zhang

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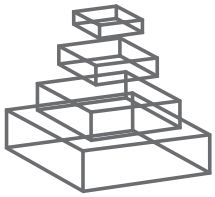

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ENDOPLASMIC RETICULUM STRESS RESPONSE AND TRANSCRIPTIONAL REPROGRAMMING

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Endoplasmic reticulum (ER) is an intracellular organelle responsible for protein folding and assembly, lipid and sterol biosynthesis, and calcium storage. A number of biochemical, physiological, or pathological stimuli can interrupt protein folding process, causing accumulation of unfolded or misfolded proteins in the ER lumen, a condition called "ER stress". To cope with accumulation of unfolded or misfolded proteins, the ER has evolved a group of signaling pathways termed "Unfolded Protein Response (UPR)" or "ER stress response" to align cellular physiology. To maintain ER homeostasis, transcriptional regulation mediated through multiple UPR branches is orchestrated to increase ER folding capacity, reduce ER workload, and promote degradation of misfolded proteins. In recent years, accumulating evidence suggests that ER stress-triggered transcriptional reprogramming exists in many pathophysiological processes and plays fundamental roles in the initiation and progression of a variety of diseases, such as metabolic disease, cardiovascular disease, neurodegenerative disease, and cancer. Understanding effects and mechanisms of ER stress-associated transcriptional reprogramming has high impact on many areas of molecular genetics and will be particularly informative to the development of pharmacologic avenues towards the prevention and treatment of modern common human diseases by targeting the UPR signaling. For these reasons, ER stress response and transcriptional reprogramming are a timely and necessary topic of discussion for Frontiers in Genetics.

The important topics in this area include but not limited to:

(1) ER-resident transcription factors and their involvements in ER stress response and cell physiology;
(2) Physiologic roles and molecular mechanisms of ER stress-associated transcriptional regulation in lipid and glucose metabolism;
(3) In vitro and in vivo models for ER stress-associated transcriptional reprogramming;
(4) ER stress-associated transcriptional regulation in human disease;
(5) Therapeutic potentials by targeting ER stress response pathways.

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EDITORIAL
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Endoplasmic reticulum stress response and transcriptional reprogramming

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Keywords: ER stress, unfolded protein response, transcriptional reprogramming, metabolism, inflammation, oncogenes

Endoplasmic reticulum (ER) is an intracellular organelle responsible for protein folding and assembly, lipid and sterol biosynthesis, and calcium storage. As a protein folding compartment, the ER provides a high-fidelity quality control system to ensure that only correctly folded proteins are released from the ER and that unfolded and/or misfolded proteins are retained and degraded. A number of biochemical, physiological, or pathological stimuli can interrupt protein folding process, causing accumulation of unfolded or misfolded proteins in the ER lumen, a condition called "ER stress." To cope with accumulation of unfolded or misfolded proteins, the ER has evolved a group of signaling pathways termed "Unfolded Protein Response (UPR)" or "ER stress response" that leads to transcriptional and translational reprogramming to either cope with the aberrant proteins or, under prolonged ER stress conditions, initiate programed cell death (Kaufman, 1999; Ron and Walter, 2007). To maintain ER homeostasis, transcriptional regulation mediated through multiple UPR branches is orchestrated to increase ER folding capacity, reduce ER workload, and promote degradation of misfolded proteins. The basic UPR pathways in mammalian cells consist of three main signaling cascades initiated by three ER-localized protein stress sensors: IRE1α (inositol-requiring 1α), PERK (double-strand RNA activated kinase-like ER kinase), and ATF6 (activating transcription factor 6). Relevant to transcriptional reprograming, the primary UPR transducer IRE1α functions as an RNase to splice the mRNA encoding X-box binding protein 1 (XBP1), which functions as a potent transcriptional activator that is functionally involved in immune response, cell metabolism, and cell survival. Alternatively, IER1α RNase can process select mRNA or pre-microRNA substrates, leading to degradation of mRNAs or microRNAs, a pathway was termed "Regulated IRE1-Dependent Decay (RIDD)" (Hollien and Weissman, 2006; Upton et al., 2012).

In recent years, accumulating evidence suggests that ER stress-triggered transcriptional reprogramming exists in many pathophysiological processes and plays fundamental roles in the initiation and progression of a variety of diseases, such as metabolic disease, cardiovascular disease, neurodegenerative disease, and cancer. Understanding effects and mechanisms of ER stress-associated transcriptional reprogramming has high impact on many areas of molecular genetics and is particularly informative to the development of pharmacologic avenues toward the prevention and treatment of modern common human diseases. To this direction, we have assembled a collection of timely research and review articles with a focus on ER stress response and transcriptional reprogramming in health and disease.

In this issue, Birk et al. contributed a method article describing the experimental framework to monitor stress-induced, time-resolved changes in ER reduction-oxidation (redox) states by using ER-targeted fluorescent biosensors (Birk et al., 2013). In the paper, advantages and drawbacks of existing techniques are discussed, and the power of these techniques was demonstrated in the context of selected cell culture models for ER stress. In regard to transcriptional reprogramming by ER stress response, a research paper by Arensdorf et al. described that the temporal clustering of gene expression links the metabolic transcription factor HNF4α to the ER stress-dependent gene regulatory network (Arensdorf et al., 2013a). In this work, they utilized a "bottom-up" approach to study the metabolic gene regulatory network controlled by the UPR in the liver, and provided a unique resource for the community to further explore the temporal regulation of gene expression during ER stress. Additionally, a review article from the same group discussed the non-canonical mechanisms and physiological consequences of the transcriptome induced by ER stress (Arensdorf et al., 2013b). As for non-canonical mechanism of stress-induced reprogramming, a review article by Coelho and Domingos summarized the physiological significance of transcriptional reprogram by RIDD, the ER stress pathway by which IRE1α processes and degrades mRNAs or pre-microRNAs, in several biological paradigms, including photoreceptor differentiation in Drosophila, mammalian liver, and endocrine pancreas function (Coelho and Domingos, 2014). They highlighted the importance of RIDD in tissues undergoing intense secretory function. Further, Horne et al. presented a newly emerging genome theory that unifies different types of stress and functional relationships from a genome-defined system point of view (Horne et al., 2014). They discussed the evolutionary relationship between stress and somatic cell adaptation under physiological, pathological, and somatic cell survival conditions, and advised to defocus from specific stresses and mechanisms by redirecting attention toward studying underlying general mechanisms.

In term of pathophysiological consequences of transcriptional reprogramming by ER stress response, an interesting

research article by Han et al. described altered methylation and expression of ER-associated degradation (ERAD) factors in long-term alcohol-induced murine hepatic tumors (Han et al., 2013). Based on the data from ER chaperone BiP conditional knockout mice, they concluded that long-term alcohol consumption and aging may promote liver tumorigenesis in females through interfering with DNA methylation and expression of genes involved in the ERAD pathway. A review article by White-Gilbertson et al. discussed the current understanding of the involvement of ER stress response in multiple myeloma, an incurable plasma cell neoplasm cancer (White-Gilbertson et al., 2013). This paper highlighted that myeloma cells utilize ER stress response to gain unique metabolic signature, and therefore, inhibition of the ER stress response may represent a promising therapeutic strategy for multiple myeloma. As for metabolic disease, Zhou and Liu reviewed the current understanding of the involvement and mechanisms of ER stress response in dysregulation of hepatic lipid metabolism and hepatic lipotoxicity (Zhou and Liu, 2014). It is anticipated that understanding the roles and mechanism of ER stress response in hepatic lipid metabolism may lead to novel therapeutic strategies toward the control of metabolic disorders. Additionally, a review article from Guo and Li discussed the progress in understanding ER stress response in hepatic steatosis and inflammatory bowel diseases (Guo and Li, 2014). They highlighted the potential signaling pathways connecting ER stress with inflammation, and depicted the interplay between ER stress and inflammation in the pathogenesis of hepatic steatosis, inflammatory bowel diseases and colitis-associated colon cancer.

In summary, we anticipate that this collection of research and review articles from the front-running scientists will help the scientific community, clinical professionals, pharmaceutical industry, and public at large understand the mechanisms and pathophysiological significance of ER stress response. In particular, delineation of the molecular components of transcriptional reprogramming driven by ER stress response will shed new light on potential therapeutic strategies toward the control of modern common human diseases, such as metabolic disease, cardiovascular disease, immune disease, and cancer. While much work in ER stress-induced transcriptional reprogramming remains to be done, we hope that this topic will trigger more interest from the biomedical community in this important research direction.

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REVIEW ARTICLE
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The role of endoplasmic reticulum stress in maintaining and targeting multiple myeloma: a double-edged sword of adaptation and apoptosis

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Increased cellular protein production places stress on the endoplasmic reticulum (ER), because many of the nascent proteins pass through the ER for folding and trafficking. Accumulation of misfolded proteins in the ER triggers the activation of three well-known pathways including IRE1 (inositol requiring kinase 1), ATF6 (activating transcription factor 6), and PERK (double stranded RNA-activated protein kinase-like ER kinase). The activity of each sensor modulates the overall ER strategy for managing protein quality control as cellular needs change due to growth, differentiation, infection, transformation, and host of other possible physiological states. Here we review the role of ER stress in multiple myeloma (MM), an incurable plasma cell neoplasm. MM is closely linked to dysregulated unfolded protein response in the ER due to the heightened production of immunoglobulin and the metabolic demands of malignant uncontrolled proliferation. Together, these forces may mean that myeloma cells have an "Achilles heel" which can be exploited as a treatment target: their ER stress response must be constitutively active at a remarkably high level to survive their unique metabolic needs. Therefore, inhibition of the ER stress response is likely to injure the cells, as is any further demand on an already over-worked system. Evidence for this vulnerability is summarized here, along with an overview of how each of the three ER stress sensors has been implicated in myeloma pathogenesis and treatment.

Keywords: endoplasmic reticulum, unfolded protein response, multiple myeloma, ER stress, apoptosis

INTRODUCTION

Multiple myeloma (MM) is a cancer of plasma cells, the antibody-producing end stage of B cell development. Plasma cells are the originating cell for a variety of diseases, collectively known as plasma cell dyscrasias, including systemic light-chain amyloidosis, monoclonal gammopathy of undermined significance (MGUS), solitary plasmacytoma, smoldering myeloma, and MM. In each case, the hallmark of the pathology is the overproduction of a secreted protein by a diseased plasma cell population (Barlogie et al., 1992). In this review, we will focus on MM, a cancer with over 20,000 new diagnoses expected in 2013 by the National Cancer Institute in the United States (http://www.cancer.gov/cancertopics/types/myeloma/). MM typically presents as an incurable disease, almost inevitably recurring after therapy (Munshi and Anderson, 2013). Nonetheless, the introduction of the proteasome inhibitor bortezomib to treatment regimens represented a breakthrough for myeloma patients by increasing survival time significantly (Moreau, 2012). The sensitivity of myeloma cells to bortezomib may be due in part to the specialized metabolism of plasma cells, which are adapted to generate large volumes of secreted immunoglobulins and operate with an elevated baseline demand on the endoplasmic reticulum (ER). This may be a liability for myeloma cells, which are additionally burdened with the protein production necessary for malignant proliferation. The resulting vulnerability to further perturbation in protein metabolism may offer a partial explanation for the success of bortezomib (Landowski et al., 2005; Obeng et al., 2006; Meister et al., 2007). Efforts to understand and target the integrated ER stress response in myeloma will be summarized here, with a focus on the three ER stress sensors that coordinate this response: inositol requiring kinase 1 (IRE1; Sidrauski and Walter, 1997; Yoshida et al., 2001), double stranded RNA-activated protein kinase-like ER kinase (PERK; Harding et al., 2000), and the transcription factor activating transcription factor 6 (ATF6; Yoshida et al., 2000). Each of these sensors is located at the apex of a pathway, and each is capable of inducing the expression of several major ER heat shock proteins and enhancing protein folding machinery (Malhotra and Kaufman, 2007).

All three ER stress response sensors are embedded in the ER membrane where they are normally bound by the ER chaperone grp78 (alias BiP; Ma et al., 2002; Sommer and Jarosch, 2002; Kimata et al., 2004). This binding inhibits the activity of each sensor. Grp78 releases the sensors in response to mounting ER stress as its chaperone functions are required (Lee, 2005). However, this is not a uniform method of control over the three combined sensors; different cellular conditions result in differing patterns of sensor activation. For example, during B cell differentiation only two sensors, IRE1 and ATF6 are activated while the third, PERK, is not (Ma et al., 2010). Using a

B cell line capable of induction of all three ER stress sensors and capable of differentiation into plasma cells, Ma et al. (2010) demonstrated that IRE1 is activated quickly upon exposure to differentiation-inducing lipopolysaccharide (LPS) treatment, with ATF6 activation following secondarily. In contrast, PERK activation could not be elicited from these cells upon differentiation, even when treated with the ER stressor thapsigargin, although this treatment could stimulate PERK activity before differentiation (Ma et al., 2010).

Crosstalk between the sensor systems provides additional control over the cellular response. For example, one effect of IRE1 activation is the transcription of a PERK inhibitor named p58ipk (Iwakoshi et al., 2003; Ma et al., 2010). In addition, ATF6 and PERK appear to converge on signaling through the transcription factor CHOP (C/EBP homologous protein; Okada et al., 2002). Thus, both re-enforcement and antagonism exist between the sensors, allowing a highly tunable response based on cellular needs (**Figure 1**).

Due to the baseline ER stress present in untransformed plasma cells, myeloma is a particularly complex disease in which to examine ER stress. Several excellent reviews have addressed ER stress response more generally (Woehlbier and Hetz, 2011; Logue et al., 2013; Schonthal, 2013), so here we will provide brief overviews of the components and focus on experimental data which elucidates their role in myeloma disease, including responsiveness to chemotherapeutics.

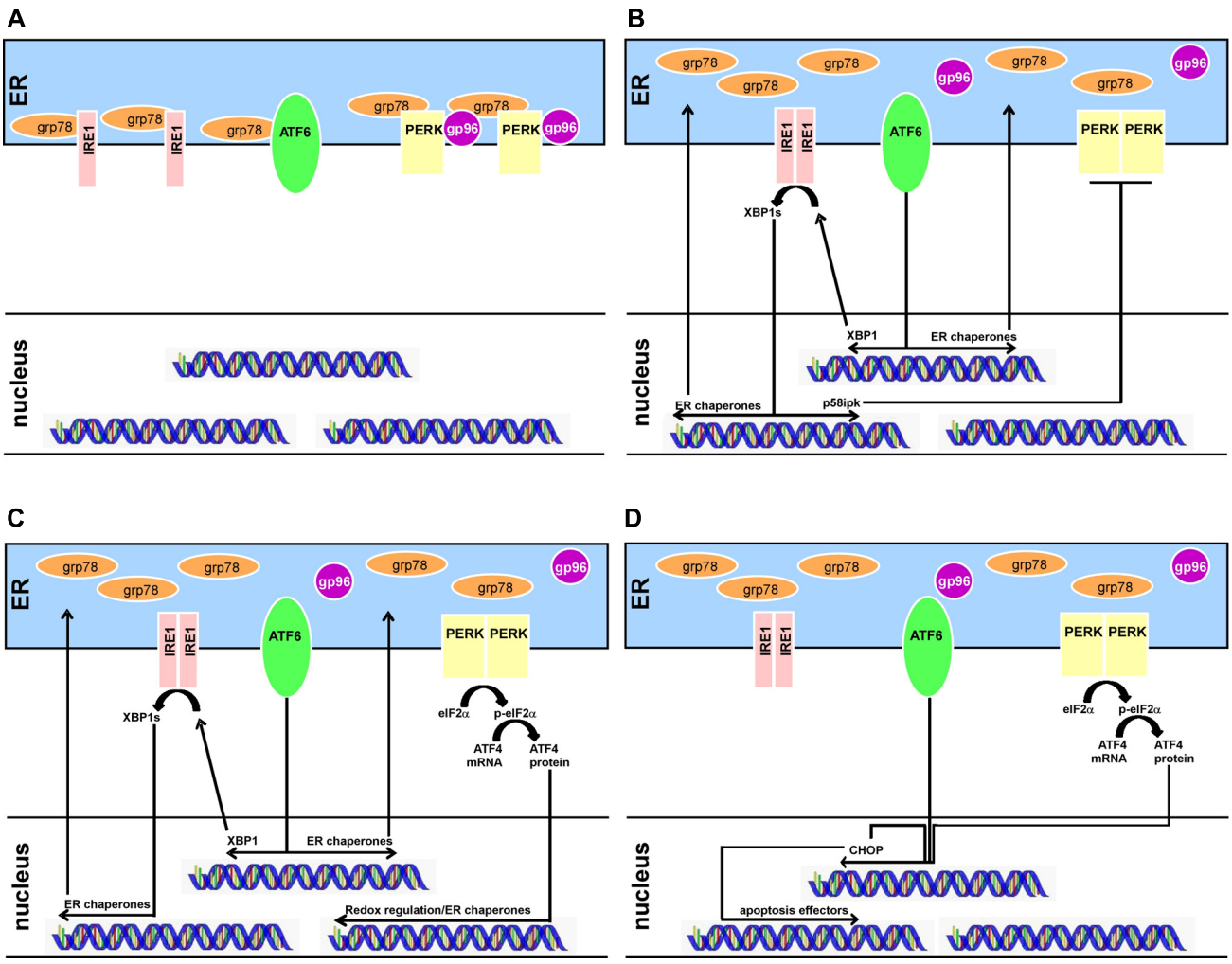

FIGURE 1 | The progression of ER stress responses in multiple myeloma development and treatment. (A) Schematic of B-cell ER stress activity before differentiation. The ER stress sensors IRE1, ATF6, and PERK are bound to ER chaperones which inhibit either homodimerization (for IRE1 and PERK) or translocation to the nucleus (ATF6). Transcriptomes downstream of the ER sensors are inactive. **(B)** Upon differentiation into a plasma cell, protein production is increased in a regulated fashion. Chaperones release the ER stress sensors but PERK remains inhibited through its IRE1-controlled inhibitor p58ipk. IRE1 and ATF4 upregulate ER chaperones to assist with the protein production involved in secreting antibody. **(C)** Myeloma cells further activate the ER stress programs, involving PERK activation. All three arms are used by myeloma cells, although the CHOP response is limited. **(D)** Myeloma cells can be induced to further increase their ER stress response, tipping the system towards cell death. When ATF4 and ATF6 coordinate to induce transcription of CHOP, a pro-apoptosis transcription factor, the ER stress response moves from adaptive to destructive. This is the therapeutic goal of some drug regimens, such as bortezomib.

IRE1

OVERVIEW OF IRE1 FUNCTION

Inositol requiring kinase 1 is a bifunctional transmembrane kinase and endoribonuclease. It was first identified in yeast, called Ire1p, which is correlated with unfolded protein response (UPR; Cox et al., 1993). Upon activation of the UPR, Ire1p oligomerizes, phosphorylates, and initiates splicing of homologous to ATF/CREB1 (HAC1; Shamu and Walter, 1996; Sidrauski and Walter, 1997). IRE1 is conserved in all eukaryotic cells. In mammalian cells, there are two forms of IRE1, IRE1α, and IRE1β. Most cells and tissues express IRE1α, while only intestinal epithelial cells express IRE1β. IRE1α and IRE1β have similar cleavage specificities (Tirasophon et al., 1998; Wang et al., 1998; Patil and Walter, 2001). Previous studies have demonstrated that *X box binding protein 1 (XBP1)* mRNA is a substrate for the endoribonuclease activity of IRE1. Upon activation of the UPR, the IRE1 RNase activity initiates and removes a 26 nucleotide intron from *XBP1* mRNA (Yoshida et al., 2001; Calfon et al., 2002; Lee et al., 2002). This splicing form of XBP1, denoted XBP1s, is a transcriptional activator that plays an important role in activation of a variety of UPR target genes, which include ERdj4, $p58^{IPK}$, DnaJ/Hsp40-like genes, ER degradation enhancer, mannosidase alpha-like (EDEM), human ER-associated DNAJ (HEDJ), protein disulfide isomerase-P5 (PDI-P5), and ribosome-associated membrane protein 4 (RAMP4; Lee et al., 2003).

IRE1/XBP1 PATHWAY IS ESSENTIAL FOR PLASMA CELL DIFFERENTIATION

Both IRE1 and XBP1 are critical for plasma cell differentiation. Genetic deletion of XBP1 causes lack of plasma cells, with concomitantly decreased baseline and antigen specific serum level of immunoglobulin (Reimold et al., 2001; Iwakoshi et al., 2003; Shaffer et al., 2004). In addition, IRE1α is required to splice XBP1 for terminal differentiation of mature B cells into antibody-secreting plasma cells as demonstrated by using an IRE1α-deficient chimeric mouse model (Zhang et al., 2005). Furthermore, in IRE1α conditional knockout mice, the serum levels of IgM and IgG1 are reduced by half compared with the control mice. However, the IgM^+, IgD^+, and $B220^+$ populations are similar between IRE1α conditional knockout mice and control mice. This result suggests that IRE1α is required for efficient plasma cell production of antibodies, and is critical for final B cell differentiation into a plasma cell (Iwawaki et al., 2010). These studies suggest that the IRE1/XBP1 pathway is required for differentiation and survival of cell types that secrete high levels of protein.

IRE1/XBP1 IS POTENTIAL THERAPEUTIC TARGET FOR MULTIPLE MYELOMA

In addition to the critical roles of IRE1/XBP1 in plasma cell differentiation, a picture has emerged for the roles of UPR in myeloma. Indeed, XBP1s and downstream ER chaperones are consistently up regulated in myeloma patients. Patients with a low XBP1 spliced/unspliced ratio ($\leq$1.33) have a longer overall survival compared with those with a higher ratio ($p = 0.03$, median, 56 vs 40 months; HR = 1.75; 95% CI = 1.07–2.85; Bagratuni et al., 2010). Moreover, transgenic expression of XBP1s in mice also leads to plasma cell dyscrasia with evidence of increased monoclonal antibodies ("M-spike"), lytic bone lesions, plasmacytosis, and kidney damage (Carrasco et al., 2007). Given this information, IRE1/XBP1 could be a potential therapeutic target for MM.

To investigate whether blocking the IRE1/XBP1 pathway is a therapeutic for MM, researchers performed chemical library screening and they identified a small-molecule, STF-083010, that specifically blocks the endonuclease activity of IRE1 without affecting its kinase activity (Papandreou et al., 2011). Furthermore, they treated different myeloma cell lines with different doses of STF-083010 *in vitro* and demonstrated that this compound causes myeloma cell death. Importantly, STF-083010 is also selectively cytotoxic to freshly isolated $CD138^+$ plasma cells from myeloma patients compared with $CD19^+$ B cells, $CD3^+$ T cells, and $CD56^+$ NK (natural killer) cells. Finally, treatment of human myeloma xenografts in NSG (NOD scid gamma) mice was performed. STF-083010 was given by intraperitoneal injection on day 1 and day 8 and this compound significantly inhibited the growth of these tumors *in vivo* (Papandreou et al., 2011). In addition, another small-molecule, MKC-3946, also blocks the IER1α endoribonuclease domain. MKC-3946 inhibits multiple human myeloma cell lines without toxicity to normal mononuclear cells. MKC-3946 also blocks ER stress induced by both bortezomib and heat shock protein 90 inhibitor 17-AAG. In addition, MKC-3946 significantly enhanced cytotoxicity induced by bortezomib or 17-AAG (Mimura et al., 2012). A similar result was found by using an XBP1 inhibitor, toyocamycin, which was identified from the culture broth of an Actinomycete strain. Toyocamycin has been shown to suppress the XBP1 mRNA splicing in HeLa cells which is induced by thapsigargin, tunicamycin, and 2-deoxyglucose. It does not, however, affect ATF6 and PERK activation. Although toyocamycin does not inhibit IRE1α phosphorylation, it prevents IRE1α-induced XBP1 mRNA cleavage and inhibits constitutive activation of XBP1 expression in myeloma cell lines as well as in samples from myeloma patients *in vitro*. Toyocamycin also induces apoptosis of myeloma cells, including bortezomib-resistant myeloma cells *in vitro*, and it also inhibits myeloma cell growth in a human myeloma xenograft model (Ri et al., 2012). Taken together, these results demonstrate that blockade of IRE1/XBP1 pathway by small-molecule compounds is a potential therapeutic for treatment of human myeloma.

ATF6

OVERVIEW OF ATF6 FUNCTION

Among the three ER stress sensors, only ATF6 does not dimerize to potentiate enzymatic activity. Instead, under ER stress conditions, ATF6 translocates to the Golgi apparatus and it is processed by site 1 protease (S1P) and site 2 protease (S2P) to release an active form of ATF6 (ATF6f). ATF6f translocates to the nucleus and activates target genes (Chen et al., 2002). In this capacity, ATF6 works in partnership with IRE1, as one of the target genes of ATF6 is XBP1, the key substrate of IRE1 (Yoshida et al., 2001). In addition to fueling the IRE1 arm of the ER stress response, ATF6 also functions as a transcription factor for ER chaperone proteins, thereby easing ER burden (Arai et al., 2006). These contributions to the ER stress response complement IRE1 activation and are generally adaptive, allowing such upregulation of protein production as is seen in

plasma cell development. However, prolonged ATF6 activation can also result in transcription of CHOP, another transcription factor which enacts a largely apoptotic program of gene expression (Matsumoto et al., 1996). This effect of ATF6 activity occurs in conjunction with PERK activation, in contrast to the protective program that ATF6 and IRE1 jointly support.

One group has made an attempt in HeLa cells to describe the genetic modulation downstream of ATF6 activation and to distinguish it from the genetic signature of PERK activation (Okada et al., 2002). The group examined this question by comparing the cellular pool of mRNA in HeLa cells treated with the general ER stress inducer tunicamycin with that of cells stably expressing the nuclear form of ATF6. From this experiment, the ATF6 contribution to the integrated ER stress response was extracted for HeLa cells. The primary targets identified were the expected ER chaperones grp78, gp96, and calreticulin (Okada et al., 2002). In addition, proteins which directly modify disulphide bonds to assure proper folding of nascent proteins were identified, such as ERp62 and ERp71 (Okada et al., 2002). Unfortunately, the authors concluded that this cell system was not conducive to the study of XBP1 transcription, which is critical for understanding myeloma development and progression. However, the research revealed that ATF6 and PERK both converge on CHOP transcription, confirming this as a locus of crosstalk between the two sensors (Okada et al., 2002).

CHOP (C/EBP homologous protein, alias GADD153) is a pro-apoptotic transcription factor routinely used as a read-out for activation of the ER stress response (Kawabata et al., 2012; Mimura et al., 2012; Schonthal, 2013). The Mori group has proposed that CHOP transcription is most efficiently activated upon binding by both the nuclear form of ATF6 and ATF4, the transcription factor effector of PERK activation (Okada et al., 2002). The convergence of ER stress signals results in CHOP binding to its target genes, with inhibitory effects on some targets and transcriptional effects on others. CHOP activity results in the downregulation of the anti-apoptotic Bcl2 (B-cell lymphoma 2) as well as the upregulation of the ER-resident oxidase ERO1-alpha (Marciniak et al., 2004). CHOP is also its own target, suggesting that its activation constitutes a commitment to programmed cell death (Marciniak et al., 2004).

ATF6 IN MULTIPLE MYELOMA

Surprisingly little has been written about the role of ATF6 in MM, especially considering the important role it plays in the generation of the IRE1 substrate XBP1 (Lee et al., 2002). Indeed, the transcriptome of ATF6 should itself be a discrete target of research in the myeloma field.

One group has performed specific knockdown of ATF6 in myeloma cells and shown that, as is also the case for the other ER stress sensors, targeted loss resulted in significant cell death (Michallet et al., 2011). In addition, increased baseline signaling through the PERK sensor was enhanced upon knockdown of ATF6. Thus, the three sensors appear to all be required for baseline survival for myeloma cells, although crosstalk may allow for some limited compensation between the sensor systems.

Certainly, the crosstalk between ATF6 and the other two ER stress sensors suggests that ATF6 plays the role of a "swing vote." When activated in conjunction with IRE1, growth and adaptation to protein production is reinforced. When linked to PERK, ATF6 activity can support a programmed cell death response. This duality indicates a potentially powerful target, identifying ATF6 as an understudied aspect of myeloma.

PERK

OVERVIEW OF PERK FUNCTION

The pancreatic eIF2-alpha kinase (PERK, alias EIF2αK3) is the third known sensor of ER stress and like the other two, it is embedded in the ER membrane. As the only such sensor left inactivated in the normal development of plasma cells, it has been of particular interest in the study of myeloma (Ma et al., 2010). We will therefore provide a summary of its canonical function and then review studies testing the role of PERK in baseline myeloma biology and in response to drug treatment.

Like the other two ER stress sensors, the activation of PERK requires its release by grp78. In addition, the chaperone gp96 (alias grp94) has been shown to bind PERK at baseline and release it during ER stress conditions (Ma et al., 2002). Upon release, PERK is free to homodimerize and activate as a kinase. Active PERK has three interacting mechanisms, allowing gradations of cellular effects ranging from protective to destructive. These effects are mediated by eIF2-alpha, ATF4, and CHOP. First, the direct phosphorylation target of PERK is eIF2-alpha, a protein needed for ribosomal translation of mRNA (Wek and Cavener, 2007). The phosphorylation of eIF2-alpha inhibits its activity, resulting in global repression of protein production. This strategy of translation repression reduces the load of nascent proteins being delivered to the ER for processing and is an effective short-term answer to the problem of ER stress. However, the side effects of halting protein production are myriad, and the phosphorylation of eIF2-alpha does allow exceptions. For instance, mRNA with IRES (internal ribosome entry site) sequences can still be translated under these conditions (Gerlitz et al., 2002). In addition, the transcription factor ATF4 is translated and subsequently translocates to the nucleus. The mechanism allowing such translation during eIF2α phosphorylation has been of significant interest and research has identified a double upstream open reading frame structure in the ATF4 mRNA which is preferentially translated when ribosomal processing is slowed (Lu et al., 2004; Kilberg et al., 2009). ATF4 then binds to genetic sequences with CCAAT (cytidine-cytidine-adenosine-adenosine-thymidine) motifs, many of which can be translated under the phosphorylated eIF2a condition which is downstream of PERK activation, likely due to upstream ORFs (open reading frames) that function like the ones present in ATF4 mRNA (Lu et al., 2004; Kilberg et al., 2009). This activation of the ATF4 transcriptome is the second major arm of PERK response to ER stress.

ATF4 facilitates the transcription mRNAs coding for proteins with functions specific to ER stress conditions. For instance, redox-management genes are turned on, as well as additional chaperones for the ER (Harding et al., 2003; Liu et al., 2008; Ye and Koumenis, 2009). Again, this strategy is adaptive for the cell and may allow the cell to cope with short-term challenges. However, the third arm of PERK signaling involves activation of CHOP, already described as a target of ATF6. The CHOP promoter includes

binding sites for both ATF4 and ATF6, which appear to synergize (Okada et al., 2002). In addition, the CHOP mRNA includes an upstream inhibitory ORF that is preferentially translated during ER stress (Jousse et al., 2001; Lee et al., 2011). Expression of this protein is very tightly regulated and eventual convergence on CHOP activation signals a likely shift into macroautophagy and/or apoptosis (Gomez-Santos et al., 2005; Kim et al., 2006; Emdad et al., 2011). Thus, PERK has protective functions, especially when first activated, but it can also induce cell death pathways if it is too strongly activated or active for too long. This temporal change in PERK effect has been described in a recent review, identifying PERK as a protein with significant characterization left to be done (Woehlbier and Hetz, 2011).

PERK AS PROTECTIVE MECHANISM IN MULTIPLE MYELOMA

As previously referenced, Michallet et al. (2011) used RNAi (RNA interference) to individually knock down IRE1, ATF6, and PERK expression. They observed that loss of any one sensor tended to increase the activation read outs of the remaining sensors, confirming crosstalk between the systems. Their specific knockdown of PERK yielded two important findings. First, this single change resulted in an autophagic cell death response, implicating PERK activation as a necessary part of the metabolic shift from plasma cell to myeloma cell. Second, the loss of PERK impeded the apoptotic response. Therefore, PERK activity was implicated in both viability of myeloma cells and in the apoptotic potential of the cells (Michallet et al., 2011). This complex finding may shed light on idea of PERK activity as a potential danger to the cell, which will be discussed in the following section.

PERK status is also a likely factor in myeloma cell response to drug. A report last year noted that myeloma cells demonstrate a baseline degree of UPR that, when inhibited by an HSP90 inhibitor, could result in an apoptotic response (Patterson et al., 2008). This dependence on UPR can therefore be considered an addiction and inhibition of the sensors could constitute a rational drug target. However, a larger body of work has suggested that the downstream effects of PERK activation are identifiable as effectors of cell death in myeloma. It may be that myeloma cells have optimized their ER stress response to survive their unique metabolism as both secretory and rapidly dividing cells. If so, repression of the ER stress response could lead to cell death as surely as stimulation of the same system.

PERK AS A CELL DEATH EFFECTOR IN MULTIPLE MYELOMA

Activation of PERK has been implicated in a wide variety of cancers as a mediator of response to chemotherapy (Kraus et al., 2008; Lust et al., 2009; Yan et al., 2010; Fribley et al., 2011; Qiao et al., 2012; Sailaja et al., 2013). Most convincingly, small interfering RNA (siRNA) against PERK or dominant negative models can ameliorate chemotherapy-induced death in many types of cancer cells (Lai and Wong, 2008; Yacoub et al., 2008; Kahali et al., 2010; Pan et al., 2012). It is therefore perhaps unsurprising that this effect has also been seen in myeloma cells, which already have baseline ER stress and may not be able to tolerate perturbations to the system. In particular, researchers have been interested in the role of PERK in myeloma cell response to the proteasome inhibitor bortezomib, the most effective myeloma therapy. Obeng et al. (2006) have reported that Bortezomib treatment upregulates PERK activity as measured by ATF4 and downstream CHOP expression. Further, they correlated ER stress to bortezomib response by measuring the retention of immunoglobulin protein accumulating in treated cells. Myeloma cells that retained more of their secretory protein load, one hallmark of ER stress, showed more activation of ER stress markers and more sensitivity to the drug (Obeng et al., 2006).

This pathway has been further probed in myeloma cells by induction of ER stress through inhibition of heat shock proteins, the family of ER chaperones that includes both grp78 and gp96. Most commonly, heat shock protein 90 is targeted experimentally with the drug 17-AAD. A 2007 paper compared 17-AAG and bortezomib effects on myeloma cells and found that both drugs produced upregulation of grp78, gp96, and CHOP, all of which are downstream effects of PERK activation (Davenport et al., 2007). These effects were ultimately joined by an apoptotic response, suggesting that PERK activation culminated in a cell death program (Davenport et al., 2007).

The key component of apoptosis-induction by PERK was investigated to better understand the unfortunate phenomenon of bortezomib resistance in myeloma. Schewe and Aguirre-Ghiso (2009) demonstrated that the phosphorylation of eIF2α is an indispensable aspect of PERK-mediated apoptosis. They studied a bortezomib-resistant subpopulation of myeloma cells and found that resistance could be reversed by inhibition of the eIF2α phosphatase or by competitive inhibition of the phosphatase via overexpression of a mutant phosphorylated eIF2α. In both conditions, cells with experimentally enhanced levels of endogenous phosphorylated eIF2α regained sensitivity to bortezomib (Schewe and Aguirre-Ghiso, 2009).

The global repression of protein translation has far-reaching consequences, even if subsets of mRNAs are selectively processed. For instance, the balance of proteins in the cell quickly changes as proteins with short half-lives are degraded but not replaced. One system affected by such a change is the anti-apoptotic network, comprised of such anti-apoptotic proteins as survivin, Mcl-1, and FLIP (FLICE-like inhibitory protein), all of which are eliminated from the protein pool if not continuously generated (White-Gilbertson et al., 2009). This time-dependent shift in cellular fitness may be another axis on which PERK activation is titrated, so that short-term activation is beneficial while long-term activation is ultimately detrimental to the cell.

CONCLUSION

The integrated ER stress response is composed of all three sensor systems and their interplay determines the overall cellular strategy and the outcome of stress. Myeloma cells may harbor an Achilles heel in their baseline metabolism, as shown by varied treatments which induce death by either inhibiting or exacerbating the ER stress response. This metabolic addiction to pathways that prevent UPR-induced death program may be a key to myeloma treatment, and deserves more focused attention. One example of such effort is the possibility of PERK inhibitors as cancer therapeutics (Bi et al., 2005; Hart et al., 2012). It is also possible that a unique adaptive UPR program is adopted by individual myeloma patients, having diseases with different vulnerabilities. An individualized.

strategy with an array of tools to inhibit or push ER stress may be needed to match therapeutic response to this adaptive disease.

In summary, the UPR mechanism can be exploited for the treatment of MM (**Figure 1**). The shift from naïve B cell to plasma cell already involves the activation of two of the three ER stress sensors and their downstream signaling partners (**Figures 1A,B**). These cells engage IRE1 and ATF6 in order to cope with regulated antibody production, although PERK is inhibited. However, upon transformation, myeloma cells require the further support of PERK, allowing transcription of the ATF4 targets that ameliorate oxidative stress (**Figure 1C**). This is a potentially risky strategy for the cell, because ATF4 and ATF6 can cooperate to transcribe the pro-apoptotic CHOP (**Figure 1D**). Thus, if chemotherapeutic interventions can tip the balance of the ER stress response into supporting programmed cell death, they would be leveraging the intrinsic weakness of the disease, to have a desired treatment outcome for MM.

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Green fluorescent protein-based monitoring of endoplasmic reticulum redox poise

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Pathological endoplasmic reticulum (ER) stress is tightly linked to the accumulation of reactive oxidants, which can be both upstream and downstream of ER stress. Accordingly, detrimental intracellular stress signals are amplified through establishment of a vicious cycle. An increasing number of human diseases are characterized by tissue atrophy in response to ER stress and oxidative injury. Experimental monitoring of stress-induced, time-resolved changes in ER reduction-oxidation (redox) states is therefore important. Organelle-specific examination of redox changes has been facilitated by the advent of genetically encoded, fluorescent probes, which can be targeted to different subcellular locations by means of specific amino acid extensions. These probes include redox-sensitive green fluorescent proteins (roGFPs) and the yellow fluorescent protein-based redox biosensor HyPer. In the case of roGFPs, variants with known specificity toward defined redox couples are now available. Here, we review the experimental framework to measure ER redox changes using ER-targeted fluorescent biosensors. Advantages and drawbacks of plate-reader and microscopy-based measurements are discussed, and the power of these techniques demonstrated in the context of selected cell culture models for ER stress.

Keywords: endoplasmic reticulum, endoplasmic reticulum stress, glutathione, green fluorescent protein, hydrogen peroxide, unfolded protein response

INTRODUCTION

The largest endomembrane compartment in the eukaryotic cytoplasm, the endoplasmic reticulum (ER), has attracted increasing research interest over the past two decades (Schuldiner and Schwappach, 2013). The reason for this appears not to be the ER's long recognized function as the "founding organelle" of the secretory pathway, which involves the co-translational folding and post-translational processing of native polypeptide chains destined for cellular membranes or for secretion (Gidalevitz et al., 2013). The proliferation of ER-centered research is mostly due to the discovery of the signaling pathways of the unfolded protein response (UPR), which emanate from the ER in response to diverse protein folding stresses (Cox et al., 1993; Mori et al., 1993; Hetz, 2012). Indeed, the ER is now being recognized as a multifaceted signaling station with vital links to other cellular communication networks (Zhang and Kaufman, 2008; Appenzeller-Herzog and Hall, 2012; Deegan et al., 2012; Claudio et al., 2013; Kiviluoto et al., 2013). Furthermore, the ER maintains physical contact sites to the plasma membrane and essentially every other cell organelle, new functions of which are constantly being discovered (Helle et al., 2013).

The physiological outputs of the UPR are diverse and almost certainly dependent on cell type and the nature of the triggering stress. In general, they are thought to promote pro-survival mechanisms, until—upon prolonged and severe stress—the UPR switches to a network of signals culminating in the execution of the intrinsic, mitochondria-dependent apoptosis pathway (Hetz, 2012). Moreover, in most, if not all, contexts, UPR signaling is accompanied by the accumulation of intracellular oxidants (including reactive oxygen species, ROS), which is commonly referred to as oxidative stress and contributes to the detrimental outcome of exaggerated ER stress (Malhotra and Kaufman, 2007; Santos et al., 2009). As ROS also challenge protein homeostasis in the ER and, therefore, constitute an upstream trigger of ER stress (Buytaert et al., 2006; Malhotra and Kaufman, 2007; Santos et al., 2009), a vicious cycle can develop. This cycle is a critical element in the pathogenesis of various protein folding disorders, e.g. in the central nervous system (Matus et al., 2011). A key molecule counteracting reduction-oxidation (redox) imbalance in the stressed ER is the endogenous tripeptide glutathione (GSH) (Jessop and Bulleid, 2004; Molteni et al., 2004). By means of its cysteinyl side chain, GSH provides critical reducing power for catalyzed or non-catalyzed neutralization of oxidants, which, in the majority of reactions, results in generation of the dimeric, oxidized form of GSH, glutathione disulfide (GSSG) (Appenzeller-Herzog, 2011). Consequently, the manipulation of cellular GSH leads to UPR activation in response to ER hyperoxidation (in case of GSH depletion) (Cuozzo and Kaiser, 1999; Hansen et al., 2012) or ER hypooxidation (in case of GSH overload) (Kumar et al., 2011).

In spite of the established relationship between ER stress and oxidative stress, the origin of ER-stress-induced ROS is still being debated (Appenzeller-Herzog, 2011). For improved mechanistic understanding of underlying oxidative insults, tools for the specific quantification of ER redox conditions are therefore required. It is important to note though that the ER harbors

so many redox couples (most of which are not in equilibrium with each other) that the precise definition of "ER redox conditions" is not possible (Appenzeller-Herzog, 2012). Nevertheless, the status of the GSH–GSSG redox couple is probably a vital measure for the thiol-disulfide homeostasis in the ER—a parameter critical for the formation of native disulfide bonds in substrate proteins and for the prevention of stress. Recently, the electrochemical reduction potential of GSH–GSSG (E_{GSH}) in the ER was measured as −208 mV (Birk et al., 2013), confirming that the impact of GSH on the ER is fairly reducing. This was achieved using a glutaredoxin-fused redox-sensitive green fluorescent protein (roGFP) sensor (Hanson et al., 2004; Gutscher et al., 2008; Lohman and Remington, 2008), which was targeted to the ER of HeLa cells. Previous real-time measurements of redox changes in the mammalian ER were performed using GFP-based probes without defined specificity (van Lith et al., 2011; Kolossov et al., 2012). Another interesting redox-active molecule in the ER is the ROS hydrogen peroxide (H_2O_2), as it can diffuse through the ER membrane and thereby directly transmit a possible redox imbalance in the ER to other cell compartments (Appenzeller-Herzog, 2011; Bertolotti et al., 2013). H_2O_2 is generated *in situ* by the action of ER-resident oxidases such as endoplasmic oxidoreductin 1α (Ero1α) (Ramming and Appenzeller-Herzog, 2012). Changes in $[H_2O_2]$ in the ER can be visualized by monitoring the dithiol-disulfide state of the fluorescent protein-based probe HyPer (Belousov et al., 2006; Enyedi et al., 2010; Wu et al., 2010), although this readout is doubtlessly also influenced by the ER-resident machinery for disulfide-bond formation and is therefore not a *bona fide* measure for $[H_2O_2]$ (Mehmeti et al., 2012; Ruddock, 2012).

In this paper, we outline detailed protocols for the assessment of ER redox conditions using targeted fluorescent protein sensors in either plate-reader- or microscopy-based fluorescence readouts. The suitability of each method for specific experimental problems is discussed.

ER-TARGETED roGFP SENSORS

For the measurement of dynamic redox changes in the ER of mammalian cells, two codon-optimized roGFP sensors with suitable midpoint reduction potentials are now available: roGFP1-iE_{ER} and Grx1-roGFP1-iE_{ER} (Birk et al., 2013). The latter probe specifically reports E_{GSH} (Meyer and Dick, 2010; Birk et al., 2013). Oxidized roGFPs harbor a disulfide bond in the GFP beta-barrel, formation of which changes the excitation spectrum of the protein (see below). Thus, the dithiol-disulfide ratio of transiently transfected, ER-targeted roGFPs can be measured by fluorescence excitation analysis. An alternative, relatively easy approach is the trapping of the roGFP redox state by treatment of cells with a membrane-permeable alkylating agent followed by immunoprecipitation, SDS-PAGE, and immunoblotting where the oxidized fraction of roGFP is identified by its increased gel mobility (Birk et al., 2013). However, this approach yields a redox distribution, which is consistently more reduced than when assessed by means of fluorescence readouts (Birk et al., 2013) (**Table 1**). Accordingly, the two methods for fluorescence excitation analysis outlined in the following sections are preferable when precise quantification of the extent of oxidation of ER-targeted roGFP probes is desired.

Table 1 | OxD values (percentage ± s.d.) of roGFP1-iE_{ER} and Grx1-roGFP-iE_{ER} obtained by different methods.

	Western blot	Microscopy	Excitation spectra analysis
roGFP1-iE_{ER}	54% ± 6	81% ± 13	81% ± 7
Grx1-roGFP1-iE_{ER}	70% ± 5	93% ± 2	90% ± 3

DETERMINATION OF THE REDOX STATE OF roGFP1-IE_{ER} IN MAMMALIAN CELLS USING A FLUORESCENCE PLATE READER

REAGENTS

- HeLa cells (ATCC).
- Mammalian expression plasmid containing the genetically encoded roGFP sensor.
- FuGene HD (Promega).
- Dulbecco's modified Eagle medium (DMEM) high glucose (Sigma), containing 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin.
- Trypsin-EDTA.
- HEPES-buffer (20 mM Hepes, 130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM Glucose, pH 7.4).
- Dithiothreitol (DTT, AppliChem), 1 M Stock in HEPES-buffer (freshly prepared).
- Diamide (Sigma), 0.5 M stock in H_2O, or 2,2′-dithiodipyridine (DPS, Sigma), 10 mM stock in H_2O.

EQUIPMENT

- Fluorescence plate reader equipped with a monochromator with an excitation wavelength range from 350 to 500 nm and an emission wavelength of 530 nm. A bottom-up reader should be used such as Gemini EM fluorescence microplate reader (Molecular Devices) with SoftMax Pro software.
- Clear, flat-bottomed 96 well plate.

PROCEDURE

Cell transfection

1. Trypsinize cells and seed them into two 60 mm dishes at a density of 9×10^4 cells per dish.
2. Transfect cells with 4 µg of sensor plasmid using FuGene HD according to the manufacturer's recommendation. Researchers are encouraged to choose the optimal transfection reagent and protocol for their particular cell line.

Transient transfection can only be used when the transfection efficiency is high enough. For other cell types, it might be difficult to obtain sufficient transfection efficiency, necessitating the generation of a cell line stably expressing the sensor (see also below).

Cell preparation

1. Trypsinize cells 48 h post-transfection and resuspend in complete DMEM.

2. Spin 3 min at 200 × g and carefully aspirate medium.
3. Wash cell pellet with HEPES-buffer and spin again 3 min at 200 × g.
4. Aspirate HEPES buffer and resuspend cells in 1 ml HEPES buffer. Avoid harsh pipetting to prevent shearing of the cells.
5. Distribute the cells to 9 wells of a 96 well plate, (100 µl of cell suspension per well) as outlined in **Figure 1**.
6. Pipet 200 µl of HEPES buffer in an additional well (**Figure 1**) for blank control. The background fluorescence excitation spectra of buffer-filled wells and of wells containing untransfected cells in buffer were found to be identical (our unpublished data).
7. Spin plate 2 min at 200 × g.

Setting up the plate reader

1. Pre-warm the instrument to 37°C.
2. Choose "bottom read."
3. Set the excitation spectrum from 350 to 500 nm with 5 nm intervals. Emission is detected at 530 nm with a cutoff at 515 nm.

Measurement

1. To the wells in the first lane (**Figure 1**) carefully add 100 µl HEPES-buffer without disturbing the monolayer.
2. Prepare a 20 mM DTT solution in HEPES-buffer and carefully add 100 µl to the wells in the second lane.
3. Incubate for 5 min.
4. Prepare a 10 mM diamide or a 1 mM DPS solution (from the respective stock solution) in HEPES-buffer and add 100 µl to the wells of the third lane.
5. Start the measurement.

CALCULATION OF THE DEGREE OF roGFP OXIDATION (OxD)

To obtain blank-corrected excitation spectra from untreated, reduced, and oxidized cells, triplicate emission intensity values at all excitation wavelengths are averaged, the corresponding blank values subtracted and plotted against the excitation wavelength. In the resulting spectra, the values at 390 and 465 nm are extracted to calculate OxD using the following equation:

$$\mathrm{OxD}_{\mathrm{roGFP}} = \frac{R - R_{\mathrm{red}}}{\frac{I_{390\,\mathrm{nm\,ox}}}{I_{390\,\mathrm{nm\,red}}}(R_{\mathrm{ox}} - R) + (R - R_{\mathrm{red}})}$$

where R, R_{red}, and R_{ox} represent the 390:465 nm fluorescence ratios at steady state, after complete reduction or oxidation, respectively. I390 nm is the average fluorescence emission at 390 nm excitation under oxidized or reduced conditions and serves as calibration factor. The obtained OxD value is expressed as percentage of sensor oxidation.

EXPECTED RESULTS

Depicted in **Figure 2** is a typical redox state analysis of roGFP1-iE$_{\mathrm{ER}}$ carried out on a fluorescence plate reader. The peak values extracted from these spectra are to be used to calculate the OxD value as described above.

An advantage of this method is its high reproducibility, which allows reasonable estimates of the redox conditions in the ER. The fact that the measurement is done over a whole cell population comprising different expression levels of the sensor results in a robust readout. The 96-well format is useful for testing the influence of several conditions like, for example, different pharmacological compounds or knockdown of different genes on the same plate. Furthermore, the quality of the experiment and the sensor performance can readily be appraised by inspection of the three excitation spectra (**Figure 2**). However, the plate reader method is not suitable for live experiments where temporal resolution of the measurement under physiological conditions is of special interest. This holds true for the monitoring of both rapid redox changes, which are difficult to visualize in the plate-reader setup, and long-lasting redox trends, which requires analysis of adherent cells under preferably standard growth conditions.

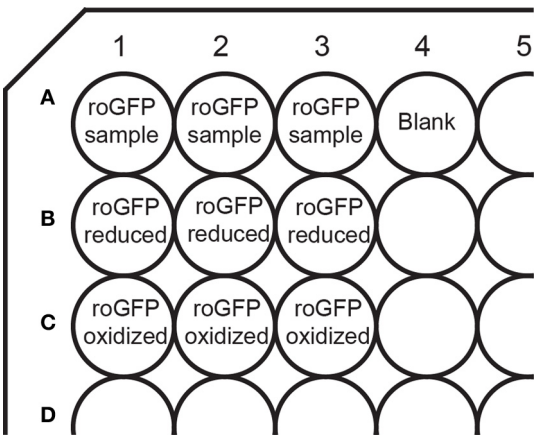

FIGURE 1 | Layout of a 96-well plate for measurements on a plate reader. The plate contains wells for fully oxidized and fully reduced controls, as well as a blank well containing buffer only for blank subtraction.

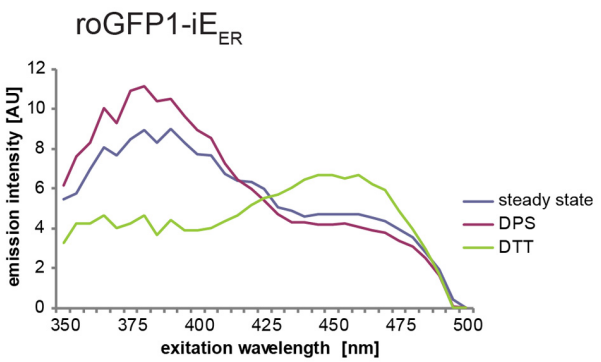

FIGURE 2 | Exemplary spectra of roGFP1-iE$_{\mathrm{ER}}$ transiently transfected into HeLa cells. The lines in the graph represent the averaged and blank corrected fluorescence intensities from cells at steady state (blue), upon oxidation with DPS (red), or reduction with DTT (green). The redox-dependent ratiometric behavior of the two peaks of roGFP1-iE$_{\mathrm{ER}}$ at 390 and 465 nm is visible.

DYNAMIC AND STEADY STATE roGFP1-iE$_{ER}$ MEASUREMENTS IN MAMMALIAN CELLS USING RATIOMETRIC VIDEO MICROSCOPY

Live cell imaging can be used to obtain information about the redox state of roGFP1-iE$_{ER}$ as well as to monitor dynamic changes thereof. Assessment of the steady-state ER redox environment is achieved by conducting a three point measurement consisting of a series of three pictures representing (1) the ground state, (2) the completely oxidized, and (3) the completely reduced state of the sensor. **Figure 3** shows a real-time experiment where the three states of Grx1-roGFP1-iE$_{ER}$ were induced and imaged by ratiometric video microscopy with the sequential addition of oxidant (diamide) and reductant (DTT) to the specimen. The OxD values obtained by this method are comparable with those obtained when using the fluorescence plate reader assay (**Table 1**) (Birk et al., 2013), which validates the data acquired under the less physiological conditions in the plate reader setup.

REAGENTS

- HeLa cells (ATCC).
- Mammalian expression plasmid containing the genetically encoded roGFP sensor.
- FuGene HD (Promega).
- Dulbecco's modified Eagle medium (DMEM) high glucose (Sigma), containing 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin.
- Trypsin-EDTA.
- HEPES-buffer (see above) or DMEM without phenol red. Phenol red can be extracted from conventional DMEM by addition of 0.5 g charcoal per 50 ml of medium and incubation for 2 h under agitation. The charcoal is then removed by filtration. Alternatively, phenol red-free DMEM is commercially available.
- DTT, 1 M Stock in HEPES-buffer.
- Diamide, 0.5 M stock in H_2O.

EQUIPMENT

- Olympus Fluoview 1000 laser scanning confocal microscope equipped with a 60× oil immersion objective (NA 1.40), a 405 nm laser diode, and a 440 nm laser diode.
- Climate chamber with CO_2 vent and humidifier.
- Automated focus control to correct for thermic drift.
- 35 mm dishes with glass bottom (MatTek Corporation).

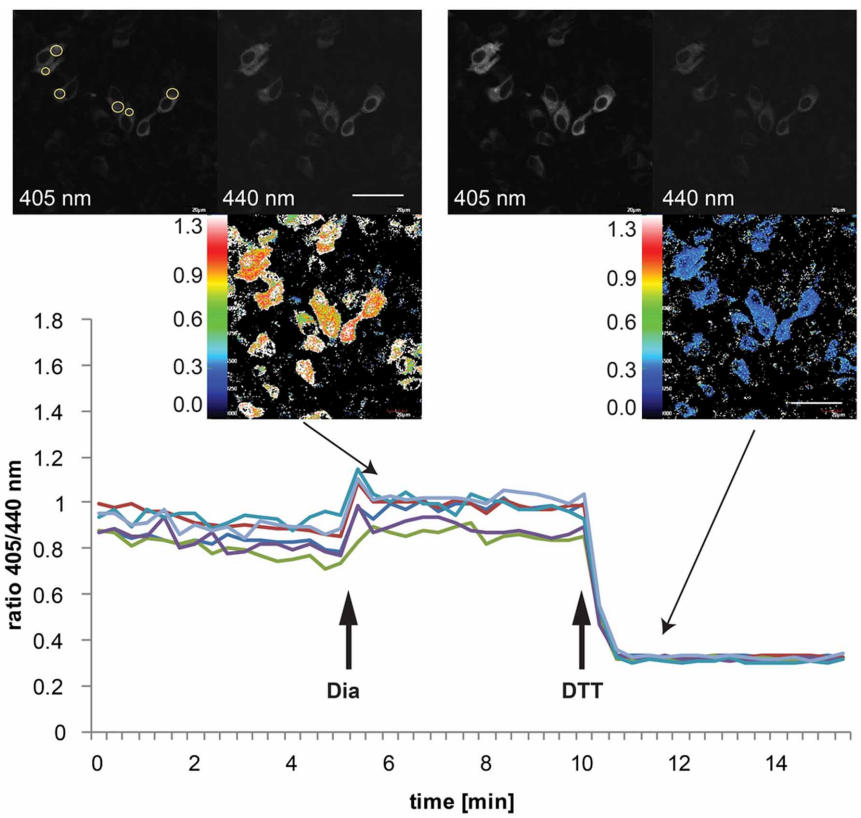

FIGURE 3 | Imaging of dynamic changes in the Grx1-roGFP1-iE$_{ER}$ excitation ratio in transiently transfected HeLa cells. HeLa cells were seeded to MatTek glass bottom dishes and transfected on the following day with Grx1-roGFP1-iE$_{ER}$. Forty eight hours post-transfection, the cells were analyzed by ratiometric laser scanning microscopy. The dynamic changes of the ratios of emission intensities (405 nm excitation):(440 nm excitation) obtained from individual cells upon treatment with 5 mM diamide (Dia), followed by addition of 10 mM DTT are plotted against time. The images in the insets show exemplary fluorescence pictures in the 405 nm or 440 nm channel, respectively, as well as the corresponding ratiometric images. In the upper left micrograph, the positions of ROIs for ratiometric image analysis are indicated as yellow circles.

PROCEDURE

Cell transfection

1. Trypsinize cells and seed to glass bottom dish at a density of 2×10^5 cells per dish in 400 µl DMEM only onto the glass bottom in the middle of the plate.
2. Carefully add 1.6 ml DMEM on the following day.
3. Transfect cells with 2 µg of sensor plasmid using FuGene HD according to the manufacturer's recommendation.

Microscope settings

Based on the excitation maxima of roGFP1-iE$_{ER}$ (see **Figure 2**), the 405 and 440 nm laser lines are used. The emission window is set to 500–600 nm. Images are acquired in sequential frame mode, separating the two channels. The scan speed is set to 8 µs/pixel. If available, use an automated focus control to correct for drift.

It is important to control the surrounding climate of the sample, especially when measurements are performed over a long period of time. Accordingly, the climate chamber needs to be closed, and usage of CO_2 and a humidifier is advised. To facilitate addition of compounds like for example oxidants or reductants despite the closed lid, it is recommendable to install a feed pipe.

To prevent saturated signals, calibration of the microscope with cells cultured in separate wells and treated with reductant or oxidant, as explained by Morgan et al. (2011), is possible when using stably expressing cell lines. However, in our own experience, when using transiently transfected cells, such calibration is not feasible owing to varying expression levels between different cells and samples. We therefore routinely pre-estimate the maximal emission levels of the sensors (i.e., after addition of reductant or oxidant) in the two channels in order to determine suitable gain settings. In practice, this means that the sensitivity of the photomultiplier tube of the 440 nm channel is adjusted to a very low signal, since complete reduction of the sensor will strongly increase the fluorescence signal in this channel. However, since the photomultiplier of the 405 nm channel has to be adjusted equally, a setting is required in which the 440 nm channel emits the lowest signal possible, but the signal in the 405 nm channel is still visible. Furthermore, in order to be able to work with lower laser power, we recommend opening the pinhole completely. This will decrease the risk of light-induced artifactual oxidation. On the flip side, this measure lowers spatial resolution.

Measurement

1. Fill the humidifier with water and open the CO_2 vault when performing a long-term measurement. For steady-state measurements, preheating of stage and objective are sufficient.
2. Set the temperature of the climate chamber to 41°C, preheat the lid to 44°C and the objective to 37°C.
3. Let the system equilibrate for one hour.
4. Wash the cell monolayer in the imaging dish two times with DMEM without phenol red. Steady-state measurements can also be performed in HEPES-buffer instead.
5. Add 1 ml medium without phenol red or HEPES-buffer to the cells and mount the dish with the probe onto the heated stage.
6. Let the system equilibrate for at least 15 min.
7. Use transmitted light to find an appropriate field for imaging.
8. *For steady state measurements:* set the time intervals to 5 min and take three pictures, one as starting point, one after addition of oxidant, and one after the addition of reductant (see below).
   For dynamic long-term measurements: choose an appropriate time interval between different frames (depending on the length of measurement and the kinetics of the redox response to be analyzed). Typically, for 3 h we recommend to use 10 min.
9. Adjust the photomultiplier tube of both channels to equal voltage following the guidelines discussed in section Microscope settings.
10. Start the measurement.
11. Add 10 mM diamide in 1 ml DMEM without phenol red or HEPES-buffer.
12. Add 60 mM DTT in 1 ml DMEM without phenol red or HEPES buffer on top.

Image Analysis

1. Image analysis can be done using different programs including ImageJ (Morgan et al., 2011); we generally use the background subtraction and the series analysis tool of the FV-100 software (Olympus) installed on our microscope.
2. Set regions of interest (ROIs) within selected cells (**Figure 3**). Exclude cells with saturated pixels. We usually set one ROI per cell and analyze as many cells in the field of view as possible, at least 10. Especially when performing long-term measurements, it is recommendable to use relatively small ROIs, since the cells may move during image acquisition. Using a smaller ROI will minimize the risk of "loosing" the cell due to migration.
3. Export the fluorescence intensity data to Microsoft excel and use the formula provided in section "Calculation of the degree of roGFP oxidation (OxD)" for calculation of OxD values.
4. The ratio between the channels is calculated as emission intensity at 405 nm excitation divided by the emission intensity at 440 nm excitation.

LIVE IMAGING OF roGFP1-IE$_{ER}$ IN THE STRESSED ER

Induction of ER stress can be achieved by treatment of cells with different compounds including DTT, which prevents disulfide-bond formation, thapsigargin, an inhibitor of the ER Ca^{2+} ATPase, or tunicamycin, an inhibitor of protein glycosylation. Induction of the full UPR upon application of these drugs to tissue culture cells typically occurs after 10–30 min (DuRose et al., 2006). Accordingly, possible effects of ER stress on the ER redox environment can readily be monitored by real-time video microscopy. As an example, **Figure 4** shows, how live cell imaging can be used to assess the effect of tunicamycin treatment on the redox state of roGFP1-iE$_{ER}$ in transiently transfected HeLa cells. Cells react heterogeneously to tunicamycin-induced ER stress. While many of them show no discernible changes in the ER redox environment, a subpopulation of cells display moderate hyperoxidation of roGFP1-iE$_{ER}$ after a lag phase of ∼100 minutes [compare also to the experiment published in Birk et al. (2013)].

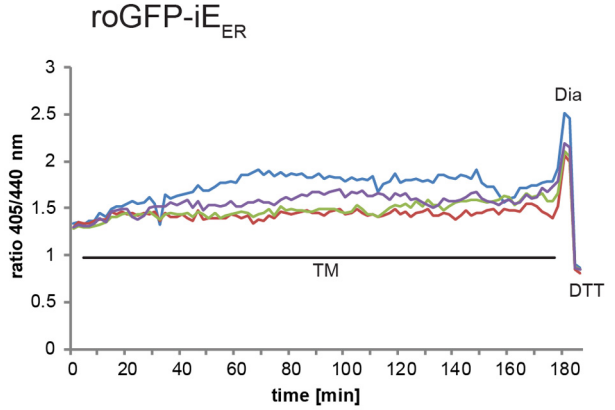

FIGURE 4 | Live imaging of roGFP1-iE$_{ER}$ upon tunicamycin treatment. HeLa cells transiently expressing roGFP1-iE$_{ER}$ were analyzed by ratiometric laser scanning microscopy 48 h post-transfection. Where indicated, 1 μg/ml tunicamycin (TM), 0.5 mM diamide (Dia), and 20 mM DTT were added to the specimen. The dynamic ratio changes of individual cells were plotted against time.

DETERMINATION OF ER REDOX CHANGES IN FlipIn TRex 293 CELLS WITH DOXYCYCLINE-INDUCIBLE GENE EXPRESSION USING HyPer$_{ER}$

Besides pharmacological induction, ER stress can also be a result of oxidative challenge. For instance, a recent study demonstrated UPR induction in response to doxycycline-induced expression of a hyperactive mutant (C104A/C131A) of Ero1α in FlipIn TRex 293 cells (Hansen et al., 2012). This ER-resident oxidase generates disulfide bonds, which entails the concomitant production of equimolar amounts of the reactive oxidant H_2O_2 (Ramming and Appenzeller-Herzog, 2012). Fluorescence-based readouts for characterization of the shift in the ER redox balance resulting from Ero1α-C104A/C131A induction, however, are not straightforward. Doxycycline displays a fluorescence excitation peak at around 375 nm, which significantly overlaps with the roGFP excitation spectrum (**Figure 5**) and cannot be eliminated by repeated washing of the cell monolayer (our unpublished data). To overcome this problem, the use of a different genetically encoded, ratiometric redox probe, HyPer (Belousov et al., 2006), shall be described in this section. In contrast to roGFP, the red-shifted excitation spectrum of HyPer does not overlap with the 375 nm peak of doxycycline and, hence, fluorescence spectrum analysis of HyPer is applicable in doxycycline-inducible cell systems. Alternatively, doxycycline-dependent changes in the redox state of roGFP-based probes can be visualized by an immunoprecipitation/Western blot approach, as has been published (Birk et al., 2013).

A peculiarity of the HyPer probe is its ability to rapidly and directly react with H_2O_2 (Belousov et al., 2006). This feature has led to the suggestion that HyPer is a specific H_2O_2 probe. While this may be true for cell compartments like the cytosol or the nucleus, which harbor potent disulfide-reducing machinery, the relatively oxidized redox state of HyPer in the environment of the ER (Enyedi et al., 2010; Wu et al., 2010; Malinouski et al., 2011) most likely does not exclusively reflect the presence of H_2O_2, but also of other oxidizing factors such as protein disulfide isomerases (Appenzeller-Herzog and Ellgaard, 2008; Mehmeti et al., 2012; Ruddock, 2012).

PROCEDURE

The protocol to determine the fluorescence excitation spectrum of HyPer in FlipIn TRex 293 cells is largely identical to the excitation spectrum analysis of roGFPs in HeLa cells (described in Section "Determination of the redox state of roGFP1-iE$_{ER}$ in mammalian cells using a fluorescence plate reader"). Thus, only explicit differences between the two protocols will be discussed in this section.

- In contrast to HeLa cells, FlipIn TRex 293 cells require stable transfection of the redox probe to reach an acceptable signal-to-noise ratio for fluorescence measurements in a plate reader. Therefore, for the experiments described further below, FlipIn TRex 293:Ero1α-C104A/C131A cells (Hansen et al., 2012) were transfected with HyPer$_{ER}$ (Enyedi et al., 2010) followed by clonal selection with G418.
- 7.5×10^5 cells are seeded into 35 mm dishes (e.g., in 6-well plates) and the expression of the inducible cDNA initiated on the following day by addition of 1 μg/ml doxycycline (from a 1000× aqueous stock solution) 24 h ahead of analysis.
- In order to yield the fully oxidized form of HyPer$_{ER}$ immediately prior to fluorescence scanning in the plate reader, cells in the 96-well plates are treated with 100 μM H_2O_2 instead of DPS or diamide. As the presence of 5 mM glucose in the cell resuspension buffer lowers the oxidizing effect of H_2O_2 (our unpublished data), presumably due to glucose-mediated reduction of H_2O_2, glucose needs to be omitted from the HEPES-buffer.
- The plate reader settings need to be adjusted to the excitation spectrum of HyPer. Thus, excitation wavelength ranges from 410 to 510 nm with 5 nm steps. Emission is detected at 535 nm with a cutoff at 530 nm.

EXPECTED RESULTS EXEMPLIFIED BY CONDITIONAL EXPRESSION OF Ero1α-C104A/C131A

Depicted in **Figure 6** is a typical outcome of an excitation spectrum analysis of HyPer$_{ER}$ stably expressed in FlipIn TRex 293:Ero1α-C104A/C131A cells. As expected, the steady-state excitation ratio of HyPer$_{ER}$ (blue line) was slightly shifted toward the spectrum obtained from completely oxidized cells (red line) upon expression of Ero1α-C104A/C131A (see **Figure 8A** for quantification).

Because the fluorescence excitation spectrum of HyPer is not only influenced by reduction/oxidation, but also by changes in pH (Belousov et al., 2006; Forkink et al., 2010; Schwarzlander et al., 2011), appropriate controls are required. To this end, the pH-sensitive C199S mutant of HyPer (termed "SypHer") (Poburko et al., 2011), which does not respond to redox changes, can be employed. As illustrated in **Figure 7**, SypHer$_{ER}$ spectra obtained from untreated (blue line) or H_2O_2-treated cells (red line) are virtually identical. However, in the case of DTT-treated cells (green line), a decrease in fluorescence emission affecting the entire

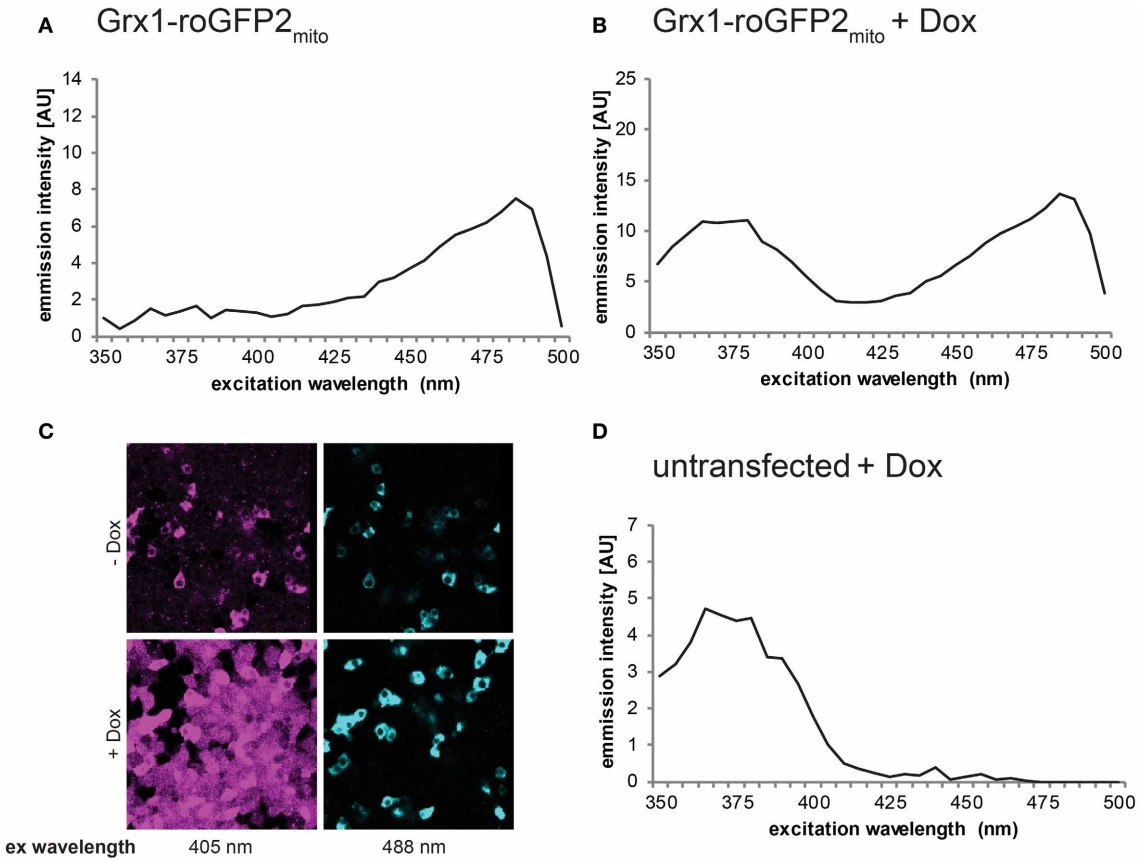

FIGURE 5 | Fluorescence of doxycycline prohibits roGFP-based redox analysis. Cells were transiently transfected with mitochondrial Grx1-roGFP2 (Grx1-roGFP2$_{mito}$) (Gutscher et al., 2008) and induced with doxycycline (Dox) for 24 h. While non-induced cells displayed a normal roGFP spectrum **(A)**, Dox treated cells showed an altered peak at 375 nm excitation **(B)**, which overlapped with the fluorescence produced by Dox alone when added to untransfected cells **(D)**. The same effect was observed using ratiometric laser scanning fluorescence microscopy, as evidenced by ubiquitous fluorescence upon excitation (ex) with the 405 nm laser line **(C)**.

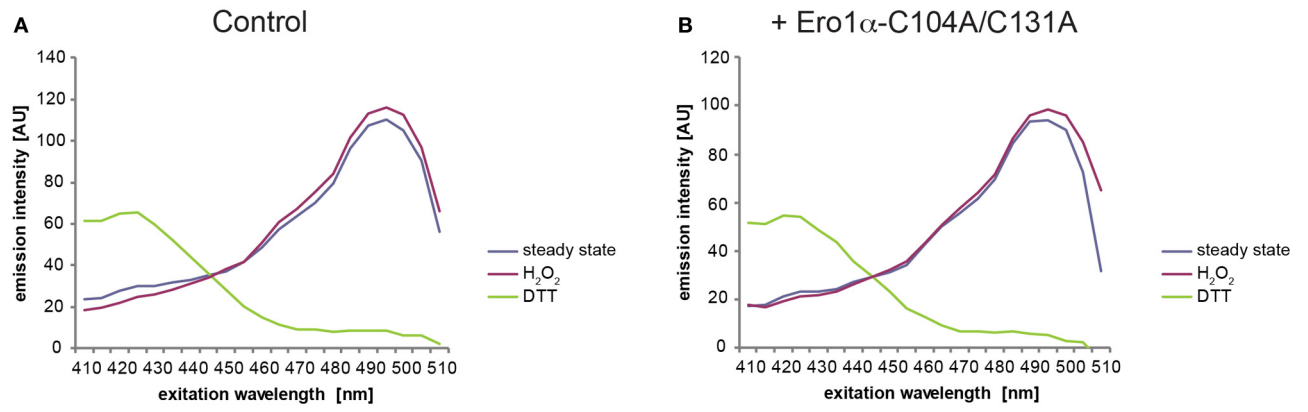

FIGURE 6 | Steady-state oxidation of HyPer$_{ER}$ changes upon expression of Ero1α-C104A/C131A. Example spectra from experiments conducted with FlipIn TRex 293:Ero1α-C104A/C131A cells stably transfected with HyPer$_{ER}$. Cells were left untreated **(A)** or Ero1α-C104A/C131A expression was induced for 24 h by addition of 1 μg/ml doxycycline **(B)**. The lines in the graphs represent the averaged and blank corrected fluorescence intensities recorded from cells at steady state (blue), upon oxidation with H_2O_2 (red) or reduction with DTT (green). The redox-dependent ratiometric behavior of the two peaks of HyPer$_{ER}$ at 420 and 500 nm can readily be appreciated.

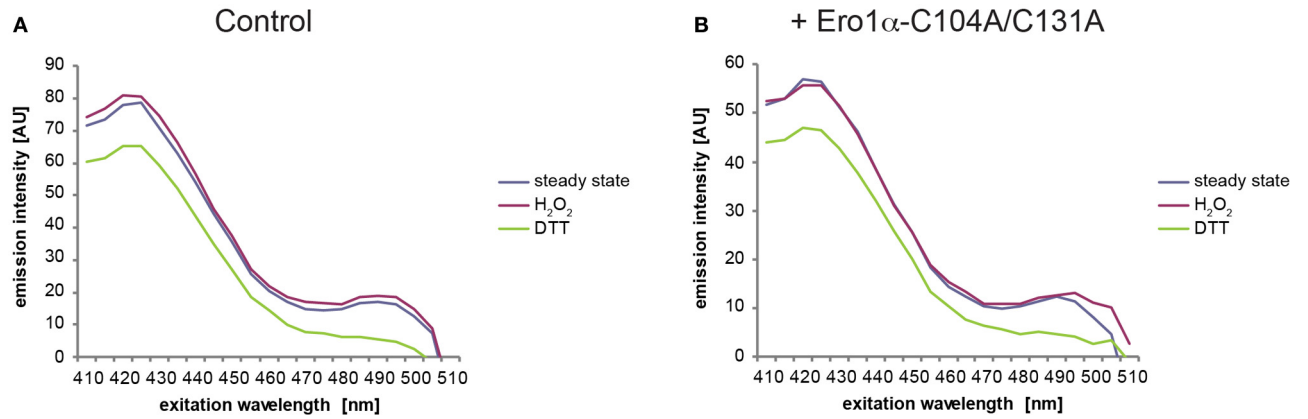

FIGURE 7 | DTT treatment changes the fluorescence excitation spectrum of SypHer$_{ER}$. Fluorescence excitation spectra were recorded from uninduced **(A)** or induced **(B)** FlipIn TRex 293:Ero1α-C104A/C131A cells stably transfected with SypHer$_{ER}$ as in **Figure 6**. Note the lack of redox-dependent ratiometric behavior and the decreased fluorescence intensity in the case of DTT-treated samples.

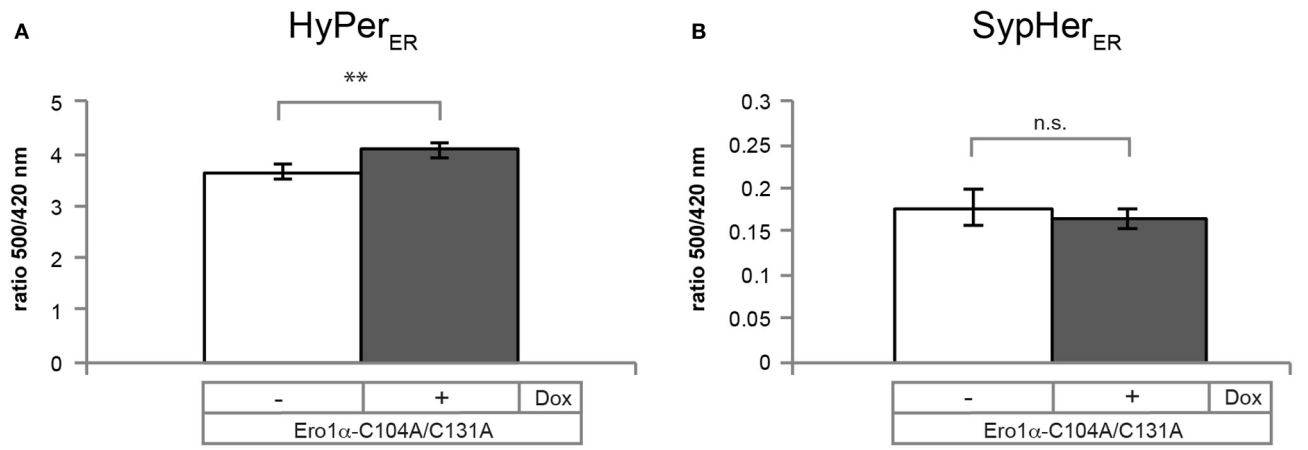

FIGURE 8 | Ero1α-C104A/C131A-induced changes in the 500/420 nm ratios of HyPer$_{ER}$ and SypHer$_{ER}$. Plotted are the ratios of the 500 and 420 nm peak amplitudes of the steady state spectra of HyPer$_{ER}$ **(A)** or SypHer$_{ER}$ **(B)**, which were obtained as shown in **Figures 6**, **7**, respectively. Data sets were analyzed for statistical significance using student's *T*-test (two-tailed distribution; heteroscedastic) ($n \geq 3$; mean ± SD). Dox, doxycycline; $^{**}p = 0.009$.

spectrum is evident. Unfortunately, this effect renders the calculation of OxD of HyPer$_{ER}$ obsolete, at least when employing DTT as a reducing agent. We therefore recommend the use of the HyPer fluorescence intensity ratio upon excitation at 500 nm over excitation at 420 nm of the steady state spectra (blue line), which serves as a robust readout of HyPer$_{ER}$ oxidation (Enyedi et al., 2010; Wu et al., 2010; Malinouski et al., 2011). Analysis of the 500:420 nm fluorescence ratios readily allows the detection of the oxidative shift in the ER elicited by Ero1α-C104A/C131A induction (**Figure 8**).

CONCLUDING REMARKS AND FUTURE CHALLENGES

Redox sensing is a complicated issue. A first layer of complexity is given by the often times short-lived nature of intracellular redox changes, which are typically rapidly reverted by dedicated machinery. For the same reason, oxidative signals are usually locally restricted so that they can be barely detected by global monitoring methods. Because redox-sensitive fluorescent proteins can easily be targeted to subcellular compartments and monitored at real-time resolution, this spatiotemporal problem can now be tackled (Meyer and Dick, 2010). More elaborate targeting of the biosensors to organelle substructures such as membrane contact sites with other cell organelles (Helle et al., 2013) is a future challenge, which will likely lead to a more sophisticated understanding of the context and function of redox changes.

A second layer of complexity in redox monitoring arises from the issue of specificity (or lack of specificity) of the readout. Thus, a sensor protein, which can become oxidized to form a disulfide bond, can in principle react with any other redox couple present so that the OxD of the sensor results from the integration of a variety of oxidizing and reductive inputs. It is important to note that the weight of these different inputs does not reflect their degree of impact on "ER redox conditions" but rather the specific

reaction kinetics of the probe with the available redox couples. Based on this principle, quasi-specific probes have been developed by selectively increasing the reaction rate toward a redox couple (e.g., GSH) through intramolecular fusion of a catalyzing enzyme [e.g., glutaredoxin (Meyer and Dick, 2010)]. While such specific readouts certainly constitute a major advance, they also open our view on the wealth of information we do not have yet. For instance, ascorbate–dehydroascorbate (Zito et al., 2012) or reduced–oxidized pyridine nucleotides (Lavery et al., 2008), which at present cannot be measured directly, are other ER redox couples with a reported impact on ER homeostasis.

Finally, it is important to mention that the use of genetically encoded redox biosensors is not restricted to cell culture models, as described herein. Recently, cytosolic and mitochondrial roGFPs sensitive for E_{GSH} or $[H_2O_2]$ were expressed and imaged in fruit flies, which led to the stunning conclusion that these two redox systems are surprisingly uncoupled *in vivo* (Albrecht et al., 2011). Clearly, it will also be interesting to determine changes in one or more ER redox couples *in vivo*, both under physiological and pathological situations known to involve the UPR.

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Temporal clustering of gene expression links the metabolic transcription factor HNF4α to the ER stress-dependent gene regulatory network

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The unfolded protein response (UPR) responds to disruption of endoplasmic reticulum (ER) function by initiating signaling cascades that ultimately culminate in extensive transcriptional regulation. Classically, this regulation includes genes encoding ER chaperones, ER-associated degradation factors, and others involved in secretory protein folding and processing, and is carried out by the transcriptional activators that are produced as a consequence of UPR activation. However, up to half of the mRNAs regulated by ER stress are downregulated rather than upregulated, and the mechanisms linking ER stress and UPR activation to mRNA suppression are poorly understood. To begin to address this issue, we used a "bottom-up" approach to study the metabolic gene regulatory network controlled by the UPR in the liver, because ER stress in the liver leads to lipid accumulation, and fatty liver disease is the most common liver disease in the western world. qRT-PCR profiling of mouse liver mRNAs during ER stress revealed that suppression of the transcriptional regulators C/EBPα, PPARα, and PGC-1α preceded lipid accumulation, and was then followed by suppression of mRNAs encoding key enzymes involved in fatty acid oxidation and lipoprotein biogenesis and transport. Mice lacking the ER stress sensor ATF6α, which experience persistent ER stress and profound lipid accumulation during challenge, were then used as the basis for a functional genomics approach that allowed genes to be grouped into distinct expression profiles. This clustering predicted that ER stress would suppress the activity of the metabolic transcriptional regulator HNF4α—a finding subsequently confirmed by chromatin immunopreciptation at the *Cebpa* and *Pgc1a* promoters. Our results establish a framework for hepatic gene regulation during ER stress and suggest that HNF4α occupies the apex of that framework. They also provide a unique resource for the community to further explore the temporal regulation of gene expression during ER stress *in vivo*.

Keywords: ER stress, fatty liver, functional genomics, gene regulatory network, lipid metabolism

INTRODUCTION

Originally identified as a program for improving protein folding during ER stress, the vertebrate UPR is composed of three separate but functionally overlapping pathways that culminate in transcriptional upregulation of genes involved in ER protein folding and processing (Ron and Walter, 2007). The ER-resident endoribonuclease IRE1, which is conserved among eukaryotes and exists as α [GenBank:NM_023913.2] and β [GenBank:NM_012016.2] paralogs in mammals, catalyzes splicing of *Xbp1* mRNA [GenBank:NM_013842.3 and NM_001271730.1] to remove a 26 base intron and allow for the translation of a transcriptional activator of the bZIP family. The PERK kinase [GenBank:NM_010121.2] is metazoan-specific, and it phosphorylates the translation initiation factor eIF2α [GenBank:NM_001005509.2] when activated, resulting in transient inhibition of protein synthesis but also specific translation of *Atf4* mRNA [GenBank:NM_009716.2] to produce the bZIP transcriptional activator ATF4. Other ER stress-independent eIF2α kinases exist, and phophorylation of eIF2α and the attendant consequences of that event are known as the integrated stress response. ATF6, also metazoan-specific and with α [GenBank:NM_007348.3] and β [GenBank:NM_017406.4] paralogs, is resident to the ER but transits to the Golgi during stress, where it is cleaved by regulated intramembrane proteolysis to liberate an active bZIP transcriptional activator. Together, these bZIPs coordinate enhancement of protein synthesis, degradation, folding, modification, and trafficking through gene regulation.

An oft overlooked feature of UPR activation is that between 20 and 50 percent of regulated genes are actually suppressed by ER stress depending on the conditions, yet much less is

Abbreviations: bZIP, basic leucine zipper; ER, endoplasmic reticulum; TM, tunicamycin; UPR, unfolded protein response; VLDL, very low density lipoprotein.

known about the mechanisms responsible for this suppression and the physiological consequences thereof. A portion of this suppression can be attributed to regulated IRE1-dependent decay, in which the IRE1 endonuclease degrades ER-associated mRNAs (Hollien and Weissman, 2006). Transcriptional mechanisms for suppression have been identified as well, including direct suppression by the bZIP C/EBP family member CHOP [GenBank:NM_007837.3] (Ron and Habener, 1992), titration of the coactivator CRTC2 [GenBank:NM_028881.2] (Wang et al., 2009), and translational regulation of the suppressive LIP isoform of C/EBPβ [GenBank:NM_009883.3] (Li et al., 2008; Arensdorf and Rutkowski, 2013). Each of these mechanisms was identified through the behavior of target genes, so the extent to which any of them contributes to global gene suppression is not clear.

In the liver, the most evident consequence of ER stress is lipid accumulation (Rutkowski et al., 2008; Yamamoto et al., 2010; Zhang et al., 2011). This lipid accumulation, or steatosis, is accompanied by suppression of a host of genes involved in hepatic lipid metabolic processes, including fatty acid oxidation, lipogenesis, cholesterologenesis, and VLDL production. Given that some of these processes are mutually antagonistic (e.g., fatty acid oxidation and lipogenesis), it seems likely that some are suppressed as primary responses to ER stress, and others as secondary consequences of feedback mechanisms.

Some regulation of hepatic lipid metabolism can be attributed directly to the action of canonical UPR signaling. XBP1 can bind to the promoters and stimulate transcription of lipogenic genes (Lee et al., 2008) and of the ER oxidoreductase PDI [GenBank:NM_001032.2] (Wang et al., 2012), the latter of which stimulates VLDL secretion by virtue of its interaction with the microsomal triglyceride transfer protein (MTTP) [GenBank:NM_001163457.1]. IRE1α can also directly degrade mRNAs encoding lipogenic genes when *Xbp1* is ablated through its regulated IRE1-dependent decay activity (So et al., 2012), thus acting at cross-purposes with its downstream target XBP1. The ER-localized transcription factor CREBH [GenBank:NM_145365.3] also contributes to lipid homeostasis (Zhang et al., 2012), although it is not yet clear whether that function is direct or indirect.

Steatosis arises as a common phenotype in response to ER stress when any of the UPR signaling pathways is ablated, or when UPR signaling is intact but ER protein folding is compromised by deletion of the ER cochaperone $p58^{IPK}$ [GenBank:NM_008929.3] (Rutkowski et al., 2008). This steatosis is likely at least partially caused by impaired secretion of triglyceride-rich VLDL particles from the stressed ER (Ota et al., 2008; Rutkowski et al., 2008; Caviglia et al., 2011) and enhanced uptake (Jo et al., 2013). However, ER stress also elicits extensive alterations in the expression of genes involved in lipid metabolism, and these alterations are more severe and persistent when any branch of the UPR—or when ER protein folding—is compromised (Rutkowski et al., 2008). Thus, these alterations correlate with the development of steatosis, although it is not known which events precede lipid accumulation and which follow as a consequence. That they emerge irrespective of which UPR pathway is ablated argues that most such metabolic genes are not directly regulated by ATF4, ATF6, or XBP1, but by some mechanism or mechanisms that indirectly tie metabolic gene regulation to the ER stress burden. To some degree the dysregulation of lipid metabolism can be attributed to CHOP (Chikka et al., 2013), which is redundantly regulated by each of the three UPR pathways and which is expressed more robustly when stress is more severe—as when the UPR or ER protein folding is disrupted (Rutkowski et al., 2008). However, $Chop^{-/-}$ animals are only partially protected from hepatic steatosis during ER stress (Rutkowski et al., 2008), suggesting that other as yet uncovered pathways exist as well.

Given the extensive nature of metabolic gene regulation during ER stress, there likely exists a mechanistic hierarchy of regulation, with some metabolic genes being more proximally connected to UPR pathways and others lying downstream of these initial events. However, the global organization of lipid metabolic gene regulation during ER stress has not been studied. Thus, our goal in this work was to begin to decipher the structure of ER stress-mediated metabolic gene regulation by establishing the temporal progression of such events in the mouse liver, and to infer hierarchical relationships using a functional genomics approach, based upon the behavior of coordinately regulated groups of genes in wild-type mice vs. mice lacking the ER stress sensor ATF6α.

MATERIALS AND METHODS

ANIMAL EXPERIMENTS

All protocols for animal use were reviewed and approved by the University Committee on Use and Care of Animals at the University of Iowa or the University of Michigan. Animals were bred in house, and were fed standard rodent chow and housed in a controlled pathogen free environment with 12 h light and dark cycles. Animals used were of varying ages and genders, with control and experimental groups having similar composition. Animals were fasted for 4 h prior to sacrifice, which was carried out in daytime hours.

LIPID ANALYSIS

ADRP immunostaining was as described (Chikka et al., 2013). For the trigylceride assay, a 100 mg piece of liver was homogenized in 1 mL of ice cold extraction buffer (1 mM Tris pH 7.6, 1 mM EGTA, 1 mM $MgCl_2$) containing protease inhibitors. A 200 μL aliquot of the homogenate was placed into a new 1.5 mL tube on ice, and an additional aliquot was set aside to determine the protein concentration of each sample. A 750 μL aliquot of a Chloroform:Methanol mixture (1:2 ratio) was added to the 200 μL homogenate sample and vortexed vigorously for 15 s. The samples were incubated at room temperature for 1 h, with the samples vortexed every 15 min. Following the hour incubation, 250 μL of Chloroform was added to each sample and vortexed for 15 s, then incubated at room temperature for 15 min. Two hundred μL of distilled water was then added to the sample and vortexed as above. The samples were centrifuged at 5000 rpm for 10 min, and the bottom organic layer was collected and placed in a fresh tube. The sample was evaporated under nitrogen gas, and the remaining lipids were dissolved in 200 μL of isopropanol. From these samples, the triglyceride levels were determined using the Infinity Triglycerides Reagent (Thermo Scientific, TR22421) per

manufacturer's instructions. The Cayman triglyceride standard (Cayman Chemical, 10010509) was used to generate a standard curve. Oil Red O staining was as described (Rutkowski et al., 2008).

RNA ANALYSIS

The 8 h microarray has been published (Rutkowski et al., 2008). For the 34 h microarray, mice were injected with 1 mg/kg TM, and mRNA was prepared from isolated livers and analyzed by Affymetrix microarray in exactly the same way. The NCBI GEO accession number for both arrays is GSE48939. Expression categories for each probeset are provided in **Table S1**. RT-PCR and qRT-PCR analysis, including validation of all primer sets, was as previously described (Rutkowski et al., 2006; Tyra et al., 2012), except that gene expression was normalized against the average expression of two housekeeping genes (*Btf3* and *Ppia*) rather than only one. Primer sequences can be found in (Rutkowski et al., 2006, 2008; Wu et al., 2007; Tyra et al., 2012) and **Table S2**.

BIOINFORMATIC ANALYSIS

Pathway enrichment analysis was performed using FunNet software (Prifti et al., 2008). Data represent GO biological process annotations with a decorrelated enrichment computation and a 5% false discovery rate correction. Transcription factor binding site analysis was performed using oPOSSUM software (Ho Sui et al., 2005). Data represent a single site analysis of vertebrate transcription factor binding sites within 2000 base pairs upstream or downstream of the transcription start site. The results of this analysis were visualized using Cytoscape software. The network was limited to genes with GO annotations involved in lipid metabolism and transport using the BiNGO plug-in (Maere et al., 2005).

CHROMATIN IMMUNOPRECIPITATION

Livers were isolated from 6–8 week-old mice and prepared for ChIP using the ChIP Tissue Chromatin Shearing Kit with SDS (Covaris). Samples were sonicated using a S220 focused ultrasonicator (Covaris) to produce DNA bands between 100 and 1000 bp. Following sonication, the immunoprecipiation was carried out as described in (Arensdorf and Rutkowski, 2013) using HNF4α antiserum (H-171, Santa Cruz) or non-specific IgG (12-370, Millipore).

RESULTS AND DISCUSSION

SUPPRESSION OF METABOLIC TRANSCRIPTIONAL MASTER REGULATORS PRECEDES LIPID ACCUMULATION DURING ER STRESS

Lipid accumulation can be induced in the liver by exposure to the inhibitor of N-linked glycosylation tunicamycin (TM) or the proteasomal inhibitor bortezomib, or by overexpression of a misfolded ER client protein such as coagulation Factor VIII [GenBank:NM_001161373.1] (Rutkowski et al., 2008; Zhang et al., 2011; Chikka et al., 2013). Each of these treatments activates either the UPR or integrated stress response and each leads to qualitiatively similar changes in the expression of key metabolic genes in the liver. These genes include both transcription factors and cofactors involved in controlling metabolism as well as the downstream targets of these factors that encode the key functional enzymes involved in lipid catabolism, anabolism, storage, and secretion (Rutkowski et al., 2008).

The temporal organization of these gene regulatory changes prior and subsequent to the onset of lipid accumulation has not been analyzed. It is likely that the earliest of these regulated events are directly mechanistically connected to the UPR and/or integrated stress response. Thus, we examined the time course of hepatic lipid accumulation in wild-type mice in response to TM, which is a more robust inducer of ER stress and steatosis than other stimuli (Chikka et al., 2013). We monitored lipid accumulation by three distinct criteria: accumulation of Oil Red O in

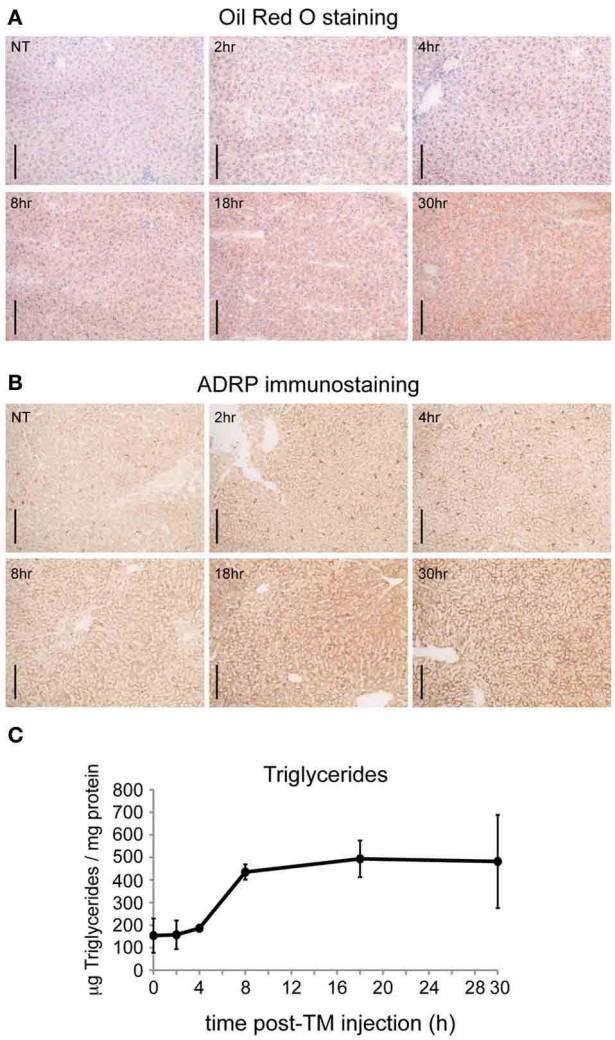

FIGURE 1 | ER stress causes substantial hepatic lipid accumulation within 8 h. (A) C57BL/6J mice were challenged with 1 mg/kg TM for the indicated times, livers were frozen in OCT, and lipids were stained with Oil Red O. Scale bar = 50 μm. **(B)** Same as **(A)**, except lipid content was assessed by immunohistochemical staining for the lipid droplet marker protein ADRP. Scale bar = 50 μm. **(C)** Same as **(A)**, except triglyceride content was measured by colorimetric assay after extraction of neutral lipids. $p < 0.001$ by One-Way ANOVA. $n = 3$ samples per time point. Error bars here and elsewhere show means ± SDM.

lipid droplets of fresh frozen liver sections (**Figure 1A**); immunohistochemical detection of the lipid-droplet associated protein adipophilin (ADRP) [GenBank:NM_007408.3] (**Figure 1B**); and direct biochemical assessment of hepatic triglyceride levels (**Figure 1C**). Results from all three assays were similar: hepatic lipid content increased most substantially at the 8 h time point. Therefore, the key genetic regulatory events responsible for altering lipid metabolism are likely to occur prior to 8 h, while those that occur later than 8 h after challenge are more likely secondary effects that contribute to lipid disruption tangentially, if at all.

Next, we examined the timing of UPR activation and of the regulation of metabolic genes. TM led to maximal IRE1α-dependent splicing of *Xbp1* mRNA within 2 h; this splicing persisted through 8 h but was diminished at later time points (**Figure 2A**). Likewise, every UPR-regulated target gene was significantly upregulated by 2 h, peaked at 4–8 h, and diminished thereafter (**Figure 2B**). These genes depend to varying extents on activity of each of the three UPR pathways (Harding et al., 2003; Lee et al., 2003; Wu et al., 2007). That they are uniformly upregulated by 2 h suggests that each of the three canonical UPR-regulated transcriptional activators—ATF6α, ATF4, and XBP1—is functionally active by this very early time point.

We then analyzed expression of genes involved in lipid metabolism, including both transcriptional regulators and key rate-limiting metabolic enzymes. We grouped the genes according to the time point at which they were first down-regulated. We immediately observed that, in contrast to conventional UPR target genes, the regulation of metabolic genes occurred in stages. The earliest group regulated included select transcription factors and cofactors. The coregulator *Pgc1b* [GenBank:NM_133249.2] was downregulated by 2 h although not at 4 h (**Figure 2C**), making the true timing of its suppression ambiguous. In contrast, by

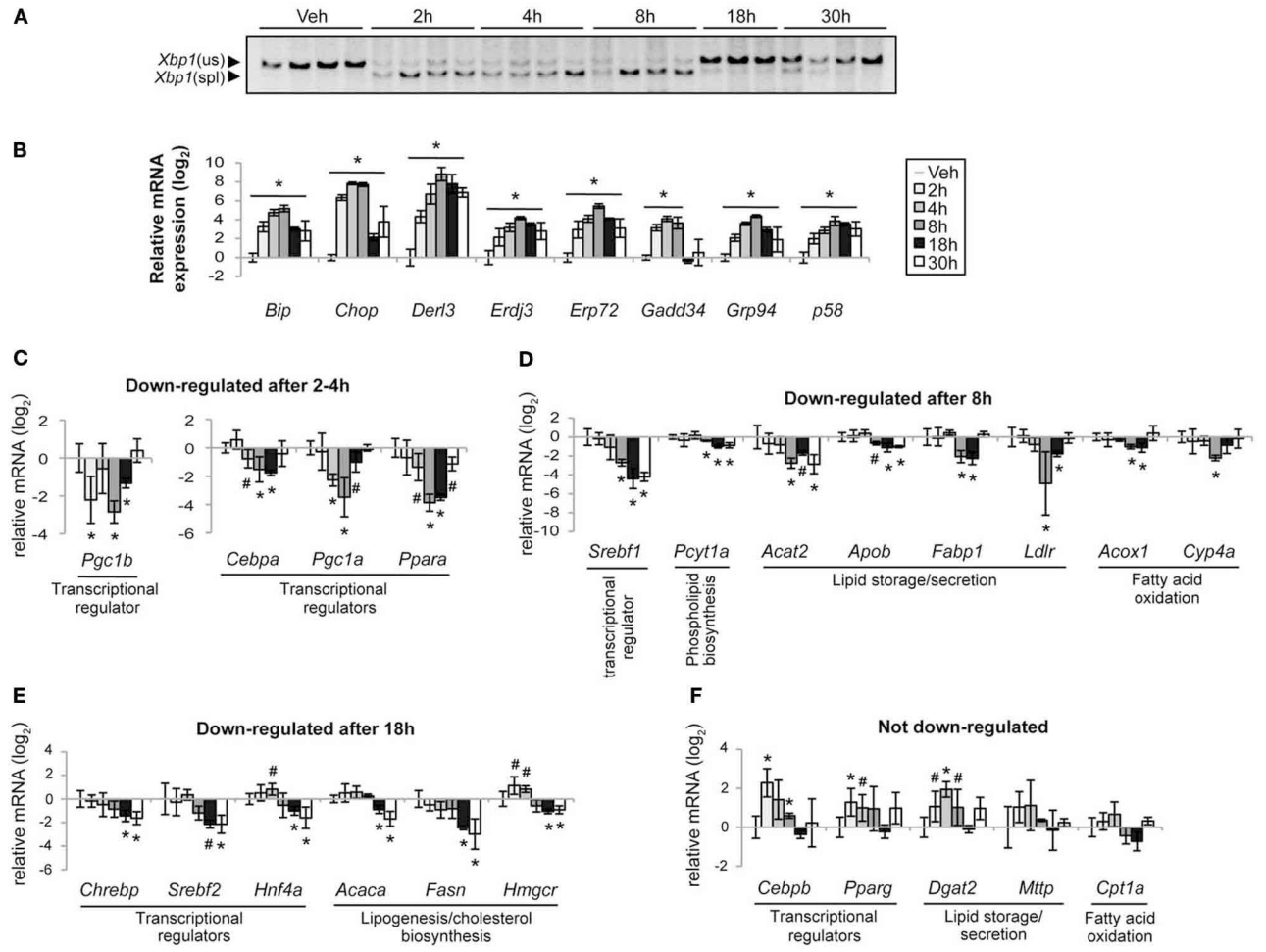

FIGURE 2 | Staggered suppression of metabolic genes during ER stress. **(A)** The spliced (spl) and unspliced (us) forms of *Xbp1* mRNA were detected by RT-PCR of total RNA isolated from the livers of mice treated with vehicle (veh) or 1 mg/kg TM for the indicated times. Each lane shows a separate animal. Image is shown in black-to-white inverted form for greater visual clarity. **(B)** Expression of the indicated UPR target genes was determined by qRT-PCR from the animals shown in **(A)**, with *Btf3* and *Ppia* used as normalizing controls. Expression here and in subsequent figures is given on a $\log_2$ scale relative to the vehicle-treated condition. Here and elsewhere unless noted: $^{*}p < 0.05$; $^{\#}p < 0.1$ by two-tailed student's *t*-test. **(C–F)** Expression of metabolic genes was assessed by qRT-PCR as in **(B)**, and genes were grouped according to the time point at which downregulation ($p < 0.1$) was first observed. The process in which each gene participates is listed.

4 h the coregulator *Pgc1a* [GenBank:NM_008904.2] and the transcriptional activators *Cebpa* [GenBank:NM_007678.3] and *Ppara* [GenBank:NM_001113418.1] were suppressed (**Figure 2C**). PGC-1β contributes to lipogenesis, fatty acid oxidation, and VLDL production and secretion (Lee et al., 2003; Lin et al., 2003, 2005a,b; Wolfrum and Stoffel, 2006), and PGC-1α contributes to the latter two of these processes (Louet et al., 2002; Lin et al., 2005a; Rhee et al., 2006). C/EBPα has general roles in energy

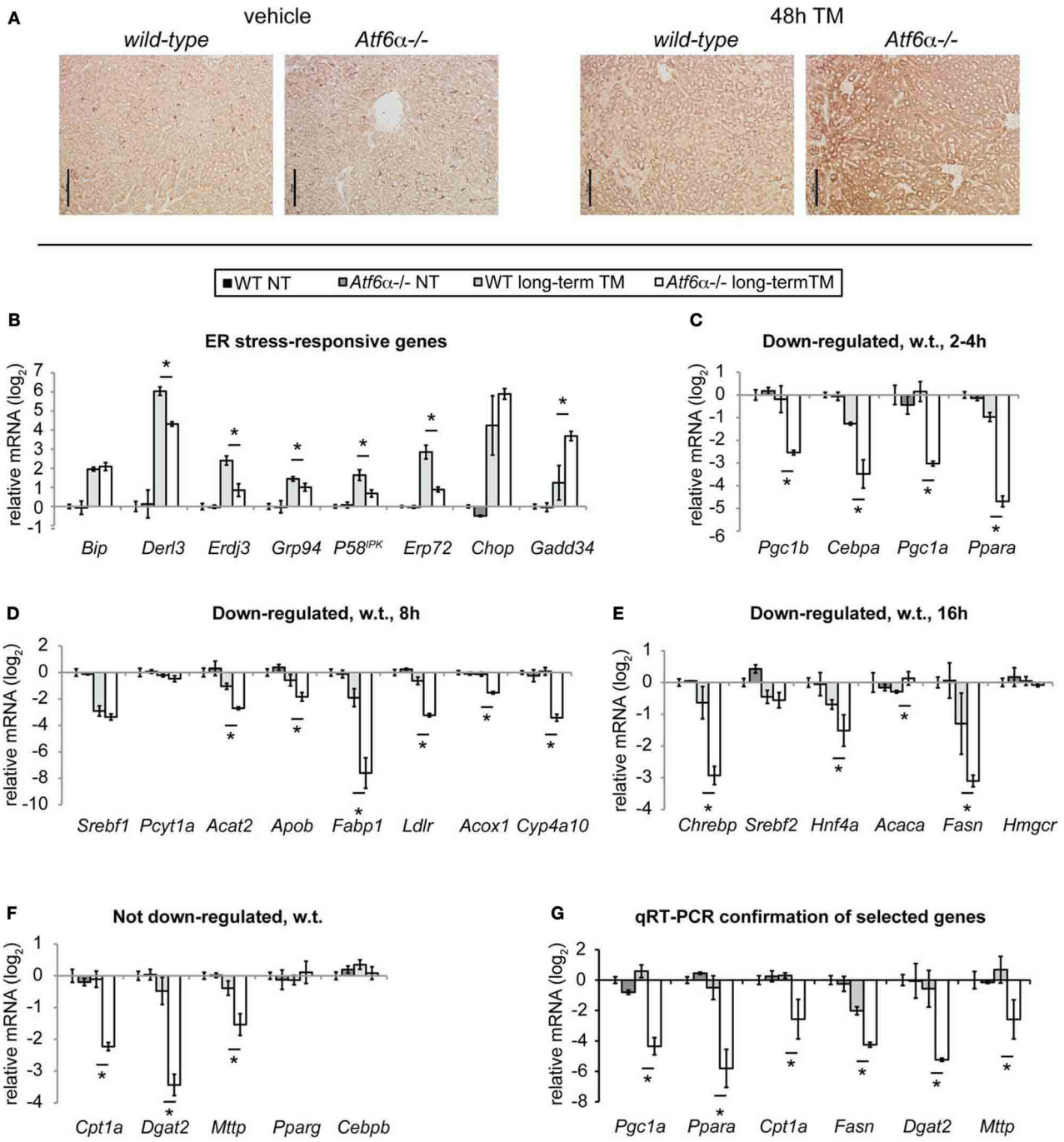

FIGURE 3 | Loss of *Atf6*α exacerbates steatosis and long-term metabolic gene suppression. (A) Wild-type or *Atf6*α$^{-/-}$ mice were challenged with 1 mg/kg TM or vehicle for 48 h, and ADRP immunostaining was carried out as in **Figure 1**. Scale bar = 50 μm. **(B–F)** Wild-type or *Atf6*α$^{-/-}$ mice were challenged with 1 mg/kg TM or vehicle for 34 h, and global mRNA expression was assessed by Affymetrix microarray. Array-determined expression of the indicated genes is shown. Genes were arranged in the same groupings as in **Figure 2**. Statistical significance was calculated by two-tailed student's *t*-test, comparing expression in TM-treated *Atf6*α$^{-/-}$ animals against TM-treated wild-type animals. $n = 3$ animals per group. **(G)** Mice were treated with TM or vehicle for 48 h as in **(A)**, and expression of the indicated genes was determined by qRT-PCR. Significance was determined as in **(B–F)**.

homeostasis including lipid and glucose metabolism, while PPARα is a master regulator of fatty acid oxidation (Desvergne et al., 2006).

The genes downregulated at later times—i.e., after the onset of pronounced lipid accumulation—included both transcriptional regulators and downstream genes encoding rate-limiting enzymes in metabolic processes, as indicated in **Figures 2D–F**. These results suggest that PGC-1α, C/EBPα, and PPARα (and possibly PGC-1β) are most likely the first lipid metabolic genes regulated by ER stress. Further, the processes downstream of these factors—namely, fatty acid oxidation and lipoprotein biogenesis and transport—are the first affected via gene regulatory mechanisms. In addition, because lipogenic genes are not altered until much later (18 h), they suggest that inhibition of lipogenesis is a secondary consequence of ER stress, perhaps occurring as a result of negative feedback when lipids begin to accumulate. In addition, even though XBP1 splicing is induced by ER stress, we found no evidence that lipogenic gene expression was *stimulated* under these conditions.

DELETION OF ATF6α EXACERBATES METABOLIC GENE SUPPRESSION

Mice lacking ATF6α, while otherwise apparently normal and healthy, exhibit dramatic steatosis upon challenge with TM (Rutkowski et al., 2008; Yamamoto et al., 2010). This phenotype is illustrated by ADRP staining in **Figure 3A**; it arises from an inability to restore ER homeostasis upon challenge. Therefore, we reasoned that these mice could be used to expose the stress-regulated gene expression changes that truly underlie lipid dysregulation, since those changes should be amplified in *Atf6*$\alpha^{-/-}$ animals. A second benefit of these animals is that they can be used to identify truly stress-responsive expression changes; those that result from ER stress-independent properties of TM would not be expected to differ between wild-type and *Atf6*$\alpha^{-/-}$ animals, since ATF6α is not thought to act outside the context of ER stress, and its deletion does not alter the apparent pharmacological activity of TM (Rutkowski et al., 2008).

We approached this goal by challenging wild-type and *Atf6*$\alpha^{-/-}$ animals for an extended period (34 h) with TM, and then profiling global gene expression by microarray. We chose this approach in part because we had previously used microarray profiling to characterize gene expression in these same mouse strains following 8 h of TM challenge (Rutkowski et al., 2008). Conducting a second microarray study with an identical gene chip at a later time point would provide us with the unique opportunity to determine, for every gene on the array, its expression in two different genotypes, under two different treatment regimens (vehicle and TM) at two different times.

By having 8 distinct combinations of experimental conditions, we anticipated that we could begin to identify clusters of coordinately regulated genes, including those metabolic genes most responsive to ER stress. This relied upon the assumption that genes which are part of a common functional pathway (in this case, lipid metabolism) should be regulated similarly. This approach has been used to great effect in yeast, and more recently in cultured mammalian cells, to expose previously hidden components of the secretory apparatus and other pathways of interest (Schuldiner and Weissman, 2013). Here, our goal was to infer hidden transcriptional regulators using temporal patterns of gene expression as an output.

From the 34 h microarray, we examined the expression of the UPR and metabolic genes shown in **Figure 2**. Consistent with the role of ATF6α in contributing to chaperone expression (Wu et al., 2007; Yamamoto et al., 2007), most ER chaperones and cochaperones known to be regulated by ATF6α, such as *Derl3* [GenBank:NM_024440.2], *Erdj3* [GenBank:NM_001190804.1], *Grp94* [GenBank:NM_011631.1], $p58^{IPK}$, and *Erp72* [GenBank:NM_009787.2], were not upregulated to as great an extent in *Atf6*$\alpha^{-/-}$ animals as in wild-type animals (**Figure 3B**). Conversely, expression of *Gadd34* [GenBank:NM_008654.2], which largely depends upon the PERK axis of the UPR (Marciniak et al., 2004), was elevated in *Atf6*$\alpha^{-/-}$ mice, consistent with persistent ER stress and activation of the other limbs of the UPR.

Amongst the metabolic genes examined, none was upregulated by ER stress in either genotype at 34 h. Two genes—the lipogenic and cholesterologenic regulators *Srebf1* [GenBank:NM_011480.3] and *Srebf2* [GenBank:NM_033218.1]—were equally downregulated in both genotypes (**Figures 3D,E**). However, the large majority of genes were expressed at normal or near-normal levels in wild-type animals, but deeply suppressed in *Atf6*$\alpha^{-/-}$ animals (**Figures 3C–F**). qRT-PCR profiling of a sampling of these genes from an independent experiment confirmed these findings (**Figure 3G**). These data are consistent with the idea that acute ER stress inhibits fatty acid oxidation and lipoprotein biogenesis at the level of gene expression, and does not stimulate lipogenic gene expression.

FUNCTIONALLY RELATED GENE GROUPS CLUSTER TEMPORALLY

Having these 8 distinct experimental conditions enabled us to compare the global behavior of hepatic gene expression in response to ER stress at early vs. late times in normal animals vs. those with a compromised UPR. A heatmap showing TM-regulated genes makes two observations clear: First, gene expression differences between the two genotypes were far more extensive at 34 h than at 8 h. Second, at 34 h, many genes have returned to normal or near-normal expression in wild-type animals, but have remained regulated, or become even more regulated in the same direction, in *Atf6*$\alpha^{-/-}$ animals.

Each gene was then categorized based on whether it was upregulated, downregulated, or unchanged by ER stress in wild-type and *Atf6*$\alpha^{-/-}$ animals after 8 or 34 h of stress. Thus, a gene could fall into any of 81 ($3 \times 3 \times 3 \times 3$) expression profiles. The assignments for all probesets are provided in **Table S1**. We were able to analyze the genes in this way in part because very few genes differed in their basal (i.e., unstressed) expression between genotypes; almost all genotype-dependent changes in expression were caused by TM, so basal expression differences were not confounding (Rutkowski et al., 2008).

Because *Atf6*$\alpha^{-/-}$ animals became more steatotic than wild-type animals, genes that were not differentially expressed between the two genotypes upon 34 h of TM treatment were not pursued further, as these were unlikely to be major contributors to the

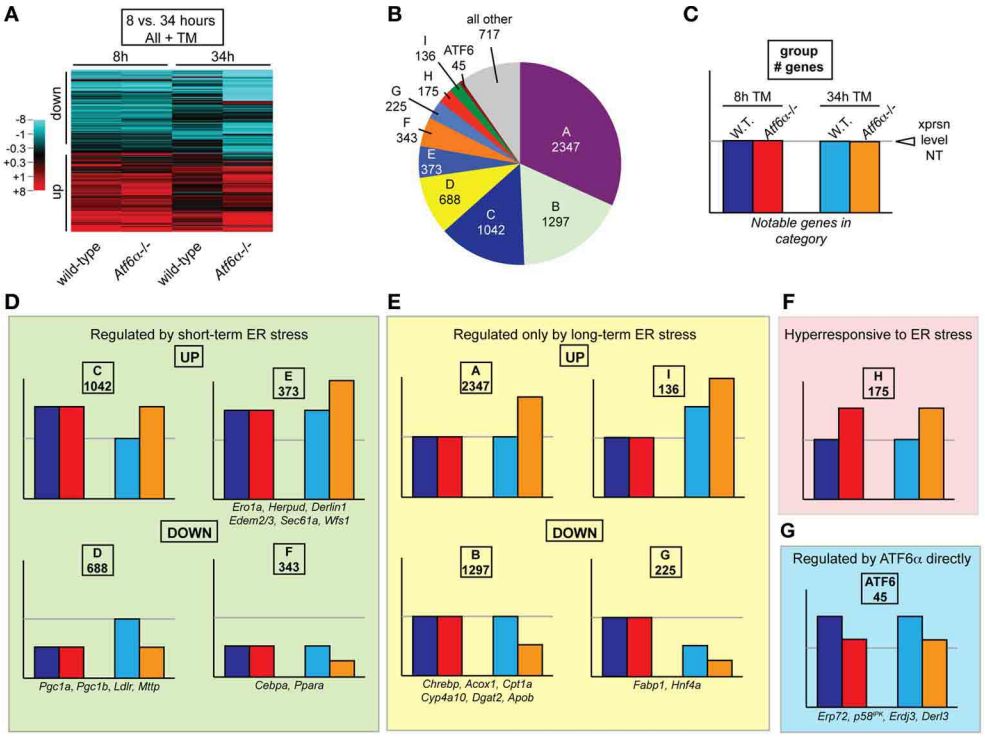

FIGURE 4 | Clustering of genes according to temporal regulation in wild-type and *Atf6*$\alpha^{-/-}$ animals. (A) The expression of every probeset on the Affymetrix microarray described in **Figure 3** was aggregated with expression data from a previously published identical array comparing gene expression in wild-type and *Atf6*$\alpha^{-/-}$ animals 8 h after challenge with vehicle or 2 mg/kg TM (Rutkowski et al., 2008). For each time point, expression was determined using a $\log_2$ scale relative to vehicle-treated wild-type animals at that time point. Only probesets showing significant ($p < 0.05$) expression differences (1.5-fold, or ± 0.58 on the $\log_2$ scale) in one or more of the four of the four conditions (wild-type or *Atf6*$\alpha^{-/-}$ at 8 or 34 h) are shown by heatmap, which accounted for ~7000 of the ~45,000 probesets on the array. Extent of up- or downregulation is shown by intensity of red or blue coloration, respectively. Each column depicts expression level averaged among the three animals per group. **(B,C)** Every probeset on the array was characterized by its expression in the following four ways, with differences defined as > 1.5-fold, $p < 0.05$: (1) up, down, or unchanged in wild-type TM-treated animals at 8 h relative to vehicle-treated wild-type; (2) up, down, or unchanged in *Atf6*$\alpha^{-/-}$ TM-treated animals at 8 h relative to TM-treated wild-type; (3) same as (1) but 34 h; and (4) same as (2) but 34 h. The genes that showed a difference by criterion (4) were broken down into groups based on their behavior with respect to these criteria, and the number of genes in the nine most populated groups is shown in **(B)**. The group of genes that were upregulated by ER stress in wild-type animals at both time points, but were less upregulated in *Atf6*$\alpha^{-/-}$ animals—i.e., genes that could be understood as directly ATF6α-dependent—is also accounted for. **(C)** provides a key for illustration of gene expression patterns. **(D–G)** Expression pattern for each of the gene groups shown in **(B)**. These include genes shown in **Figure 2** (those that did not fall into one of these groups are illustrated in **Figure S1**) as well as genes involved in ER protein processing found in Group E. For genes represented by more than one probeset, the behavior most commonly represented and/or most consistent with qRT-PCR data is shown.

steatotic phenotype. Although 54 remaining combinations of expression were possible ($3 \times 3 \times 3 \times 2$), 90 percent of the genes fell into only 9 categories (A–I, **Figure 4B**). Each category of genes was then given a graphical representation of the expression pattern of its members (**Figures 4C–G**). The two most populated groups of genes (Groups A and B) were those that were unaffected by stress at 8 h in either genotype, or in wild-type animals at 34 h, but were up- or down-regulated, respectively, at 34 h in *Atf6*$\alpha^{-/-}$ animals (**Figure 4E**). Among the metabolic genes depicted in **Figures 2**, **3**, a number of these populate Group B, and they include genes encoding rate-limiting enzymes in each pathway of fatty acid oxidation—*Acox1*, *Cpt1a*, and *Cyp4a10* [GenBank:NM_015729.3, NM_013495.2, and NM_010011.3], which control peroxisomal, mitochondrial, and microsomal oxidation, respectively. As the expression of these genes is not altered until the later timepoint, they are unlikely to be proximally connected to the UPR, but more likely represent indirect effects—for example, of suppression of PPARα. The next two most populated groups (Groups C and D) included those genes that were either up- or down-regulated early in both genotypes, and whose expression returned to normal levels in wild-type animals by 34 h but which remained regulated in *Atf6*$\alpha^{-/-}$ animals. Among metabolic genes, Group D included the coregulators *Pgc1a* and *Pgc1b*. Closely related to these groups were Groups E and F, which included genes that were up- or downregulated early in both genotypes, and for which this regulation was enhanced in *Atf6*$\alpha^{-/-}$ animals at 34 h. The other two rapidly regulated metabolic genes, *Cebpa* and *Ppara*, were found in Group F.

The lipogenic genes *Fasn* [GenBank:NM_007988.3] and *Acaca* [GenBank:NM_133360.2] and the cholesterologenic genes *Acat2* [GenBank:NM_009338.3] and *Hmgcr* [GenBank:NM_008255.2] showed no evidence of upregulation in the 8 or 34 h array data, and *Fasn* and *Acat2* were actually suppressed, as were *Srebf1* and

Srebf2 themselves (**Figure S1**). While lipogenesis can be stimulated by non-transcriptional mechanisms—most notably processing of SREBP-1c and SREBP-2—this processing would result in stimulation of their downstream target genes, which is not evidenced here.

Groups D and F were of the most interest to us because they represented those genes most likely to be proximally mechanistically connected to UPR signaling, since they were regulated rapidly by ER stress and since their long-term expression coincided with the persistent ER stress and worsening lipid accumulation seen in *Atf6α*$^{-/-}$ animals. Group F in particular was noteworthy because its counterpart cohort of upregulated genes—Group E—included a number of genes known to be direct targets of UPR transcription factors and which are thus proximally connected to UPR signaling (**Figure 2D**).

Also supporting the validity of the approach was the group of genes that were upregulated by ER stress in wild-type animals at both time points, but that were not as upregulated in *Atf6α*$^{-/-}$ animals at both time points (**Figure 4G**). These genes would fit the expected profile of direct targets of ATF6α. While there were relatively few genes in this group, they included those already described as direct ATF6α targets, including *Erp72*, *p58*^{IPK}, *Erdj3*, and *Derl3* (Wu et al., 2007; Yamamoto et al., 2007). This finding supports the idea that coregulated genes can be discriminated based on their expression profile in this setup.

This idea was reinforced by pathway analysis. The genes from each of the ten groups (A–I and ATF6) were analyzed for functional enrichments of Gene Ontology (GO) pathways using FunNet (Prifti et al., 2008). As proof-of-concept, pathway analysis of Group ATF6 genes yielded "unfolded protein response" as the most significantly enriched process, which would be expected of a group encompassing ATF6α direct targets (**Figure 5A**). Genes representing processes relevant to lipid metabolism were enriched in all of the downregulated groups—B, D, F, and G—but not in the upregulated groups A, C, E, H, and I (**Figures 5B,C**). Conversely, each of the upregulated groups was enriched in genes representing pathways relevant to protein synthesis, trafficking, and degradation. UPR activation during ER stress is known to transcriptionally augment the cellular protein biogenesis machinery through the action of the major UPR-regulated transcriptional activators XBP1, ATF4, and ATF6 (Harding et al., 2003; Lee et al., 2003; Wu et al., 2007). This pathway analysis thus suggests that, at least in the liver, suppression of genes involved in lipid metabolism represents a concerted focus of UPR activation.

FUNCTIONAL GENOMIC ANALYSIS IDENTIFIES HNF4α AS A PROXIMAL REGULATOR OF METABOLIC GENE EXPRESSION DURING ER STRESS

We wished to harness the statistical power of our microarray comparisons in order to predict regulatory transcription factors whose activity was altered by ER stress; these would be the most likely to be proximally mechanistically connected to the UPR. To accomplish this, we subjected the genes in each group to analysis using oPOSSUM (Ho Sui et al., 2005), which searches the promoter/enhancer region of each gene for consensus transcription factor binding sites from the JASPAR CORE database. Establishing the validity of this approach, the genes in Group ATF6 yielded the transcription factor NFYA [GenBank:NM_001110832.1] as the sole statistically significant hit (**Figure 6A**). ATF6α is known to dimerize with NFYA to regulate transcription from promoters containing ERSE and ERSE II elements (Yoshida et al., 2000, 2001; Kokame et al., 2001). NFYA was not enriched in any other group, underscoring the specificity of the algorithm.

Because genes involved in lipid metabolism were found in groups B, D, F, and G, we sought hits found in those groups and no others, with a particular emphasis on groups D and F, since these groups contain the early-responding genes. Remarkably, binding sites for two transcription factors—HNF4α and NR2F1 [GenBank:NM_010151.2]—were enriched in Groups B, D, and F but not in any other group (**Figures 6B,C**). These factors have similar consensus binding sites (Kimura et al., 1993; Ellrott et al., 2002; Schmidt et al., 2010), explaining why they segregate together in this analysis. This finding suggests that the activity of these two transcriptional activators is altered during ER stress, since genes containing binding sites for these factors are downregulated by ER stress, and their downregulation is exacerbated by the ongoing stress seen in *Atf6α*$^{-/-}$ mice. Conversely, groups A, C, and E were enriched for genes with potential binding sites for ELK4, also known as SRF Accessory Protein (SAP)-1 [GenBank:NM_007923.2].

Group I, encompassing genes upregulated by long-term ER stress in wild-type animals and further upregulated in *Atf6α*$^{-/-}$ animals, was enriched for binding by CREB1 [GenBank:NM_001037726.1], which shares a consensus binding sequence with the ER-localized transcription factor CREBH (Zhang et al., 2006). CREBH is activated by proteolysis induced by, among other stimuli, ER stress, and it regulates expression of acute phase response genes and metabolic genes (Zhang et al., 2006, 2012; Luebke-Wheeler et al., 2008). Accordingly, the genes in Group I have a profile expected for CREBH targets—namely that they are upregulated by ER stress, and further upregulated in *Atf6α*$^{-/-}$ animals, in which ER stress is exacerbated. However, lipid metabolic genes were not enriched in Group I, and the CREB1 binding site was not enriched in the groups containing lipid metabolic genes. In addition, other than the gene encoding serum amyloid P-component (*Apcs*) [GenBank:NM_011318.2], most genes encoding acute phase response proteins (SAA proteins, CRP, coagulation and clotting proteins, etc.) were not significantly upregulated at either time point in either genotype, suggesting that CREBH activity during *bona fide* ER stress (as opposed to endotoxin, pro-inflammatory cytokines, or other stimuli) might be minimal, and the genes in Group I might instead be regulated by another factor from the CREB family.

ELK4 is part of the Ternary Complex Factor (TCF) family of transcription factors that interact with Serum Response Factor (SRF) (Dalton and Treisman, 1992). A role for ELK4 in hepatic gene expression has not been described, nor has ELK4 been directly linked to ER stress. ELK4 has been shown to be activated by JNK-dependent phosphorylation (Janknecht and Hunter, 1997), and JNK [GenBank:NM_016700.4] is activated by ER stress (Urano et al., 2000). Thus, we speculate that ELK4 activity might be regulated during ER stress in the liver by JNK or other MAP kinases to promote expression of genes in groups A,

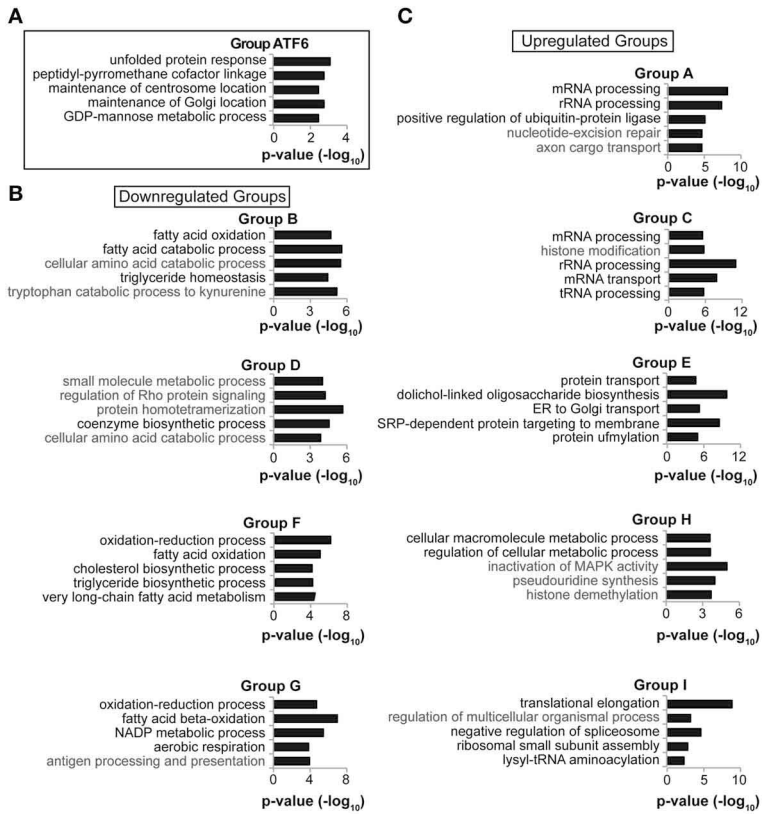

FIGURE 5 | Functionally related genes cluster by temporal regulation. (A–C) Each of the gene groups in **Figure 4** was subjected to Gene Ontology pathway analysis using FunNet. The top seven most significant pathway enrichments were then reordered by the number of genes from that group that were pathway "hits," with pathways having the most "hits" listed higher. For reasons of space, five of these seven pathways from each group are shown. In no case was a lipid metabolism process enriched among the upregulated gene groups. In **(B)**, pathways relevant to lipid metabolism are listed in black, and other pathways in gray. In **(C)**, pathways relevant to protein processing are listed in black.

C, and E. NR2F1, also known as COUP-TF, is an orphan receptor. Deletion leads to perinatal lethality with extensive dysregulation of neuronal differentiation (Qiu et al., 1997). Its function in the liver is less clear, although it has been shown to coactivate transcription synergistically with HNF4α (Ktistaki and Talianidis, 1997; Yanai et al., 1999).

HNF4α is expressed most strongly in the liver, intestine, kidney, and pancreas. Deletion is lethal during gastrulation (Chen et al., 1994). Mice with a liver-specific deletion of HNF4α develop steatosis concomitant with impaired *ApoB* and *Mttp* expression and VLDL production (Hayhurst et al., 2001). Hepatic knockdown of HNF4α by adenoviral delivery resulted in steatosis and in impaired VLDL production, along with essentially uniformly diminished expression of a host of genes involved in lipid metabolism, including many of those reported here (Yin et al., 2011). Thus, loss of HNF4α phenocopies many of the lipid metabolic genetic changes that are induced by ER stress. Overexpression of HNF4α in primary hepatocytes resulted in upregulation of many of these same genes, though not of *Srebf1* nor *Srebf2* (Yin et al., 2011). This exception is notable because *Srebf1* and *Srebf2* are conspicuous among the metabolic genes analyzed here in the fact that their downregulation is *not* exacerbated at 34 h in *Atf6α*$^{-/-}$ mice (**Figures 3D,E**). HNF4α binding sites in the mouse liver genome have been characterized by ChIP-seq (Schmidt et al., 2010), and genes in Groups B, D, and F are confirmed HNF4α targets (**Figure 6D**).

Our results, together with the known activity of HNF4α, predict that ER stress leads to diminished activity of HNF4α, and that this occurs as an early event in metabolic gene regulation by ER stress. To test this prediction, we analyzed the binding of HNF4α to ChIP-seq-defined sites in the promoter/enhancer regions of three of the four earliest regulated metabolic genes—the transcription regulators *Cebpa*, *Pgc1a*, and *Ppara* (the proximal *Pgc1b* promoter/enhancer did not contain a predicted or validated HNF4α binding site). We found that treatment of animals with TM for 8 h did not change either the total expression (**Figure 7A**) or nuclear localization (**Figure 7B**) of HNF4α. Yet, consistent with our prediction, we found that HNF4α chromatin binding decreased significantly at several sites in the *Cebpa* and *Pgc1a* promoters upon treatment of animals with TM (**Figure 7C**). This diminishment was not uniform; several sites within the three promoters showed unaltered HNF4α binding (**Figure 7A**). Together, these findings suggest that ER stress reduces the activity of HNF4α at specific sites in the genome through a mechanism that is independent of the HNF4α expression level.

A

Group	TF	Z-score	Fisher score	Target gene hits	Target gene non-hits	Background gene hits	Background gene non-hits
ATF6	NFYA	33.81	4.98E-05	16	10	3627	11523

B

Upregulated							
Group	TF	Z-score	Fisher score	Target gene hits	Target gene non-hits	Background gene hits	Background gene non-hits
A	**ELK4**	21.23	9.21E-06	367	1007	3268	11882
	TP53	16.5	9.61E-03	6	1368	17	15133
C	**ELK4**	18.58	1.33E-03	172	471	3268	11882
E	**ELK4**	11.42	4.64E-03	68	166	3268	11882
H	ELK1	12.74	8.51E-03	86	39	8780	6370
I	CREB1	12.9	1.05E-03	32	43	3892	11258

C

Downregulated							
Group	TF	Z-score	Fisher score	Target gene hits	Target gene non-hits	Background gene hits	Background gene non-hits
B	NR2F1	15.46	3.03E-04	142	641	2058	13092
	HNF4A	15.14	4.32E-04	235	548	3723	11427
	HNF1A	10.79	1.85E-03	102	681	1466	13684
D	**HNF4A**	12.39	4.47E-07	154	281	3723	11427
	NR2F1	14.66	2.31E-05	91	344	2058	13092
	PAX4	15.78	7.19E-03	9	426	113	15037
F	NR2F1	17.29	6.34E-05	49	157	2058	13092
	HNF4A	21.13	6.37E-05	76	130	3723	11427
G	HNF1A	15.93	2.98E-03	24	112	1466	13684

D

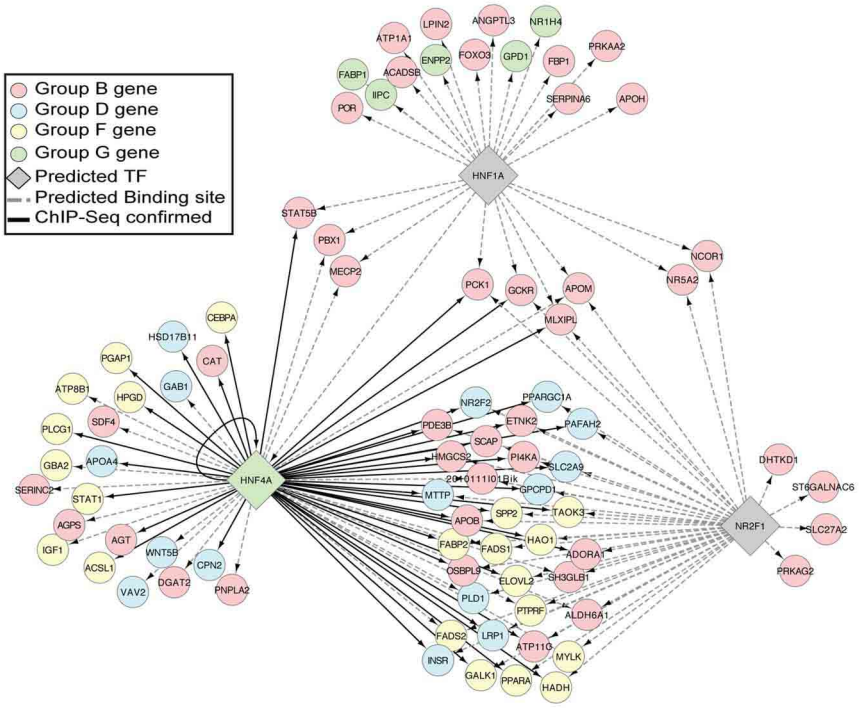

FIGURE 6 | Transcription factor prediction implicates ELK4, HNF1α, NR2F1, and HNF4α as hidden regulatory nodes in hepatic stress-dependent gene regulation. (A–C) Each of the gene groups in **Figure 4** was subjected to oPOSSUM single-site analysis, which searches regulatory regions (in this case, ±2000 bp from the transcriptional start site) for potential binding sites of transcription factors identified in the JASPAR CORE database. The results were limited to a *Z*-score > 10 and a Fisher score < 0.01. **(D)** The data from **(C)** were visualized using the BINGO plug-in for Cytoscape software, considering only genes relevant to lipid metabolism as annotated from GO analysis. oPOSSUM-predicted binding sites are shown using dashed lines, while genes with confirmed HNF4α-binding sites are shown by solid lines.

CONCLUSIONS

The hepatic lipid dysregulation that is elicited by ER stress is accompanied by sweeping alterations to the expression of genes involved in the process. With few exceptions, these genes are downregulated by ER stress, and encompass numerous metabolic pathways including fatty acid oxidation, lipogenesis, triglyceride storage and secretion, and phospholipid synthesis. The downregulated genes include both those encoding the key enzymes of each of these processes as well as the upstream transcription factors that regulate them. However, it would be unlikely that these genes would be directly suppressed by canonical UPR-regulated transcription factors, since these are, for the most part, transcriptional activators rather than repressors. Further, ablation of the ATF6α pathway of the UPR compromises recovery from ER stress and leads to an exacerbated steatotic phenotype, yet ATF6α does not directly act upon genes involved in lipid metabolism—no such genes populate either group ATF6 or its downregulated converse. Therefore, other mechanisms linking UPR activation to metabolic gene regulation must be at work. The extensive nature of metabolic gene regulation during ER stress also suggests a hierarchical organization of gene regulatory events, with most genes regulated as an indirect consequence of earlier events. Such an organization predicted that the temporal ordering of gene regulation could be used to identify the transcriptional events most proximal to UPR signaling.

With these facts in mind, we hypothesized that a "bottom-up" approach could be used to establish which ER stress-dependent metabolic gene expression changes occurred earliest, and to identify common regulators of those genes. To that end, our analysis predicted altered activity of HNF4α, which we then confirmed experimentally. We can conclude that its diminished binding to the promoters of *Cebpa* and *Pgc1a* is likely to contribute to the suppression of these genes, because knockdown of HNF4α is already known to have similar effects on metabolic genes to those reported here (Yin et al., 2011). We have combined the experimental and bioinformatic experiments described here with existing literature on the roles of HNF4α, C/EBPα, PGC-1α, and PPARα into a working model describing the genetic hierarchy of lipid metabolic gene regulation during stress (**Figure 8**). Although these relationships were elicited using TM, the observation that proteasome inhibition or overexpression of a misfolded secretory protein leads to similar genetic changes and lipid accumulation (Rutkowski et al., 2008), together with the fact that they were elicited using animals with a specific lesion in ER stress signaling, argue that they are likely to apply to ER stress in general.

Two key questions immediately arise for further study. The first of these is whether all of the metabolic gene regulatory changes downstream of ER stress are subordinate to HNF4α and subsequent changes in the expression of just one or a small number of rapidly suppressed master regulators such as

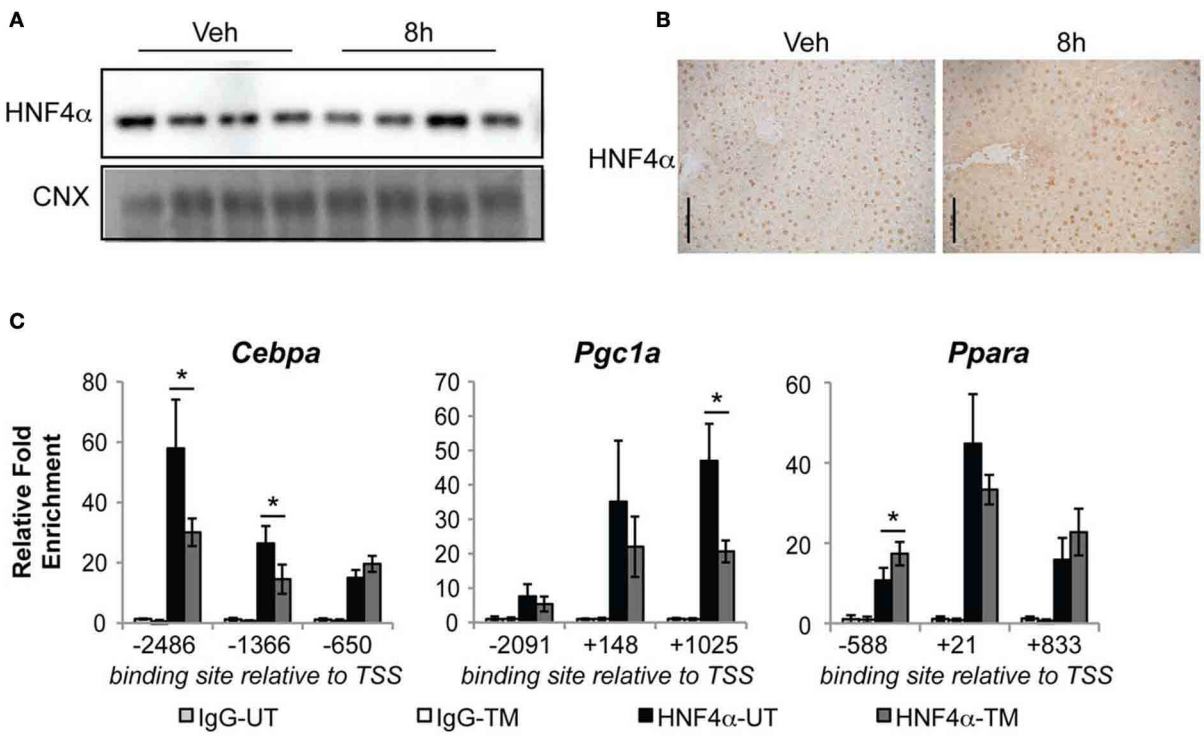

FIGURE 7 | Diminished HNF4α binding at *Cebpa* and *Pgc1a* promoters during ER stress. (A,B) Wild-type mice were challenged with 1 mg/kg TM for 8 h, and expression of HNF4α was assessed by immunoblot **(A)** or immunohistochemistry **(B)**. Scale bar = 50 μm. **(C)** HNF4α binding to the regulatory regions of the indicated genes was assessed by chromatin-IP. Regions are given relative to the transcriptional start site, and correspond to regions identified by ChIP-seq analysis (Schmidt et al., 2010). $n = 3$–4 animals per group. Typical recovery of genomic material in samples containing HNF4α antibody was in the range of 0.1–1 percent of total input. $^*p < 0.05$ by *t*-test.

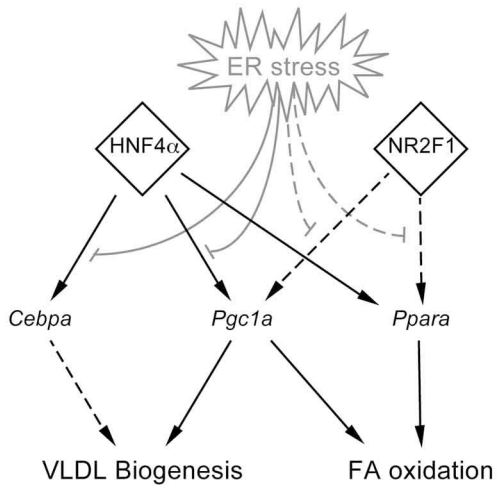

FIGURE 8 | Model for organization of lipid metabolic gene regulation during ER stress. Experimentally demonstrated (here or elsewhere) relationships are shown using solid lines, while as-yet unvalidated relationships suggested by this work are shown by dashed lines.

Cebpa, *Pgc1a*, etc. Testing this hypothesis requires systematic overexpression of different metabolic transcriptional regulators, to see which blunt lipid accumulation during ER stress and how downstream metabolic genes respond. It is possible that HNF4α regulates both the proximal transcriptional regulators (C/EBPα, PGC-1α, etc.) and also genes encoding the downstream metabolic enzymes (CPT1A, ACOX1, etc.), which would provide a feedforward mechanism for suppression of these pathways. The second question is how ER stress influences HNF4α activity. Absent changes in HNF4α expression level or localization (**Figure 7**), the most likely scenarios are either covalent modification of HNF4α itself or modification, change in expression, or sequestration of a binding partner of HNF4α.

It is possible that the preponderance of bZIP factors produced during ER stress (ATF6α, ATF6β, ATF4, XBP1, and CHOP) could titrate a coregulator away from HNF4α. Persistent ER stress experienced in *Atf6α*$^{-/-}$ animals causes prolonged UPR activation and expression of the other non-ATF6α UPR-regulated bZIPs (Rutkowski et al., 2008), which might therefore cause continued cofactor sequestration even as wild-type animals recover from stress. A sequestration model predicts that the strength and persistence of the stress will be a key factor in altering the activity of non-UPR transcription factors like HNF4α. Accordingly, chronic stresses would be expected to elicit different patterns of metabolic gene regulation than acute stresses, and strong stresses different patterns from milder ones. In support of this idea, exposure of zebrafish larvae to chronic but mild TM results in efficient induction of steatosis in the larval livers, and knockdown of ATF6α ameliorated this lipid accumulation. In contrast, a more acute but stronger ER stress—perhaps most akin to the exposures used in this work—led to less efficient steatosis that was exacerbated by ATF6α knockdown (Cinaroglu et al., 2011). The effects of stresses of different strengths and persistence have not yet been tested in a mammalian system, and will be important in validating or refuting the idea that pathophysiological conditions like obesity are in effect states of chronic ER stress. We also note that this work warrants an exploration of the roles of NR2F1 and ELK4 in hepatic gene expression. In fact, with all genes broken down into 81 possible expression profiles, we predict that other testable hidden regulatory nodes will emerge, and that searching these groups for conserved sequences will reveal nodes beyond the relatively small number that are linked to transcription factors in the JASPAR CORE database.

Finally, the scope of this work was limited to exploring the gene regulatory network linking acute ER stress to metabolism. Our gene expression results suggest that genetic suppression of VLDL production and fatty acid oxidation likely contribute to the steatotic phenotype while lipogenesis does not contribute, and might even be inhibited as a feedback mechanism to offset lipid accumulation. However, it remains to be determined whether these changes in mRNA expression are reflected in protein levels and consequent biochemical activities of the various pathways, and the extent to which they are also seen during physiological ER stresses such as obesity.

In summary, we have provided proof-of-principle that a bottom-up approach sheds light on the organization of metabolic gene regulation during ER stress and makes testable predictions about this organization. As a consequence, we have identified a novel regulatory node in the process. Beyond revealing a likely role for HNF4α in this regulation, it provides a resource for regulators of other coordinated gene expression groups to be discovered.

AUTHORS' CONTRIBUTIONS

AMA carried out qRT-PCR, ChIP, immunoblot, and bioinformatic analysis, and analyzed data. DDM carried out lipid analysis and immunohistochemistry, and analyzed data. RJK participated in the design and coordination of the microarray study. DTR conceived the study, participated in the design and coordination of all experiments, and drafted the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Genomic_Endocrinology/10.3389/fgene.2013.00188/abstract

Figure S1 | Figure illustrates the gene expression profiles of lipid metabolic genes not part of Groups A–I.

Table S1 | Table is a spreadsheet listing each of the probesets from the Affymetrix microarray used in this study, and the average expression values and group classification for each.

Table S2 | Table lists the oligonucleotides used for qRT-PCR and ChIP analysis.

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Altered methylation and expression of ER-associated degradation factors in long-term alcohol and constitutive ER stress-induced murine hepatic tumors

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Mortality from liver cancer in humans is increasingly attributable to heavy or long-term alcohol consumption. The mechanisms by which alcohol exerts its carcinogenic effect are not well understood. In this study, the role of alcohol-induced endoplasmic reticulum (ER) stress response in liver cancer development was investigated using an animal model with a liver knockout (KO) of the chaperone BiP and under constitutive hepatic ER stress. Long-term alcohol and high fat diet feeding resulted in higher levels of serum alanine aminotransferase, impaired ER stress response, and higher incidence of liver tumor in older (aged 16 months) KO females than in either middle-aged (6 months) KOs or older (aged 16 months) wild type females. In the older KO females, stronger effects of the alcohol on methylation of CpG islands at promoter regions of genes involved in the ER-associated degradation (ERAD) were also detected. Altered expression of ERAD factors including derlin 3, Creld2 (cysteine-rich with epidermal growth factor-like domains 2), Herpud1 (homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member), Wfs1 (Wolfram syndrome gene), and Yod1 (deubiquitinating enzyme 1) was co-present with decreased proteasome activities, increased estrogen receptor α variant (ERα36), and enhanced phosphorylations of ERK1/2 (extracellular signal-regulated protein kinases 1 and 2) and STAT3 (the signal transducers and activators of transcription) in the older KO female fed alcohol. Our results suggest that long-term alcohol consumption and aging may promote liver tumorigenesis in females through interfering with DNA methylation and expression of genes involved in the ERAD.

Keywords: alcohol, aging, unfolded protein response, ERAD, hepatocellular tumorigenesis

INTRODUCTION

Liver cells are rich in the essential organelle-endoplasmic reticulum (ER), which assumes synthesis of a large amount of secretory and membrane proteins and lipids, maintains intracellular calcium homeostasis, and detoxifies drugs (Dara et al., 2011; Cao and Kaufman, 2013). For the protein synthesis and modifications, the ER ensures correct protein folding and maturation. Unfolded proteins are normally retained in the ER and targeted for retro translocation to the cytoplasm for rapid removal through the ER-associated protein degradation (ERAD). Malfunction of the ER leads to ER stress and accumulation of unfolded proteins triggering the unfolded protein response (UPR). The UPR is essentially mediated by molecular chaperones such as the glucose-regulated protein 78 (GRP78/BiP), which interact with three ER membrane resident stress sensors: inositol-requiring enzyme-1 (IRE1α), activating transcription factor-6 (ATF6), and protein kinase R (PKR)-like eukaryotic initiation factor 2α kinase (PERK; Walter and Ron, 2011). The UPR reduces protein translation, enhances protein folding capacity, and accelerates degradation of unfolded proteins, restoring ER homeostasis. However, persistent or prolonged UPR leads to impaired hepatic lipid synthesis, aberrant immune response, and eventually an attempt to eliminate the over-stressed cells, causing liver injuries (Zhang, 2010; Fu et al., 2012).

Alcohol is the most socially accepted drug that is mainly metabolized in the liver. Alcohol is oxidized by alcohol dehydrogenase (ADH) or cytochrome P450IIE1 (CYP2E1) to acetaldehyde. Acetaldehyde dehydrogenase (ALDH) converts acetaldehyde to acetate which enters the circulation. Alcohol overdose leads to overproduction of highly reactive acetaldehyde, reactive oxygen species (ROS) and intracellular NADH, all of which collectively play etiological roles in alcoholic pathologies (Zakhari, 2006; Zakhari and Li, 2007; Gao and Bataller, 2011). Growing evidence indicates that alcohol-induced liver ER stress contributes to liver disease (Ji, 2012). ER proliferation and liver injury is associated with microsomal alcohol oxidations by CYP2E1 in rats and humans (Cinti et al., 1973; Lieber, 1987). Multiple alcohol consumption-related factors including free radicals, acetaldehyde, toxic lipid species, oxidative stress, excessive homocysteine or *S*-adenosyl methionine (SAH) from impaired one carbon metabolism, disruption of calcium homeostasis, and insulin resistance are reported to disturb ER homeostasis and induce hepatic ER stress in cultured hepatocytes as well as in the livers of several species including mouse, rat, minipigs, zebrafish, and humans

(Ji and Kaplowitz, 2003; Nishitani and Matsumoto, 2006; Passeri et al., 2009; Esfandiari et al., 2010; Magne et al., 2011; Galligan et al., 2012; Kao et al., 2012; Longato et al., 2012; Ramirez et al., 2012, 2013). However, the importance of alcohol-induced ER stress in liver injury may depend on other genetic and environmental factors, patterns of alcohol exposure, and stages of liver disease (Ji, 2012). Alcohol-induced liver cirrhosis and hepatocellular carcinogenesis (HCC) is often enhanced by high fat diet (HFD) feeding or by aberrant epigenetic factors such as methylation of genome DNA (Seitz and Becker, 2007; Shukla et al., 2008; Philibert et al., 2012; Loomba et al., 2013; Tsuchishima et al., 2013). It is unclear whether the alcohol-induced ER stress is also involved in the development of liver cancer and whether epigenetic modifications of ER stress factors contribute to alcohol-induced advanced liver injury. Considering that epigenetic inactivation of genes play a critical role in many important human diseases such as cancer and that methylation of CpG islands of the genomic DNA is in general a core mechanism for epigenetic inactivation of genes (Rakyan et al., 2011), we hypothesize that alcohol consumption affects DNA methylation of genes pertinent to the UPR/ER stress response and we tested the hypothesis in a liver tumor-prone mouse model under constitutive hepatic ER stress.

MATERIALS AND METHODS

ANIMAL EXPERIMENTS

Mouse models with a liver-specific deletion of the immunoglobulin heavy chain-binding protein (BiP), also known as glucose-regulated protein 78 (Grp78) were previously created through the LoxP-Cre strategy (Luo et al., 2006; Ji et al., 2011). Briefly, the established BiP floxed females ($BiP^{f/f}$) were crossed with male mice carrying the *Cre* transgene under the control of the rat albumin promoter (*Alb-Cre*). The resulting heterozygous mice carrying the floxed alleles and the *Alb-Cre* gene were back-crossed with the BiP floxed founders to yield mice with liver-specific BiP deletion. The littermates carrying homozygous floxed alleles without the *Alb-Cre* gene were used as wild type (WT) controls. PCR genotyping with tail or liver genomic DNA was performed to distinguish BiP alleles of WT and knockout (KO). The presence of the *Alb-Cre* transgene was determined by duplex quantitative PCR using *Cre*-specific primers. Animal breeding, genotyping, daily inspection, and maintenance of the colonies were described previously (Ji et al., 2011; Lau et al., 2013). The animals were grouped into two age groups. One group was 6–8 months old termed middle-aged (Mid) group; the other group was 12–16 months old termed older (Old) group that corresponds to humans aged of approximately 50. The older KOs with suspected spontaneous liver tumor development without alcohol were excluded from the experiments. Animals that were moribund, unable to move or failure to respond to gentle stimuli, with labored breathing or diarrhea, and inability to eat and drink were eliminated from the experiments. For long-term alcohol treatment, mice were fed orally a liquid HFD (AIN-93G #710301; Dyets, Inc., Bethlehem, PA, USA) mixed with alcohol at a dose of 4 g alcohol/kg body weight or an isocaloric HFD (#710301) without alcohol for 12 months. Pair feeding was conducted by feeding the alcohol group in the first day of the experiment and by measuring amount of alcohol diet consumed by each animal in the next day, which was used to calculate isocalorically matched control diet for the control group. Occasionally, there were a couple of hours' waiting time for the control mice since some of the mice tended to consume the control diet without alcohol more and faster than the diet with alcohol. All animals were treated in accordance with the Guide for Care and Use of Laboratory Animals approved by a local committee for animal care and use.

PARAMETERS OF LIVER INJURY

At the time of killing, serum samples were collected and liver tissues were either snap frozen in liquid nitrogen and stored at −80°C or fixed immediately for histological staining. Serum alanine aminotransferase (ALT) and liver histology for hematoxylin and eosin (H&E) staining and immunohistochemistry were described previously (Kao et al., 2012). Histological changes were checked by a pathologist blinded to the genotypes. The Betazoid DAB Chromogen kit and ancillary reagents (BioCare Medical, CA, USA) were used for the immunohistochemistry. Primary antibodies against the molecular marker of proliferation Ki-67 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Hepatocytes stained positive with anti-Ki-67 were counted under a microscope at 100× magnification.

IMMUNOBLOTTING OF LIVER PROTEINS AND PROTEASOME ACTIVITIES

Proteins (whole or nuclear) were extracted respectively from WT liver tissues, KO without liver tumors and the normal liver portion and the tumor portion from tumor bearing livers of KOs, which were analyzed according to the methods described previously (Kao et al., 2012; Lau et al., 2013). Immunoblotting was conducted using horseradish peroxidase-labeled secondary antibodies (1:2000 dilutions), in which the intensity of protein bands on the immunoblots was quantified with the NIH software, ImageJ. Primary antibodies against BiP, CCAAT-enhancer-binding proteins (C/EBP) homologous protein (CHOP; sc-7351), ATF6 (sc-22799), GRP94, protein disulfide isomerase (PDI), cysteine-rich with epidermal growth factor (EGF)-like domains 2 (Creld2), Der1p-like protein (derlin), cyclin D, estrogen receptor α, homocysteine-induced ER protein (HERP), phosphorylated extracellular signal-regulated protein kinases 1 (p-ERK1/2), and phosphorylated signal transducers and activators of transcription 3 (p-STAT3) were from Santa Cruz Biotechnology Inc. Primary antibodies against the transcription activator 4 (ATF4) were from Aviva System (San Diego, CA, USA). Primary antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were from Millipore (Billerica, MA, USA). Primary antibodies against β-actin were from Sigma. Proteasome activities were assessed with the 20S Proteasome Activity Assay Kit from MILLIPORE (Billerica, MA, USA) and the relative fluorescent units were recorded with the Omega Microplate Readers from BGM LABTECH (Gary, NC, USA) using 355/460 nm filter set.

PCR ANALYSIS OF PROMOTER METHYLATION

For analysis of promoter methylation of ER stress marker genes, genomic DNA was extracted from the mouse liver tissues using the QIAGEN DNeasy Tissue Kit (Valencia, CA, USA). Methylation was analyzed with a methylation promoter PCR kit (Panomics; Fremont, CA, USA). Briefly, the isolated genomic DNA was

digested with *Mse*I, and the resulting DNA fragments were incubated with the methylation binding protein MeCP2 (a.k.a. MBP). The methylated DNA fragments were isolated with a spin column and then amplified with PCR using promoter specific primers for gene markers of ER stress. The Tag PCR Master Mix kit from QIAGEN was used for the PCR. The PCR products were visualized through agarose gel electrophoresis and were semi-quantified by Image J after normalized against corresponding input PCR products from the genomic DNA fragments without the MeCP2 incubation. The following primer pairs were used:

Atf6, 5′-CTTCTTTAGGAGGTAAGTGCG-3′; 5′-TGAGTAACC TGAAACGGCG-3′;

Chop, 5′-AGAGAAGCGGGTGGACTATC-3′; 5′-TAACTGACC TCAAGAGCGG-3′;

Gapdh, 5′-AAGCAAAGGTTATCACCAGG-3′; 5′-TACGCCAT AGGTCAGGATG-3′;

Grp94, 5′-ACTCAGAGACATTTCCCGC-3′; 5′-GAACTCACC AATCGTGCCTC-3′;

PDI, 5′-AGCCACCCAAATCTCCATC-3′; 5′-TGCTGCTCCCA GGAATAAG-3′.

For real-time PCR analysis of promoter methylation of ERAD factors, genomic DNA was extracted with Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) from mouse liver tissues. Then the DNA was fragmented with Episonic Multifunctional Bioprocessor (Epigentek, Farmingdale, NY, USA) into average size of 400 bp with an average power delivery of 170∼190 W for 40 cycles. The size and quality of the fragment were confirmed with gel electrophoresis and Nanodrop, respectively. The methylated DNA was enriched with *MethylMiner*™ Methylated DNA Enrichment Kit (Invitrogen) and the resulting DNA fragments were isolated by binding to magnetic beads conjugated with methylation binding protein MeCP2 and eluted with high concentration of NaCl followed by purification with ethanol precipitation in the presence of glycogen. The promoter methylation was quantified by qPCR with ABI qPCR system and levels of methylation were calculated after normalized with input. The following primer pairs were used:

β-actin, 5′-GTTCCGAAAGTTGCCTTTTATG-3′; 5′-CAACGA AGGAGCTGCAAAGAA-3′;

Creld2, 5′-CCGATAGAAGATTACGGTTCTG-3′; 5′-CTGATG TGGACCAATTGAGG-3′;

Derl3, 5′-GATTCTAGAGTTTTACAGAATGTCA-3′; 5′ATCTA GAAAAGAACCAATAGCAAG-3′;

Herpud1, 5′-GTTCCGAAAGTTGCCTTTTATG-3′; 5′-AAATT GTGCCCTCACAAAGC-3′;

Wfs, 5′-CACACACACTTTTTGTACTCG-3′; 5′-GCTATTACA ATACTGACTAAGGTC-3′;

Yod1, 5′-CCATGATGAAGTGTCTTCCTA-3′; 5′-GCTATTACA ATACTGACTAAGGTC-3′.

MICROARRAY ANALYSIS OF TRANSCRIPTIONAL EXPRESSION OF GENES

Total hepatic RNA was isolated from fresh liver tissues using the RNeasy Mini Kit from QIAGEN following the manufacturer's instructions and with an addition of 500 U of an RNase inhibitor (RNAguard, Amersham Pharmacia Biotech) to each starting material of 300 mg. Gene profiling and analysis was performed in the Cancer Center Microarray Core Facility of Keck School of Medicine of USC using Illumina's Sentrix MouseRef-8 V2.0 Expression BeadChip (Illumina, San Diego, CA, USA). The quality of total RNA from liver samples was evaluated using an Experion apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Total RNA (0.5 μg) from each sample was labeled and the hybridized biotinylated cRNA was detected with streptavidin-Cy3 and quantitated using Illumina's BeadArray Reader Scanner in accordance with the manufacturer's instructions. Microarray data were processed and analyzed with the Illumina BeadStudio software. Data of the average signal was filtered with a p-value (<0.05) and normalized via rank invariant normalization, after which significant changes (2- to 10-folds) were clustered for ER stress pathways and exported for heat-mapping comparisons.

STATISTICAL ANALYSIS

Values are expressed as means ± SD unless otherwise indicated. Statistical analyses were performed using ANOVA for comparison of multiple groups or the Student's t-test for pair-fed groups. $p < 0.05$ was considered significant.

RESULTS

EFFECTS OF LONG-TERM ALCOHOL FEEDING ON LIVER TUMOR DEVELOPMENT

Previous studies demonstrated that genetic ER stress predisposition with a liver-specific deletion of BiP led to fatty liver injury in both male and female mice and hepatic tumorigenesis in a significant portion of female mice at age of greater than 17 months (Ji et al., 2011; Lau et al., 2013). To know effects of alcohol on the liver tumorigenesis in the BiP KOs, we fed the mice with an alcohol HFD abbreviated as alcohol diet. Premature death was observed in greater than 50% of the KO mice fed a standard high dose of alcohol diet (6.5 g alcohol/kg body weight). Alcohol doses at less than 4 g/kg body weight were thus adopted for the experiments. At the reduced alcohol doses, liver tumors were not observed in either WT or KO males during an experimental period of 2.5 years. Thus, all subsequent studies and comparisons were focused on females. **Figure 1** demonstrates that liver tumors were observed in less than 2% of WT females fed alcohol at 12–16 months old (Old) but not in those at 6–8 months old (Mid). Alcohol induced liver tumors in 30% of the Mid female KOs and 70% of the Old female KOs (**Figure 1A**). The tumor occurrence was associated with severity of liver injury that was indicated by increased serum ALT. The alcohol feeding increased ALT by less than threefold in WT. ALT levels were constitutively higher in the KOs than in WT (**Figures 1B,C**), which were further increased by more than fivefold in response to alcohol (**Figure 1C**). Interestingly, ALT levels were significantly higher in the female KOs of older age than those of middle-aged. Histologically, mild to moderate lipid accumulation was observed in the WT females fed alcohol (**Figure 2**), which was consistent with previous findings (Ji et al., 2011). In contrast, two or more tumor masses were observed in the livers of the middle-aged female KOs fed alcohol and multiple proliferative nodules were observed in the livers of the older female KOs fed alcohol (**Figure 2**). Neutrophil infiltration was observed in the liver tumors of KOs fed alcohol. The number of Ki-67 positive hepatocytes was significantly increased in the KO compared to the

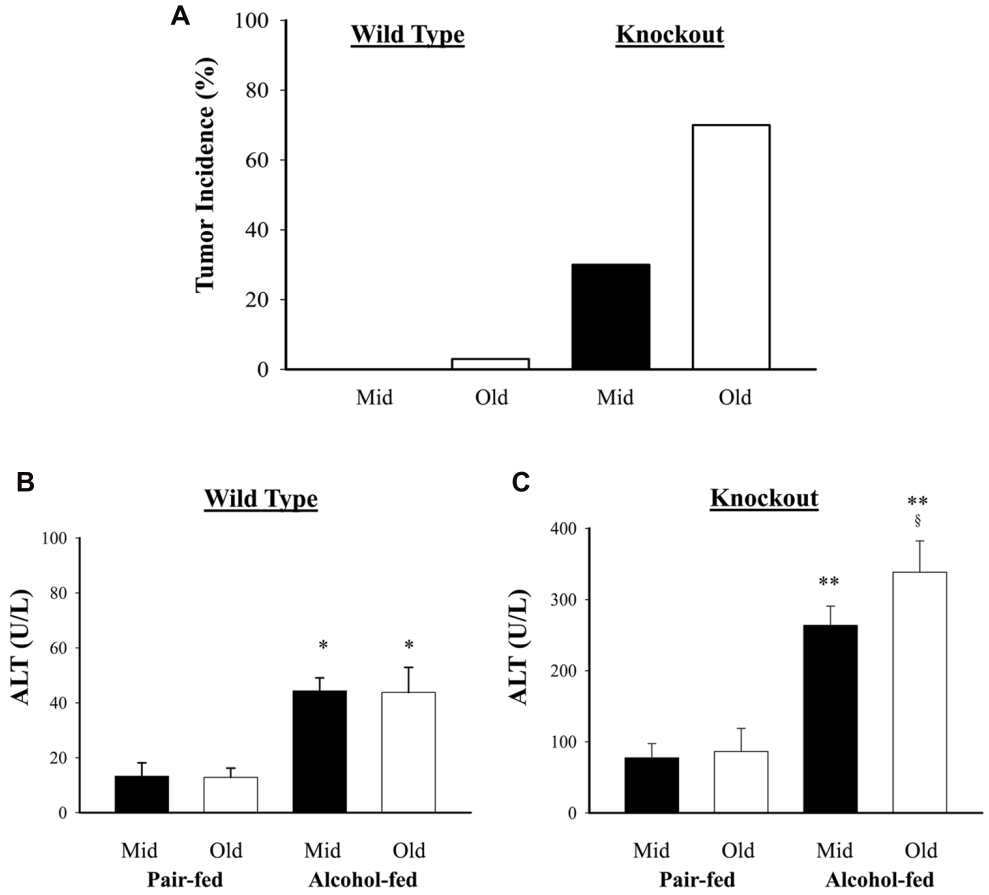

FIGURE 1 | Effects of alcohol on liver tumorigenesis in mice under constitutive endoplasmic reticulum (ER) stress. Female mice at age of 6–8 months (Mid) and at age of 14–16 months (Old) were fed alcohol respectively for 12 months. **(A)** Liver tumor occurrence in mice fed alcohol and high fat diet; **(B)** serum alanine aminotransferase (ALT) levels in wild type mice fed alcohol; **(C)** ALT in knockout mice fed alcohol. $^{*}p < 0.05$; $^{**}p < 0.01$ compared to pair-fed control; $^{\S}p < 0.05$ compared between mid and old age groups, $n = 5$.

WT (**Figures 2C,D**). More proliferative cells were found in the older KO mice fed alcohol than in the middle-aged KO fed alcohol (**Figure 2D**).

EFFECTS OF CONSTITUTIVE ER STRESS ON METHYLATION OF DNA PROMOTERS OF UPR MARKERS

DNA methylation of cytosine residues at CpG dinucleotides is a commonly occurring modification of human DNA. Aberrant methylation of CpG islands is often related with cancer (Rakyan et al., 2011). Evidence is emerging for aberrant methylation of hepatic ER stress pathways (Lenz et al., 2006; Leclerc and Rozen, 2008; Esfandiari et al., 2010). In order to seek evidence to support a potential role of DNA methylation in constitutive ER stress-induced liver tumorigenesis in mice of different ages, we focused on examining the methylation of DNA promoters of selective UPR stress marker genes: Hsp90b1 (Grp94), Ddit3 (Chop or Gadd153), Atf6, and Pdia3 (PDI). The CpG island regions of Grp94, Chop, Atf6, and PDI genes were methylated in DNA isolates from the livers of WT mice of both age groups while only low and moderate levels of the DNA methylation were observed in the livers of KO mice of both age groups (**Figure 3**). There was no difference in the methylation of the DNA promoters of the UPR marker genes between the middle-aged and older WT mice (**Figure 3**). However, in KO mice without liver tumors, the methylation of Grp94, Chop, and PDI was lower in the older group than in the middle-aged group and the methylation of Atf6 was increased in the older group than in the middle-aged group. In the older KO mice with liver tumors, overall methylation of Grp94, Chop, or PDI was increased compared to middle-aged KO and there was a significant methylation difference between the normal liver portion and the tumor portion of the tumor bearing livers. The methylation of Grp94 and PDI tended to be lower in the tumor portion of KO mice with liver tumors than in the normal liver portion whereas the methylation of Chop and Atf6 tended to be higher in the tumor portion of the tumor bearing livers than the normal liver portion. These data indicate differential or abnormal methylation patterns of the UPR factors in the BiP KOs of different ages.

EFFECTS OF LONG-TERM ALCOHOL FEEDING ON PROTEIN EXPRESSION OF ER STRESS MARKERS

Although both chronic (1–2 months) and acute (1–7 days) treatments with high doses of alcohol (i.e., 6.5 g/kg body weight) have

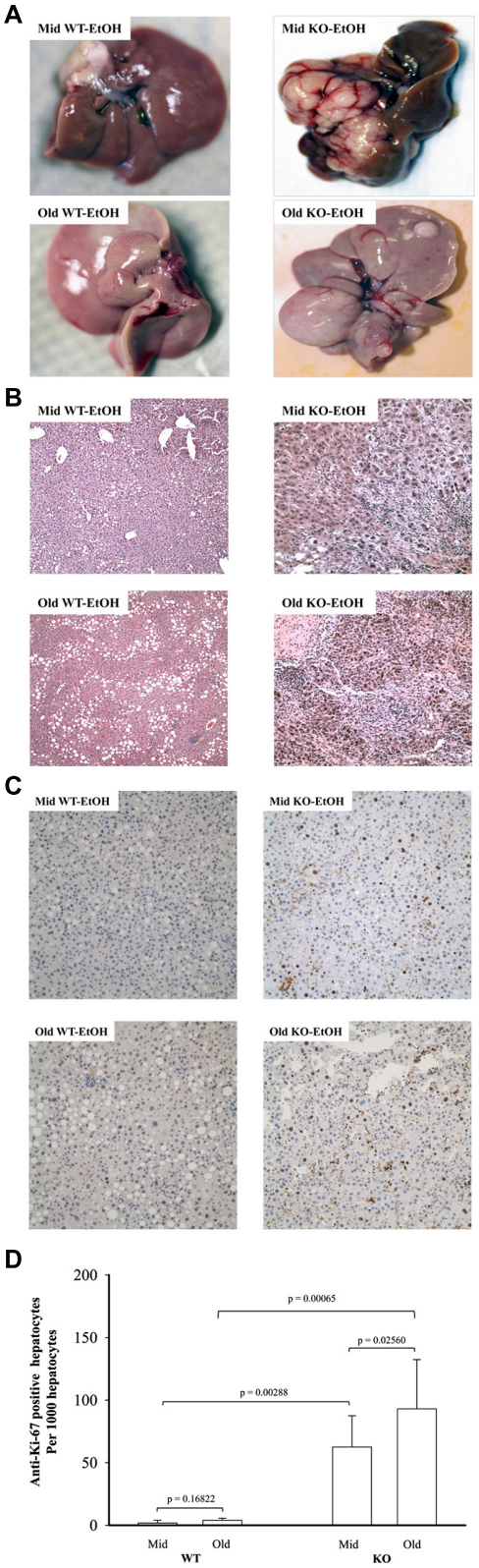

FIGURE 2 | Histology of alcohol-induced liver tumors in female mice under endoplasmic reticulum stress. Female mice at age of 6–8 months *(Continued)*

FIGURE 2 | Continued
(Mid) and at age of 12–16 months (Old) were fed alcohol (EtOH) respectively for 12 months. **(A)** Liver images showing alcohol-induced liver tumors in knockout (KO) mice. WT, wild type; **(B)** hematoxylin and eosin (H&E) staining of the liver tissues reveal alcohol-induced moderate lipid accumulation in WT mice of middle age, severe lipid accumulation in older WT mice, neutrophil infiltration and nodular formation in KO mice of middle age, and severe inflammation and multiple neoplastic hepatic lesions in older KO mice; original magnification: ×100; **(C)** liver immunohistochemistry with anti-proliferative cell nuclear antigen Ki-67 antibodies. Original magnification: ×200. **(D)** Quantitation of anti-Ki-67 positive hepatocytes.

been reported to induce ER stress response that contributed to liver injury (Ji, 2012; Galligan et al., 2012; Longato et al., 2012; Ramirez et al., 2013), it is not clear whether long-term (i.e., 1 year) alcohol feeding at moderate doses induces ER stress response and contributes to the observed hepatic tumorigenesis as well. To know that, we examined protein expression of the ER stress markers: GRP94, CHOP, active ATF6 (nATF6), and PDI in the liver of WT versus KO animals of different age groups. **Figure 4** demonstrates that moderate alcohol increased GRP94 expression in the KO mice but not in the WT mice. There was no difference of GRP94 expression between different age groups treated with alcohol. CHOP that mediates ER stress-induced cell death was low abundant in the liver tissues in general and was increased in response to alcohol feeding in WT of both age groups and in KO of the middle-aged group. CHOP expression in the older KO was significantly different from that of the middle-aged KO. Both nATF6 and PDI were increased in the middle-aged KO and the inductions of nATF6 and PDI appeared to be suppressed in the older KO in response to alcohol.

MARKED EFFECTS OF ALCOHOL ON TRANSCRIPTIONAL EXPRESSION OF GENES OF ERAD

DNA microarray analysis of approximately 19,000 transcripts of known genes was further performed to identify genes that were related to UPR/ER stress and induced by the long-term moderate alcohol feeding. Three hundred eighty two transcripts were altered significantly in the alcohol-fed animals. Among them, molecular chaperones including Grp170, oxygen-regulated protein 150 (ORP150), PDI, Dnajc3 (DnaJ homolog, subfamily C, member 3, also known as p58IPK), Grp94, ERdj5 (ER-resident protein containing DnaJ and thioredoxin domains), and calreticulin; ubiquitin and protein degradation factors including Usp 4 and 18, Ube3b, EDEM2, and Der1p-like protein 3 (Derl3), transcription factors regulating apoptosis including Nupr1 (nuclear protein 1), Chop, Trib3 (tribbles homolog 3), Gadd45, and FoxO, some nuclear factor-kappaB (NFκB) targeted genes including tumor necrosis factor (TNF) related protein 1 and TNF receptor-1 (TNFR1) were increased, whereas Biklk and hepcidin 1 were decreased in response to the long-term alcohol feeding. Interestingly, the long-term alcohol feeding seemed to have strong effects on transcriptional expression of genes involved in ERAD in the KO mice (**Figure 5**). Two- to eightfold increase in derl3, Chop, and Ccnd1 (cyclin D1) was detected in the alcohol-fed KO in comparison with the pair-fed WT. Two- to eightfold decrease in Eif2ak2 (eukaryotic translation initiation factor 2α kinase 2), Wfs1 (Wolfram syndrome gene), Xbp1

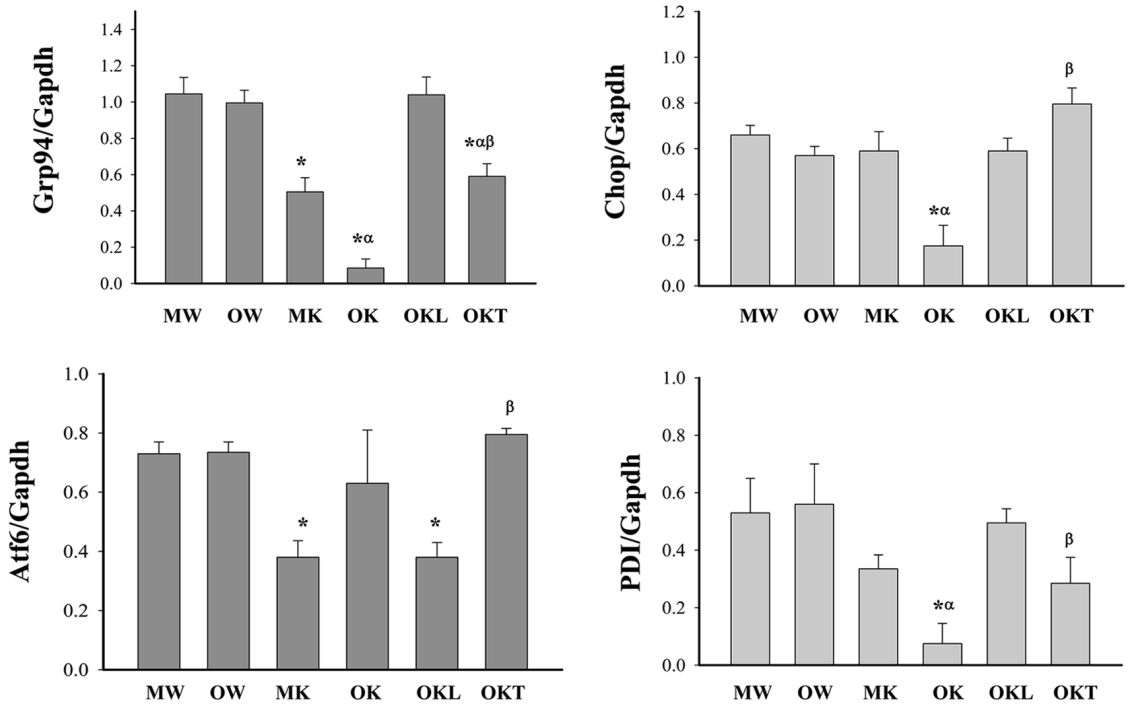

FIGURE 3 | Methylation of CpG islands of ER stress gene promoters in the livers of mice at different ages. Grp94, glucose-regulated protein 94; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Chop, DNA damage-inducible transcript 3, also known as C/EBP homologous protein; Atf6, activating transcription factor 6; PDI, protein disulfite isomerase; MW, wild type of middle age; OW, wild type of older age; MK, BiP knockout of middle age; OK, BiP knockout of older age without liver tumors; OKL, normal liver portion from tumor bearing livers of older BiP knockouts; OKT, tumor portion from tumor bearing livers of older BiP knockouts; $^{*}p < 0.05$, compared between wild type of same age; $^{\alpha}p < 0.05$, compared between middle and older mice of same genotype; $^{\beta}p < 0.05$, compared between normal liver portion and tumor portion of tumor bearing livers of knockouts of same age. $n = 3$.

(X-box binding protein 1), Creb3 (cAMP responsive element binding protein 3), Nfe2I2 (NF-E2-related factor 2), Vapb (the vesicle-associated membrane protein B), Casp12 (caspase-12), Herpud1 (homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member also known as Herp), Aars (alanyl-tRNA synthetase), Amfr (autocrine motility factor receptor), E3 ubiquitin protein ligase, Stc2 (stanniocalcin 2 also known as Hrd1), and Yod1 (hydrolase that removes conjugated ubiquitin from proteins and participates in ERAD was detected in the alcohol-fed KO in comparison with the pair-fed WT.

DEFERENTIAL EFFECTS OF LONG-TERM ALCOHOL FEEDING ON DNA METHYLATION OF ERAD FACTORS

The above strong effects of long-term alcohol on the expression of ERAD prompted us to examine further methylation of the promoters of selective ERAD factors. In the middle-aged group, the methylation of the promoters of Derl3, Creld2, Herp, and Yod1 was not significantly changed in the KO compared to the WT (**Figure 6**). In contrast in the older mouse group, the methylation of the promoters of Derl3, Creld2, Herp, Wfs, and Yod1 was lower in the KO than in the WT. In addition, the methylation of Derl3, Herp and Yod1 was increased in the normal liver portion of tumor bearing livers of older KO compared to older KO without liver tumors. In the tumor bearing livers, the methylation of Derl3, Herp, and Yod1 was reduced in the tumor portion compared to the normal liver portion whereas the methylation of Creld2 or Wfs was not significantly changed in the tumor portion compared to the liver portion. The data suggest a potential association of impaired methylation of the ERAD factors in the livers of BiP KOs with aging and liver tumor development.

CO-OCCURRENCE OF ALTERED ERAD AND TUMORIGENESIS SIGNALING IN THE LIVER OF STRESSED MICE

From our previous research with feeding of a diet contained much higher purified fat, we found both ERK (the Ras-dependent extracellular signal-regulated kinase) and Jak-Stat pathways were likely involved in stress induced liver tumorigenesis in this KO model (Lau et al., 2013). To know also whether the ERAD alterations by long-term alcohol feeding activate the two signaling pathways of liver tumorigenesis, we examined protein expression of ERAD and phosphorylation of ERK1/2 and STAT3. Increased expression of the transcription factor-ATF4 was detected in the liver of both middle-aged and older KOs (**Figure 7**). However, ATF4 was inhibited in the tumor portion compared to the liver portion. Cyclin D was slightly inhibited in all KOs compared to WT. Consistent with previous findings, ERα36 (estrogen receptor α variant 36) was increased in the middle-aged KO and was greatly increased in older KO and in the tumor portion. DERL3 was increased in both older and the tumor portion. CRELD2 was increased in both middle-aged and older KOs. The expression pattern of HERP was similar

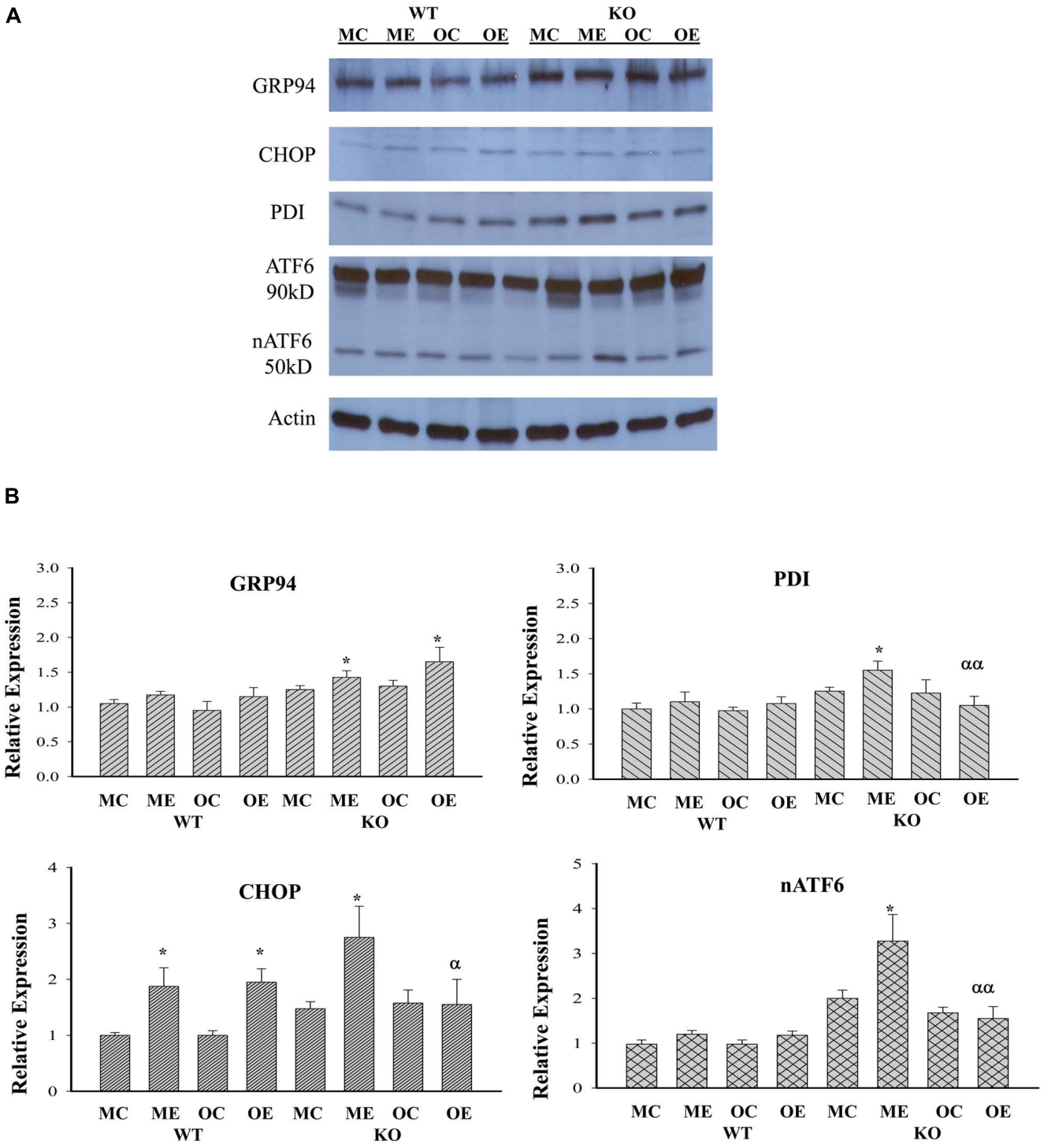

FIGURE 4 | Immunoblotting analysis of liver proteins of ER stress markers from alcohol-fed mice. KO, liver-specific knockout of immunoglobulin heavy chain-binding protein (BiP), also known as glucose-regulated protein 78 (GRP78); WT, wild type littermate; GRP94, glucose-regulated protein 94; CHOP, DNA damage-inducible transcript 3, also known as C/EBP homologous protein; nATF6, activated form of the activating transcription factor 6; PDI, protein disulfide isomerase; MC, pair-fed wild type of middle age; ME, alcohol-fed wild type of middle age; OC, pair-fed knockout of older age; OE, alcohol-fed knockout of older age; **(A)** representative western blots of the selective ER stress markers; **(B)** relative expression of each marker protein; $^{*}p < 0.05$ compared between pair-fed and alcohol-fed; $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$ compared between alcohol-fed middle and older mice of same genotype. $n = 3$.

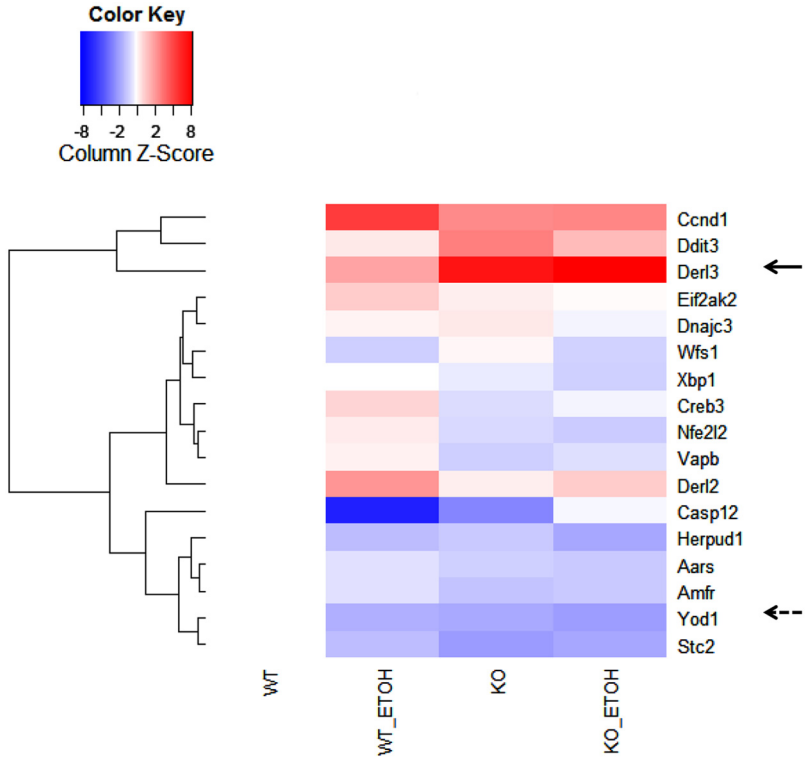

FIGURE 5 | Effects of alcohol consumption on mRNA expression of ER-associated degradation (ERAD) factors in the liver of wild type versus BiP knockout. Ccnd1, cyclin D1; Ddit3, DNA damage-inducible transcript 3, also known as C/EBP homologous protein (CHOP); Derl2 and 3, Der1p-like protein called derlin; Eif2ak2, eukaryotic translation initiation factor 2-α kinase 2; Dnajc3, DnaJ (Hsp40) homolog, subfamily C, member 3, also known as p58IPK; Wfs1, Wolfram syndrome gene; Xbp1, X-box binding protein 1; Creb3, cAMP responsive element binding protein 3; Nfe2l2, NF-E2-related factor 2; Vapb, the vesicle-associated membrane protein B; Casp12, caspase-12; Herpud1, homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member; Aars, alanyl-tRNA synthetase; Amfr, autocrine motility factor receptor, E3 ubiquitin protein ligase; Yod1, hydrolase also known as Otud2 that removes conjugated ubiquitin from proteins and participates in ERAD; Stc2, stanniocalcin 2. The solid arrow indicates strong induction by alcohol; the dashed arrow indicates strong inhibition by alcohol.

to CRELD2. Phosphorylation of ERK1/2 was detected in older WT and KOs whereas phosphorylation of STAT3 was observed only in the KOs with liver tumors (**Figure 7**). The mRNA expression of Hrd1 and Yod1 was increased by three- to sixfold in the older KO with liver tumors (**Figure 8A**). In addition, the 20S proteasome activities were reduced by 44% in older KO compared to middle-aged KO, by 45% in older KO compared to older WT, and by 53% in the tumor portion compared to the liver portion (**Figure 8B**). There were no significant differences in the proteasome activities between middle-aged KO and middle-aged WT or between middle-aged and older WT.

DISCUSSION

Alcohol consumption is well known to be a risk factor for chronic liver disease, from steatosis or fatty liver to steatohepatitis to fibrosis to cirrhosis and even liver cancer (HCC; Gao and Bataller, 2011; Brandon-Warner et al., 2012; Testino et al., 2012). Alcohol attributes to cancer related death significantly (Morgan et al., 2004). Alcohol metabolism directly contributes to the initiation of cancer. For instance, the first metabolite of alcohol-acetaldehyde is highly reactive, forming DNA-acetaldehyde adducts that can incorporate into the genome, leading to mutagenesis and transformation of healthy cells into tumor cells. Alcohol consumption induces CYP2E1 and results in the production of ROS, directly damaging DNA or generating lipid peroxidation products capable of forming mutagenic DNA adducts. ROS promotes inflammatory environments damaging to healthy host tissue leading to the development of cancer through mutagenesis (Jerrells, 2012). Alcohol-induced organelle stress, especially ER stress has been associated with a spectrum of liver diseases (Dara et al., 2011). Evidence for ER stress-induced hepatic tumorigenesis is emerging (Wang et al., 2010; Lau et al., 2013). However, how alcohol influences ER stress and liver tumorigenesis is not clear. Our current study using the animal model with a liver KO of the chaperone BiP and under constitutive hepatic ER stress may reveal a few critical clues with respect to alcohol-induced cancer. First, the long-term moderate alcohol-induced liver tumor development was observed only in the ER stress-predisposed KO animals. This suggests that additional insults such as genetic and environmental stresses may be required for the alcohol-induced hepatic tumorigenesis. This can also explain why, to date, no rodent model has demonstrated the formation of HCC in the setting of chronic alcohol consumption alone. Alcohol-induced HCC is often reported under circumstantial conditions such as an

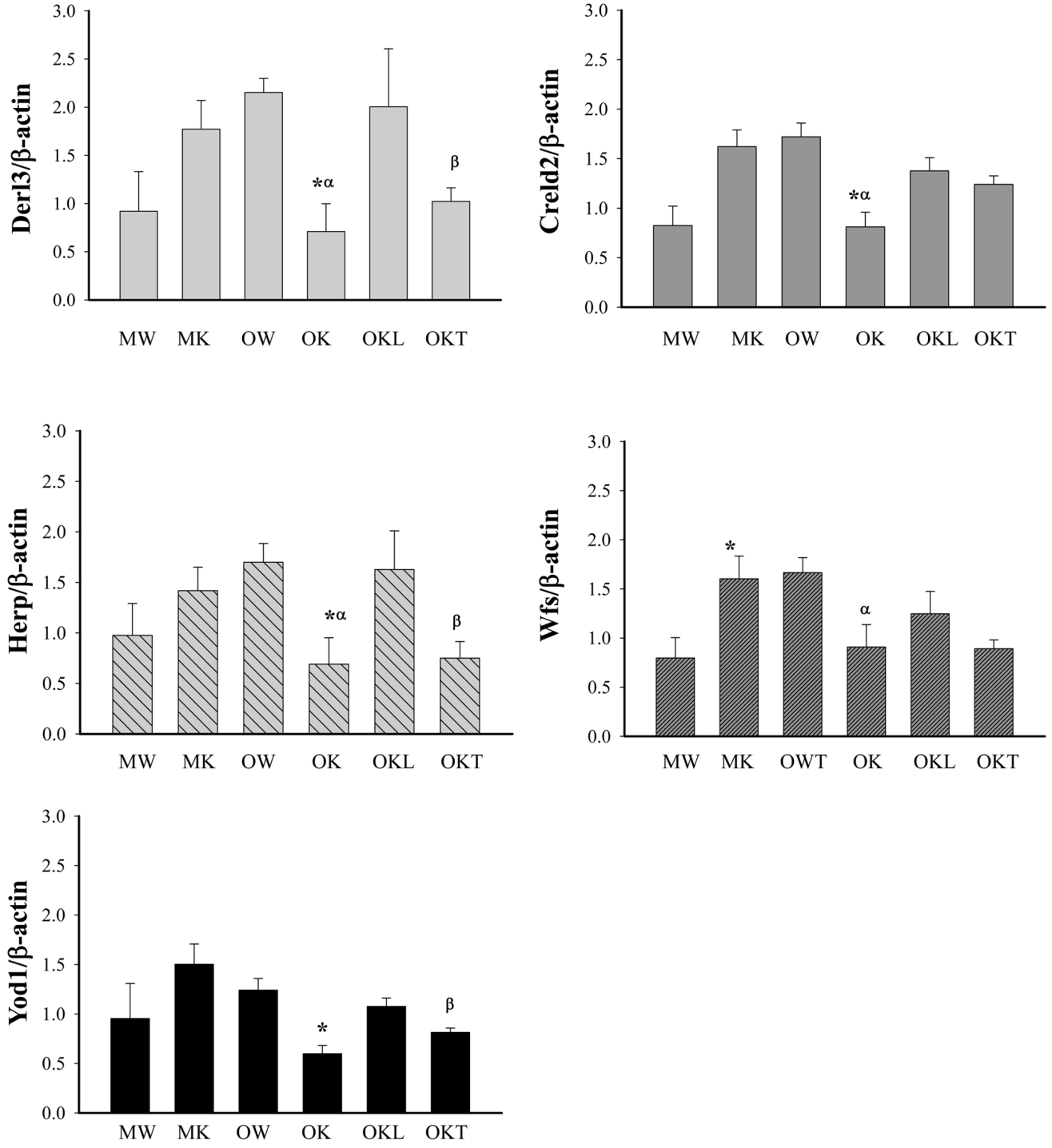

FIGURE 6 | Methylation of CpG islands of ERAD gene promoters in the liver of alcohol-fed mice of different ages. Derl3, Der1p-like protein; Creld2, cysteine-rich with EGF-like domains 2; Herp, homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member also known as Herpud1; Wfs1, Wolfram syndrome gene; Yod1, hydrolase that removes conjugated ubiquitin from proteins and participates in ERAD. MW, middle-aged wild type; MK, middle-aged knockout; OW, older wild type; OK, older knockout without liver tumor; OKL, the normal liver portion of tumor bearing livers of KO; OKT, the tumor portion of tumor bearing livers of KO. $^{*}p < 0.05$, compared between wild type of same age; $^{\alpha}p < 0.05$, compared between middle and older mice of same genotype; $^{\beta}p < 0.05$, compared between normal liver portion and tumor portion of tumor bearing livers of knockouts of same age. $n = 3$.

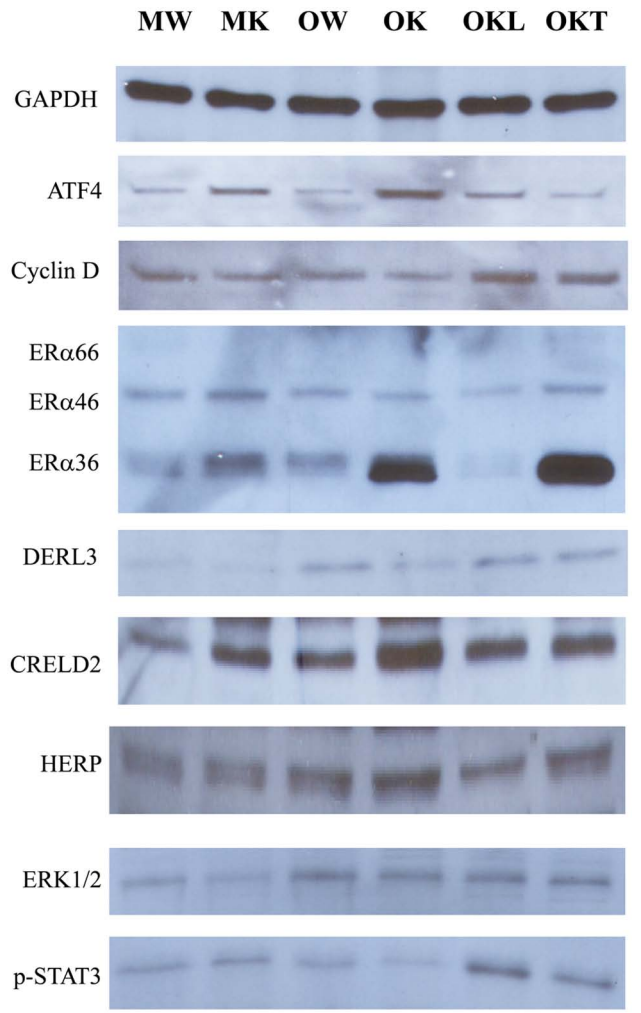

FIGURE 7 | Effects of alcohol and aging on ERAD and tumorigenesis pathways. ERα, estrogen receptor α variants (ERα36, 46, and 66); p-ERK1/2, phosphorylated extracellular signal-regulated protein kinases 1 and 2; p-STAT3, phosphorylated signal transducers and activators of transcription; MW, middle-aged WT; MK, middle-aged KO; OW, older WT; OK, older KO; OKL, the liver portion of tumor bearing livers of older KO; OKT, the tumor portion of tumor bearing livers of older KO.

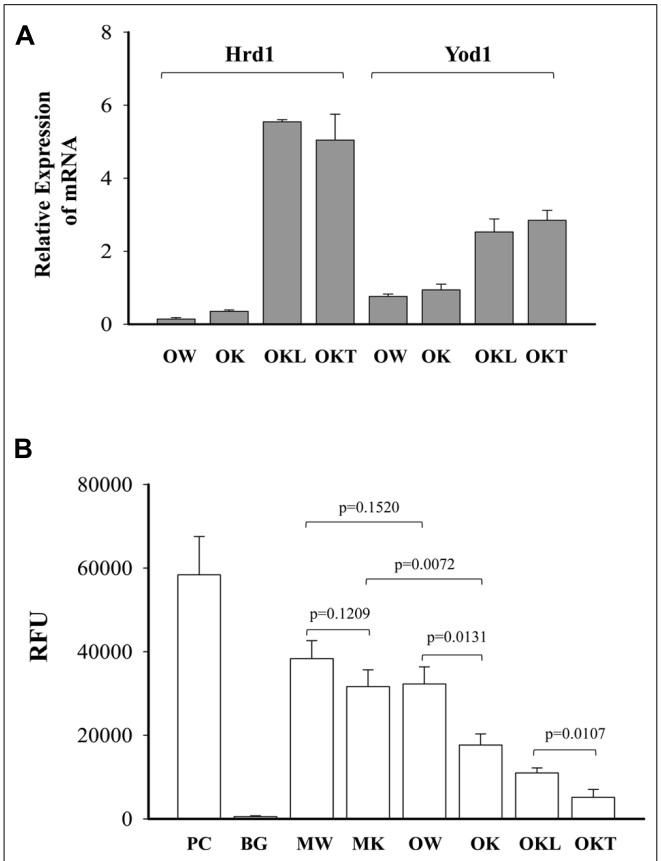

FIGURE 8 | Effects of alcohol and age on mRNA expression of ERAD factors and proteasome activities. (A) Relative expression of mRNA of Hrd1 (synoviolin, an E3 ubiquitin ligase) and Yod1 analyzed with quantitative PCR and normalized with Gapdh; **(B)** comparison of 20S proteasome activities between wild type (WT) and knockout (KO). Data are presented as RFU (relative fluorescent unit) recorded with a fluorometer, $n = 5$. PC, positive control from analysis kit; BG, negative background; MW, middle-aged WT; MK, middle-aged KO; OW, older WT; OK, older KO; OKL, the liver portion of tumor bearing livers of older KO; OKT, the tumor portion of tumor bearing livers of older KO.

alcohol-preferring (P) rat line that voluntarily drinks large quantities of alcohol (Yip-Schneider et al., 2011), in combination with obesity (Thompson et al., 2013) or co-dosing with carcinogenic diethylnitrosamine (DEN; Brandon-Warner et al., 2012), in the presence of expression of hepatitis C virus (HCV) components (Machida et al.; 2009), or consuming alcohol for an excessively long period of more than 70 weeks (Tsuchishima et al., 2013). While some of the observations reported by others may not be clinically relevant, our results support the concept of necessity of additional insults for the alcoholic HCC development and are significant since alcohol-induced ER stress occurs in human alcoholics and emerging evidence has already demonstrated that polymorphic responses (SNPs) of BiP are associated with alcohol, HCC, and other types of cancer in the human population (Zhu et al., 2013).

Second, alcohol-induced ER stress and liver cancer may also depend on aging of this animal model. We found in the present study that a tendency for liver cancer development was higher in ER stress-predisposed (KO) mice fed alcohol at older age (12–16 months) than at middle age (4–6 months). Remarkably robust and consistent impacts on ALT levels and the ER stress were detected in the older mice. Long-term alcohol apparently suppressed the protective UPR, i.e., inhibition of GRP94, PDI, and ATF6 and promoted ER stress-mediated elimination of injured cells, i.e., increase of CHOP. Aging might deteriorate the shift from adaption by the UPR to injury by alcohol. The underlying mechanism is currently not known and may be complex. In other systems, aging had a prominent role in determining genomic DNA methylation and aberrant methylation of CpG islands has often been related with cancer (Rakyan et al., 2011; Ozen et al., 2013). Alcohol is known to affect DNA methylation by its interference with one carbon metabolism and by alteration of the methylation of specific promoters (Medici and Halsted, 2013; Ozen et al., 2013).

In relevant to the animal model with constitutive ER stress, we assumed that aging might impair methylation of DNA promoters of the UPR components. As we expected, there was no difference in the methylation of the DNA promoters of the UPR marker genes between the middle-aged and older WT mice (**Figure 3**). However, differential effects of alcohol on the methylation of ER components were observed in the KO mice. The methylation of Grp94, Chop, and PDI was lower in the older KO group than in the middle-aged KO group whereas the methylation of Atf6 was higher in the older KO group than in the middle-aged group. In addition, there were significant differences between older KO with and without liver tumors and between normal liver portion and tumor portion of tumor bearing livers. For instance, hypomethylation of Grp94 and hypermethylation of Chop were seen in the tumor portion of older KO mice, which were respectively consistent with increased protein expression of GRP94 and decreased protein expression of CHOP in the tumors. Thus, our findings indicate that alterations of methylation patterns of the UPR/ER stress factors in the aging BiP KOs are likely contribute to liver tumor development.

Third, proteins that fail to fold and assemble into their mature forms are usually removed by the ERAD process that depends on activities of ubiquitin and proteasome. Although it is not clear based on the current data whether the methylation of UPR causally influences the methylation of ERAD or vice versa, the constitutive ER stress in the liver of animals without the chaperone BiP must burden the ERAD, which may be worsened by additional stress such as altered cellular levels of *S*-adenosyl-L-methionine (the principal biological methyl donor) as a consequence of chronic alcohol consumption (Kharbanda, 2013). We support this assumption with the observations that the effects of the long-term alcohol on transcriptional and translational expression of the ERAD related genes including derlin 3, Creld2, Herpud1, and Wfs1 were stronger than on the expression of the UPR related genes such as Chop, cyclin D, and Xbp-1. The alterations of ERAD expression corresponded to decreased proteasome activities and were age-related. In the middle-aged groups, methylation of the promoters of Derl3, Creld2, Herpud1, and Yod1 was not altered significantly in the KO than in the WT (**Figure 6**) whereas in the older mouse groups, the methylation of the promoters of these ERAD genes was lower in the KO than in the WT. Particularly, the methylation of Derl3 and Herp was reduced in the tumor portion of older KOs, methylation of Creld2, Wfs, and Yod1 was not changed in the normal liver portion, and mRNA expression of Hrd1 and Yod1 was remarkably increased in both liver and tumor portions of KO with liver tumors. These differential effects of alcohol and aging on the ERAD factors may reflect a severe impairment of protein processing in the liver under long-term stress. Therefore, we speculate that long-term alcohol has profound effects on protein quality control in aging animals, which in general, affects protein turnover leading to accumulation of excessive unfolded proteins, which continuously stimulates pathological changes leading to tumorigenesis. One identified potential tumorigenic factor in this study is the abundant estrogen receptor α variant ERα36, which might result either from malfunctioning of proteasomal degradation, impaired physical interactions between cyclin D and the authentic ERα, or alternative splicing of internal exons of ERα (Zwijsen et al., 1997; Fu et al., 2004; Rao et al., 2011; Lau et al., 2013). ERα36, perhaps together with other improperly processed proteins yet to be identified, interfered with phosphorylation of ERK1/2 and STAT3 in the older KO female fed alcohol resulting in high incidence of tumors. The exact molecular mechanisms up and downstream of ERα36 pertinent to UPR/ER stress signaling or abnormal methylation await further investigations.

Fourth, there are reports that human males are more likely developing HCC than females in some regions of the world (Venook et al., 2010; Center and Jemal, 2011). However, the male prevalence of HCC is circumstantial and not contradictory to our findings for a couple of reasons. The male prevalence usually occurs in areas such as Asia where men tend to expose themselves more to additional HCC risk factors such as hepatitis B virus (HBV) and aflatoxin from contaminated maize and peanut. The other reason is that higher levels of estrogen in young- and middle-aged females may play some protective role against HCC development, which might be age-dependent. There are epidemiological data demonstrate that the incidence of HCC drops significantly in old individuals of both genders (El-Serag, 2011). Since the age range of the experimental animals of this study corresponds to humans aged of greater than 50, which is generally a post-menopause age for women, the possible protective effects of estrogen are diminishing and there should be equal odds of HCC development for aged men and women without additional gender-specific risks. In this respect, the impaired expression of estrogen receptor α caused by long-term ER stress in females consists of a gender-specific risk and is most likely responsible for the high incidence of liver tumors observed in aged females.

In summary, in ER stress-predisposed older animals fed alcohol for a prolonged period, we observed marked alterations in expression and promoter methylation of ERAD genes that were co-present with development of liver tumors. We propose that long-term alcohol consumption and aging may promote liver tumorigenesis through interfering with DNA methylation and expression of genes related to the ERAD.

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GENERAL COMMENTARY
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Erratum: Green fluorescent protein-based monitoring of endoplasmic reticulum redox poise

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A commentary on

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An error has been identified in the equation to calculate roGFP oxidation (OxD) after our article was published in Frontiers in Genetics. The current equation wrongly features the ratio of ox/red average fluorescence emissions at 390 nm excitation in the denominator. This should be the ox/red ratio of average fluorescence emissions at 465 nm. Accordingly, the correct equation should read:

$$\mathrm{OxD}_{\mathrm{roGFP}} = \frac{R - R_{\mathrm{red}}}{\frac{I_{465\ \mathrm{nm}\mathrm{ox}}}{I_{465\ \mathrm{nm}\mathrm{red}}}(R_{\mathrm{ox}} - R) + (R - R_{\mathrm{red}})}$$

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Regulation of the transcriptome by ER stress: non-canonical mechanisms and physiological consequences

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The mammalian unfolded protein response (UPR) is propagated by three ER-resident transmembrane proteins, each of which initiates a signaling cascade that ultimately culminates in production of a transcriptional activator. The UPR was originally characterized as a pathway for upregulating ER chaperones, and a comprehensive body of subsequent work has shown that protein synthesis, folding, oxidation, trafficking, and degradation are all transcriptionally enhanced by the UPR. However, the global reach of the UPR extends to genes involved in diverse physiological processes having seemingly little to do with ER protein folding, and this includes a substantial number of mRNAs that are suppressed by stress rather than stimulated. Through multiple non-canonical mechanisms emanating from each of the UPR pathways, the cell dynamically regulates transcription and mRNA degradation. Here we highlight these mechanisms and their increasingly appreciated impact on physiological processes.

Keywords: ER stress, unfolded protein response (UPR), mRNA stability, Transcription Factors, gene regulatory networks (GRN)

INTRODUCTION

The ER is best known as the gateway to the secretory pathway. As the site of synthesis for nascent secretory proteins and resident lumenal and transmembrane proteins of the endomembrane system, the ER shepherds the folding, oxidation, modification, and assembly of approximately one-third of the cellular proteome—or more in cell types specialized for protein secretion such as antibody-secreting plasma B lymphocytes or endocrine or exocrine cells (Huh et al., 2003; Tagliavacca et al., 2003). As such, the ER is replete with chaperones, cochaperones, oxidases, and thiol isomerases to facilitate protein folding, and utilizes an elaborate quality control system to recognize terminally misfolded proteins and purge them from the ER for degradation (Araki and Nagata, 2012). With this system in place, the low level of protein misfolding that arises because of the inherent error rate in the process can presumably be managed. However, the quality control machinery can be overwhelmed either by an overload of nascent client proteins or by any exogenous disruption to the protein folding and trafficking system—so-called "ER stress" (Ron and Walter, 2007). The consequences of a pervasive defect in ER protein folding can be grave for both the cell and the organism. A cell with overwhelmed ER quality control machinery will, at best, fail to maintain secretory pathway integrity and to effectively sense and respond to the extracellular milieu. At worst, accumulated misfolded proteins might seed the formation of toxic protein aggregates (Matus et al., 2011). Thus, the cell has in the UPR a signal transduction system that augments the protein folding capacity of the ER. While the UPR improves ER function by several short-term mechanisms, it ultimately culminates in gene regulation for longer-lasting enhancement of the ER folding environment. Classically, this regulation constitutes a self-contained system in which ER stress leads to transcriptional induction of genes encoding ER chaperones and other proteins that grease the wheels of secretory pathway function, thereby alleviating ER stress and shutting the response off (Travers et al., 2000). However, it is now becoming clear that the UPR is much more deeply entwined in cellular physiology than this simple view would suggest. In mammals, it regulates genes involved in a number of cellular processes that have little on their face to do with ER function, including metabolism and inflammation (Fu et al., 2012; Garg et al., 2012). The regulation of many of these genes cannot be explained by the canonical mechanisms of UPR signaling. The aim of this review is to highlight emerging concepts in the non-canonical regulation of mRNA expression by the UPR. Rather than providing an exhaustive account of all possible means by which mRNA abundance might be controlled by the UPR, here we describe the general principles by which such regulation can occur and provide illustrative examples that emphasize the diverse physiological consequences of such pathways.

THE CANONICAL UPR

The idea that there must be a signal transduction pathway emanating from the ER first emerged from the observation that expression of misfolded influenza hemagglutinin (HA) in mammalian cultured cells led to upregulation of the ER chaperones *Bip* (aka GRP78, the product of the *Hspa5* gene) and GRP94 (the product of the *Hsp90b1* gene) (Kozutsumi et al., 1988). This finding allowed previous reports of *Bip* and *Grp94* induction

in response to chemical perturbants (Drummond et al., 1987; Kim and Lee, 1987) to be tied specifically to disrupted ER protein folding. The general applicability of the phenomenon was extended to other misfolded ER client proteins (Dorner et al., 1989), and to the upregulation of ER oxidases and thiol isomerases as well (Dorner et al., 1990). As with many fundamental cellular processes, an analogous response was soon discovered in yeast (Normington et al., 1989), and a *cis*-acting unfolded protein response element (UPRE) within the yeast *Bip* (aka *KAR2*) promoter was discovered (Mori et al., 1992; Kohno et al., 1993). The ER stress-responsiveness of *KAR2* served as the springboard for a classic series of genetic and biochemical studies describing the mechanistic basis of what had become known as the unfolded protein response (Cox et al., 1993; Mori et al., 1993; Cox and Walter, 1996; Sidrauski et al., 1996; Sidrauski and Walter, 1997). Together, these studies identified Ire1p (for Inositol-requiring enzyme) as an ER-resident transmembrane kinase that became autophosphorylated during ER stress, activating a cytosolic endoribonuclease activity that catalyzed the removal of an inhibitory intron from *HAC1* mRNA. This splicing event allowed the mRNA to be translated into the Hac1p transcription factor, which subsequently bound to the *KAR2 UPRE* and stimulated *KAR2* transcription. A survey of the breadth of UPR targets (Travers et al., 2000) took advantage of then-new microrarray technology, and revealed two fundamental features of the yeast UPR that have heavily influenced the subsequent portrayal of the response in both yeast and mammals: (1) It is predominantly an inductive response, with a bias for upregulated genes; and (2) its scope is not limited solely to ER chaperones and phospholipid synthesis enzymes, as had been originally thought, but encompasses genes involved in other aspects of secretory pathway function, including ER-associated protein degradation (ERAD), vesicular trafficking, and protein translocation into the ER, among other processes. Thus, even though the response was more expansive than anticipated, it could still be considered as a discrete transcriptional program designed to upregulate the expression of genes with a common *cis*-element, all of which influenced ER protein folding either directly or indirectly.

Once Ire1p was described in yeast, its mammalian homologs (IRE1α and an intestine-specific paralog IRE1β) were discovered (Tirasophon et al., 1998; Wang et al., 1998), as was a gene encoding a transmembrane protein with a lumenal domain homologous to IRE1α but a cytosolic aspect homologous to other kinases such as PKR that phosphorylate the translation initiation factor eIF2α. The protein encoded by this gene was named PERK (PKR-like ER kinase) (Shi et al., 1998; Harding et al., 1999). And, much as the *UPRE* had been instrumental in identification of Ire1p in yeast, so also a mammalian ER stress response element (*ERSE*) in the promoters of *Bip* and *Grp94* was characterized and used to identify ATF6 (of the activating transcription factor family) as another ER-resident stress sensor (Yoshida et al., 1998; Haze et al., 1999). All three pathways are conserved throughout metazoa, but the PERK and ATF6 pathways appear to assume more modest roles in invertebrates such as flies and worms (Shen et al., 2001; Ryoo and Steller, 2007).

As for yeast Ire1p, activation of each of the three mammalian UPR pathways culminates in production of a transcriptional activator and attendant rearrangement of chromatin structure (Baumeister et al., 2005; Donati et al., 2006; Gal-Yam et al., 2006) and recruitment of RNA Polymerase II (Sela et al., 2012) to stimulate gene transcription. These pathways have been reviewed exhaustively elsewhere (Schröder and Kaufman, 2005) and so will only be described briefly here. Mammalian IRE1α is activated by autophosphorylation and catalyzes the splicing of *Xbp1* mRNA, resulting in excision of a 26-base intron and thus allowing in-frame translation of the downstream transcriptional activation domain of XBP1 (Yoshida et al., 2001a; Lee et al., 2002). Translocation and activity of the bZIP protein produced from spliced *Xbp1* mRNA are regulated by the protein produced by the unspliced mRNA (Lee et al., 2003a; Tirosh et al., 2006), by the regulatory subunits of PI3 Kinase (Park et al., 2010; Winnay et al., 2010), and by acetylation (Wang et al., 2011). In the nucleus, XBP1 binds to *UPRE* sequences (distinct from yeast *UPRE* elements) in, among others, the promoters of genes encoding ERAD factors (Yoshida et al., 2001b; Lee et al., 2003b; Yoshida et al., 2003; Yamamoto et al., 2007). XBP1 can also bind to additional non-*UPRE* sequences and regulate genes involved in phospholipid biosynthesis (Sriburi et al., 2007), lipogenesis (Lee et al., 2008) and myogenic differentiation (Acosta-Alvear et al., 2007). Mice lacking XBP1 die prenatally due to liver defects (Reimold et al., 2000), and mice lacking IRE1α die even earlier with both liver and lymphocyte differentiation defects (Zhang et al., 2005) that might be secondary to placental failure (Iwawaki et al., 2009). Liver-specific rescue of XBP1 deficiency only postpones death into the neonatal period, when dysmorphogenesis of the exocrine pancreas leads to digestive failure (Lee et al., 2005).

PERK activation and autophosphorylation lead to eIF2α phosphorylation which, while having the immediate (and transient) effect of inhibiting the translation of most mRNAs, stimulates translation of the *Atf4* mRNA due to the presence of upstream open reading frames (uORFs) in the *Atf4* 5′ UTR (Lu et al., 2004; Vattem and Wek, 2004). This effect is a consequence of the inefficient ribosome assembly brought on by eIF2α phosphorylation, which allows certain mRNAs—2 to 8 percent by one estimate—to be translationally stimulated rather than inhibited, based on the presence of uORFs (Ventoso et al., 2012; Barbosa et al., 2013). Like XBP1, ATF4 is a bZIP transcription factor; it binds to amino acid response elements (*AARE*s) in target gene promoters. eIF2α can be phosphorylated by other kinases in response to various cellular stresses, and this pathway of signal transduction is known as the integrated stress response (ISR) (Harding et al., 2003). *Perk*$^{-/-}$ mice develop progressive postnatal diabetes and exocrine pancreas disruption (Harding et al., 2001).

Finally, ATF6 is an ER-localized transmembrane transcription factor. ER stress releases it from the ER to the Golgi, where it is cleaved by regulated intramembrane proteolysis (RIP) to liberate the transcriptionally active cytosolic domain—itself a bZIP family member—that dimerizes with the constitutive factors NFY and YY1 and binds to *ERSE* and *ERSE-II* sequences in target genes (Li et al., 2000; Ye et al., 2000; Yoshida et al., 2000; Kokame et al., 2001; Baumeister et al., 2005). ATF6 can also heterodimerize with XBP1 on *UPRE* sites (Yamamoto et al., 2007); indeed, the mammalian *UPRE* was first identified by virtue of its binding by ATF6

(Wang et al., 2000). Mice lacking ATF6 are overtly normal (Wu et al., 2007; Yamamoto et al., 2007), and no basal phenotype has yet been reported, although this surprising absence might be due to functional redundancy between ATF6 and its paralog ATF6β (discussed in more detail later).

The phenotypes of mice with constitutive deletions of UPR components mesh with the narrative of the UPR as a self-contained program for maintaining ER protein folding homeostasis. Where basal phenotypes are evident, disrupted cell types in the various animals show evidence of grossly altered ER structure and impaired secretory pathway function. In addition, constitutive deletion of *Xbp1*, *Atf4*, and *Atf6* allowed the transcriptional programs downstream of each to be examined by microarray in the most convenient cell type, mouse embryonic fibroblasts (MEFs)—the cell type in which most of the basic pathways of the UPR were elucidated. Similarly to yeast, MEFs respond to ER stress with an upregulation of genes encoding ER chaperones and cochaperones, ERAD factors, lipid synthesis enzymes, and other proteins of importance to secretory pathway function and general protein biosynthesis. Subsets of these genes were found to depend on each of the three UPR-regulated bZIP transcription factors. Although there is considerable overlap in the sets of genes regulated by these factors, to a first approximation ATF4 coordinates the upregulation of genes involved in protein anabolism and redox defense (Harding et al., 2003), XBP1 appears to regulate ERAD (Lee et al., 2003b), and ATF6 contributes to upregulation of chaperones and ERAD factors (Wu et al., 2007; Adachi et al., 2008). For the purposes of this review, we shall refer to this mechanistic framework, culminating in production of XBP1, ATF4, and ATF6—along with the direct actions of these factors on target genes—as the canonical UPR, and it is indicated in green in the accompanying figure to highlight the non-canonical mechanisms that are the focus of this review (**Figure 1**).

A deeper look at these microarray studies reveals that the transcriptional output of the UPR is not so simple and self-contained. The emphasizing of the UPR as a program for transcriptional induction meant that the downregulated genes were not characterized in each of these analyses; yet between one quarter and one half of mRNAs regulated by ER stress are actually suppressed, with few mechanisms to account for them. In contrast to upregulated genes, the suppressed genes cluster among a number of cellular processes having apparently little to do with ER protein folding (Arensdorf and Rutkowski, 2013). Further, even among genes upregulated by ER stress, only a relatively small number can be tied definitively to ATF4, XBP1, and/or ATF6. The mechanisms responsible for regulation of the majority of genes during ER stress even in a presumptively "generic" cell type such as the MEF are not understood, and quite possibly fall outside the scope of the canonical UPR.

TEMPORAL DYNAMICS OF mRNA REGULATION

There are four ways in which mRNA abundance might be regulated by the UPR: stimulation or inhibition of transcription, and enhancement or compromise of mRNA stability. The historical view of the UPR as a program for upregulation of ER chaperones has shone the most attention on the first mechanism, but the other three contribute as well. Indeed, they probably collectively contribute to a substantial fraction of the observed changes in mRNA expression upon ER stress, or perhaps even the majority of it. The mechanism by which an mRNA is regulated has implications for the timing and persistence of that event and, by extension, the window of time during which the protein product translated from that mRNA is able to influence cellular function.

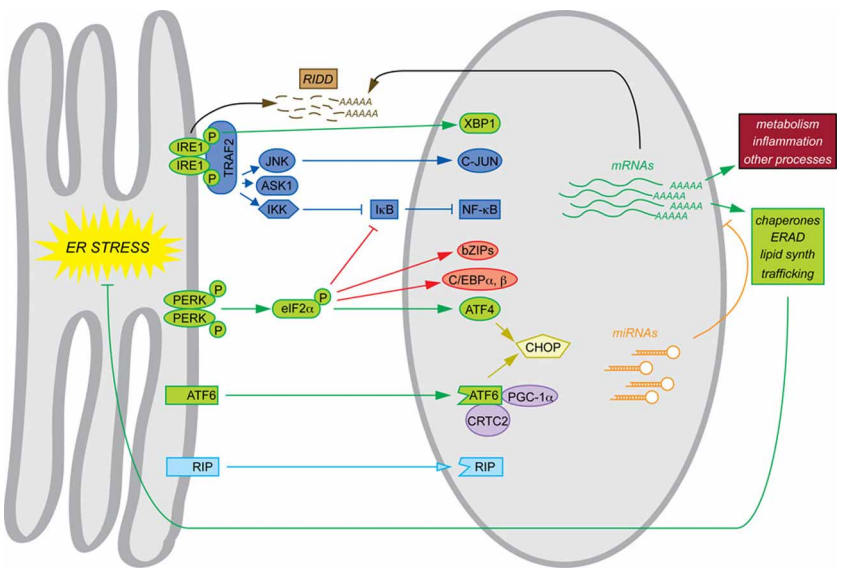

FIGURE 1 | Canonical and non-canonical pathways of mRNA regulation. Examples of each of the pathways of mRNA regulation discussed in this review are shown. The canonical UPR pathways are shown in green. Also depicted are translational regulation (red), scaffolding (dark blue), transcriptional cascades (yellow), cofactor titration (purple), alternate RIP substrates (light blue), RIDD (brown), and miRNAs (orange). Together, these processes result in the regulation not only of ER protein folding function (green) but also other cellular processes such as metabolism and inflammation (maroon).

To illustrate how the mode of regulation impacts the kinetics of mRNA expression, we provide here a simple computational model of a gene regulatory event, where the expression of a target gene is controlled by the expression of an upstream factor that can either stimulate transcription of the target gene, inhibit transcription, stimulate degradation of the target gene mRNA, or inhibit degradation (**Figure 2A**). The regulatory step is modeled as a non-cooperative interaction obeying simple Michaelis-Menten kinetics. Transcriptional effects depend upon the synthesis rate constant of the target transcript, the concentration of the upstream factor, and the affinity constant of that factor for its target gene. mRNA stability effects depend upon the same values, and also, as with any first-order decay process, on the concentration of the target mRNA itself. (The equations and parameters are given in the Supplemental Material).

The point of this exercise is to illustrate how—all other variables being held constant—the mechanism of mRNA regulation influences the rapidity with which the regulation is executed and its persistence. Thus, the hypothetical scenario shown here proposes that the action of the upstream factor has an effect on the target mRNA that results in its regulation (either up- or down-) by approximately ten-fold at its peak. With that stipulation, it then tests how the expression of an mRNA with an otherwise fixed rate of synthesis and rate of degradation behave in response to the expression of the upstream factor.

For this particular example, we modeled the response so that its peak effect was close to its saturation level, but the results were similar when the association/dissociation rate constants were varied over a wide range. This analysis reveals two salient features *that are largely independent of the actual parameters (rate constants and affinity constants) chosen*: First, mRNA levels can be more rapidly altered by stimulatory processes (of either transcription or degradation) than by inhibitory ones. Second, downregulated genes as a group return to basal expression levels more rapidly than do upregulated genes (**Figure 2B**). Further, the stability of the target mRNA influences the window of time when its expression is regulated, with shorter half-lives causing mRNAs to more directly mirror the expression of the upstream controlling factor (**Figure 2C**). In other words, while factors intrinsic to an mRNA (its synthesis and degradation rate constants) influence the window of time in which the mRNA is expressed, "stretching" its expression curve to the right or left, the extrinsic mode of regulation determines whether the expression of an mRNA experiences a lag either as stress is first experienced (i.e., if transcription or degradation is inhibited) or when the response begins to resolve (i.e., when transcription or degradation is stimulated). These observations imply that varied mechanisms for mRNA regulation ensure that the UPR is a dynamically evolving amalgam of outputs rather than a single output that simply varies in intensity over time. Practically, they also imply that the timing of mRNA regulatory events will reveal important clues about the mechanisms responsible.

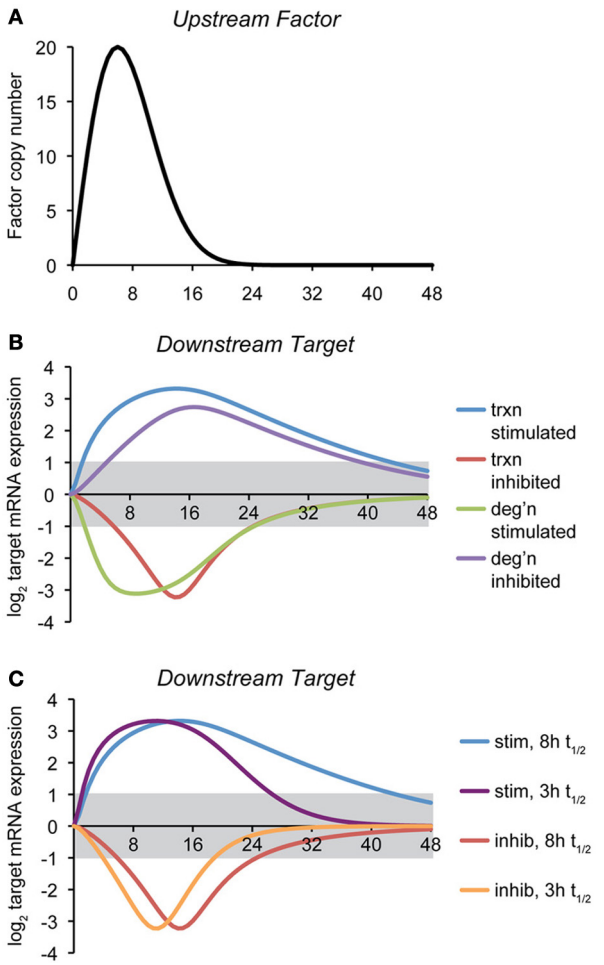

FIGURE 2 | Temporal dynamics of mRNA regulation by different mechanisms. (A) A computational model was created in which the expression of a downstream target mRNA is directly controlled by the induction of an upstream factor, the expression of which is shown. The behavior of this factor is modeled loosely off of the dynamics of ATF4, ATF6, and XBP1 upon a level of ER stress to which cells can successfully adapt (Rutkowski et al., 2006). **(B)** Expression of the downstream target mRNA was modeled based on the upstream factor either stimulating or inhibiting transcription, or stimulating or inhibiting degradation, under conditions where maximal expression of the factor approaches the saturation level. For this simulation, the intrinsic (i.e., unregulated) half-life of target mRNA was chosen to be 8 h, and the parameters were then chosen to elicit 10-fold maximal regulation in expression, and varying the rate- and affinity-constants did not change the essential behavior of the model. The gray region indicates changes in mRNA levels that are less than two-fold (the threshold most frequently used to identify regulated genes in microarray experiments). The various curves illustrate two principles: (1) the window of time in which the expression of an mRNA will appear to be regulated (based on the two-fold criterion) depends on the mechanism of regulation, and (2) downregulation of mRNA either by inhibition of transcription or stimulation of degradation is necessarily shorter-lived than is upregulation, implying that changes in expression of downregulated genes might be easily overlooked. **(C)** The effect on transcriptional regulation of decreasing the half-life of the target mRNA form ~8 to ~3 h is shown. While the median half-life of cellular mRNA is 8–9 h, many of those encoding transcription factors have shorter half-lives (Schwanhausser et al., 2011). This includes that of *Chop*, which is itself transcriptionally regulated by the UPR.

NON-CANONICAL PATHWAYS OF TRANSCRIPTIONAL REGULATION

Non-canonical transcriptional outputs may arise as offshoots of the framework of the canonical UPR by three general

mechanisms: (1) UPR pathway branching that results in the production of additional transcriptional regulators; (2) transcriptional cascades that expand the repertoire of targeted genes; and (3) alteration of the activity of constitutively expressed transcription factors through competition or cooperativity in the nucleus. In addition, UPR transcriptional output can be enhanced by the existence of parallel non-canonical stress-sensing pathways. Below, we provide examples of each of these modes of transcriptional regulation.

NON-CANONICAL REGULATION EMANATING FROM CANONICAL PATHWAYS

The PERK and IRE1α cascades of the UPR in particular present multiple points at which additional signaling cascades could be initiated. First, both molecules are kinases, raising the possibility that other substrates exist. Both also self-associate during activation (Bertolotti et al., 2000), potentially forming stress-dependent scaffolds that can seed the assembly of signaling modules and that culminate in transcriptional regulation. In addition, the effects of eIF2α phosphorylation on protein synthesis potentially allow for the production of transcription factors in addition to ATF4.

Translational regulation

Phosphroylation of eIF2α initially suppresses the translation of 90% of cellular mRNA, which decreases to 50% within the first 3 h of stress (Ventoso et al., 2012). The effect of this suppression on the expression level of a given protein depends on the half-life of that protein and the duration of eIF2α phosphorylation, which is regulated by both constitutive (CreP) and inducible (GADD34) phosphatases (Jousse et al., 2003; Marciniak et al., 2004). Accordingly, the expression of proteins with short half-lives diminishes more rapidly than does that of long-lived proteins, as demonstrated initially for the cell cycle regulator Cyclin D1, the loss of which upon eIF2α phosphorylation leads to cell cycle arrest (Brewer et al., 1999). Translational inhibition offers the opportunity to transiently alter the composition of the transcription factor network based on the expression of both the factors themselves and upstream proteins that regulate transcriptional cascades. While transcription factors as a class tend to have short half-lives, the range of their half-lives nonetheless spans an order of magnitude or more, meaning that inhibition of protein synthesis will have a more pronounced effect on the protein levels of some transcription factors than others (Schwanhausser et al., 2011, 2013).

UPR activation converges on inflammatory signaling in part through translation-dependent regulation of NF-κB. Members of the NF-κB/Rel family of transcription factors (i.e., NFKB1, NFKB2, c-REL, RELA, RELB) dimerize to form the NF-κB transcriptional complex. The transcriptional activity of this complex is determined by its composition (Elsharkawy et al., 2010); and the complex regulates the transcription of genes involved in immunoregulation, growth regulation, inflammation, carcinogenesis and apoptosis (Hoesel and Schmid, 2013). NF-κB is sequestered in the cytoplasm by IκB; in order for NF-κB to be activated, the inhibitory subunit must be removed through phosphorylation and degradation (Ahn and Aggarwal, 2005).

PERK—through the phosphorylation of eIF2α—affects NF-κB signaling by suppressing the translation of IκB family members (i.e., NFKBIE, NFKBIB), which have a shorter half-life than NF-κB/Rel family members (Jiang et al., 2003; Deng et al., 2004). Thus, eIF2α phosphorylation decreases the amount of IκB relative to NF-κB. In addition, the translation of certain NF-κB/Rel family members is suppressed during ER stress (i.e., REL, RELA, RELB) while others are not (i.e., NFKB1, NFKB2) (Ventoso et al., 2012), suggesting that eIF2α phosphorylation might not simply stimulate NF-κB activity, but might instead regulate the formation of specific NF-κB complexes. The physiological significance of NF-κB activation during ER stress is unknown; however, a number of NF-κB target genes (Pahl, 1999) are found among ER stress-regulated genes, pointing to a contribution of NF-κB to UPR transcriptional output.

While NF-κB activity appears to be regulated passively by eIF2α phosphorylation through simple loss of an unstable inhibitor, translational control is also used to stimulate the translation of specific proteins, including several transcription factors beyond ATF4. Indeed, while the majority of mRNA translation is suppressed by eIF2α phosphorylation, the translation of ∼2–8% of cellular transcripts is increased (Ventoso et al., 2012). Among the transcripts whose translation is stimulated are a number of transcription factors, including several among the bZIP family in addition to ATF4. These include ATF5 (Zhou et al., 2008), ATF3, CHOP, JUN, JUNB, FOS, FOSB, and CREB1 (Ventoso et al., 2012). How the translation of these transcripts is regulated during conditions of eIF2α phosphorylation is an area of active research; however, the presence of alternative uORFs within these transcripts is thought to dictate their translation during ER stress (Morris and Geballe, 2000).

In the case of the constitutively expressed transcription factors C/EBPα and C/EBPβ, translational control allows for the production of a truncated inhibitory form at the expense of a full-length activating form. C/EBP family members are bZIP transcription factors that participate in diverse physiological processes including differentiation, proliferation, metabolism, and inflammation (Ramji and Foka, 2002). Both α and β forms possess a cluster of potential translation initiation sites that are highly conserved among mammals. One of the start codons within this cluster is out of frame with respect to the others, and introduces a short open reading frame just upstream of the start codons that initiate translation of the full-length forms of α and β. The presence of this uORF is essential for translation of the truncated forms of both proteins, which is stimulated by eIF2α phosphorylation (Calkhoven et al., 2000; Wethmar et al., 2010). The truncated versions of both proteins have intact DNA binding domains but lack transactivation domains, making them potentially dominant-negative inhibitors of transcription (Descombes and Schibler, 1991).

Like *Atf4*, *Cebpa*, and *Cebpb* mRNAs both have uORFs that regulate the translation of alternate isoforms, but their expression appears to be controlled by a somewhat different mechanism. While the mechanism of C/EBPα translational regulation by eIF2α phosphorylation is still somewhat unclear, the expression of the long (aka Liver Activating Protein or LAP) and short (Liver Inhibiting Protein or LIP) forms of C/EBPβ are regulated by both

eIF2α phosphorylation and dephosphorylation. Synthesis of the LIP form of C/EBPβ is translationally inhibited by eIF2α phosphorylation, with levels diminishing starkly because of its short half-life (Li et al., 2008). However, translational recovery promotes both increased synthesis and increased stability of the LIP form, causing its expression to predominate over that of LAP at later times after the induction of ER stress (Li et al., 2008; Arensdorf and Rutkowski, 2013). Thus, C/EBPβ, and probably C/EBPα as well, are more indirectly tied to eIF2α phosphorylation than is ATF4.

Translational regulation of both C/EBPα and C/EBPβ has been linked to diminished lipogenesis and improved glucose tolerance in mice fed a high-fat diet (Oyadomari et al., 2008). Mice with liver-specific overexpression of a constitutively active fragment of GADD34 that were raised on a high fat diet were resistant to weight gain and showed enhanced insulin sensitivity and reduced hepatic triglyceride accumulation. The reduced steady-state phosphorylation of eIF2α seen in these animals corresponded with lower expression of both C/EBPα and C/EBPβ (only the long forms of each were studied) and their downstream target genes, including the lipogenic mediator PPARγ. While a direct role for C/EBPα and β was not tested in GADD34-overexpressing mice, these findings illustrate a potentially significant physiological consequence of non-canonical ER stress-mediated transcription—namely, that eIF2α phosphorylation promotes hepatic lipid accumulation through enhanced or altered synthesis of C/EBPα and β.

C/EBPβ regulates inflammatory cascades in diverse cell types; it is alternatively named NF-IL6 based on its ability to regulate expression of the pro-inflammatory cytokine IL-6 (Akira et al., 1990). Preferential production of the LIP form of C/EBPβ during ER stress in cultured cells was found to result in transcriptional repression of a number of inflammatory genes, including IL4RA, which is an essential component of the IL-4 and IL-13 receptors (Arensdorf and Rutkowski, 2013). Most cell types express either the IL-4 or IL-13 receptor, and signaling through IL4RA stimulates pro-inflammatory processes as diverse as B cell proliferation, IgE class-switching, T_H2 cell differentiation, smooth muscle contraction, mucus hypersecretion, eosinophil requirement, fibrotic deposition, and chemokine expression (Hershey, 2003; Wynn, 2003; Wills-Karp and Finkelman, 2008; Holgate, 2011). Remarkably, while ER stress suppressed IL-4/IL-13-dependent downstream signaling, this suppression was lost in *Cebpb*$^{-/-}$ cells, indicating that the translational regulation of C/EBPβ can influence responsiveness to inflammatory signals. This finding suggests that UPR or ISR activation could influence the natural history of parasitic infections and allergic responses, both of which are accompanied by extensive IL-4 and IL-13 signaling (McKenzie, 2000).

Scaffolding

Both PERK and IRE1α self-associate during ER stress (Bertolotti et al., 2000). For yeast Ire1p, this self-association leads to the formation of oligomeric Ire1p clusters (Korennykh et al., 2009). While the dynamics of self-association for mammalian IRE1α and PERK are less understood, it seems likely that, here too, higher ordered multimeric complexes of both proteins form, resulting in the creation of potential stress-specific scaffold supports on the cytosolic face of the ER membrane (Li et al., 2010). IRE1α can seed the formation of cytosolic signaling modules, at least some of which culminate in transcriptional regulation (Hetz and Glimcher, 2009).

Among the proteins recruited to phosphorylated and oligomerized IRE1α is TNF receptor-associated factor 2 (TRAF2) (Urano et al., 2000). This association is thought to be required for activation of several kinases including IKK, JNK, ASK1, p38 MAPK, and ERK that contribute to cell fate decisions (i.e., survival vs. apoptosis) during ER stress (Urano et al., 2000; Nishitoh et al., 2002; Nguyen et al., 2004; Hu et al., 2006; Li et al., 2010; Tam et al., 2012). For IKK regulation, the subsequent activation of NF-κB (IKK phosphorylates IκB, leading to its degradation) was found to lead to induction of TNF-α expression (Hu et al., 2006). Likewise, JNK activation leads to phosphorylation of the bZIP transcription factor C-JUN (Urano et al., 2000), which has been implicated in ER stress-mediated regulation in cultured neurons of the gene encoding methylenetetrahydrofolate reductase, which participates in folate and homocysteine metabolism (Leclerc and Rozen, 2008), and of *Gpt1* and *Got1* in the liver, which encode the liver enzymes AST and ALT that are released from the liver upon damage (Josekutty et al., 2013). These examples notwithstanding, however, the contributions of IRE1α/TRAF2-dependent signaling to the sum of ER stress-mediated transcriptome control are poorly understood.

PERK also has the potential to recruit other molecules upon autophosphorylation and oligomerization, but there are fewer known parallel pathways arising from PERK activation than from IRE1α. PERK activation is necessary and sufficient for phosphorylation of the transcription factor NRF2 [Nuclear Factor (Erythroid-Derived 2)-Like 2] and can phosphorylate NRF2 *in vitro* (Cullinan et al., 2003). Cells lacking PERK or NRF2 do not effectively upregulate the NRF2-target genes *Gclc* or *Nqo1* upon ER stress (Cullinan and Diehl, 2004). PERK was also found to be required for activation of MAP kinase- and phospholipase C-dependent gene expression in response to ER calcium depletion (Liang et al., 2006a). However, as with IRE1α-dependent signaling modules, the global contribution of these pathways to mRNA regulation during ER stress is not clear.

TRANSCRIPTIONAL CASCADES

The output of the UPR is dramatically expanded by hierarchically arranged gene regulatory networks, in which the expression of subordinate transcription factors is targeted for regulation by the canonical UPR factors. Beyond conferring stress-responsiveness to genes not directly bound by ATF4, XBP1, or ATF6, this expansion also allows for transcriptional suppression, to the extent that ATF4, XBP1, or ATF6 enhance the expression of repressive transcription factors.

The best-characterized example of a secondary UPR-regulated transcription factor is C/EBP Homologous Protein (CHOP). CHOP is a direct target of ATF4 and ATF6 (Ma et al., 2002), and its translation is stimulated by eIF2α phosphorylation (Palam et al., 2011). Phosphorylation of CHOP by p38 MAP kinase appears to be required for its full activity (Wang and Ron, 1996). A member of the C/EBP family of transcriptional regulators,

CHOP can form heterodimers with other C/EBP proteins, and was proposed to act as a dominant-negative inhibitor of C/EBPα and β in particular (Ron and Habener, 1992). It is now clear that CHOP possesses both activating and repressing potential, and so its effect on the transcriptome is complex.

CHOP is strongly functionally associated with cell death; both cells and animals lacking CHOP are protected from a diverse array of stressful stimuli (Zinszner et al., 1998; Oyadomari and Mori, 2004). Due at least in part to the very short half-life of both the protein and its mRNA, CHOP expression is strongly correlated with the ER stress burden in real-time (Rutkowski et al., 2006). Hence, the window of time in which CHOP can exert a direct effect on the transcriptome is tightly controlled, as one might expect *a priori* for a factor that potentiates cell death. As a transcriptional regulator rather than a conventional pro-apoptotic effector, CHOP promotes cell death through the regulation of several classes of downstream genes. CHOP has been proposed to regulate the expression of both anti-apoptotic and pro-apoptotic genes of the *Bcl2* family (McCullough et al., 2001; Puthalakath et al., 2007). In addition, CHOP regulates expression of the bZIP factor ATF5, which is itself translationally regulated by eIF2α phosphorylation (Watatani et al., 2008; Zhou et al., 2008), and the targets of ATF5 include the pro-apoptotic protein NOXA (Teske et al., 2013). However, ChIP-seq analysis revealed that its direct targets are most prominently enriched for genes involved in protein synthesis, which are co-regulated by ATF4 (Han et al., 2013). Among these genes is GADD34, which indicates that CHOP controls a negative feedback loop allowing for the dephosphorylation of eIF2α and resumption of protein synthesis, even if the ER is ill-equipped to handle nascent protein influx (Marciniak et al., 2004). A consequence of restored protein synthesis is increased production of reactive oxygen species (ROS), likely resulting from oxidative protein folding in the ER; indeed, CHOP also regulates expression of the ER oxidase ERO1α (Li et al., 2009), indicating that CHOP promotes oxidative folding in tandem with increased ER influx. Blunting either ROS accumulation or protein synthesis neuters CHOP's pro-apoptotic potential (Marciniak et al., 2004; Malhotra et al., 2008; Li et al., 2009).

The strong association of CHOP with cell death in both cell and animal models raises the question of whether CHOP is intrinsically apoptotic, or instead whether this role is only manifested in the context of severe stress, masking other roles for CHOP in maintaining normal physiologic homeostasis. Indeed, the restoration of protein synthesis following stress is essential to maintain vital cellular functions, and the existence of a constitutive phosphatase ensures that even *Chop*$^{-/-}$ or *Gadd34*$^{-/-}$ cells are able to resume protein synthesis (Harding et al., 2009; Tsaytler et al., 2011); CHOP, therefore, merely accelerates the process, and it is possible that the kinds of stresses encountered in normal (i.e., non-pathologic) physiology are sufficiently mild that the benefits to cellular function of restoring protein synthesis outweigh the cost of increased ROS production. In addition, while CHOP induction is largely suppressed in some instances of physiological UPR induction such as B lymphocyte differentiation (Gass et al., 2002) and toll-like receptor ligation (Woo et al., 2009), it occurs in others such as feeding after a fast (Pfaffenbach et al., 2010).

CHOP likely contributes directly to the suppression of several metabolic transcriptional regulators during ER stress in the liver (Chikka et al., 2013). This finding suggests that CHOP might serve a role in regulating lipid metabolism *in vivo*, which is consistent with the steatosis observed in *Chop*$^{-/-}$ mice (Maris et al., 2012). To the extent that CHOP (or other secondary stress-regulated transcription factors) regulates metabolic transcription factors such as *Cebpa*, *Ppara*, and *Srebf1c*, there exists a multistep gene regulatory network during ER stress in hepatocytes that culminates in changes in the expression of genes encoding rate-limiting enzymes of intermediary metabolism (Rutkowski et al., 2008). CHOP also promotes inflammation (Maris et al., 2012; DeZwaan-McCabe et al., 2013; Malhi et al., 2013), although whether this is a consequence of direct CHOP action on inflammatory genes, or instead a secondary consequence of the other functions of CHOP is not yet clear.

The actions of CHOP on the transcriptome and on the accompanying physiological processes highlight the ability of the UPR to expand its reach through the regulation of secondary transcription factor expression, but CHOP is certainly not the only transcription factor whose transcription is regulated by ER stress. In fact, a search through several published microarrays from ER stress-treated MEFs (Marciniak et al., 2004; Wu et al., 2007; Rutkowski et al., 2008) reveals expression changes for several dozen transcription factors and cofactors, including both activators and repressors (**Table 1**). It follows that such transcriptional cascading will create a temporal hierarchy of gene regulation, with the earliest regulated genes being most proximally connected to UPR pathways. Such cascading likely contributes to the regulation of metabolic genes in the liver [(Arensdorf et al., 2013) *this issue*].

HETEROMERIC INTERACTIONS AND COFACTOR TITRATION

The major transcription factors of the UPR, both canonical and secondary, are bZIPs (ATF4, ATF6, XBP1, CHOP, JUN, ATF3, ATF5). The dozens of members of this family form homotypic and heterotypic dimers, typically within functionally related subclasses (Vinson et al., 2006). Thus, the complement of genes regulated during ER stress can be influenced by the formation of novel regulatory complexes not possible when the UPR is inactive, containing one UPR-regulated member and one constitutively expressed member. For instance, as a C/EBP family member, CHOP can interact with C/EBPα and C/EBPβ, altering the transactivation potential of each of these (Fawcett et al., 1996; Ubeda et al., 1996; Chiribau et al., 2010). Likewise, ATF6 was recently shown to interact with C/EBPβ upon stimulation with the inflammatory cytokine IFN-γ to transcriptionally upregulate the autophagy-promoting gene *Dapk1* (Gade et al., 2012).

Stress-regulated transcription factors can also influence global gene expression beyond the genes they directly regulate through the titration of coregulatory molecules shared with constitutive transcription factors. Such a mechanism was demonstrated recently for the coactivating factor CRTC2, which was shown to lose its costimulatory interaction with the gluconeogenic bZIP transcription factor CREB in favor of an interaction with ATF6 (Wang et al., 2009). The consequence of this titration was inhibition of hepatic gluconeogenesis during acute ER stress,

Table 1 | Transcription factors and cofactors whose mRNA expression is regulated by the UPR[a].

Likely activator	Likely repressor	Both activities demonstrated
UPREGULATED		
Aatf	*Cry1*	*Atf2*
Arnt1	*Cry2*	*Atf3*
Atf4	*Hey2*	*Cebpg*
Atf6	*Mybbp1a*	*Ddit3 (Chop)*
Ets2	*Sin3a*	*Myc*
Fubp1	*Zfp57*	*Nfil3*
Hoxa1		*Rbpj*
Hoxa11		
Myst4		
Nfya		
Nr4a2		
Rxrb		
Snip1		
Tfcp2		
Zbtb7b		
DOWNREGULATED		
Foxq1	*Id1*	*E2f8*
Nfkbiz	*Nr1d1*	*Elk3*
	Nfkbia	*Hipk2*
		Stat3

[a] *1.5-fold or more, $p < 0.05$, in at least 2 of the arrays described in Marciniak et al. (2004); Wu et al. (2007); and Rutkowski et al. (2008).*

and gluconeogenesis and hyperglycemia could be suppressed in diabetic animals by ATF6 overexpression.

ATF6 has also been shown to interact with the coactivator PGC-1α. In skeletal muscle, this interaction promoted the full upregulation of canonical UPR target genes upon exercise (Wu et al., 2011). Likewise, a PGC-1α/ATF6 interaction stimulated expression of the ERRγ orphan nuclear receptor/transcription factor in a hepatocyte cell line (Misra et al., 2013). Roles for ERRγ in glucose and alcohol metabolism in the liver have recently emerged (Kim et al., 2012, 2013). PGC-1α has been implicated in the transcriptional regulation of many key metabolic processes including gluconeogenesis, fatty acid oxidation, and mitochondrial biogenesis, based on its ability to coactivate a number of transcription factors (Lin et al., 2005). Therefore, it is possible that ER stress will disrupt or otherwise influence the regulation of such gene networks based on competition for PGC-1α binding, although this has not yet been demonstrated.

EXPANSION OF UPR SIGNALING PATHWAYS

The status of PERK, IRE1, and ATF6 as canonical UPR regulators arises from the primacy of their early discoveries and their ubiquity. However, a number of other stress-signaling pathways have since been discovered that extend the scope of the UPR in both general and context-specific ways.

While IRE1α is ubiquitously expressed, its paralog IRE1β, expressed in mucin-producing cells of the gut (Bertolotti et al., 2001) and airway (Martino et al., 2013), was identified around the same time (Wang et al., 1998). Like its paralog, IRE1β can catalyze the splicing of *Xbp1* (Calfon et al., 2002). Mice lacking IRE1β are sensitive to colitis induced by dextran sodium sulfate challenge (Bertolotti et al., 2001), as are animals lacking XBP1 (Kaser et al., 2008), ATF6, or the ER cochaperone $p58^{IPK}$/ERDJ6 (Cao et al., 2013). The phenotypic similarities among these animals suggests that, like its paralog, IRE1β contributes to ER homeostasis largely through *Xbp1* splicing and upregulation of the ER folding and quality control machinery. However, the endonuclease domain of IRE1β displays less activity toward *Xbp1* than does that of IRE1α and has an enhanced specificity for *28s* rRNA, which can contribute to suppression of protein synthesis (Imagawa et al., 2008). In addition, in contrast to *Ire1*$\alpha^{-/-}$ animals, *Ire1*$\beta^{-/-}$ mice showed elevated expression of *mucin 2* mRNA, impaired MUC2 secretion, and exacerbated ER stress, including, paradoxically, increased *Xbp1* splicing (Tsuru et al., 2013). These findings suggest that IRE1α and β have at least partially separable functions, and raise the question of how strong a contribution IRE1β makes to mRNA regulation in the cells where it is expressed.

IRE1β aside, the reach of the UPR has been most expanded by the discovery of ER localized proteins that, like ATF6, are activated by regulated intramembrane proteolysis. First among these was a paralog of ATF6 known as ATF6β (ATF6 is also known as ATF6α) that is 36 percent identical to ATF6 over 93 percent of its length (Haze et al., 2001). Cells lacking ATF6β show no apparent defect in upregulation of canonical UPR target genes (Yamamoto et al., 2007). However, like ATF6, ATF6β binds to ERSE sequences in conjunction with NFY (Yoshida et al., 2001a). Mice lacking both ATF6 and ATF6β die during embryogenesis (Yamamoto et al., 2007), as do similarly manipulated medaka fish (Ishikawa et al., 2012). Overexpression of *Bip* could partially rescue impaired notochord development in these fish, suggesting that ATF6 and ATF6β converge on chaperone mRNA regulation, albeit with somewhat different kinetics (Haze et al., 2001). Whether ATF6β regulates the expression of any unique genes is not yet known.

In addition to ATF6 and ATF6β, there are at least 5 additional ER-resident transmembrane bZIP transcription factors that are cleaved by RIP, including CREBH, Luman, OASIS, BBF2H7, and CREB4 [reviewed in (Asada et al., 2011)]. These 5 proteins are not highly homologous to each other or to ATF6, and each is expressed in a unique subset of tissues, but they are all known or thought to be cleaved by S1P (Raggo et al., 2002; Murakami et al., 2006; Stirling and O'Hare, 2006; Zhang et al., 2006). These proteins can be activated by conventional ER stress and/or can regulate the expression of UPR target genes through traditional ER stress-responsive *cis*-acting sequences (Kondo et al., 2005; Liang et al., 2006b; Stirling and O'Hare, 2006; Zhang et al., 2006; Kondo et al., 2007). However, they may be activated more strongly by physiological signals that influence ER homeostasis in ways other than the simple perturbation of or excess demand upon the protein folding machinery, and might also be retained in the ER by distinct mechanisms; at least CREBH appears to be retained by virtue of its cytosolic membrane-proximal segment rather than by lumenal *Bip* binding as for ATF6 (Llarena et al., 2010). In addition, they also appear to regulate expression of distinct sets

of genes, suggesting that they diversify the responsiveness of the UPR and the scope of genes that it regulates in various tissues rather than simply augmenting these processes.

The predominantly liver-specific RIP substrate CREBH illustrates the complex relationship between these substrates and the canonical UPR. *Crebh* mRNA is upregulated by conventional ER stress in the liver, and cleavage of the protein is modestly stimulated as well; the cleaved form is capable of associating with ATF6 and upregulating *UPRE*- or *ERSE*-dependent reporters (Zhang et al., 2006). However, both its expression and cleavage are also strongly induced by inflammatory stimuli such as LPS exposure or IL-6 treatment, and the genes encoding the inflammatory modulators CRP and SAP were also identified as likely CREBH targets. While inflammatory stimuli such as LPS appear capable of inducing ER stress, they spawn a modified eIF2α-independent response (Woo et al., 2009), suggesting that, even if CREBH is activated simply by the accumulation of unfolded proteins in the ER, it would be so in the context of a modified UPR. CREBH also regulates the expression of hepicidin, leading to dysregulation of iron homeostasis upon ER stress in wild-type mice but not *Crebh*$^{-/-}$ animals (Vecchi et al., 2009). Therefore, CREBH can also contribute to the expansion of mRNA expression even during exposure to a conventional ER stressor. More recently, CREBH was shown to directly regulate genes involved in gluconeogenesis (Lee et al., 2010) and lipid metabolism (Zhang et al., 2012). The latter of these processes showed a stronger CREBH dependence in the context of a high-fat atherogenic diet, raising the question of the extent to which physiological stimuli such as obesity impact gene expression through the canonical UPR vs. through pathways that enlist the action of molecules like CREBH to produce a unique response.

Knockout of each of the RIP substrates [or, in the case of Luman, a regulator of the pathway, LRF; (Martyn et al., 2012)] yields a discrete phenotype attributable to dysfunction of the major tissue in which the substrate is expressed (Asada et al., 2011). In contrast, mice lacking ATF6, which have compromised ER chaperone induction upon ER stress, show no apparent basal phenotype (Wu et al., 2007; Yamamoto et al., 2007). Therefore, simple failure to upregulate ER chaperones to the maximum extent is not sufficient to elicit a phenotype, making it unlikely that the RIP substrates merely augment chaperone induction; more likely, the phenotypes induced by their deletion are attributable to non-redundant actions on specific genes, be they chaperones or something else entirely. However, their activation signals and the global effects on transcriptome regulation remain unclear.

mRNA STABILITY

Although each UPR pathway culminates in production of a transcription factor, mRNA abundance can just as readily be regulated by enhanced or diminished stability. Indeed, one estimate of the relative contribution of transcriptional and post-transcriptional mechanisms to mRNA abundance during ER stress—based on the comparison of mRNA levels in nuclear run-off assays against total mRNA pools—suggested that ∼75 percent of mRNAs were regulated at least in part at the level of stability (Kawai et al., 2004). Pathways linking ER stress to mRNA stability are much less understood than transcriptional mechanisms, but are emerging as important influences on UPR output and physiological responses.

GENE REGULATION BY RIDD

A subset of ER-localized mRNAs are degraded directly by the endonuclease activity of IRE1 in a process called regulated IRE1-dependent decay (RIDD). The process was first observed in *Drosophila* cells, in which a subset of mRNAs was rapidly suppressed by ER stress in an IRE1α-dependent but XBP1-independent manner (Hollien and Weissman, 2006). This group of mRNAs was highly enriched for those encoding proteins with in-frame ER targeting sequences, but the cleavage of these mRNAs was otherwise non-sequence-specific. It is possible that RIDD represents an ancient activity of IRE1, since Ire1p in the fission yeast *S. pombe* catalyzes a RIDD activity but not a *HAC1* splicing activity (Kimmig et al., 2012). More recently, a sequence necessary for mRNA cleavage by IRE1α *in vitro* was identified (Oikawa et al., 2010), although it is not yet clear whether these findings extend to RIDD targets *in vivo* as well. These findings led to the idea that activated IRE1α directly cleaves some mRNAs that are brought to proximity with the ER membrane by virtue of their association with translating ribosomes synthesizing signal peptide- or signal anchor-encoding proteins. This finding was subsequently extended to mammalian cells (Han et al., 2009; Hollien et al., 2009). The RIDD and *Xbp1* splicing activities of IRE1α are functionally separable (Han et al., 2009; Hollien et al., 2009), implying that each process plays a distinct role in UPR-mediated control over the transcriptome. Where these functions overlap—i.e., in the expression of ER chaperones and other ER-localized proteins that facilitate recovery from stress but whose mRNAs are RIDD targets by virtue of their localization—the transcriptional induction must be sufficient to overcome degradation by RIDD.

Although the RIDD pathway has not yet been as well characterized in mammalian cells, its targets in that context are involved in processes ranging from signaling cascades (e.g., *Pdgfrb*, *Efnb2*, *Ncam1*, *Raptor*) to transcription (e.g., *Pbxip1*, *Hoxb4*, *Srsf3*) to lysosomal degradation (e.g., *Bloc1s1*, *Tpp1*, *Hgsnat*) to xenobiotic metabolism (Cytochrome p450s-encoding genes) and energy production (e.g., *Oxct1*) (Hollien et al., 2009; Hur et al., 2012). RIDD appears now not to be solely limited to genes encoding ER-translocated proteins but includes mRNAs encoding cytosolic factors as well (Hollien et al., 2009; Oikawa et al., 2010; Ventoso et al., 2012). This ability of the ER-tethered IRE1α to degrade mRNAs encoding cytosolic proteins might arise from the localization of specific cytosolic mRNAs in the process of translation to the ER membrane (Stephens et al., 2005); indeed, localization of an mRNA to the ER membrane, irrespective of whether it encodes a protein of the endomembrane system, appears sufficient to target that mRNA for RIDD for the large majority of mRNAs, at least in insect cells (Gaddam et al., 2012). In addition, even mRNAs not stably associated with the ER membrane can still be targeted for RIDD if they contain an Xbp1-like stem-loop structure that allows them to associate directly with IRE1 (Moore et al., 2013).

Irrespective of whether RIDD acts on specific mRNAs or more generally on most of those associated with the ER membrane, its activation has distinct physiological consequences. IRE1β (but not α) might control efflux of absorbed lipids through its RIDD activity. Lipids absorbed from the diet are packaged by enterocytes into lipoprotein particles known as chylomicrons, and a key step in this packaging is lipidation of apolipoproteins in the ER by microsomal triglyceride transfer protein (MTTP) (Hussain, 2000). IRE1β was found to cleave *Mttp* mRNA, and *Ire1*β$^{-/-}$ mice on a high fat diet had elevated MTTP expression, elevated chylomicron production, and hyperlipidemia (Iqbal et al., 2008). The RIDD pathway also can regulate lipid metabolism in the liver; IRE1α-dependent degradation of genes involved in lipogenesis and lipoprotein synthesis was elicited as a feedback mechanism in mice lacking XBP1 (So et al., 2012). An analogous pathway of feedback elicited IRE1α-dependent suppression of proinsulin processing in pancreatic β cells of *Xbp1*$^{-/-}$ mice (Lee et al., 2011). These findings are consistent with the idea that RIDD is most active on ER-localized mRNAs, since lipoprotein formation, lipogenesis, and proinsulin processing all take place in the ER or at the ER membrane. A conservative estimate puts the frequency of RIDD targets at ∼5 percent of all ER stress-regulated mRNAs in cultured mammalian cells (Hollien et al., 2009), and it might in fact be substantially higher (Gaddam et al., 2012). Whether RIDD is more or less active during physiological stimuli *in vivo* and whether its specificity for certain groups of substrates can be meaningfully regulated are not yet understood.

mRNA REGULATION BY miRNAs

Another common source of mRNA regulation occurs through microRNAs (miRNAs). miRNAs are short (∼22 nt) single-stranded RNAs which bind to complementary mRNAs and promote their degradation or, less frequently, inhibit their translation (Valencia-Sanchez et al., 2006). The UPR-mediated regulation of miRNA is a rapidly emerging area of investigation and a potential mechanism for fine-tuning of mRNA abundance. The pathways leading from UPR activation to miRNA regulation and the consequences of this regulation for mRNA abundance and downstream cellular processes have been reviewed extensively in (Maurel and Chevet, 2013), to which we direct the reader for details. Each of the three canonical UPR pathways transcriptionally regulates the expression of discrete miRNAs (Bartoszewski et al., 2011; Belmont et al., 2012; Byrd et al., 2012; Chitnis et al., 2012; Gupta et al., 2012). To date, the best-described functions of ER stress-regulated miRNAs are in tuning UPR sensitivity (Byrd et al., 2012; Maurel et al., 2013; Zhang et al., 2013) or in regulating cell proliferation and apoptosis during stress (Chitnis et al., 2012; Duan et al., 2012; Gupta et al., 2012; Muratsu-Ikeda et al., 2012).

As an emerging area of study, the physiological roles of ER stress-mediated miRNA regulation are largely unknown; however, it was recently demonstrated that IRE1α activation causes degradation of miR-17, which in turn leads to upregulation of the miR-17 target mRNA encoding thioredoxin-interacting protein (TXNIP) (Lerner et al., 2012). TXNIP promoted inflammasome assembly, caspase-1 activation, and cell death, and *Txnip*$^{-/-}$ mice were protected from pancreatic β cell death induced by production of misfolded insulin. Given the ability of miRNAs to coordinately regulate the stability of many target mRNAs, it seems likely that this mechanism will emerge as a major contributor to noncanonical UPR output, and that further physiological roles will be discovered.

ER stress has been associated with changes in the stability of individual mRNAs through undetermined mechanisms (Pereira et al., 2010; Park et al., 2012), and so it is possible that other pathways for regulating mRNA turnover exist as well. One suggested mechanism is the sequestration of translationally inhibited mRNAs in stress granules (Kimball et al., 2003). mRNA stabilization through sequestration would thus decouple mRNA abundance from protein abundance (Kawai et al., 2004). This possibility illustrates the caution that must be exercised when interpreting changes in mRNA abundance in general: mRNA and protein expression are only loosely correlated (Gygi et al., 1999), and understanding the mechanisms of mRNA regulation by the UPR only illuminates one component in the regulation of gene expression.

A WAY FORWARD: DISSECTING UPR-RESPONSIVE GENE REGULATORY NETWORKS

The abundance of non-canonical mechanisms of mRNA regulation by the UPR suggest that the number of mRNAs whose regulation is attributable to direct binding by XBP1, ATF4, or ATF6 is likely to represent only a small portion of all the regulated genes. How, then, can the complex gene regulatory network of the UPR be best studied moving forward? The explosion of high throughput methodology and increasingly sophisticated bioinformatic tools holds promise for both "top-down" and "bottom-up" approaches to this problem. The application of microarray technology to the yeast UPR first revealed the complexity of the transcriptional response (Travers et al., 2000); similar microarray-based approaches (Harding et al., 2003; Lee et al., 2003b; Wu et al., 2007) in mammals revealed the dependencies of subsets of genes on each UPR pathway, but could not separate direct from indirect influences. Next-generation sequencing methodologies, including mRNA-seq and ChIP-seq, will now be used to piece together regulatory hierarchies; these techniques were recently combined to elucidate the gene networks regulated by XBP1 (Acosta-Alvear et al., 2007) and CHOP and ATF4 (Han et al., 2013). A complementary approach will be to find groups of genes that are coordinately regulated and use bioinformatic analysis to predict previously hidden upstream regulators. As proof-of-principle, we have shown that the temporal organization of metabolic gene regulation upon ER stress in the liver identifies the transcription factor HNF4α as a key link between UPR activation and the expression of genes involved in lipid metabolism [(Arensdorf et al., 2013); this issue]. Such functional genomics approaches have until recently been restricted to studies in simple organisms like yeast. However, the ability to probe and experimentally manipulate the entire mammalian genome has now made these techniques feasible in higher eukaryotes as well (Kampmann et al., 2013), and these approaches have been used to understand secretory pathway function (Bassik et al., 2013) and the ERAD network (Christianson et al., 2011).

Although best known as the gateway to the secretory pathway, the ER participates in many cellular processes that have little or nothing to do with protein folding *per se*. While augmentation of the ER protein folding capacity is certainly a significant consequence of UPR activation, it remains to be seen whether most of the genes regulated by the UPR ultimately redound to this capacity, or whether the UPR has been co-opted in the homeostatic regulation of other cellular processes—particularly those, such as lipid metabolism, that involve the ER. Deciphering the pathways leading from UPR activation to mRNA regulation will allow the functional significance of non-canonical UPR signaling mechanisms to be understood in the contexts of normal and pathological physiology.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fgene.2013.00256/abstract

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Physiological roles of regulated Ire1 dependent decay

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Inositol-requiring enzyme 1 (Ire1) is an important transducer of the unfolded protein response (UPR) that is activated by the accumulation of misfolded proteins in the endoplamic reticulum (ER stress). Activated Ire1 mediates the splicing of an intron from the mRNA of Xbp1, causing a frame-shift during translation and introducing a new carboxyl domain in the Xbp1 protein, which only then becomes a fully functional transcription factor. Studies using cell culture systems demonstrated that Ire1 also promotes the degradation of mRNAs encoding mostly ER-targeted proteins, to reduce the load of incoming ER "client" proteins during ER stress. This process was called RIDD (regulated Ire1-dependent decay), but its physiological significance remained poorly characterized beyond cell culture systems. Here we review several recent studies that have highlighted the physiological roles of RIDD in specific biological paradigms, such as photoreceptor differentiation in *Drosophila* or mammalian liver and endocrine pancreas function. These studies demonstrate the importance of RIDD in tissues undergoing intense secretory function and highlight the physiologic role of RIDD during UPR activation in cells and organisms.

Keywords: Ire1, Xbp1, RIDD, endoplasmic reticulum stress, unfolded protein response

ENDOPLASMIC RETICULUM STRESS AND THE UNFOLDED PROTEIN RESPONSE

The endoplasmic reticulum (ER) is the entry site for the secretory pathway; all proteins targeted to the plasma membrane, extracellular space, and some organelles are translated into the ER, where they are folded and modified (Cooper, 2000). Proteins that fail to fold into their native conformation are targeted for ER-associated degradation (ERAD). Proteins marked as terminally misfolded are dislocated to the cytoplasm, where they are degraded by the ubiquitin−proteasome system (Smith et al., 2011; Claessen et al., 2012).

A number of cellular stress conditions such as low glucose levels, redox stress, or abnormal ER calcium content may perturb protein maturation in the ER or interfere with the capacity of the folding machinery in the ER (Marciniak and Ron, 2006). Many physiological processes may further challenge the ER by imposing suddenly large amounts of "client" proteins (Moore and Hollien, 2012). The imbalance between the ER folding capacity and the burden of incoming proteins may lead to the accumulation of misfolded proteins, causing ER stress. Adaptation to ER stress is mediated by the unfolded protein response (UPR; Ron and Walter, 2007; Hetz, 2012; Gardner et al., 2013). The UPR is a collection of integrated signaling pathways activated by ER-localized transmembrane protein sensors, which have luminal domains that detect misfolded proteins in the ER and cytoplasmatic effector domains that transduce signaling to the transcriptional and/or translational apparatus.

The UPR was first described in budding yeast, where it is represented by a single linear pathway (Mori et al., 1992). In higher eukaryotes the UPR is more complex and is mediated by three ER transmembrane sensors: pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (Ire1; Harding et al., 2002). The UPR outcomes are temporally coordinated: first, translation is attenuated to reduce the load of proteins into the ER; second, genes encoding ER chaperones and enzymes are up-regulated to increase the ER folding capacity; and third, genes encoding ERAD components are induced to enhance degradation of misfolded proteins from the ER.

If the mechanisms activated by the UPR are insufficient to decrease ER stress and restore ER homeostasis, cells may undergo apoptosis (Rasheva and Domingos, 2009). Chronic ER stress and defective activation of the UPR have been involved in the pathology of several human diseases, such as cancer, diabetes, neurodegenerative disorders, and chronic inflammation (Wang and Kaufman, 2012). Therefore, there has been increasing interest in controlling the ER stress pathways and discover new therapeutic targets to treat these diseases.

Ire1 SIGNALING

Being the most evolutionarily conserved arm of the UPR, Ire1 is a type I ER-resident transmembrane protein with a ER luminal dimerization domain and a cytoplasmic domain with Ser/Thr kinase and endoribonuclease activities (**Figure 1**; Cox et al., 1993; Mori et al., 1993; Shamu and Walter, 1996; Tirasophon et al., 1998; Wang et al., 1998; Liu et al., 2000; Koizumi et al., 2001; Shen et al., 2001; Korennykh and Walter, 2012).

In the budding yeast the only known substrate of Ire1 is the mRNA of the bZIP transcription factor Hac1 (Cox and Walter, 1996; Mori et al., 1996; Nikawa et al., 1996). In case of ER stress, Ire1 associates in oligomers after Binding immunoglobulin protein (BiP) release and activates its RNase domain by autophosphorylation (Shamu and Walter, 1996; Welihinda and Kaufman, 1996;

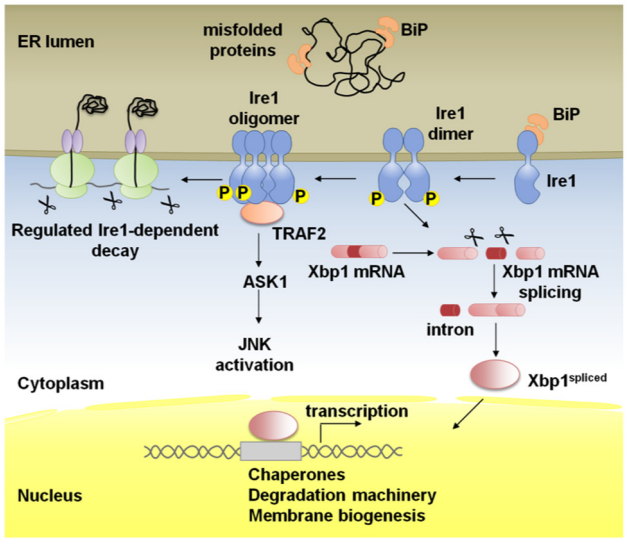

FIGURE 1 | Schematic representation of Ire1 signaling. Binding immunoglobulin protein (BiP) binds Ire1 luminal domain and maintains it in a monomeric inactive form. In stressed cells, BiP is recruited to misfolded proteins and Ire1 is activated following conformational changes induced by dimerization of monomers in the plane of the membrane and trans-autophosphorylation. Higher order oligomers, which might form upon additional stress stimuli, reinforce Ire1 RNase activity. Activated Ire1 mediates the splicing of Xbp1 mRNA in higher eukaryotes (or Hac1 in yeast). Splicing of the intron from the Xbp1 transcript results in a frame-shift and the production of a potent transcription factor, Xbp1-spliced, that regulates many UPR target genes to promote protein folding in the ER lumen, ER-associated degradation (ERAD) of misfolded proteins and ER biogenesis. Ire1 can also act by alternative pathways; phosphorylated Ire1 associates with TRAF2 and activates the JNK pathway via ASK1. In the regulated Ire1 dependent decay (RIDD) pathway, Ire1 degrades mRNAs localized to the ER membrane through its RNase activity leading to a reduction in the amount of proteins imported into the ER lumen.

Liu et al., 2000; Papa et al., 2003; Lee et al., 2008; Korennykh et al., 2009, 2011a). Activated Ire1 recognizes a double stem loop flanking a 252bp intron in Hac1 mRNA and cleaves it twice (Sidrauski and Walter, 1997; Korennykh et al., 2011b), while a transfer RNA ligase joins the exons (Sidrauski et al., 1996). This Ire1-mediated unconventional splicing event releases the translational repression exerted by the 252bp intron and allows the $Hac1^{spliced}$ protein to act as a transcription factor (Chapman and Walter, 1997; Rüegsegger et al., 2001). The functional homolog of Hac1 in mammals is Xbp1 (Yoshida et al., 2001; Calfon et al., 2002), which is also only active as a transcription factor after the Ire1-mediated splicing of the Xbp1 mRNA. In this case, however, $Xbp1^{unspliced}$ is translated and originates a protein that is rapidly degraded (Calfon et al., 2002; Yoshida et al., 2006).

Genetic profiling and analyses revealed that Hac1/Xbp1 control the expression of genes related to the UPR including chaperone induction, up-regulation of ERAD machinery, membrane biogenesis, and ER quality control (Lee et al., 2003; Shaffer et al., 2004; Shoulders et al., 2013). In mammals, Xbp1 also activates the expression of cell type specific targets linked to cell differentiation, signaling, and DNA damage (Acosta-Alvear et al., 2007; Lee et al., 2003).

TARGETING OF mRNAs TO Ire1

The mechanism of recruitment of Hac1/Xbp1 mRNAs to the ER membrane seems to differ considerably between yeast and mammals. Under non-stressed conditions, unspliced Hac1 mRNA is found mostly in the cytoplasm, in association with stalled ribosomes. Upon ER stress, Hac1 mRNA is recruited to Ire1 clusters in the ER membrane, in a process that depends on translational repression and on a bipartite element (BE) present at the 3′ untranslated region of the Hac1 mRNA (Aragón et al., 2009).

In mammals, the $Xbp1^{unspliced}$ mRNA is translated under normal conditions and originates a protein that associates with membranes via two hydrophobic regions (HR1 and HR2). The HR2 is a conserved region predicted to form a α-helix that has propensity to interact with the lipid bi-layer of the membrane (Yanagitani et al., 2009, 2011). Presumably, upon Xbp1 mRNA translation, HR1 and HR2 on the nascent polypeptide associate with the ER membrane and bring the Xbp1 mRNA-ribosome-nascent chain (RNC) complex to the vicinity of Ire1, facilitating Ire1-mediated splicing of Xbp1 mRNA.

Xbp1 INDEPENDENT FUNCTIONS OF Ire1

Non-overlapping defects in Ire1 or Xbp1 mutant *Caenorhabditis elegans* first supported the existence of alternative roles for Ire1, besides Xbp1 mRNA splicing (Shen et al., 2005). Ire1 is thought to regulate apoptosis, autophagy, and ERAD through interaction with cytoplasmic partners independently of its RNase activity (Hetz and Glimcher, 2009). The cytosolic domain of Ire1 interacts with Traf2 (TNFR-associated factor 2) and activates ASK1 (Apoptosis signal-regulating kinase 1), triggering the JNK (cJun-N terminal kinase) pathway (Urano et al., 2000; Nishitoh et al., 2002). This Ire1/Traf2 interaction may lead to the activation of apoptosis under irreversible ER stress (Mauro et al., 2006). Ire1 may also control levels of autophagy under ER stress through activation of the JNK pathway (Ogata et al., 2006). The phosphorylation of the anti-apoptotic BCL2 at the ER by JNK stimulates autophagy by modulating the activity of Beclin1. Dissociation of the complex formed by BCL2 and Beclin1 induces autophagy (Pattingre et al., 2009).

The cytoplasmic domain of unphosphorylated (inactive) Ire1 physically interacts with the ubiquitin specific protease 14 (USP14), and this association is inhibited by ER stress and Ire1 activation (Nagai et al., 2009). USP14, which is recruited to the ERAD machinery via interaction with Ire1α, inhibits ERAD through a deubiquitination-independent mechanism (Nagai et al., 2009). Finally, non-muscle myosin IIB interacts with Ire1 during ER stress, revealing that interaction of Ire1 with the cytoskeleton is required for optimal regulation of Ire1 activity (He et al., 2012).

REGULATED Ire1-DEPENDENT DECAY (RIDD)

A breakthrough report uncovered that the Ire1 RNase has broad range of mRNA substrates besides Xbp1 mRNA in *Drosophila* S2 cells. The group of Jonathan Weissman found through gene profiling experiments that a subset of mRNAs are degraded during ER stress by a mechanism that is dependent on Ire1 but not Xbp1 (Hollien and Weissman, 2006). The degraded mRNAs encoded mostly proteins with signal peptide/transmembrane domains that

would represent an additional challenge to the ER folding machinery under ER stress. This mechanism was named RIDD, for Regulated Ire1-Dependent Decay, and was later also described in mammalian cells and in the fission yeast (which lacks any Hac1/Xbp1 homolog; Hollien et al., 2009; Cross et al., 2012; Kimmig et al., 2012). While in *Drosophila* S2 cells RIDD down-regulates many RNAs by 5−10 fold, in mammals the magnitudes of the changes in expression were smaller, twofold or less for many targets (Hollien and Weissman, 2006; Hollien et al., 2009).

The mechanism of targeting mRNAs to RIDD seems to have diverged throughout evolution (Hollien, 2013). In *Drosophila* S2 cells, RIDD has a broad scope of targets and there is a strong correlation between interaction of a mRNA with the ER membrane and the extension of its degradation by RIDD (Hollien and Weissman, 2006; Gaddam et al., 2013). In fact, deletion of the signal peptide from a known RIDD target prevents its degradation and conversely, addition of a signal peptide to GFP is sufficient to promote its degradation by RIDD. One interesting exception is the mRNA of PlexinA, which is strongly associated with the ER membrane, but it is protected from RIDD and is continuously translated, even during ER stress (Gaddam et al., 2013). PlexinA mRNA has regulatory upstream ORFs, which are necessary for its protection from RIDD. Another interesting exception is the mRNA encoding Smt3, a homolog of SUMO (small ubiquitin-like modifier), which is cleaved by RIDD on a stem loop structure, despite not being stably associated to the ER membrane (Moore et al., 2013).

In mammalian cells, RIDD targets are enriched for mRNAs containing a cleavage site with a consensus sequence (CTGCAG) and a predicted secondary structure similar to the conserved Ire1 recognition stem−loop of the Xbp1 mRNA (Han et al., 2009;Oikawa et al., 2010; Hur et al., 2012). Deletion of the stem−loop or mutagenesis of the conserved bases abrogated RIDD (Oikawa et al., 2010).

PHYSIOLOGICAL RELEVANCE OF RIDD

The physiological relevance of RIDD has been recently demonstrated in several different biological models, with specific cellular and/or developmental conditions. RIDD has a role controlling the expression of lipogenic enzymes and modulating levels of lipids in the serum. Ire1β, which is specifically expressed in the epithelial cells of the gastrointestinal tract, has a protective role against hyperlipidemia in mice fed with a high fat or high cholesterol diet by decreasing the absorption of lipids in the intestine (Iqbal et al., 2008). Ire1β promotes the post-transcriptional degradation of the ER chaperone microsomal triglyceride transfer protein (MTP), involved in the assembly of apolipoproteins B and biosynthesis of chylomicrons (Iqbal et al., 2008). In the liver, Xbp1 deficiency provokes Ire1α hyperactivation, which contributes to a hypolipogenic phenotype in mice characterized by reduced plasma cholesterol and triglycerides (So et al., 2012). A comprehensive comparative microarray analysis identified 112 genes induced by Ire1α siRNA treatment in Xbp1-deficient mice. Among these genes are the ones encoding Angiopoietin-like protein 3 (Angptl3) and ces1 genes, which are involved in lipid metabolism and were further validated as RIDD substrates (So et al., 2012). The targeting of Xbp1 may be a therapeutic approach in dyslipidemic diseases, as Xbp1 deficiency in the liver, in leptin-deficient ob/ob mice, lowers hepatic triglycerides and plasma cholesterol levels (So et al., 2012).

Ire1β was also found to have a homeostatic role in the secretory goblet cells of the intestine through the down-regulation of mRNA levels of the major secretory product mucin 2 (Tsuru et al., 2013). The knock-out of Ire1β isoform in mouse colon results in disorganization of the ER in the goblet cells at early stages of maturation with accumulation of a precursor form of mucin 2 in the expanded ER lumen and induction of ER stress. Remarkably, Ire1α seems to have a distinct role in goblet cells mediating Xbp1 splicing and promoting the activation of UPR targets like BiP (Tsuru et al., 2013). Ire1α has a protective role in rodents against the liver damage caused by an overdose of the analgesic drug acetaminophen through the degradation of the mRNA of two P450 enzymes, Cyp1a2 and Cyp2e1, that are responsible for metabolizing the drug into a cytotoxic metabolite (Hur et al., 2012). Again, hyperactivation of Ire1α, caused by the liver specific deletion of Xbp1, prevents prolonged JNK activation and improves the morphology of the liver in mice injected with acetaminophen (Hur et al., 2012).

Several studies demonstrate that Ire1α plays an important role regulating pancreatic β-cells homeostasis by controlling the levels of insulin synthesized in the ER. Treatment of β cells with high levels of glucose hyperactivates Ire1, which correlates with a decrease of insulin mRNA expression (Lipson et al., 2008). Later, Ire1 was shown to cleave Insulin 1 and Insulin 2 mRNAs at specific sites *in vitro* (Han et al., 2009). Chronic stimulation of β-cells with high glucose concentrations might impose insurmountable levels of ER stress and promote the shift from a protective response (Xbp1 splicing and up-regulation of chaperones) to a deleterious response (RIDD and degradation of insulin). Supporting this hypothesis, islets from mice heterozygous for Ire1α are more resistant to chronic high glucose and had higher gene expression for both Insulin 1 and Insulin 2 (Lipson et al., 2008). Deletion of Ire1 may be beneficial in the case of diabetes type II models.

Maturation of insulin is also inefficient in β-cells deficient for Xbp1 due in part to RIDD. Ire1α is activated by Xbp1 silencing in Min6 insulinoma cells and activated Ire1α reduces the levels of components of the insulin secretory pathway, namely PC1, PC2, and CPE enzymes, by cleaving the respective mRNAs (Lee et al., 2011). Xbp1 deficient islets of the pancreas present morphological abnormalities, including disorganized structure, few insulin granules, and distended ER, consistent with Xbp1 being required for expression of ER chaperone genes such as BiP (Hspa5), ERdj4 (Dnajb9), and p58IPK (Dnajc3; Lee et al., 2011).

Regulated Ire1-dependent decay was also associated with innate and adaptive immunity. Ire1 is activated by binding part of the cholera toxin to induce an inflammatory response (Cho et al., 2013). In this case, Xbp1 is dispensable for signaling but RIDD is required for the activation of RIG-1 (retinoic acid inducible gene 1), NF-kB and interferon pathways (Cho et al., 2013). RIDD is necessary in $CD8\alpha^+$ dendritic cells for cross-presentation of cell-derived antigens via MHC-class I to CD8+ T cells (Osorio et al., 2014). Xbp1 is necessary to maintain a normal morphology of the ER in $CD8\alpha^+$ conventional dendritic cells, whereas RIDD has a critical function in regulating the expression of

integrins and components of the major histocompatibility complex class I antigen-presentation machinery in these cells (Osorio et al., 2014). Moreover, RIDD is also active in B cells, where it cleaves the mRNA of secretory μ chains (Benhamron et al., 2014).

Regulated Ire1-dependent decay has an essential role in the differentiation of the photoreceptor cells in *Drosophila* to maintain the homeostasis of the secretory pathway during the morphogenesis of the light gathering organelle, the rhabdomere, which depends on the massive synthesis of membrane and proteins during the second half of pupal development (Coelho et al., 2013). Ire1 mutant photoreceptors show a very dramatic phenotype with atrophy of the rhabdomeres and collapse of the interrhabdomeral space (caused by a defect in the delivery of Rhodopsin1 and spacemaker protein, respectively), resulting in progressive degeneration of the retina in adult flies (Coelho et al., 2013). Remarkably, Xbp1 mutant photoreceptors show rhabdomeres with almost a normal morphology, evidencing Xbp1-independent roles of Ire1 in this specific biological context (Coelho et al., 2013). RIDD is activated in the *Drosophila* photoreceptors and promotes the degradation of several mRNA substrates (**Figure 2**), among them Fatp (Fatty acid transport protein), a previously described regulator of Rhodopsin1 levels in photoreceptor cells (Dourlen et al., 2012). Fatp mediates the uptake of fatty acids into cells and fatty acids are precursors for the biosynthesis of phosphatic acids. Increased levels of phosphatic acids were previously shown to disrupt rhabdomere morphogenesis (Raghu et al., 2009), causing a phenotype very similar to the one of Ire1 mutant photoreceptors.

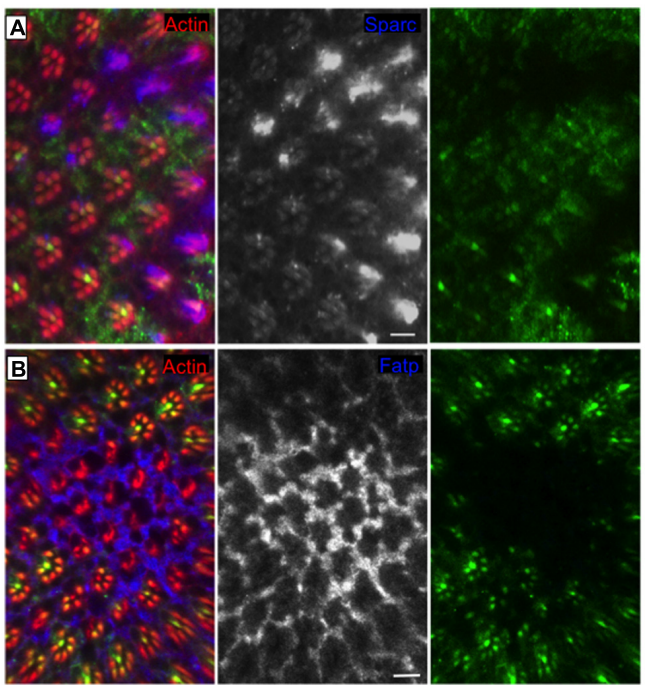

FIGURE 2 | RIDD "in action" in the *Drosophila* eye. Clones of cells homozygous for Ire1 mutant chromosome (*PBac{WH}Ire1*^{f02170}) are labeled by the absence of myrGFP. The protein levels of **(A)** Sparc (blue and monochrome) and **(B)** Fatp (blue and monochrome) are elevated in Ire1 mutant tissue, in comparison with the surrounding control tissue. Sparc and Fatp are two RIDD substrates in the *Drosophila* eye. Actin is in red. Scale bars represent 10 μm. Adapted from Coelho et al. (2013).

Under conditions of overwhelming ER stress induction or chronic low level stress mRNAs encoding secretory cargo proteins and secretory pathway resident proteins start to decay (Han et al., 2009). Indeed, ER chaperone BiP and Golgi-localized glycosylating enzyme Gyltl1b are targets of RIDD. Unmitigated ER stress may deplete important cell surface proteins or secretory pathway proteins by continuous decay and promote apoptosis. Expression of wild-type Ire1 in Xbp1$^{-/-}$ MEF triggers apoptosis, but expression of a Ire1 kinase active/RNase dead mutant does not induce apoptosis, arguing that an active RNase is required to induce pro-apoptotic signals independent of Xbp1 mRNA splicing (Han et al., 2009). Indeed, RIDD can also promote the cleavage of selected microRNAs (miRs – 17, 34a, 96, 125b) that normally repress translation of Caspase 2, to control induction of apoptosis upon continued ER stress (Upton et al., 2012).

The two Ire1 functions, RIDD and splicing of Xbp1 mRNA, can be uncoupled *in vitro*, allowing a better understanding of the physiological output of each pathway. The Ire1^{I642G}mutant has an enlarged kinase pocket that prevents autophosphorylation and activation of the RNase. The need for ATP binding can be bypassed by incubation with 1NM-PP1, an ATP analog that binds specifically to the designed pocket of Ire1^{I642G} and activates the RNase by an allosteric mechanism (Papa et al., 2003; Hollien et al., 2009). Addition of the 1NM-PP1 is sufficient to induce Xbp1 splicing when Ire1^{I642G} is over-expressed, even in the absence of ER stress. However, RIDD function can only be engaged by 1NM-PP1 in the presence of ER stress, suggesting different activation modes of Ire1 (Han et al., 2009; Hollien et al., 2009). Other compounds, known as KIRAs (kinase inhibiting RNase attenuators) can bypass the need for autophosphorylation to activate wild-type Ire1, stimulating Xbp1 splicing, and tempering RIDD (Han et al., 2009).

CONCLUSION

A variety of recent articles demonstrated that RIDD has several physiological roles in different experimental conditions and paradigms. In most cases, RIDD couples the load of ER targeted mRNAs with the ER folding capacity, maintaining the ER homeostasis during cell differentiation and ER expansion. In cases of extreme ER stress, RIDD may trigger apoptosis since it can promote the degradation of ER resident proteins and de-repress Caspase 2. As always, questions remain and should be addressed by additional studies. For example, is it possible to control the RIDD vs. Xbp1 splicing activities of Ire1 or to control the substrate specificity of RIDD to cause different biological outcomes? Stay tuned for further developments.

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Stress, genomic adaptation, and the evolutionary trade-off

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Cells are constantly exposed to various internal and external stresses. The importance of cellular stress and its implication to disease conditions have become popular research topics. Many ongoing investigations focus on the sources of stress, their specific molecular mechanisms and interactions, especially regarding their contributions to many common and complex diseases through defined molecular pathways. Numerous molecular mechanisms have been linked to endoplasmic reticulum stress along with many unexpected findings, drastically increasing the complexity of our molecular understanding and challenging how to apply individual mechanism-based knowledge in the clinic. A newly emergent genome theory searches for the synthesis of a general evolutionary mechanism that unifies different types of stress and functional relationships from a genome-defined system point of view. Herein, we discuss the evolutionary relationship between stress and somatic cell adaptation under physiological, pathological, and somatic cell survival conditions, the multiple meanings to achieve adaptation and its potential trade-off. In particular, we purposely defocus from specific stresses and mechanisms by redirecting attention toward studying underlying general mechanisms.

Keywords: genome theory, genome instability, chromosomal instability, stress response, somatic evolution

UNIFYING THE WIDE VARIETY OF CELLULAR STRESSES

Under either normal physiological or pathological conditions, cells are subject to a wide variety of internal and external stresses, which have been associated with a variety of biological responses. For example, responses to endoplasmic reticulum (ER) stress include cell death, inflammatory signaling, insulin resistance, and lipogenesis (Kaufman, 1999; Schroder, 2008; Zhang and Kaufman, 2008; Lee et al., 2012). Exposure to ROS stress can result in transient growth arrest, increase in cellular proliferation, permanent growth arrest or senescence, and cell death (Pelicano et al., 2004). While ongoing efforts are being placed on identifying stress-associated molecular mechanisms and their interactions (Rutkowski and Hegde, 2010; Walter and Ron, 2011; Hetz, 2012) and linking their contributions to system homeostasis and many common diseases, the complexity, heterogeneity, and combinations of these stresses and cellular responses can challenge the characterization of a specific gene's or pathway's role in disease onset and progression.

Despite gaining deeper understanding regarding each specific stress response pathway, the introduction of various large-scale omics technologies has provided conflicting information in understanding functions of individual pathways in the entire system context (Heng et al., 2009, 2011a; Abu-Asab et al., 2011). The cellular stress response is a reaction to any form of macromolecular damage that exceeds a set threshold, independent of the underlying cause, and the fragmented knowledge of the stress response needs to be unified at the conceptual level to explain its universality for many different species and types of stress (Kultz, 2003). In fact, many aspects of the cellular stress response are not stressor-specific, because cells monitor stress based on macromolecular damage without regard to the type of stress that causes such damage (Kultz, 2005). There is also limited pathway specificity for stress response during somatic cell evolution, especially under pathological conditions where stochastic genetic alteration plays an important role.

To establish a common mechanism of stress response, rather than continuing to link more genes to different pathways by studying gene regulations and interactions in more linear experimental models, research efforts need to be focused on the genome dynamics during somatic cell evolution, as the stress response represents a key component of somatic cell evolution, impacting on many physiological and disease conditions (Heng et al., 2011b, 2013a). To achieve this goal, two major changes are needed. First, we need new strategies to monitor the stress response at the cellular system level. Despite source and degree variance, stress clearly results in system change. Thus, we will generalize stress to encompass the wide variety of internal and external stressors and pathways, as increased system dynamics is the common consequence. This holistic approach can provide understanding regarding the impact of stress to the cellular system and its implications to common disease without attempting to decipher massive amounts of potentially conflicting molecular data. Second, in contrast to the misconception that stress is bad and the stress-response mainly is a means to overcome "negative" influence, the stress response is essential for biological function. ER stress is required in B cell lymphopoiesis (Zhang et al., 2005), certain degrees of hypoxic

Abbreviations: CCAs, clonal chromosome aberrations; CIN, chromosomal instability; NCCAs, non-clonal chromosome aberrations; UPR, unfolded protein response.

stress can increase the homing of tissue-specific stem cells, and stress-induced genome variations are important for short-term evolutionary adaptation (Heng et al., 2013b). While the stress response is essential for life by creating heterogeneity-mediated robustness, it also generates biological damage for the system, in particular, when stress is high. These damages represent the trade-off to adapt under stress.

Clearly, to study the general stress response mechanism, the appropriate evolutionary framework is needed. Since many reviews have discussed gene- and pathway-specific mechanisms, we will focus on the genome perspective.

STRESS INDUCES SYSTEM DYNAMICS AT MULTIPLE LEVELS

Currently, most molecular characterization of stress focuses on the gene and pathway levels. Great progress in the field has achieved the understanding of the regulatory mechanisms and signaling crosstalk of the three branches of the unfolded protein response (UPR; Hetz, 2012). It is known that ER stress is buffered by the activation of UPR, and failure to adapt to ER stress leads to apoptosis. Increased studies revealed many layers of interaction/crosstalk and molecular heterogeneity. Many novel physiological outcomes of the UPR that are not directly related to protein-folding stress have been discovered, including metabolism, innate immunity, cell differentiation, functional composition, and somatic cell evolution. Many diseases with different molecular mechanisms are also linked to ER stress, further complicating this issue.

Since genetic organization can be divided into gene and genome levels, and genome-level alteration plays a key role in cancer evolution (Heng, 2009; Heng et al., 2011a,b), it is necessary to investigate the often-ignored linkage between ER stress and genome aberrations. One interesting window is to study cell death-mediated karyotype changes. Many current researchers analyze how ER stress results in cell death, as if when apoptosis occurs, the story ends. When the results of cell death are under investigation, however, a new picture emerges: not all cells under ER stress-mediated cell death will die, some of them do survive, but display altered genomes (Stevens et al., 2007, 2010, 2011, 2013, 2014; Stevens and Heng, 2013; Liu et al., 2014). Furthermore, stress in general, even before reaching the point of cell death, results in many infrequent genome alterations (Heng et al., 2006).

These seemingly random non-clonal chromosome aberrations (NCCAs), encompassing all random structural and numerical aberrations, have been ignored as insignificant genetic noise. However, elevated NCCA frequency represents increased genome-level system dynamics and can be linked to virus infection, drug treatment, many types of environmental stress, and tumorigenicity (Ye et al., 2009; Heng et al., 2013a).

Thus, the general mechanism of stress is to trigger alteration of system dynamics at multiple levels. In addition to the fact that whether or not a specific change is good or bad is context-dependent, the trade-off can be reflected at multiple levels in addition to cell death. Stress-response can be classified into three types. The first type is caused by a low-level of stress, resulting in increased non-genetic dynamics that only require an energy cost for recovery. The second type is caused by an intermediate level of stress, resulting in gene and/or epigenetic alteration. The third type is caused by the highest level of stress that can result in genome-level reorganization. In addition to the level of stress, the duration of the stressful condition also contributes to how multiple genetic and non-genetic factors respond. For example, long-term low-level stress could also trigger genome-level alterations. In general, under lower-levels of stress, system recovery can be achieved even though epigenetic and gene mutations may be involved. In contrast, high-levels of stress can lead to genome alteration, the point of no return for the individual cell. Lower-levels of stress often create stepwise evolutionary adaptation whereas high-level stress can lead to massive death or occasionally successful punctuated macroevolution. Finally, when the cell population is dominated by altered genomes, the disease will become obvious. Previous studies have supported that high-levels of stress can induce genome chaos, characterized by rapid, stochastic genome shattering and reorganization (Heng, 2007b, 2014; Liu et al., 2014). This results in network restructuring and rewiring, as evidenced by observed transcriptome elevation associated with karyotypic alteration (Stevens et al., 2014). Therefore, cells that survive this process display altered karyotypes and systems (**Figure 1**). Linking these different degrees of stress in this scheme would suggest that for a system to sense a particular stress, specificity might be less important than its degree or intensity. In addition, due to stochasticity, there may not be a specific response or end product to a particular stress or degree. This is especially important for disease research that focuses of the long-term consequences of stress, as stochastic genome variation has been associated with common disease.

STOCHASTIC GENOME VARIATION IS ASSOCIATED WITH COMMON DISEASE AND WITHIN NORMAL TISSUE

The search for molecular causative mechanisms of common diseases has resulted in the identification of high-level genome alterations. Autism and Alzheimer's disease are associated with altered karyotypes (Ye et al., 2007; Iourov et al., 2008). CGH analysis revealed that 80% of children with intellectual disability, epilepsy, autism, and congenital anomalies exhibited CNVs, chromosomal imbalances, or meiotic genome instability (Iourov et al., 2012a). Aneuploidy has been detected in several brain diseases (Iourov et al., 2012b). Stochastic genome alterations have been observed in Gulf War Illness and chronic fatigue syndrome patients (Heng et al., 2013b), and these diseases have been linked to elevated genome instability (Heng et al., unpublished data). Celiac and Crohn's disease patients display significantly increased numbers of chromosomal aberrations in peripheral blood lymphocytes (Hojsak et al., 2013). Increased polyploidy was observed in cardiomyocytes associated with hypertension, cardiac overloading, and congenital heart disease (Davoli and de Lange, 2011).

This association suggests similarities to cancer (McClellan and King, 2010), where most cancer arises from stochastic genome alterations rather than common gene mutations (Heng et al., 2006; Heng, 2007c, 2010). Unlike single gene-driven disease, in which highly penetrant genetic defects are detectable within a patient population, the molecular evolution of most cancers can only be explained by the evolutionary mechanism that is equal to all

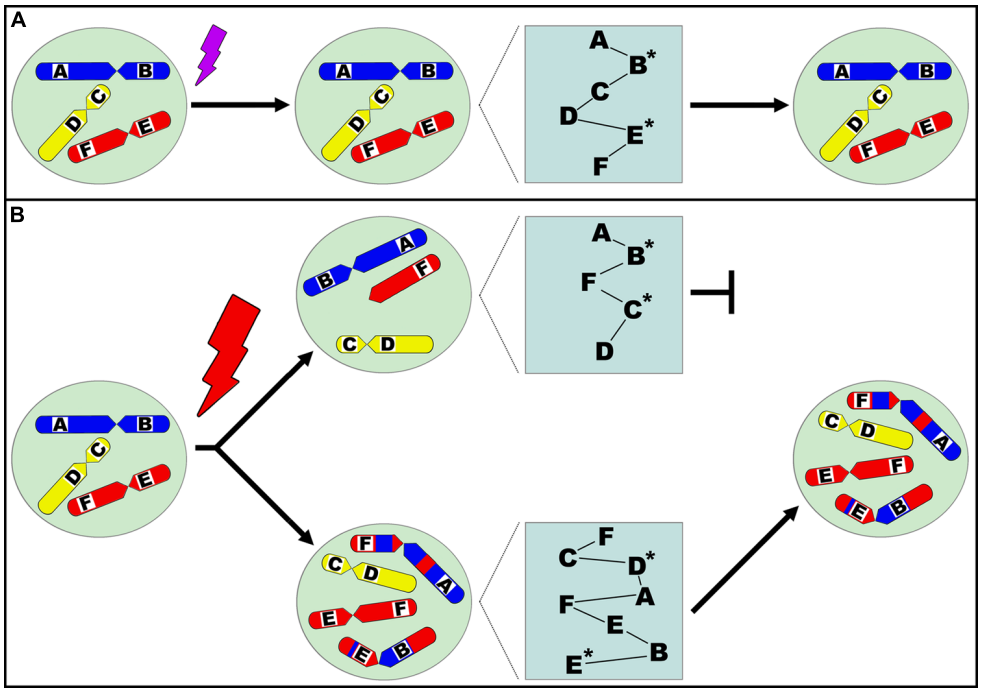

FIGURE 1 | Diagram illustrating the relationship between stress, genome topology alteration, resulting genetic network reorganization, and successful evolutionary selection. Different chromosomes are designated by color (red, yellow, blue) and drawn within the nucleus, representing the genome, and genes are designated A, B, C, D, E, F within the chromosomes. Corresponding protein networks are illustrated by the relationships between proteins A, B, C, D, E, F. A cell is exposed to a moderate level of stress **(A)**, resulting in genetic and/or epigenetic alteration as indicated by asterisks (*) next to impacted proteins. The cell survives the stress event without genome-level alteration. When a cell is exposed to a high-level of stress **(B)**, this results in genome topology alteration represented by numerical aberrations (e.g., aneuploidy) and/or structural aberrations (e.g., translocations). This directly affects the physical three-dimensional relationship between genes and changes the overall genetic network structure, resulting in drastic systemic changes beyond the influence of genetic and/or epigenetic alterations that may concurrently occur. As a consequence, the corresponding protein network changes are shown by altered relationships between proteins. These new genomic systems then undergo evolutionary selection, and those that are stochastically selected upon may clonally expand and dominate the cell population.

molecular mechanisms in the entire patient population (Ye et al., 2009; Heng et al., 2010). It is also known that the *de novo* locus-specific rate of genomic rearrangement is at least 100- to 10,000-fold greater than the rate of point mutations (Lupski et al., 2010).

Surprisingly, genome alterations have been reported in normal, healthy tissues, including the polyploidization of liver cells, skeletal muscle, and Purkinje neurons, as well as blastocyst mosaicism and trisomy 21 mosaicism in the general population (Celton-Morizur and Desdouets, 2010; Davoli and de Lange, 2011; Fragouli and Wells, 2011; Hulten et al., 2013). An increase of genome-level alterations in healthy individuals has been revealed by whole-genome sequencing application (Abecasis et al., 2012). Chromosomal aneuploidy, chromosome non-disjunction, and micronuclei formation in peripheral lymphocytes are associated with age (Ohshima and Seyama, 2010). Polyploidy increases with age in hepatocytes (Gentric et al., 2012). Somatic mosaicism as a result of chromosome instability and aneuploidy has been proposed to play a role in brain aging (Faggioli et al., 2011; Iourov et al., 2012b).

What is the difference between normal and disease tissue in terms of genome alterations? Overall, in pathological conditions, the frequencies of stochastic genome change are elevated and coupled with the presence of specific clonal chromosome aberrations (CCAs). In addition, the degree of genome alteration is much higher for each cell.

GENOME THEORY OFFERS EXPLANATIONS

To explain the widely detected stochastic genome alterations in normal and disease conditions, a new framework is needed, as current gene theory fails to achieve satisfactory explanations. Gene theory states that DNA sequence serves as the genetic blueprint, where information transfers from DNA to RNA to proteins. Accordingly, defective genes are the main cause of disease and should be readily identifiable. However, defective genes are rarely the common drivers of disease when considering the large number of essential genes, and only under very specific circumstances does this concept hold true, as in the cases of sickle cell anemia and chronic phase chronic myeloid leukemia (Horne et al., 2013b). Furthermore, personal whole-genome sequencing revealed high numbers of gene mutations for healthy individuals, illustrating disconnect between gene mutation and most common diseases (Abecasis et al., 2012).

In contrast, the recently introduced genome theory calls for a shift from the gene to the genome, as genes and genomes represent different levels of genetic organization with distinct coding systems (Heng, 2009; Heng et al., 2009, 2011a). The information

regarding assembly of parts is most likely not stored within the individual gene or genetic locus. DNA only encodes for the parts and some tools of the system (RNAs, proteins, regulatory elements). The complete interactive genetic network is coded by genome topology-mediated self-organization (Ye et al., 2007; Heng, 2009, 2010; Heng et al., 2010, 2011a). The genome is not merely the entire DNA sequence or the vehicle of all genes. Rather, the genome context or landscape (genomic topologic relationship among genes and other sequences within three-dimensional nuclei) defines the genetic system and ensures system inheritance (Heng, 2009). Since the interaction of genes with the environment comprise the genetic system, and that most genes are neither independent information units nor common factors in disease, it is now easier to understand the importance of the stochastic genome alteration detected within various diseases. Stochastic genome alterations can no longer be considered insignificant noise as altered genomes yield altered networks (Heng, 2009; Heng et al., 2011a).

A key to appreciating the genome theory accepting the multiple level adaptive landscape model (Heng et al., 2011a,b, 2013a; Huang, 2013). In this model, pathway switching within a given cell represents microevolution, or small adaptation through local landscape change. In contrast, genome switching among cells often represents macroevolution or huge adaptation across the global landscape. Each genome-mediated global landscape can be achieved by large numbers of pathway-mediated local landscapes. Most of the current research on transcriptional reprogramming in ER stress is likely focused on the local landscape level.

STRESS-INDUCED GENOME DYNAMICS RESULT IN ADAPTATION AND DISEASE

Genome-level alterations are more effective at drastically changing the genetic system than gene mutation or epigenetic change, as supported by a recent study where karyotypic alterations were shown to influence gene expression profiles (Stevens et al., 2014). In addition, evidence in yeast studies strongly supports that aneuploidy directly affects gene expression, resulting in phenotypic variation (Pavelka et al., 2010). Genome-level alterations at the somatic cell level generate new systems by creating new frameworks, rather than new features defined by gene mutation/epigenetic regulation. Thus, genome alteration results in new genetic networks, suggesting that somatic cell genome evolutionary dynamics provide adaptive advantages for cells against stress. Further, genome diversity within normal, healthy tissues allows for complex organ function while providing the genome heterogeneity necessary to account for organ function-associated stress, such as liver-mediated blood detoxification. This realization is of high importance as genomic alterations were previously only viewed in a negative light.

Stochastic somatic genome dynamics can also result in disease onset and promotion. Higher NCCA frequencies have been linked with genome instability, disease conditions, and drug resistance (Heng et al., 2006, 2011a,b; Heng, 2007c, 2010; Ye et al., 2009). This realization provides explanation for the many common diseases that have not yet been linked with common biomarkers within the majority of cases. Focusing on genome alterations can unify the diverse factors that have been linked to individual genes by current molecular studies.

Therefore, adaptation requires "noise" elevation or an increase in heterogeneity. However, increased system dynamics can also potentially lead to disease onset. Now the question is, how does the bio-system solve this paradox of promoting system dynamics for short-term adaptation while avoiding the accumulation of alterations that could potentially harm the species?

This paradox was addressed by re-evaluation of the main function of sex. The century-old reasoning states that sexual reproduction functions to increase genetic variation. Under the new paradigm, sexual reproduction primarily acts to reduce genomic alterations despite its secondary function of mixing genes (Heng, 2007a; Wilkins and Holliday, 2009; Gorelick and Heng, 2011). Thus, sexual reproduction functions as a filter that effectively eliminates high-levels of stochastic genome alterations. This relationship between stochastic somatic genome dynamics and genome purification through sexual reproduction solves the conflict between short-term dynamics of adaptation (for somatic cell function) and long-term system persistence (to preserve the species). Stochastic somatic genome-level aberrations provide individuals with an evolutionary advantage against stress. However, somatic genomic aberrations could also lead to the onset and progression of common disease. In contrast to increasing evolutionary potential by stochastic somatic genomic aberrations, the constraint of germ line evolution through sexual reproduction preserves system integrity. Through this separation of germ line and somatic cell genomes, somatic genome alteration ensures short-term adaptation, while the filtering process of the germ line genome ensures long-term genome system identity (Horne et al., 2013a; Heng, 2014).

CONCLUSION AND FUTURE PERSPECTIVE

By reviewing the importance of genome alteration in somatic evolution and its potential link with stress, we hope readers can grasp the rationale of studying the genome rather than pathways and understand the key relationship between stress, adaptation advantages, and the evolutionary trade-off. Even though refocusing on genome changes seems counterintuitive, as the resolution is lower at the genome-level than gene and pathway levels, it is the genome package that serves as the evolutionary selection unit in somatic cell evolution, especially in pathological conditions. As pointed out by Barbara McClintock, "in the future, attention undoubtedly will be centered on the genome. . .a highly sensitive organ of the cell that. . .senses unusual and unexpected events, and responds to them, often by restructuring the genome" (McClintock, 1984).

New strategies need to be developed to monitor system behavior under stress. More attention is needed to study the linkage of chromosomal instability (CIN) with various types of stress, as CIN serves as a general mechanism for cancer and potentially for other common diseases (Heng, 2010; Burrell et al., 2013; Heng et al., 2013a). In addition to our studies that link many stresses to CIN, ER stress has been directly linked to chromosome maintenance (Henry et al., 2010) and aneuploidy status (Sheltzer et al., 2012). Significantly, the linkage of stress-induced genome chaos

(the survival strategy induced under high-levels of stress) and molecular pathway diversity have illustrated the ultimate importance of genome re-organization in cancer (Liu et al., 2014; Stevens et al., 2014).

Fortunately, the level of stochastic genomic change is the best measuring tool. Applying single cell-based approaches to measure the population profile is key (Heng et al., 2006; Abdallah et al., 2013). Although common practice may include disregarding these "noisy" data, heterogeneity in fact provides the complexity necessary for organismal survival and adaptation after stress, thus these data are of ultimate importance (Heng, 2014). Further studies are urgently needed to compare genome-level measurements with other known methods that focus on gene or pathway levels (Tang and Amon, 2013). Despite the complexity, measuring higher-level behaviors could be simpler than measuring lower-level diversity. For example, the family history of heart disease (higher-level of phenotype) has much more prediction power than comparing individual molecular markers (Heng, 2013).

A more systematical view is needed when dealing with stress and response. We need to monitor how the genome system changes during evolution, rather than only focus on specific pathways (using linear models) within limited time scales. By drastically simplifying the system to eliminate the heterogeneity, we might be able to identify an artificial linear relationship, but this often does not reflect clinical reality where multiple levels of heterogeneity rule. To change this situation, this discussed genome-mediated evolutionary concept must be incorporated into the field of stress research.

AUTHOR CONTRIBUTIONS

Wrote the manuscript: Steven D. Horne and Henry H. Q. Heng. Participated in planning and editing: Saroj K. Chowdhury.

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ER stress and hepatic lipid metabolism

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The endoplasmic reticulum (ER) is an important player in regulating protein synthesis and lipid metabolism. Perturbation of ER homeostasis, referred as "ER stress," has been linked to numerous pathological conditions, such as inflammation, cardiovascular diseases, and metabolic disorders. The liver plays a central role in regulating nutrient and lipid metabolism. Accumulating evidence implicates that ER stress disrupts lipid metabolism and induces hepatic lipotoxicity. Here, we review the major ER stress signaling pathways, how ER stress contributes to the dysregulation of hepatic lipid metabolism, and the potential causative mechanisms of ER stress in hepatic lipotoxicity. Understanding the role of ER stress in hepatic metabolism may lead to the identification of new therapeutic targets for metabolic diseases.

Keywords: ER stress, UPR, hepatic, lipid metabolism, liver diseases

INTRODUCTION

Endoplasmic reticulum is an important intracellular organelle responsible for protein synthesis, folding, modification, and trafficking. In addition, the ER also plays a crucial role in calcium homeostasis and in regulating the biosynthesis of steroids, lipids and carbohydrates (Borgese et al., 2006). In the ER, millions of proteins are synthesized, but not all of them are able to be properly folded and processed. Under normal physiological conditions, the unfolded or misfolded proteins are directed to degradation pathways through activating an evolutionally conserved signaling pathway, the UPR. Activation of the UPR can either help eliminate the unfolded proteins and restore cellular homeostasis, or activate a cascade of intracellular events resulting in cell death (Shen et al., 2004; Merksamer and Papa, 2010). The UPR is of particular importance in hepatocytes, which are rich in ER content and responsible for the synthesis of proteins, cholesterol, bile acids, and phospholipids. The UPR and its contribution to hepatic injury have been investigated in various liver diseases including ALD, NAFLD, DILD, cholestatic liver disease, and viral hepatitis (Zhou et al., 2006; Kaplowitz et al., 2007; Colgan et al., 2011; Dara et al., 2011; Jo et al., 2013).

Abbreviations: ABCA1, ATP-binding cassette, sub-family A, member 1; ACC, acetyl-CoA carboxylase; ALD, alcoholic liver disease; ATF, activating transcription factor; C/EBP, CCAAT/enhancer-binding protein; CHOP, C/EBP homologous protein; DILD, drug-induced liver disease; eIF2α, eukaryotic translation initiation factor; ER, endoplasmic reticulum; FAS, fatty acid synthase; FXRα, farnesold X receptor α; GADD, growth arrest and DNA damage-inducible protein; HDL, high density lipoprotein; Insig, insulin-induced gene; IRE1, inositol requiring enzyme 1; JNK, c-Jun N-terminal kinase; LDL, low density lipoprotein; MCD, methionine-choline-deficient diet; NAFLD, non-alcoholic fatty liver disease; PERK, protein kinase RNA-like ER kinase; S1P, site 1 protease; S2P, site 2 protease; SCAP, SREBP cleavage activating protein; SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element binding protein; UPR, unfolded protein response; VLDL, very low density lipoprotein; VLDLR, VLDL receptor; XBP, X-box binding protein.

ER STRESS AND THE UPR

The ER is a membranous network of cisternae responsible for the synthesis and export of proteins and lipids. It is also crucial for cellular calcium homeostasis (Borgese et al., 2006; Gorlach et al., 2006). The ability of the ER to adapt to the metabolic changes, such as an increase in protein synthesis and accumulation of unfolded proteins and cholesterol in the ER lumen, is of paramount importance for the cell. When the misfolded or unfolded proteins accumulate in the ER, the ER stress and its related signaling pathways, UPR, are activated (Ron and Walter, 2007; Schroder, 2008).

Three main branches of the UPR-mediated signaling pathways have been identified so far: the IRE1 pathway, protein kinase RNA-like ER kinase (PERK) pathway, and ATF6 pathway. As illustrated in **Figure 1**, IRE1, PERK, and ATF6 are associated with the ER membrane. Under non-stressed condition, these transmembrane proteins are bound to a chaperone protein, BiP/GRP78, which is also known as the master regulator of the UPR. The binding of BiP/GRP78 to these UPR transducers prevents them from activation. When the ER is stressed by accumulation of misfolded or unfolded proteins, depletion of ER calcium content, or increase of free cholesterol in the ER lumen, BiP/GRP78 is released from the UPR transducers. The disassociation of BiP/GRP78 from the UPR transducers results in the activation of IRE1-, PERK-, and ATF6-mediated signaling pathways. PERK activation results in a rapid down-regulation of protein synthesis *via* phosphorylation of eIF-2α and inhibition of the formation of the translation initiation complex (DuRose et al., 2009). The phosphorylated eIF-2α further promotes the translation of ATF4, a member of the basic leucine zipper (bZIP)-containing protein subfamily. IRE1α has both protein kinase and endoribonuclease activities. Under ER stress conditions, IRE1α is oligomerized and autophosphorylated. The activated IRE1α removes a 26-bp intron from the XBP1 mRNA, resulting in the production of spliced XBP1 protein (XBP1s). XBP1s, which is also a bZIP transcription factor,

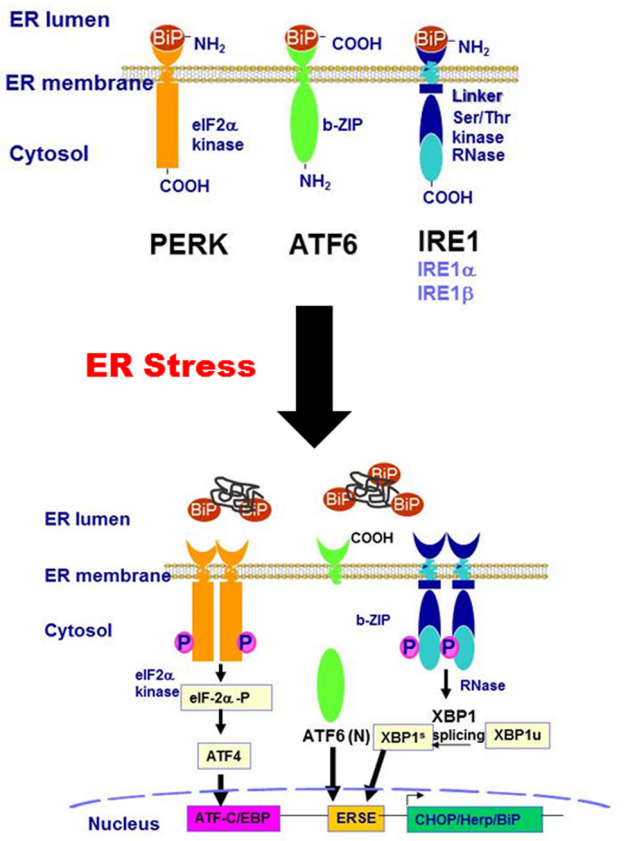

FIGURE 1 | Schematic diagram of the ER stress-activated signaling pathways.

regulates the expression of several genes involved in UPR and ER-assisted degradation (ERAD) to help restore ER homeostasis (Acosta-Alvear et al., 2007). In addition, IRE1α also induces the activation of stress kinases, JNK and p38 MAPK, that promote apoptosis (Ron and Hubbard, 2008). ATF6 is the third branch of the UPR. Dissociation of BiP/GRP78 from ATF6 leads to its translocation to the Golgi, where it is processed into its active form by cleavage of its N-terminal domain by S1P and S2P (Chen et al., 2002). The activated ATF6 (ATF-6 N terminal domain) is translocated to the nucleus and functions as a transcription factor, promoting the expression of downstream target genes involved in ER stress including XBP1, GADD153 (also known as CHOP), and ER chaperones (Oyadomari and Mori, 2004). CHOP is a proapoptotic transcription factor that plays a critical role in ER stress-mediated apoptosis (Marciniak et al., 2004).

HEPATIC LIPID METABOLISM

Liver is the central metabolic organ and plays a critical role in fatty acid and cholesterol metabolism (Hylemon et al., 2001). Several inter-dependent pathways are involved in hepatic lipid metabolism. The hepatic fatty acids can be derived from *de novo* lipogenesis, hydrolysis of triglyceride from cytoplasmic lipid droplets or direct uptake of non-esterified fatty acids from circulation (Bechmann et al., 2012). The liver is also a major organ in the processing of lipids into the various lipoproteins, in particular VLDL and LDL. Fatty acids synthesized by the liver are converted into triglyceride and exported as constituents of VLDL into the blood circulation. The VLDLs absorbed into peripheral tissues are partially digested by lipoprotein lipase into LDL and free fatty acids. LDL is transported into the cell *via* LDL receptors for its conversion into free fatty acids, cholesterol, and other components of LDL. Similarly, cholesterol also can be derived from *de novo* synthesis or absorbed from the diet, and are transported into circulation as lipoprotein particles (Horton et al., 2002a; Babin and Gibbons, 2009; van Heyningen, 2009; Musso et al., 2013; Faust and Kovacs, 2014). The cholesterol can be stored in cells as cholesterol esters or metabolized into bile acids. The hepatic triglycerides and cholesterol contents are tightly regulated by multiple interrelated signaling pathways. Under normal physiological conditions, lipid input is equal to lipid output from the body. Disruption of either the input or output pathways will result in dysregulation of lipid metabolism (Hylemon et al., 2001). Tremendous studies have been done to elucidate the extremely complex regulation network of hepatic lipid homeostasis (Bradbury and Berk, 2004; Bradbury, 2006; Weickert and Pfeiffer, 2006; Nguyen et al., 2008; Musso et al., 2009; Sparks and Dong, 2009; van Heyningen, 2009; Trauner et al., 2010; Jump, 2011; Fu et al., 2012). Here, we will focus on the current understanding regarding the role of ER stress in hepatic lipid metabolism.

UPR AND HEPATIC LIPID HOMEOSTASIS

The ER is the primary site of lipid metabolism. Many enzymes and regulatory proteins of lipid metabolism reside in the ER. Perturbation of ER homeostasis contributes to hepatic steatosis, inflammation and insulin resistance in the liver (Kaplowitz et al., 2007; Hotamisligil, 2010; Rohrl et al., 2014). Although the UPR was originally identified as a conserved signaling pathway, functioning to maintain essential ER homeostasis, numerous studies indicate that the UPR has broader functions and plays an essential role in maintaining hepatic lipid homeostasis. Recent studies have shown that the pharmacologic ER stress inducers increase *de novo* lipogenesis and lipid droplet formation in hepatocytes by up-regulating a subset of genes encoding key lipogenic trans-activators and enzymes (Lee et al., 2012).

Hepatic lipid homeostasis is controlled by numerous transcription factors and nuclear receptors. The SREBPs are master regulators of lipid homeostasis (Eberle et al., 2004) and play a critical role in *de novo* lipid biosynthesis (Amemiya-Kudo et al., 2002). SREBP-1 controls fatty acid and triglyceride biosynthesis, while SREBP-2 controls cholesterol metabolism and LDL receptor expression. SREBPs, are basic-helix-loop-helix-leucine zipper (bHLHLZ) transcription factors bound to the ER membranes as an inactive precursor (Brown and Goldstein, 1997). The regulation of SREBP activity is controlled within the ER by the interaction of SCAP with insulin regulated proteins (Insigs; Yang et al., 2002). The Insigs can cause ER retention of the SREBP-SCAP complex and prevent the activation of SREBPs. When the sterol level is low, Insigs are disassociated with SCAP, which allows the SREBP-SCAP complex to migrate to the Golgi, where SREBPs are processed into active forms by S1P and S2P (Rawson,

2003; Lee and Ye, 2004). The activated SREBPs are subsequently translocated into nucleus, where SREBPs regulate the expression of various genes involved in lipid metabolism by binding to the sterol regulatory element of their target genes (Horton et al., 2002a,b; Radhakrishnan et al., 2007). It has been shown that ER stress induces proteolytic activation of SREBPs by increasing the turnover of Insig-1 (Lee and Ye, 2004). The expression of Insigs is regulated by insulin and FXRα (Yabe et al., 2003; Hubbert et al., 2007). Overexpression of hepatic Insigs has been shown to reduce hepatic lipogenesis (Engelking et al., 2004; Takaishi et al., 2004).

ER stress and lipid metabolism are tightly intertwined. The chronic ER stress is the most important contributor to metabolic diseases. Unresolved ER stress induces dysregulation of hepatic lipid metabolism (Fu et al., 2012). It is also well-documented that excess saturated fatty acids and cholesterol can induce ER stress and disrupt lipid metabolism in hepatocytes, macrophages and adipocytes (Fribley et al., 2009; Colgan et al., 2011; Anderson et al., 2012; Fu et al., 2012; Wang et al., 2013; Zha et al., 2013). The various components of the UPR signaling pathways play a role in the regulation of lipid metabolism.

IRE1α-XBP1 PATHWAY

IRE1α-XBP1 pathway is one of three main branches of UPR, which has been identified as a critical regulator of hepatic lipid metabolism. Hepatic-specific deletion of IRE1α increased hepatic lipid levels and reduced plasma lipid by altering several genes involved in hepatic lipid metabolism under ER stress conditions such as C/EBPβ, C/EBPδ, peroxisome proliferator-activated receptor γ (PPARγ), and enzymes involved in triglyceride biosynthesis (Zhang et al., 2011). Although, these results suggest a plausible protective role of IRE1α from hepatic steatosis, the deletion of IRE1α blocks the basal level of the UPR in liver which may lead to an unresolved ER stress. Thus, it is still unclear whether the induction of lipogenesis genes in IRE1α$^{-/-}$ mice is due to the loss of IRE1α function or elevated ER stress. Studies done by Lee et al. (2008) reported that disruption of hepatic XBP-1 significantly reduced serum triglyceride, cholesterol and fatty acids levels by decreasing *de novo* hepatic lipogenesis in mice. In addition, the IRE1α-XBP1 pathway is also involved in regulation of hepatic VLDL assembly and secretion (Wang et al., 2012). IRE1α is required for efficient secretion of VLDL and LDL from hepatocytes under the condition of ER stress (Zhang et al., 2011). A most recent study done by Rohrl et al. (2014) identified novel links between ER stress and hepatic cholesterol metabolism. Activation of acute ER stress reduced ABCA1 expression and induced ABCA1 redistribution to tubular perinuclear compartments in hepatocytes, which significantly diminished cholesterol efflux to apoA-I and HDL formation (Rohrl et al., 2014).

PERK-ATF-4 PATHWAY

Protein kinase RNA-like ER kinase activation induces eIF2α phosphorylation, which causes translation attenuation that is required to protect against apoptosis in response to ER stress. Although the exact role of PERK in hepatic steatosis is still not completely understood, a recent report also suggests that antipsychotic drugs (APDs)-induced activation of PERK-p-eIF2α signaling pathway increases intracellular lipid accumulation through activation of SREBP-1c and SREBP-2 in hepatocytes (Lauressergues et al., 2012). Attenuation of eIF2α in GADD34 transgenic mice significantly altered the metabolism profile and reduced high fat diet-induced hepatic steatosis (Oyadomari et al., 2008). As a down-stream transcriptional factor of the UPR, ATF4 avoids global suppression of protein expression induced by p-eIF2α due to a different upstream signaling pathway. It has been shown that ATF4 deficiency preferentially attenuated hepatic lipogenesis *via* down-regulation of PPARγ, SREBP-1c, ACC and SCD expression without affecting hepatic triglyceride production and fatty acid oxidation (Li et al., 2011; Xiao et al., 2013). A recent study further identified that activation of PERK-eIF2α-ATF4 pathway under ER stress condition is required for hepatic VLDL receptor up-regulation in hepatocytes, which is responsible for intracellular accumulation of triglycerides and hepatic steatosis (Jo et al., 2013). Furthermore, attenuation of global translation by activation of PERK-eIF2α pathway also decreases ApoB expression, which further promotes hepatic steatosis.

ATF6 PATHWAY

Both ATF6 and SREBPs are activated by the same proteases (S1P and S2P) in the Golgi (Sakai et al., 1998; Ye et al., 2000; Horton et al., 2002b). Several independent studies have shown that ATF6 and XBP-1 share similar DNA binding specificities (Yoshida et al., 2000, 2001; Acosta-Alvear et al., 2007; Misiewicz et al., 2013). In addition, ATF6 and XBP-1 are able to form a heterodimer and regulate down-stream target genes (Yamamoto et al., 2007). Several recent studies in ATF6α knockout mice indicate that ATF6 also plays an important role in regulating hepatic lipid homeostasis (Rutkowski et al., 2008; Yamamoto et al., 2010). Similar to IRE1α, deletion of ATF6α does not result in an apparent phenotype under physiological conditions. However, under ER stress conditions, ATF6α knockout mice exhibited severe liver injury and hepatic steatosis caused by inhibition of fatty acid β-oxidation and VLDL formation (Yamamoto et al., 2010). In addition, the CHOP expression is significantly up-regulated while PPARα expression and ApoB-100 protein levels are decreased in the livers of ATF6α knockout mice (Rutkowski et al., 2008; Yamamoto et al., 2010). A recent study in zebrafish with fatty liver disease demonstrated that ER stress induces fatty liver disease. During chronic ER stress, ATF6 promotes steatosis. However, ATF6 prevents acute ER stress-induced steatosis. This study suggest that ATF6 can play both protective and pathological roles in fatty liver disease (Cinaroglu et al., 2011).

ER STRESS MASTER REGULATOR-GRP78/BiP

GRP78/BiP is a glucose-regulated protein that functions as a molecular chaperone in the ER (Mote et al., 1998). As described in previous section, GRP78 acts as a master regulator of the activation of UPR signaling pathways. Numerous studies have indicated that ER stress is an important component of the hepatic steatosis and insulin resistance in obese rodent models (Kammoun et al., 2009; Chen et al., 2013; Teodoro-Morrison et al., 2013). GRP78 plays a critical role in maintaining hepatic lipid homeostasis. Overexpression of GRP78 prevents ER stress-induced SREBP-1c proteolytic cleavage and reduced hepatic

steatosis (Kammoun et al., 2009). It also has been reported that overproduction of GRP78 prevents palmitate-induced ER stress and cytotoxicity in human HepG2 cells (Gu et al., 2010) and high fat diet-induced type 2 diabetes in mouse models (Teodoro-Morrison et al., 2013). A most recent study further indicated that GRP78 is able to prevent oxidative stress-induced injury by inhibiting lipid peroxidation (Suyama et al., 2014).

C/EBP HOMOLOGOUS PROTEIN

C/EBP homologous protein is a proapoptotic transcriptional factor downstream of all three UPR signaling pathways. Numerous evidences suggest that CHOP activation promotes cell apoptosis and induces tissue injury. In absence of CHOP, both cells and animals are protected against various pharmacological and physiological insults (Tabas and Ron, 2011). It has been shown that CHOP deficiency attenuates cholestasis-induced liver fibrosis and methionine-choline-deficient (MCD) diet-induced steatohepatitis, fibrosis, and carcinogenesis in mice (Tamaki et al., 2008; Toriguchi et al., 2013). In a murine model of intragastric ethanol feeding, CHOP null mice have remarkable absence of hepatocellular apoptosis, but no protection against alcohol-induced steatosis (Ji et al., 2005). In human liver cell lines, saturated fatty acids induce ER stress and apoptosis *via* the PERK/ATF4/CHOP (Cao et al., 2012). Our recent studies suggest that CHOP is a major player in human immunodeficiency virus protease inhibitor-induced hepatic lipotoxicity in mice (Wang et al., 2013). Studies done by Chikka et al. (2013) further suggest CHOP has a non-apoptotic role in regulating hepatic metabolic genes during ER stress. In addition, a recent study reported that CHOP expression is up-regulated in human hepatocellular carcinoma (HCC) and two mouse HCC models. CHOP expression contributes to hepatic carcinogenesis by promoting inflammation and cell apoptosis (DeZwaan-McCabe et al., 2013). These studies indicate CHOP is a common contributing factor in ER stress-induced liver injury.

THERAPEUTIC POTENTIAL TARGETING ER STRESS IN METABOLIC DISEASES

Chronic ER stress has been implicated in the pathogenesis of metabolic diseases such as a diabetes, obesity, cardiovascular diseases as well as fatty liver disease (Colgan et al., 2011; Malhi and Kaufman, 2011; Mollica et al., 2011; Bechmann et al., 2012; Cnop et al., 2012). Targeting the specific UPR signaling pathways to attenuate ER stress and UPR activation would provide opportunities in developing new therapeutic strategies in a wide array of diseases. Several studies have shown the promising effects of small chemical chaperones on alleviating the UPR activation in animal models, such as 4-phenylbutyric acid (4-PBA) and tauroursodeoxycholic acid (TUDCA; Heubi et al., 2002; Ozcan et al., 2006; Basseri et al., 2009; Engin and Hotamisligil, 2010; Lee et al., 2010). Both 4-PBA and TUDCA have been approved by US Food and Drug Administration (FDA) for treating children with urea-cycle disorders and cholestatic liver disease, respectively. However, more clinical studies are needed to validate the potential application of these chemical chaperones in treating ER stress-associated metabolic diseases.

CONCLUSION

Hepatic lipid homeostasis requires integration of multiple signals. A significant amount of evidence indicates that activation of ER stress signaling pathways play a critical role in various diseases associated with dysregulation of hepatic lipid metabolism. Although acute ER stress response helps restore ER homeostasis, prolonged or chronic ER stress activation contributes to development of various metabolic diseases including NAFLD, type 2 diabetes, and atherosclerosis by inducing widespread pathologic apoptosis (Tabas and Ron, 2011). The balance of the UPR signaling pathways, such as ATF6, IRE1/XBP1, and PERK/ATF4, is critical for maintaining cellular homeostasis. However, the exact mechanisms underlying ER stress-induced disruption of hepatic lipid homeostasis remains to be fully identified. Recent studies have shown that ER stress is also closely linked to inflammation and autophagy, which are two important players in regulating hepatic lipid metabolism (Ogata et al., 2006; Yorimitsu et al., 2006; Hoyer-Hansen and Jaattela, 2007; Yorimitsu and Klionsky, 2007; Hotamisligil, 2010; Hummasti and Hotamisligil, 2010; Mollica et al., 2011; Qiu et al., 2011; Adolph et al., 2012; Hasnain et al., 2012; Kolattukudy and Niu, 2012). The contribution of autophagy to lipid metabolism has been reviewed in several excellent reviews (Singh, 2010; Amir and Czaja, 2011; Czaja, 2011; Ding et al., 2011; Lavallard et al., 2012). Elucidating the signaling pathways of ER stress and its intertwining with other intracellular signaling components not only furthers our current understanding of lipid metabolism in a central metabolic organ, but also helps develop an effective approach that can be used to treat patients with metabolic diseases.

A growing body of evidence links ER stress and UPR activation to diseases associated with lipid metabolism. The UPR signaling pathways and activation of transcription factors such as XBP1 and ATF6 have novel roles in controlling the transcriptional regulation of lipogenesis. While IRE1α itself is protective against ER-stress-induced lipogenesis and hepatic steatosis, its downstream mediator XBP1 promotes transcription of genes involved in fatty acid and cholesterol biosynthesis. Phosphorylation of eIF2α downstream of PERK affects the transcriptional activity of C/EBPs, PPARγ, and SREBP-1c thereby leading to lipid accumulation and hepatic steatosis under high-fat-diet conditions. Similar to IRE1α, ATF6α also protects against ER stress-induced steatosis and lipid droplet formation in mice. Furthermore, nuclear ATF6 attenuates SREBP2-mediated lipogenesis. The exact mechanisms by which ER stress signaling pathways affect lipid homeostasis are incompletely understood. Given the temporal differences in the activation of the three arms of the UPR, a closer examination of each branch of the UPR will allow for a better understanding of how various components of this signaling network impact lipogenesis and disease progression. Such studies will further enhance our understanding of the biological and pharmacological tools needed to effectively treat ER-associated diseases.

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Endoplasmic reticulum stress in hepatic steatosis and inflammatory bowel diseases

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As an adaptive response to the overloading with misfolded proteins in the endoplasmic reticulum (ER), ER stress plays critical roles in maintaining protein homeostasis in the secretory pathway to avoid damage to the host. Such a conserved mechanism is accomplished through three well-orchestrated pathways known collectively as unfolded protein response (UPR). Persistent and pathological ER stress has been implicated in a variety of diseases in metabolic, inflammatory, and malignant conditions. Furthermore, ER stress is directly linked with inflammation through UPR pathways, which modulate transcriptional programs to induce the expression of inflammatory genes. Importantly, the inflammation induced by ER stress is directly responsible for the pathogenesis of metabolic and inflammatory diseases. In this review, we will discuss the potential signaling pathways connecting ER stress with inflammation. We will also depict the interplay between ER stress and inflammation in the pathogenesis of hepatic steatosis, inflammatory bowel diseases and colitis-associated colon cancer.

Keywords: ER stress, inflammation, hepatic steatosis, colitis, UPR, inflammasome, IRE1

INTRODUCTION

The endoplasmic reticulum (ER) is an intracellular organelle involving in folding of membrane and secreted proteins, synthesis of lipids and sterols, and maintenance of intracellular calcium homeostasis (Kleiner and Brunt, 2012; Milic and Stimac, 2012; Tuyama and Chang, 2012; Wierzbicki and Oben, 2012; Ibrahim et al., 2013). ER chaperones, such as glucose-regulated protein of 78 kDa (GRP78) and 94 kDa (GRP94, also termed as gp96) function as a quality control system that monitors newly synthesized proteins, and ensures only correctly folded proteins to be transported out of ER (Yang and Li, 2005; Yang et al., 2007; Zhang et al., 2011; Cnop et al., 2012; Chen et al., 2013; Lake et al., 2013). ER stress takes place when unfolded or misfolded proteins accumulate in the ER lumen. In response to stress conditions, ER initiates a series of unfolded protein response (UPR) signal transduction pathways, including inositol-requiring enzyme 1 (IRE1), double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK), and activating transcription factor-6 (ATF6) pathways, eventually, leading to changes in translational and transcriptional programs (Urano et al., 2000; McGuckin et al., 2010; Schroder and Sutcliffe, 2010; Hetz, 2012; Zha and Zhou, 2012). Cells that undergo constant ER stress include immune cells such as macrophages, plasma cells, and cells regulating metabolism such as hepatocytes, pancreatic β-cells, adipocytes, and mucosal epithelial cells. Those cells are sensitive to changes in the environments, protein traffic, and ER homeostasis (Ozcan et al., 2006; Eizirik and Cnop, 2010; McAlpine et al., 2010; Schroder and Sutcliffe, 2010; Staron et al., 2010; Ye et al., 2010; Ji et al., 2011; Pfaffenbach and Lee, 2011). While UPR is an adaptive response for cells to restore ER homeostasis, severe or prolonged ER stress leads to cell death and tissue damage. Accumulating evidence indicates that ER stress is involved in various diseases including neurodegenerative diseases, metabolic diseases, inflammatory diseases, cancer, and so on.

Recently, ER stress has been recognized to induce inflammation (McGuckin et al., 2010; Eri et al., 2011; Garg et al., 2012; Osorio et al., 2014; Shenderov et al., 2014). Interestingly, many ER stress-associated diseases also display inflammatory phenotypes (McGuckin et al., 2010; Eri et al., 2011; Garg et al., 2012; Larrain and Rinella, 2012; Petrasek et al., 2012; Szabo and Csak, 2012; Wood, 2012). Inflammatory cytokines released from stressed cells may function as alarming or danger signals to communicate with other cells or to recruit immune cells. While ER stress-induced inflammation is essential for tissue remodeling, it can cause tissue damage and contributes to the pathogenesis of many inflammatory and metabolic diseases (Eizirik and Cnop, 2010; McGuckin et al., 2010; Malhi and Kaufman, 2011; Cnop et al., 2012; Garg et al., 2012; Zha and Zhou, 2012). Inflammation can be induced directly by UPR pathways in stressed cells, or indirectly through interaction with innate immune cells. Induction of innate immunity is mediated by diverse families of Pattern Recognition Receptors (PRRs) that recognize molecular "signature" of the invading pathogens termed as pathogen associated molecular patterns (PAMPs; Takeuchi and Akira, 2010; Kawai and Akira, 2011). Additionally, innate immune cells can be activated by various endogenous ligands from damaged or dead cells. Toll-like receptors (TLRs) are a major family of PRRs mainly expressed by cells of the innate immune system. TLRs can initiate distinct innate immune responses through recruitment of different MyD88 adaptor family members. Currently, at least 13 TLRs have been cloned in mammals, and each receptor is involved in the recognition of a unique set of PAMPs. Studies from our group had demonstrated

that GRP94 is a master molecular chaperone in the ER for TLRs. Our results show that the function of most TLRs is dependent on the integrity of GRP94 in the ER (Yang et al., 2007; Liu and Li, 2008). In addition to TLRs, most nucleated cells are capable of sensing and responding to pathogens inside the cytoplasm via intracellular receptors including NOD-like receptors (NLRs) or RIG-I-like receptor family members, which recognize bacteria or viruses, respectively.

TLR activation triggers the recruitment of MyD88 via the Toll-interleukin-1 receptor (TIR) domain, allowing for subsequent recruitment of IL-1R associated kinase (IRAK) and tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6), which leads to the activation of both the NF-κB and JNK pathways. In addition, TLRs are able to activate other signaling cascades including the PI3K, p38, and ERK pathways. Activation of these pathways leads to the expression of inflammatory cytokines such as TNFα and IL-6. Recent progress demonstrates that inflammasomes, signaling complexes essential for IL-1β production, are also involved in various inflammatory and metabolic diseases (Ting et al., 2010; Davis et al., 2011; Stienstra et al., 2011; Henao-Mejia et al., 2012a). Furthermore, pathogens can also induce ER stress directly or indirectly in host cells (Joyce et al., 2009; Merquiol et al., 2011; Muralidharan and Mandrekar, 2013; Blazquez et al., 2014; Chan, 2014; Liu and Dudley, 2014; Smith, 2014). Components of pathogen can interact with TLRs to influence cellular stress response. Martinon et al. (2010) showed that stimulation with TLR4 or TLR2 ligands activated the UPR sensor IRE1α and its downstream target, X box-binding protein (XBP1). The authors indicated that TLR-induced IRE1/XBP1 activation was required for optimal and sustained production of proinflammatory cytokines from macrophages. Notably, XBP1-deficient mice had reduced production of inflammatory mediators and significantly increased bacterial burden when infected with intracellular pathogen *Francisella tularensis*. This novel finding suggests that TLR and IRE1-XBP1 pathways acted in synergy to maximize innate immune responses to pathogens. Interestingly, Woo et al. (2009, 2012) found that the ATF4/CHOP branch of ER stress signaling pathways was selectively suppressed by TLR 3 or 4 through a TRIF-dependent pathway. In mice pretreated with LPS, a TLR4 ligand, ER stress-induced CHOP expression and apoptosis in macrophages, renal tubule cells, and hepatocytes were suppressed. Accordingly, TLR engagement protected mice from ER stress-induced renal dysfunction and hepatosteatosis. Results from those studies suggest that host innate immune pathways modulate ER-stress response to enhance inflammation, thereby the host defense response, while suppress apoptosis pathways during pathogen infections. In addition to the cross-talk of TLR and UPR pathways, viral infection is frequently associated with ER stress and UPR response because of viral protein synthesis and assembly during viral life cycle. A striking example is hepatitis C virus (HCV) infection, which is a strong risk factor for chronic liver diseases and hepatocellular carcinoma. The ER stress and inflammation induced by HCV contribute to chronic liver diseases such as hepatic steatosis and cirrhosis though further studies are needed (Joyce et al., 2009; Merquiol et al., 2011; Chan, 2014).

Since there are a number of excellent reviewers on ER stress and UPR pathways (Bertolotti et al., 2000; Eizirik and Cnop, 2010; McGuckin et al., 2010; Malhi and Kaufman, 2011; Pfaffenbach and Lee, 2011; Cnop et al., 2012; Garg et al., 2012; Hetz, 2012; Logue et al., 2013; Chan, 2014; Smith, 2014; Zhou and Liu, 2014), this review will focus on ER stress-associated inflammation. ER stress can initiate several signaling pathways that induce inflammation, including ROS production, NF-κB pathway, JNK pathway, and autophagy (McGuckin et al., 2010; Garg et al., 2012; Pagliassotti, 2012). Experimental evidence has indicated that all three major UPR sensors are involved in inflammatory response. However, the IRE1α pathway may play a dominant role in the upregulation of inflammatory cytokines, chemokines, and tissue remodeling molecules. The interaction between IRE1α and TRAF2 activates NF-κB and JNK pathways, which are critical for the expression of cytokines, chemokines, and other inflammatory mediators (Urano et al., 2000; Pincus et al., 2010; Zhang et al., 2011; Lerner et al., 2012; Oikawa et al., 2012; Tam et al., 2012; Upton et al., 2012; Qiu et al., 2013). Our following discussion will be centered on hepatic steatosis and inflammatory bowel diseases to illustrate the interplay between ER stress and inflammation (**Figure 1**).

ER STRESS-INDUCED INFLAMMATION IN HEPATIC STEATOSIS

As a vital organ for protein synthesis and detoxification, liver is especially susceptible to ER stress. Non-alcoholic fatty liver disease (NAFLD), a spectrum of metabolic disorders ranging from steatosis (NAFL) to steatohepatitis (NASH) to cirrhosis, is the foremost cause of non-alcoholic and non-viral liver-associated illness and death in the US (Kleiner and Brunt, 2012; Larrain and Rinella, 2012; Milic and Stimac, 2012; Pagliassotti, 2012; Tuyama and Chang, 2012; Wierzbicki and Oben, 2012; Ibrahim et al., 2013). Hepatic steatosis or fatty liver can progress to NASH, characterized by progressive liver injury, inflammation, and fibrosis. NASH is also associated with obesity, type 2 diabetes, and liver cancer development (Kleiner and Brunt, 2012; Tuyama and Chang, 2012; Wood, 2012). While ER stress is important for maintaining liver homeostasis, dysregulated ER stress contributes to the pathogenesis of various liver diseases. Accumulating evidence suggests that the interaction between ER stress and inflammation also promote liver steatosis and injury (Malhi and Kaufman, 2011; Cnop et al., 2012; Pagliassotti, 2012; Lake et al., 2013).

ER STRESS AND HEPATIC STEATOSIS

Although clinical and experimental data implicate the involvement of ER stress in liver diseases, the role and mechanism of ER stress in liver diseases remain not fully understood. A number of studies indicate that ER stress induces liver steatosis through regulating lipid synthesis and inflammation (Malhi and Kaufman, 2011; Li et al., 2012; Pagliassotti, 2012; Wierzbicki and Oben, 2012). This notion is supported by mice carrying genetic mutations of ER stress molecules, or by directly inducing ER stress *in vivo* via administration of chemical reagents (Birkenfeld et al., 2011; Ji et al., 2011; Zhang et al., 2011; Li et al., 2012; Nagarajan et al., 2012; Petrasek et al., 2012; Chen et al., 2013; Guo et al., 2013; Hamano et al., 2013). For example, Tunicamycin (TM), a widely used ER stress inducer, is a nucleoside antibiotics that blocks N-linked glycosylation, causing accumulation of unfolded or misfolded proteins in the ER lumen. Injection of TM into

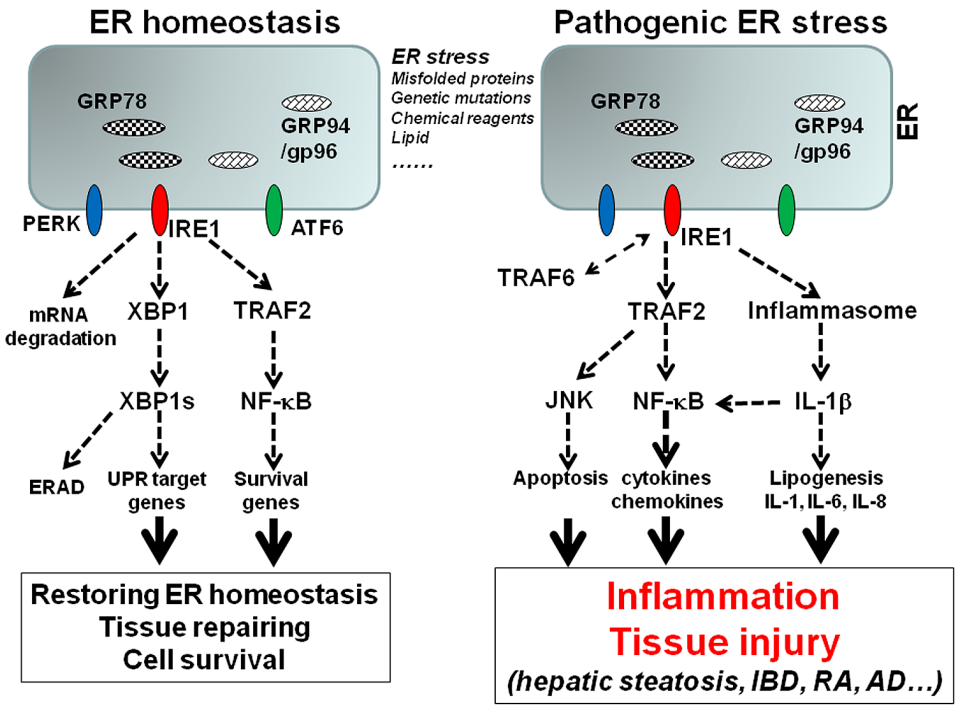

FIGURE 1 | The model of ER stress-associated inflammation. Accumulation of misfolded proteins, genetic mutations of ER stress molecules, or pharmacological compounds cause ER stress, which triggers the activation of three major UPR sensors: IRE1α, PERK, and ATF6. Activation of IRE1 pathway leads to the unconventional splicing of XBP-1, which control the transcription of a group of UPR genes including GRP78 and GRP94. The interaction of IRE1 and TRAF2 leads to NF-κB activation, which upregulates survival genes. The signaling and transcription programs initiated by IRE1 and other UPR pathways can restore the ER homeostasis. However, prolonged or unresolved ER stress leads to inflammation. Sustained interaction of IRE1 and TRAF2 leads to NF-κB and JNK activation, which promote inflammatory cytokine production and apoptosis. IRE1 is also involved in ER stress-induced inflammasome activation and IL-1β production, which in turn induces other inflammatory cytokines such as IL-6 and IL-8. In addition, TRAF6 can mediate IRE1 ubiquitination, which is required for TLR mediated optimal inflammatory cytokine production.

mice results in ER stress-mediated liver steatosis and lipogenesis (Zhang et al., 2011; Lee et al., 2012a,b). These findings indicate the potential pathological role of ER stress in the development of hepatic steatosis. On the other hand, obesity and liver steatosis have been shown to induce ER stress. For instance, mice with high fat feeding not only develop hepatic steatosis, insulin resistance, and type 2 diabetes, but also exhibit ER stress markers in liver and other tissues (Yang et al., 2010; Zhou and Liu, 2010; Birkenfeld et al., 2011). Thus ER stress and hepatic steatosis can form a positive feedback loop to further amplify liver inflammation and injury.

Several genetic strategies have been applied to tease out the roles of ER stress and chaperones in liver steatosis. The ER chaperone protein GRP78 is a critical regulator of ER homeostasis and stress responses, because it interacts and sequesters all major UPR sensors (Ye et al., 2010; Pfaffenbach and Lee, 2011). Kammoun et al. (2009) found that overexpression of GRP78 inhibited ER stress-induced sterol regulatory element binding protein (SREBP) expression and steatosis in the livers of obese (ob/ob) mice. Conversely, Ji et al. (2011) showed that GRP78 deletion led to liver fat accumulation and steatosis. Using conditional GRP78 KO mouse model, Ji et al. (2011) found that liver-specific deletion of GRP78 led to ER stress and apoptosis. These conditional KO mice also displayed liver injury and steatosis. Furthermore, the authors showed that liver-specific deletion of GRP78 exacerbated liver injury and/or steatosis induced by alcohol, high-fat diet, drugs, and toxins (Ji et al., 2011). These findings underscore the critical role of ER tress and GRP78 in liver homeostasis and viability in normal or disease conditions.

Results from Zhang et al. (2011) also demonstrate that ER is not only important for protein quality control, but also critical for lipid synthesis and metabolism. ER stress induces hepatic steatosis through upregulation of transcriptional factors essential for lipogenesis, including CCAAT/enhancer-binding protein β (C/EBPβ), peroxisome proliferator-activated receptor γ (PPARγ), and SREBP. Zhang et al. (2011) showed that the most conserved UPR sensor IRE1α protected animals from ER stress-induced hepatic steatosis. To study the role of IRE1 in liver steatosis, the authors generated a hepatocyte-specific IRE1α deficient mouse line. Deletion of IRE1α gene resulted in profound hepatosteatosis and hypolipidemia in mice under conditions of ER stress induced by proteasome inhibitor Bortezomib, or partial hepatectomy (Zhang et al., 2011). Results from this study further demonstrated that IRE1α represses the expression of transcriptional factors in lipid metabolism pathways, including C/EBPβ, C/EBPδ, and PPARγ (Zhang et al., 2011). The authors proposed that IRE1 is required for maintaining hepatic lipid homeostasis under ER stress conditions.

THE INTERACTION OF IRE1 AND TRAF PROTEINS IN NF-κB ACTIVATION

IRE1α plays a critical role in transcription of inflammatory genes due to its interaction with TRAF2, which promotes NF-κB activation and inflammatory response. The TRAF family proteins are intracellular adaptors that have been extensively studied in the signaling pathways of TNFR or IL-1/TLR superfamilies (Chung et al., 2002; Oganesyan et al., 2006; Bishop et al., 2007). All TRAF family proteins (TRAF1-7) have the most conserved TRAF domains in their carboxyl terminal region, which involve in binding to different receptor cytoplasmic tails and the formation of homo- or hetero-dimers between the family members. In addition, all TRAF proteins except TRAF1 have ring fingers, which may function as E3 ubiquitin ligase. Ubiquitination has been shown to play an important role in NF-κB and other signal pathways (Chen, 2005; Pineda et al., 2007; Ha et al., 2009). NF-κB is a homo or heterodimeric transcription factor that binds to κB sites in the promoters of a large number of genes involved in cell survival, inflammation, and immune responses (Senftleben and Karin, 2002; Chen, 2005). The activity of NF-κB is tightly regulated by members of the IκB family. In the classical NF-κB pathway, receptor engagement leads to the activation of the IκB kinase (IKK) complex, which includes IKKα, IKKβ, and IKKγ (NEMO). The activated IKKs phosphorylate IκBs such as IκBα, leading to subsequent ubiquitination and degradation of IκBs. This releases NF-κB and allows it to enter the nucleus and activate transcription of appropriate gene targets (Razani and Cheng, 2010; Dev et al., 2011). NF-κB dimers are composed of the five Rel family members that include NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel. The function of each individual NF-κB molecule is influenced by binding partners and other upstream molecules. Several early studies suggested that UPR sensor IRE1α and signaling molecule TRAF2 are required for activation of NF-κB in response to ER stress-inducing agents, Thapsigargin and TM. Kaneko et al. (2003) showed that ER stress-induced NF-κB activation was inhibited by a dominant-negative form of IRE1 or TRAF2. Hu et al. (2006) also showed that ER stress-induced NF-κB activation was impaired in IRE1α knockdown cells and IRE1 KO MEFs. The authors further demonstrated that TRAF2 provided a critical link between UPR/IRE1 signaling and downstream IKK/NF-κB activation. Biochemical experiments showed that in response to ER stress, IRE1α formed a signaling complex with IKKs though the adapter protein TRAF2 (Hu et al., 2006). Although those studies suggested that the interaction of IRE1, TRAF2, and IKK plays an important role in ER stress-induced NF-κB activation, how IRE1α activates the IKK complex is poorly characterized. Furthermore, the function of IRE1 and TRAF2 in ER stress-induced NF-κB activation *in vivo* needs to be further explored.

ER STRESS-INDUCED INFLAMMASOME ACTIVATION

IL-1β and other IL-1 family members are major mediators in inflammation. Production of mature IL-1β is regulated by at least two signals: TLR-mediated transcriptional upregulation of pro-IL-1β gene, and inflammasomes-mediated IL-1β maturation (Cassel et al., 2009; Leemans et al., 2011; Rathinam et al., 2012; Wen et al., 2012). An inflammasome is a multiprotein signaling complex, composed of NOD-like protein (NLR) such as NLRP3, the adaptor apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and caspase-1. Activation of an inflammasome triggers autoproteolytic cleavage of pro-caspase-1 into its active form, which subsequently processes pro-IL-1β into its mature form (Cassel et al., 2009; Horng and Hotamisligil, 2011; Menu and Vince, 2011; Vandanmagsar et al., 2011). Although very diverse and unrelated stimuli are found to trigger the activation of inflammasomes, the molecular mechanisms responsible for inflammasome activation remain elusive.

Recent progress suggests that inflammasomes play a critical role in a number of autoimmune and metabolic diseases. However, inflammasome activities have been shown to promote or protect liver steatosis (Imaeda et al., 2009; Petrasek et al., 2012; Szabo and Csak, 2012; Wood, 2012). Because of the important role of both ER stress and inflammasome in inflammation, we hypothesize that ER stress and inflammasome activity are interconnected during liver injury and steatosis. While several recent studies demonstrated that ER stress induces pro-IL-1β mRNA expression or NLRP3 inflammasome activation, whether or not UPR pathways are involved in inflammasome activation remains controversial (Lerner et al., 2012; Menu et al., 2012; Oslowski et al., 2012; Shenderov et al., 2014). For instance, an early study from Tschopp's group suggested that ER stress triggered NLRP3 inflammasome activation in THP-1 cells, a macrophage/monocyte cell line, in a mechanism that is independent of the classical ER stress signaling pathways (Lerner et al., 2012; Menu et al., 2012; Oslowski et al., 2012), whereas other groups suggested that IRE1α under irremediable ER stress induced pro-IL-1β mRNA expression via thioredoxin-interacting protein, which was also associated with programmed cell death of pancreatic β cells. Further studies in our and other laboratories are ongoing to elucidate the molecule mechanisms by which ER stress induces NLRP3 inflammasome activation and IL-1β production.

URP SIGNALING PATHWAYS INVOLVED IN LIVER INFLAMMATION

In addition to NF-κB pathway, IRE1/TRAF2 can activate other transcription factors such as AP-1, which is also important for the expression of inflammatory cytokines. PERK and ATF6 pathways have also been reported to activate NF-κB (Yamazaki et al., 2009; Nakajima et al., 2011; Tam et al., 2012); however, the mechanisms of PERK or ATF6-medaited NF-κB activation remain unknown. In addition, activation of UPR leads to the generation of ROS, which can induce inflammatory response. All these UPR pathways potentially contribute to liver steatosis and inflammation. However, the relative contribution of each pathway is unclear. Furthermore, NFALD progression is associated with increased apoptosis of hepatocytes. It has been suggested that severe or chronic ER stress can cause cell death via induction of the CHOP pathway (Nishitoh, 2012; Han et al., 2013). The UPR pathways PERK and ATF6 branches are responsible for the activation of CHOP. IRE1 pathway can also induce cell death through either IRE1/TRAF2-mediated JNK activation or direct interaction with Bac and Bak proapoptotic proteins (Kato et al., 2012). It is possible that cell death under severe ER stress conditions leads to sterile inflammation and tissue damage in liver.

INFLAMMATORY BOWEL DISEASES (IBD)

IBD, including Crohn's disease (CD) and ulcerative colitis (UC), is a complex intestinal tract disease involving various immune cells, epithelial cells, and intestinal microflora (Strober et al., 2007; Buonocore et al., 2010; Fritz et al., 2011; Papatriantafyllou, 2011; Jostins et al., 2012). Genetic studies have identified many genes, most related with immune response such as IL-10, TLR, NOD2, and IL-23, autophagy and ER stress, that are associated with the development of intestinal inflammation (Hugot et al., 2001; Van Limbergen et al., 2007; Okazaki et al., 2008; Abraham and Cho, 2009; Sarra et al., 2010; Begue et al., 2011; Fritz et al., 2011). Intestinal epithelial cells (IECs) are constantly exposed to gut microflora. One of major functions of intestinal Paneth cells and goblet cells is secretion of various factors essential for the intestinal homeostasis and host defense. Furthermore, macrophages and DCs actively produce inflammatory cytokines in response to translocated bacteria or bacterial components. Therefore, the intestinal system is highly susceptible to ER stress. The interaction of innate and microflora contributes to intestinal inflammation in both acute and chronic colitis. Several excellent studies using genetic mutant mice have highlight the critical role of ER stress in the pathogenesis of colitis.

GRP94 IN GUT HOMEOSTASIS AND INFLAMMATION

Recent studies from others and our group have demonstrated that ER HSP protein GRP94 is a critical chaperone for multiple TLRs and integrins (Yang et al., 2007). To study the role of ER chaperones in the intestinal homeostasis and inflammation, Liu et al. (2013) generated conditional GRP94 KO mice and intestinal tissue-specific GRP 94 KO mice. In the tamoxifen-inducible GRP94 KO mice, deletion of GRP94 led to rapid weight loss, diarrhea, and ultimately death 12–14 days post-tamoxifen injection. While not affecting embryo development, gut-specific deletion of GRP94 was associated with postnatal death of mutant mice. Pathological analysis demonstrated that GRP94 loss compromised the intestinal barrier function with significant intestinal dilatation, edema and hemorrhage, and thickening of the intestinal wall. The authors further demonstrated that GRP94 maintained gut homeostasis through direct regulation of canonical Wnt-signaling pathway. The results showed that GRP94 interacted with mesoderm development (MesD), an ER chaperone essential for the Wnt coreceptor low-density lipoprotein receptor-related protein 6 (LRP6; Liu et al., 2013). The Wnt/β-catenin signaling pathway has been shown previously to be critical for intestinal homeostasis (Krausova and Korinek, 2014). Mechanistically, GRP94 deletion impaired export of LRP6 from ER to the cell surface, leading to profound loss of gut intrinsic Wnt signaling and intestinal homeostasis. This finding underscores the importance of GRP94 in chaperoning the canonical LRP6/Wnt signal pathway.

THE IRE1-XBP1 PATHWAY IN INTESTINAL HOMEOSTASIS

Several studies implicated UPR pathways in intestinal inflammation and colitis. Bertolotti et al. (2001) showed that IRE1β deficient mice exhibited increased sensitivity to acute colitis induced by dextran sodium sulfate (DSS). The authors found that expression of IRE1β was restricted to the epithelium of the gastrointestinal tract. DSS treatment led to elevated levels of ER stress markers and increased severity of colitis in IRE1β KO mice, compared with WT mice (Bertolotti et al., 2001). It is unclear whether increased ER stress in IECs was caused by espousing of gut epithelial cells to microflora or by intestinal inflammation after DSS treatment. Nevertheless, these results suggest that deletion of UPR sensor IRE1β disrupted intestinal homeostasis in response to environmental challenge. IRE1 has two forms, IRE1α and IRE1β, encoded by two distinct genes. It is noteworthy that untreated IRE1β deficient mice showed no sign of intestinal inflammation histologically (Bertolotti et al., 2001). One of reasons might be the redundant role of IRE1α and IRE1β. However, whether specific deletion of IRE1α in the IECs enhances the susceptibility to DSS-induced colitis has not been reported.

The involvement of IRE1 pathway in colitis is also supported by XBP1 conditional KO mice (Kaser et al., 2008). As a ribonuclease, an important substrate of IRE1 is the mRNA for transcriptional factor XBP1. Activated IRE1 splices the XBP1 mRNA by the excision of a 26bp fragment via an unconventional splicing mechanism, generating spliced XBP1 (XBP1s; Adolph et al., 2012; Upton et al., 2012). XBP1s controls the transcription of a set of UPR target genes, including chaperones, protein disulfide isomerases (PDIs), and components of ERAD, essential for the maintenance of ER function. Furthermore, XBP1 plays a critical role in the development of highly secretory cells such as plasma cells and pancreatic cells (Glimcher, 2010; Sha et al., 2011). Dr. Blumberg's group reported that mice with tissue-specific deletion of XBP1 in IECs developed spontaneous enteritis and displayed increased susceptibility to DSS-induced colitis (Kaser et al., 2008). Kaser et al. (2008) showed that mice deficient in XBP-1 in intestinal tissues displayed spontaneous intestinal inflammation characterized by immune cell infiltration, loss of crypts, and ulceration. Strikingly, deletion of XBP1 in IECs also resulted in the reduced number of Paneth and goblet cells (Kaser et al., 2008; Adolph et al., 2013). A major function of Paneth cells is to secret antimicrobial peptides. Goblet cells are IECs that produce protective mucus in the microbiota–intestine interface. The diminished function or number of both cell types rendered these XBP1 deficient mice more susceptible to DSS-induced colitis. The authors also found that XBP-1 deficient intestinal tissues had increased expression of ER stress mediators such as GRP78, ATF4, and CHOP (Kaser et al., 2008). Enhanced ER stress may lead to more inflammation in response to gut microflora. In addition, upregulation of CHOP may contribute to apoptosis of Paneth cells in XBP-1 deficient mice. Thus, dysregulated ER stress in IEC compartment induces intestinal inflammation and colitis. Kaser et al. (2008) also found the association of XBP1 variants with both CD and UC, indicating XBP1 as a genetic risk factor for human IBD.

INFLAMMASOME ACTIVATION IN THE DEVELOPMENT OF COLITIS

The role of inflammasomes as well as their interaction with microbiota in intestinal inflammation is an active area of research. Up to now, a number of publications have painted a more complex picture of inflammasomes in colitis and colitis-associated colon cancer (Sands, 2007; Bauer et al., 2010, 2012; Zaki et al., 2010;

Henao-Mejia et al., 2012b). Moreover, the interaction between ER stress pathways and inflammasomes is still yet to be established. Several studies showed that mice deficient for inflammasome components including NLRP3, ASC, and caspase-1 were highly susceptible to acute colitis induced by DSS, indicating the protective role of inflammasome in acute colitis (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Hirota et al., 2011). However, Bauer et al. (2010) showed that that defective in NLRP3 inflammasome protected mice from DSS-induced acute colitis, indicating that inflammasomes contribute to the development of colitis. Our recent study showed that inflammasome activation promoted the intestinal inflammation in IL-10 KO mice, which develops chronic colitis resembling human IBD. We also found that inhibition of inflammasome activities with IL-1 receptor antagonist or caspase-1 inhibitors suppressed intestinal inflammation (Zhang et al., 2014). Our data further suggest that inflammasome-derived IL-1β promoted Th17 phenotype in intestinal tissues. Those results indicate that without inhibitory effects of IL-10, the interaction between inflammasomes and microbiota leads to intestinal inflammation and the development of colitis.

ER STRESS AND INFLAMMATION IN COLITIS-ASSOCIATED COLON CANCER

Inflammation contributes to tumor initiation and progression, especially for colitis-associated colon cancer. Because of unregulated growth and hypoxia environments, ER stress is associated with tumor development. Depending on type or stage of tumor development, ER stress can enhance or suppress tumor development (Ghiringhelli et al., 2009; Allen et al., 2010; Hu et al., 2011; Bruchard et al., 2013). Moreover, Mahadevan et al. (2011) found that ER stress from stressed tumor cells could be transmitted to other neighborhood cells via un-identified heat-stable molecules. Conditioned media derived from cancer cells with ER stress could induce ER stress markers such as XBP-1, GRP78, and CHOP in macrophages, leading to production of inflammatory molecules (Mahadevan et al., 2011). It is possible that this kind of transmissible ER stress could amplify inflammation in tumor microenvironments.

Because most studies on ER stress and tumor development are focused on tumor-intrinsic stress pathways, research on ER stress and inflammation in tumor microenvironments is limited so far. Using macrophage-specific KO mice, Morales et al. (2014) showed that mice with macrophage-specific deletion of GRP94/gp96 exhibited decreased colitis and inflammation-associated colon cancer induced by DSS/AOM, with reduced expression of inflammatory cytokines such as IL-6, IL-17, and IL-23. These results demonstrate the macrophage-intrinsic role of chaperone GRP96 in promoting colitis and inflammation-associated colon tumorigenesis.

Similar to its controversial role in colitis, inflammasomes also have a complex role in tumor development. A study by Allen et al. (2010) showed that NLRP3-deficient mice were more sensitive

Table 1 | A list of diseases linked to dysregulated ER stress.

Diseases	Reference
Metabolic diseases	
Hepatic steatosis	Malhi and Kaufman (2011), Zhang et al. (2011)
Type 2 diabetes mellitus	Papa (2012), Biden et al. (2014)
Obesity	Kawasaki et al. (2012), Tripathi and Pandey (2012)
Atherosclerosis	McAlpine and Werstuck (2013), Zhou and Tabas (2013)
Autoimmune diseases	
Arthritis	Yoo et al. (2012), Qiu et al. (2013), Savic et al. (2014)
Inflammatory diseases	Kaser et al. (2008), Liu et al. (2013)
Chronic pulmonary diseases	Blohmke et al. (2012), Blumental-Perry (2012)
Neurodegenerative diseases	
Alzheimer's disease	Cornejo and Hetz (2013), Li et al. (2014)
Parkinson's disease	Bellucci et al. (2011), Vidal et al. (2012), Mercado et al. (2013)
Multiple sclerosis	Cunnea et al. (2011), McMahon et al. (2012)
Pathogen infections	
Viral infection	Chan (2014), Tesh (2012), Blazquez et al. (2014)
Bacterial infection	Martinon et al. (2010), Lee et al. (2013)
Tumor	
Breast cancer	Martin-Perez et al. (2014), Zheng et al. (2014)
Colon cancer	Hardy et al. (2012), Morales et al. (2014)
Leukemia and lymphomas	Mahoney et al. (2013), Kharabi Masouleh et al. (2014)
Hepatocellular carcinoma	Shuda et al. (2003), Tang et al. (2012), Chen et al. (2013)
Pancreatic cancer	Kubisch and Logsdon (2008), Romero-Ramirez et al. (2009)

to colorectal tumorigenesis. The authors found that mice deficient for NLRP3 inflammasome components including NLRP3, ASC or caspase-1 had severe colitis and increased tumorigenesis in AOM/DSS colon cancer model. But NLRC4 KO mice displayed similar incidence of colitis-associated colon cancer as WT mice. The authors further demonstrated that NLRP3-inflammasome activation in bone marrow-derived cells was critical for the tumor suppression. In contrast, Hu et al. (2011) found that caspase-1 KO and NLRC4 KO mice, but not NLRP3 KO mice, had increased tumorigenesis in the AOM/DSS colon cancer model. Furthermore, NLR6 and NLR12 also have been shown to suppress tumor development in AOM/DSS colon cancer model (Chen and Nunez, 2011; Chen et al., 2011). A possible explanation is that inflammasome-processed IL-1 and IL-18 signaling in IECs provides protection against apoptosis of gut epithelial cells. Those studies imply that the inflammasomes may promote or suppress colon cancer development depending on experimental conditions, possibly gut microflora.

ER STRESS AND INFLAMMATION IN OTHER DISEASES

ER stress-induced inflammation is also associated with other metabolic (Kawasaki et al., 2012; Papa, 2012; Tripathi and Pandey, 2012; McAlpine and Werstuck, 2013; Zhou and Tabas, 2013; Biden et al., 2014), inflammatory (Blohmke et al., 2012; Blumental-Perry, 2012; Yoo et al., 2012; Qiu et al., 2013; Savic et al., 2014), neurodegenerative diseases (Bellucci et al., 2011; Cunnea et al., 2011; McMahon et al., 2012; Vidal et al., 2012; Cornejo and Hetz, 2013; Mercado et al., 2013; Li et al., 2014), pathogen infections (Martinon et al., 2010; Merquiol et al., 2011; Tesh, 2012; Lee et al., 2013; Blazquez et al., 2014; Chan, 2014), and cancer (Shuda et al., 2003; Kubisch and Logsdon, 2008; Romero-Ramirez et al., 2009; Hardy et al., 2012; Tang et al., 2012; Chen et al., 2013; Mahoney et al., 2013; Kharabi Masouleh et al., 2014; Martin-Perez et al., 2014; Shimodaira et al., 2014; Zheng et al., 2014). While this review focuses on liver steatosis and inflammatory bowl diseases, **Table 1** listed various diseases where pathogenesis has directly or indirectly been linked to ER stress.

CONCLUSION AND PERSPECTIVES

ER stress functions as an adaptive response to maintain cell homeostasis and survival when normal protein or lipid synthesis and metabolism are perturbed. Emerging evidence indicates that ER stress is also closely associated with inflammation, in addition to its classic role in protein quality control. We propose that the IRE1/TRAF2 axis represents a major pathway to link ER stress with key transcription factors such as NF-κB that controls the expression of inflammatory cytokine genes. We also speculate that the interaction between UPR pathways and inflammasomes provides another link between ER stress and inflammation. While the activation of UPR pathways can lead to resolving the ER stress and achieving new protein homeostasis, prolonged and severe ER stress can result in cell death and tissue damage, contributing to the pathogenesis of various diseases. In both scenarios, inflammation can be induced in stressed cells and surrounding cells. In a broad term, inflammatory diseases such as IBD and hepatic steatosis are the results of interaction amongst ER stress, inflammatory cytokines, metabolism, and gut microflora (Machado and Cortez-Pinto, 2012). Similarly, ER stress and inflammation can promote or inhibit the development of colitis-associated cancer depending on genetic background, environments, and intestinal microflora. A better understanding of ER stress and inflammation may lead to identification of potential therapeutic targets for the treatment of inflammatory and metabolic diseases as well as cancer.

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