

Review

Proteostasis and Beyond: ATF6 in Ischemic Disease

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Endoplasmic reticulum (ER) stress is a pathological hallmark of numerous ischemic diseases, including stroke and myocardial infarction (MI). In these diseases, ER stress leads to activation of the unfolded protein response (UPR) and subsequent adaptation of cellular physiology in ways that dictate cellular fate following ischemia. Recent evidence highlights a protective role for the activating transcription factor 6 (ATF6) arm of the UPR in mitigating adverse outcomes associated with ischemia/reperfusion (I/R) injury in multiple disease models. This suggests ATF6 as a potential therapeutic target for intervening in diverse ischemia-related disorders. Here, we discuss the evidence demonstrating the importance of ATF6 signaling in protecting different tissues against ischemic damage and discuss preclinical results focused on defining the potential for pharmacologically targeting ATF6 to intervene in such diseases.

Endoplasmic Reticulum Stress, the Unfolded Protein Response, and Ischemic Disease

Many diseases lead to impaired circulation, which can cause ischemic conditions in a variety of organs, including the brain, heart, and kidney [1]. In these settings, prolonged ischemia causes irreversible damage, which can be partially mitigated by clinical interventions that restore blood flow through reperfusion [2]. While reperfusion is necessary to mitigate the damage of continued ischemia, reperfusion itself is known to cause some additional damage, generally as a result of **reactive oxygen species** (ROS; see [Glossary](#)) formation [3]. The complex nature of cellular injury associated with ischemia or ischemia followed by reperfusion (I/R) has previously been shown to affect the levels and activities of numerous signaling pathways and transcription factors, including protein kinase C, protein kinase B (Akt), nitric oxide synthase, glycogen synthase kinase 3b, Nrf2, and HIF-1 α [4]. One I/R-activated pathway that has been more recently studied involves the disruption of endoplasmic reticulum (ER) protein homeostasis (or **proteostasis**), otherwise known as **ER stress** [3,5,6]. Pathological ER stress is associated with numerous adverse outcomes, including impaired Ca²⁺ homeostasis, disruptions in secretory proteostasis, impaired lipid metabolism, and increased apoptotic signaling [3,5–7]. To protect against pathological ER stress induced by I/R, tissues activate endogenous adaptive stress-responsive signaling pathways, such as the **unfolded protein response (UPR)**.

The UPR is the primary stress-responsive signaling pathway activated in response to ER stress. In metazoans, the UPR comprises three stress-signaling arms activated downstream of the ER stress-sensing membrane proteins PERK, IRE1, and ATF6 [8–10]. In response to ER stress, these sensors initiate well-defined signal transduction pathways that lead to translational and transcriptional remodeling of stress-responsive biological pathways involved in diverse activities, including ER proteostasis maintenance, cellular redox maintenance, and metabolite homeostasis [8–10]. The activation of these pathways is a protective mechanism to alleviate

Highlights

ATF6 activation is protective against ischemia and ischemia/reperfusion in cellular and animal models of diverse ischemic diseases, including myocardial infarction and stroke.

ATF6 activation ameliorates ischemia-associated damage through the transcriptional upregulation of genes involved in multiple, protective biological activities, including ER proteostasis maintenance, Ca²⁺ regulation, and antioxidant response.

Human genetic and animal studies indicate that moderate levels of ATF6 activation are well tolerated in adults and do not induce systemic or neurological defects.

Recently established pharmacological ATF6-activating compounds are protective against ischemia-associated damage in multiple tissues both *in vitro* and *in vivo*.

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ER stress and promote cellular adaptation following an acute insult. However, severe or chronic ER stress can initiate proapoptotic UPR signaling, primarily as a result of activating the PERK UPR signaling arm [9,11]. Thus, the UPR has a central role in dictating cellular physiology and fate in response to diverse types of ER insult.

All three arms of the UPR are activated in tissue-specific I/R rodent models and in postmortem samples from humans with ischemic diseases, including **myocardial infarction (MI)** and stroke (reviewed in [4,5]). Genetic dissection of these three UPR pathways indicates that PERK, IRE1, and **ATF6** activation distinctly influence tissue physiology in response to I/R. Signaling through the PERK pathway has been proposed to promote apoptosis during I/R through mechanisms including transcriptional upregulation of proapoptotic factors, such as CHOP [5,6,12]. Alternatively, signaling through the IRE1 and ATF6 pathways has been shown to protect against I/R in diverse tissues, including the heart and brain. The protection afforded by IRE1 during I/R is discussed briefly in **Box 1**. For the remainder of this review, we specifically focus on recent genetic and pharmacological evidence highlighting the protective benefits of ATF6 activation in ischemic disease.

The ATF6 Signaling Arm of the UPR

ATF6 is a type II ER transmembrane protein that comprises three functional domains: an N-terminal bZIP transcription factor domain, a transmembrane domain, and a luminal domain (Figure 1A). Metazoans have two different isoforms of ATF6, ATF6 α and ATF6 β , which encode proteins with similar domain architectures [13–15] (Figure 1A). In the context of ER stress and the UPR, ATF6 α is the predominant ATF6 isoform responsible for regulating the expression of ER stress-responsive genes [16]. By contrast, the functional implications of ATF6 β activity remain poorly defined, although it has been proposed that it can act as an endogenous repressor of ATF6 α and serves to fine-tune the strength and duration of ATF6 α signaling during ER stress [17–20]. Genetic ablation of either *Atf6 α* or *Atf6 β* does not overtly impact prenatal development in mice, although genetic ablation of both ATF6 isoforms is embryonic lethal [16]. This indicates that ATF6 α and ATF6 β complement each other to exert functions that are required for organismal development. Since ATF6 α is the predominant isoform responsible for regulating cellular physiology in response to ER stress, we primarily focus here on the implications of signaling through the *ATF6 α* isoform (herein referred to as ATF6 unless otherwise indicated) in ischemic diseases.

Box 1. IRE1/XBP1s Signaling Is Protective against I/R and Related Disorders

When activated by ER stress, IRE1 leads to the generation of the active form of the transcription factor XBP1, referred to as spliced XBP1 or XBP1s, through a mechanism involving the IRE1-dependent regulated splicing of *XBP1* mRNA [8–10,78]. XBP1s has been shown to be protective in several disease models, including ischemia and I/R injury in cancer, as well as in the heart, kidney, and brain [5,11,79–81]. While this protection is likely to be afforded through a multitude of pathways, depending on the model system, several common themes have emerged linking the XBP1s gene program to protection from ischemia and I/R, including the induction of protective chaperones, protein **O-GlycNACylation**, and autophagy [5,12,82–85]. For example, it was shown that key genes that regulate the synthesis of hexosamines, which are precursors used in the process of protein O-GlycNACylation, are direct targets of XBP1s [85]. In that study, genetic deletion of XBP1 increased I/R damage in the heart, which could be reduced by overexpression of hexosamine biosynthetic enzymes, indicating that XBP1-dependent regulation of the hexosamine pathway is protective during I/R. XBP1s has also been shown to regulate angiogenesis in response to ischemia through a novel mechanism. In response to ischemia, XBP1s in vascular smooth muscle cells regulates the release of key miRNAs that interact with nearby endothelial cells enhancing vascular endothelial growth factor (VEGF) signaling, endothelial cell migration, and new blood vessel formation [86]. Thus, the IRE1/XBP1 UPR signaling arm exerts broad effects on cellular processes well beyond its canonical role as a regulator of ER proteostasis that appear protective against ischemia and I/R. This suggests that these protective aspects of IRE1/XBP1s signaling could be pharmacologically accessed to intervene in ischemia-related diseases using similar approaches to those described for ATF6 in this review.

Glossary

Activating transcription factor 6

(ATF6): an UPR-associated stress-sensing protein that is activated in response to ER stress. ATF6 activation involves a regulated proteolysis event that releases the ATF6 N-terminal domain containing an active transcription factor. This active transcription factor induces expression of multiple stress-responsive genes shown to be protective against I/R.

Endoplasmic reticulum (ER)

stress: a cellular condition induced by physiological and pathological insults that disrupt ER proteostasis and function.

Ischemia/reperfusion (I/R):

a condition defined by the initial restriction of blood flow to a specific tissue (ischemia) followed by the restoration of blood flow to that tissue (reperfusion).

Myocardial infarction (MI):

a condition involving restriction of blood flow to the heart (cardiac ischemia) and the resulting damage associated with the heart.

O-GlycNACylation:

the process of adding a single O-linked N-acetylglucosamine to proteins at serine or threonine residues of proteins.

Proteolysis:

a biological process involving the breakdown of proteins into smaller fragments or amino acids.

Proteostasis:

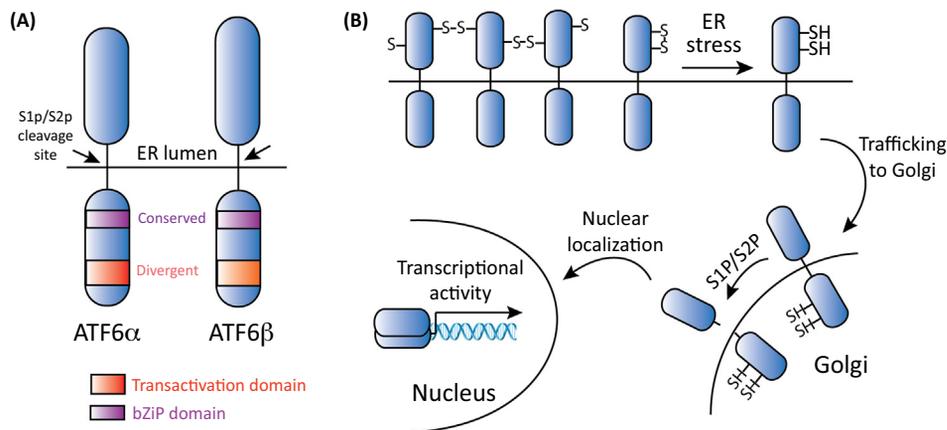
the condition of an organism, tissue, or cell having sufficiently properly folded proteins to maintain essential physiological functions while minimizing the accumulation of toxic misfolded protein species.

Reactive oxygen species (ROS):

reactive chemical species including oxygen atoms. These include peroxide and superoxide.

Unfolded protein response (UPR):

a cellular pathway that functions to regulate cellular adaptation and survival in response to physiological and pathological insults that induce ER stress.



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Figure 1. Activating Transcription Factor 6 (ATF6) Is Activated in Response to Endoplasmic Reticulum (ER) Stress through a Process Involving Regulated Proteolysis. (A) Illustration showing the domain organization of the genes encoding ATF6 α and ATF6 β highlighting the conserved and divergent features of these two genes. (B) Illustration showing the mechanism of ATF6 activation during ER stress. In the absence of ER stress, ATF6 is retained within the ER in disulfide bond-linked oligomers. In response to ER stress, these disulfides are reduced to yield reduced ATF6 monomers that traffic to the Golgi, where they are proteolyzed by the site 1 (S1P) and site 2 (S2P) proteases. This proteolysis releases the cytosolic, N-terminal ATF6 transcription factor domain that localizes to the nucleus, homo- or heterodimerizes, and promotes ATF6-regulated transcriptional activity.

Of the three arms of the UPR, the mechanism for ER stress-dependent ATF6 activation remains the least well understood. In response to ER stress, ATF6 is trafficked from the ER to the Golgi, where it is cleaved by site 1 and site 2 proteases at specific sites near or within the transmembrane domain (Figure 1B) [21,22]. This **proteolysis** liberates the cytosolic N-terminal domain of ATF6, allowing it to localize to the nucleus, where it acts as a transcription factor to regulate expression of stress-responsive genes (e.g., *BiP*) that constitute the ATF6 transcriptional program [21,22]. The details of the key step in this activation mechanism, the regulated trafficking from the ER to the Golgi, are currently poorly defined, but appear to involve regulation of both ATF6 redox status and oligomerization. In the absence of ER stress, ATF6 is retained within the ER as disulfide-linked oligomers bound to the ER chaperone BiP, a configuration that favors ER localization and prevents trafficking to the Golgi [23,24]. In response to ER stress, these disulfide bonds are reduced, facilitating the dissociation of ATF6 oligomers to reduced monomers that can traffic to the Golgi and undergo proteolytic processing by Site 1 and Site 2 proteases (S1P and S2P, respectively) [23,24]. Although the molecular factors responsible for regulating ATF6 redox status in the absence or presence of ER stress are not well defined, protein disulfide isomerases (PDIs), such as PDIA5, have been implicated in the ER stress-dependent reduction of ATF6 disulfides [25,26], suggesting a critical role for PDIs in regulating ATF6 trafficking. While the reduction and subsequent dissociation of ATF6 disulfide bonds are required for both trafficking to the Golgi and proteolytic activation, genetic disruption of ATF6 disulfide bonds alone was not sufficient to promote complete ATF6 activation [23], indicating that other mechanisms are also involved in dictating activation of this UPR signaling pathway. For example, other molecular events, including dissociation of the ER chaperone BiP from the ATF6 luminal domain [27,28] or alterations in ATF6 N-linked glycosylation [29], have been implicated in ER stress-dependent ATF6 activation and likely contribute to the regulation of ATF6 activation during ER stress. However, how, and if, these other events integrate with the regulation of ATF6 disulfides remains an open question.

Upon proteolytic activation, the cleaved version of ATF6 homodimerizes and binds to promoter regions of target genes containing canonical ATF6 binding motifs, such as the ER stress element (ERSE) [30]. The ATF6 transcriptional program has been elucidated using multiple genetic and chemical genetic approaches, all of which demonstrate that this pathway has a central role in regulating the composition of biological pathways involved in diverse functions, including ER proteostasis maintenance, protein degradation, and cellular redox regulation [30–33]. Unlike the other arms of the UPR regulated by PERK and IRE1, the ATF6 arm has not been substantially linked to proapoptotic signaling [9,11]. Instead, ATF6 primarily functions in the so-called ‘adaptive UPR’ designed to promote protective, adaptive remodeling of cellular physiology and recovery following acute physiological and pathological insults. As part of the adaptive UPR, ATF6 integrates with multiple other stress-responsive signaling pathways to sensitively adapt cellular physiology to diverse types of ER insult. In the context of the UPR, this integration can be achieved through heterodimerization of ATF6 with other UPR-regulated bZIP transcription factors, such as XBP1s (a bZIP transcription factor activated downstream of IRE1) or ATF6 β . ATF6/XBP1 heterodimers promote expression of select subsets of genes involved in biological functions, such as ER-associated degradation (ERAD) [16,31]. Alternatively, as mentioned earlier, heterodimerization of ATF6 α and ATF6 β bZIP transcription factors provides a mechanism to ‘fine-tune’ the extent and timing of the ATF6 stress response [17–20]. ATF6 also has the potential to heterodimerize with other bZIP transcription factors similarly regulated through a mechanism involving S1P/S2P-dependent proteolysis, such as CREB-H [34,35], providing additional mechanisms to sensitively adapt ATF6 signaling during ER stress. Apart from heterodimerization, ATF6 signaling also integrates with other stress-responsive signaling pathways, such as PGC1 α -dependent mitochondrial biogenesis and mTOR signaling (Box 2). The capacity for ATF6 to integrate with other signaling pathways through multiple mechanisms reflects a unique potential for this UPR signaling arm to coordinate protective cellular responses, in addition to ER proteostasis remodeling, to a range of pathological insults that induce ER stress, including I/R.

ATF6 Protects against I/R Damage in Multiple Tissues

Consistent with a protective role for ATF6 in regulating cellular physiology during pathological insults, ATF6 protects against I/R-associated damage in numerous tissues. One of the first examples of this protection was demonstrated using a chemical genetic strategy to regulate the activity of ATF6 in the heart. This strategy used a transgenic mouse line that expresses a tamoxifen-activated form of ATF6; administration of tamoxifen to these transgenic mice

Box 2. ATF6 Activity Integrates with Diverse Signaling Pathways to Coordinate Cellular Physiology during Stress

Recent studies have revealed that, in addition to its roles as a regulator of genes that fortify ER proteostasis, ATF6 influences the expression of genes that affect cellular physiology more broadly. This comes, in part, through integration with other stress-responsive signaling pathways. For example, ATF6 has been found to induce genes related to growth of skeletal [87] and cardiac muscle [88], the latter of which has been attributed to ATF6-specific induction of mTOR-activating proteins, which coordinates the upregulation of ER protein-folding resources with increased protein-folding demand during growth. The ATF6 gene program in a given cell or tissue type differs depending upon the stimulus. For example, oxidative stress leads to ATF6-dependent induction of antioxidant genes, but not growth promoting genes, while growth stimuli lead to induction of growth promoting genes but not antioxidant genes [88]. Additionally, lipotoxic stress activates lipid metabolism genes but not ER proteostasis genes, while proteotoxic stress induces proteostasis genes, but not lipid metabolism genes [89]. Such differential ATF6 target gene induction by treatments that all activate ATF6 suggests that there are yet-to-be-described regulatory layers that fine-tune the ATF6 gene program to best adapt to specific conditions. Some possible mechanisms that could contribute to this differential expression are beginning to emerge, as it has been shown that ATF6 can functionally interact with other transcription factors, such as NRF1, PGC1 α , PPAR α , and ERR γ [66,87,90,91], which changes the transcriptional programming in ways that sensitively adapt ATF6-dependent cellular responses to diverse types of pathologic insults.

induced robust activation of ATF6 in the heart, evidenced by increased expression of multiple ATF6 target genes [32]. Tamoxifen-dependent ATF6 activation *in vivo* significantly improved the recovery of cardiac contractility following *ex vivo* I/R and reduced markers of damage, such as infarct size and apoptotic signaling in response to *in vivo* I/R [32]. Consistent with these findings, inhibiting ATF6 activation using S1P inhibitors or a dominant-negative ATF6 increased cardiac damage in a mouse model of MI, reduced cardiac function, and reduced 14-day survival following MI [36]. The importance of ATF6 in protecting the heart during I/R was further confirmed using whole-body and cardiac myocyte-specific *Atf6*^{-/-} mice, both of which showed increased sensitivity to cardiac I/R damage both *ex vivo* and *in vivo* [37,38]. In both cases, exogenous overexpression of ATF6 ameliorated this cardiac sensitivity to I/R damage and improved cardiac function, both in wild-type and *Atf6*^{-/-} mice [36–38]. Apart from cardiac ischemia, *Atf6* deletion also sensitized the brain to middle cerebral artery occlusion (MCAO) models of ischemic stroke [37–39], and ATF6 overexpression in the brain ameliorated neurological defects associated with this model [40]. These results indicate that ATF6 can protect multiple tissues against ischemic insults.

The molecular mechanism by which ATF6 protects against I/R damage in different tissues appears to be tightly linked to ATF6-regulated stress-responsive genes involved in diverse biological functions (Figure 2). ATF6-dependent increases in ER chaperones, such as the ATP-dependent chaperone BiP, are likely implicated in ATF6 protection against I/R in tissues such as the heart. I/R-dependent increases in *BiP* are blocked in *Atf6*-deficient mice, confirming the specific regulation of this chaperone by ATF6 [37,38]. Mimicking ATF6-dependent increases in BiP by overexpression of this chaperone reduced ROS-associated damage and improved heart function in mice subjected to cardiac I/R [41]. Similarly, BiP has been shown to be protective in both *in vitro* and *in vivo* mouse models of ischemic stroke [42–44]. The specific mechanism by which BiP protects against I/R-associated damage remains to be fully defined. However, cellular models of simulated I/R using neonatal rat ventricular myocytes (NRVM) indicate that the protection afforded by BiP overexpression results from activation of AKT, a central kinase involved in regulating cell survival during I/R [45–48], potentially through a mechanism involving increased localization of BiP to the cell surface [41]. Alternatively, BiP overexpression in astrocytes improves Ca²⁺ regulation and mitochondrial function in cell culture models of ischemia, suggesting that BiP protects cells against I/R through mitochondrial

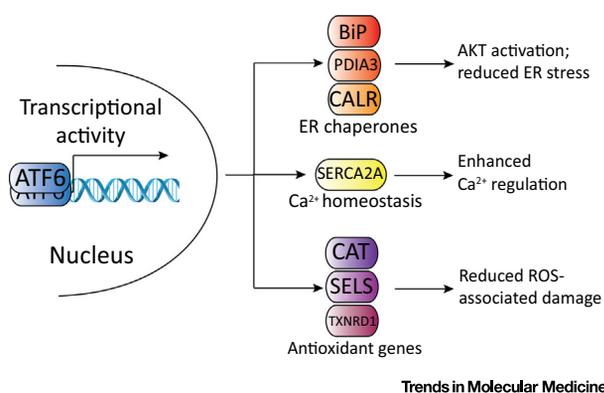


Figure 2. Molecular Mechanisms by which Activating Transcription Factor 6 (ATF6) Activation Protects Tissues against Ischemia and Ischemia/Reperfusion (I/R). Illustration showing three types of protective, stress-responsive gene induced by ATF6, including endoplasmic reticulum (ER) chaperones (e.g., *BiP*, *PDIA3*, and *CALR*) and Ca²⁺ pumps (*SERCA2A*), which are involved in canonical ER proteostasis pathways, and antioxidant genes [e.g., catalase (*CAT*), Selenoprotein S (*SELS*), and thioredoxin reductase 1 (*TXNRD1*)], which are examples of non-canonical targets of ATF6. The specific impact of these different genes on cellular physiology during I/R is highlighted. Abbreviation: ROS, reactive oxygen species.

regulation [44]. Increases in BiP also likely protect by improving ER proteostasis and attenuating ER stress-associated proapoptotic signaling through the UPR. Consistent with this, overexpression of other ATF6-regulated ER chaperones and folding enzymes, including GRP94, PDIA3, and PDIA6, also have been shown to protect cells and tissues against I/R associated injuries [49–51]. Despite the evidence indicating that these chaperones are protective against I/R, the specific dependence of ATF6-mediated protection on the increased expression of these different chaperones and folding enzymes remains to be determined.

ATF6 also transcriptionally induces the expression of the Ca^{2+} pump SERCA2a, which is involved in regulating intracellular cytosolic contractile Ca^{2+} in the heart (Figure 2) [52]. Increased expression of SERCA2a afforded by ATF6 could mitigate the dysregulation of Ca^{2+} levels in the cytosol, which are associated with adverse outcomes during cardiac I/R [53]. Consistent with this, SERCA2a overexpression improved myocardial function, decreased apoptotic signaling, and reduced damage in numerous models of cardiac I/R [54,55]. However, similar to the ER chaperones, the specific contributions of ATF6-dependent increases in SERCA2a on the protection afforded by ATF6 have not yet been defined.

Interestingly, recent evidence suggested that ATF6 attenuates I/R injury by directly decreasing ROS. Transcriptional profiling of ventricles from mice overexpressing the active ATF6 transcription factor in the heart showed that ATF6 induced expression of multiple antioxidant genes that encode proteins residing outside of the ER, such as catalase (*Cat*), *Se/S*, and thioredoxin reductase 1 (*Txnrd1*) (Figure 2) [37]. The increased expression of these antioxidant genes could explain the reduction in ROS and decreased ROS-associated I/R damage afforded by ATF6 activation [37]. Consistent with this, overexpression of one of the antioxidant genes, *Cat*, rescued the impaired cardiac function observed in *Atf6*-deficient hearts subjected to I/R [37], indicating that ATF6 activation protects against cardiac I/R, at least partly through increased antioxidant gene expression.

The finding that ATF6 regulates the expression of multiple genes the overexpression of which protects against I/R damage in preclinical models indicates that ATF6 likely confers protection through multiple complementary mechanisms. This suggests that pharmacologically activating ATF6 could provide multiple avenues for improving tissue outcomes in diverse models of I/R damage. However, the development of this approach hinges on both the safety of targeting ATF6 for disease intervention and the potential for developing compounds that selectively activate ATF6 signaling in affected tissues with limited off-target effects. We address these two specific points in the following sections.

ATF6 as a Therapeutic Target for Human Disease

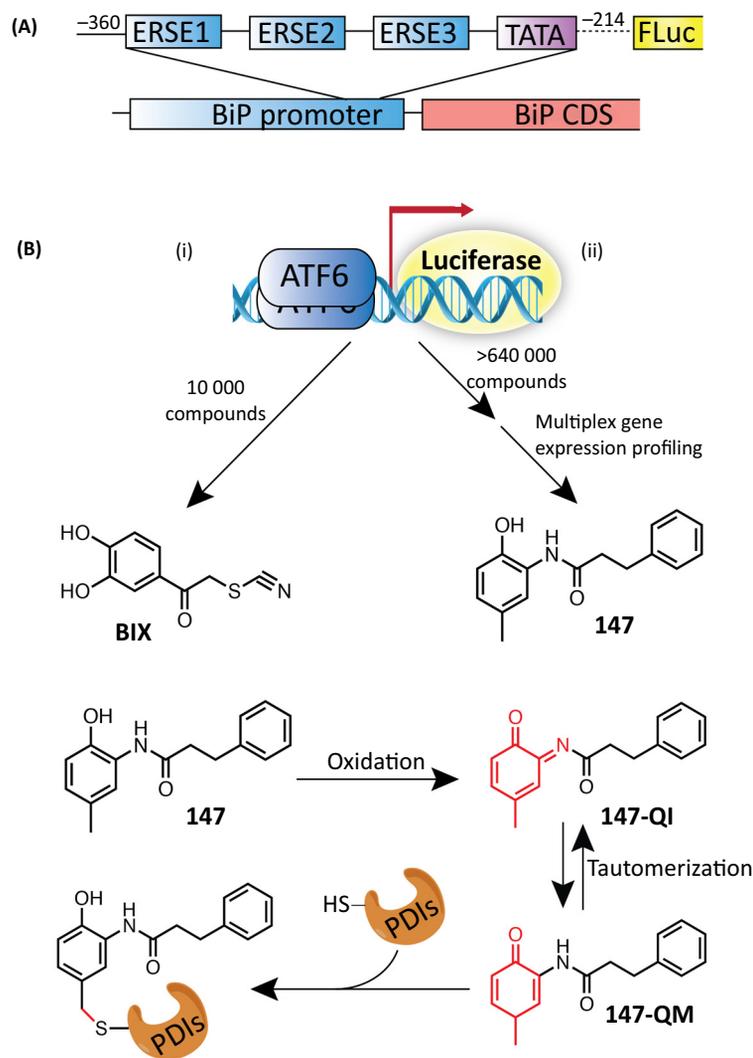
Recent human genetic data highlight the therapeutic potential for targeting ATF6 to intervene in diseases such as ischemic disorders. Polymorphisms within *ATF6 α* have been implicated in altered glucose homeostasis and plasma cholesterol levels in certain human populations, although the specific dependence of these altered phenotypes on ATF6 mutations remain to be established [56–59]. More recently, three independent groups identified mutations in *ATF6 α* that are clinically associated with the congenital retinal disease achromatopsia [60–62]. Achromatopsia is an autosomal recessive blinding disease caused by the selective dysfunction of cone photoreceptors. Disease-relevant *ATF6 α* mutations were identified by next-generation whole-exome sequencing and were shown to cause missense, nonsense, splice site, and single nucleotide perturbations throughout the entire ATF6-coding region [60–62]. These mutations can be clustered into three distinct classes based on their functional impact on ATF6 activity [63]. Class 1 and 3 *ATF6 α* mutations localize to either the ATF6 luminal domain or

the DNA-binding domain. In both cases, these mutations attenuate ATF6 activation in response to ER stress by either disrupting ER-to-Golgi trafficking or directly impairing transcriptional activity of the active ATF6 transcription factor, respectively [63]. However, class 2 *ATF6 α* mutations introduce a premature stop codon at the end of the ATF6 transcription factor domain. This leads to the production of the active ATF6 transcription factor independent of the transmembrane and luminal regulatory domains. Patients expressing these class 2 mutations have moderate levels of constitutive ATF6 activity [63]. This lower level of ATF6 activation likely reflects nonsense-mediated decay of the mutant *ATF6 α* mRNA, although this remains to be formally tested [63]. Despite presenting with achromatopsia, a developmental disorder specific to the retina, patients expressing class 2 *ATF6 α* mutations have not been reported to present with systemic or neurological defects, even though ATF6 is constitutively active in every tissue. Similarly, patients expressing class 1 and 3 *ATF6 α* mutations do not present with any severe systemic or neurological defect. Although the number of patients with achromatopsia expressing constitutively active *ATF6 α* mutants are low, these results suggest that alterations in ATF6 signaling could be well tolerated in adult humans.

These human genetic results are consistent with significant evidence from animal models showing that alterations in ATF6 activity do not induce global organismal defects. In mice, deletion of *Atf6 α* does not impair organismal development [16], although *Atf6 α* -deficient mice do show progressive rod and cone dysfunction in the eye as they age [60]. Apart from the eyes, *Atf6 α* -deficient mice do not show defects in aging, although they are more sensitive to ER stress-associated insults [16,64]. There are also numerous reports that overexpressing constitutively active or ligand-regulated ATF6 in diverse tissues of model organisms increases signaling through the ATF6 arm of the UPR to physiological levels, but does not induce global organismal toxicity. For example, while high levels of ATF6 activity in zebrafish can induce adverse outcomes, such as hepatosteatosis, lower physiologically relevant levels of ATF6 activity do not [65]. Furthermore, increasing ATF6 activity is not generally associated with adverse outcomes in mouse tissues, such as the liver, heart, brain, or kidney [32,37,38,40,66,67]. Instead, increasing the activity of ATF6 in these tissues is often associated with protection (see earlier). These results, in combination with the genetic evidence highlighting the potential for ATF6 to ameliorate I/R-related disorders in preclinical models likely through multiple mechanisms (see earlier section), suggest ATF6 as a potential target to intervene in ischemic diseases.

Pharmacological Targeting of ATF6

ATF6 is a challenging target protein due to a lack of knowledge concerning its activation mechanism and the absence of known small molecule-binding sites on ATF6 that can be pharmacologically targeted. As such, strategies to identify pharmacological activators of ATF6 have largely relied on phenotypic assays using cell-based reporters. One widely used reporter of ATF6 activity monitors the expression of firefly luciferase expressed downstream of a promoter region isolated from the well-studied ATF6 target gene *BiP* (Figure 3A). The first effort to identify an ATF6-activating compound used this reporter in a high-throughput screen (HTS) of a ~10 000 compound library to identify those that increased luciferase expression [68] (Figure 3Bi). This HTS identified a small molecule called BiP inducer X (BiX), which was shown to increase expression of the ATF6 target gene *BiP* in multiple cell models and *in vivo* in tissues, such as the brain and kidney, without increasing the expression of genes regulated by the IRE1 or PERK arms of the UPR [68–71]. BiX-dependent induction of *BiP* was ablated in cells deficient in both *ATF6 α* and *ATF6 β* [68]. This demonstrated that BiX induces *BiP* through an ATF6-dependent mechanism, although the mechanistic significance of BiX-dependent ATF6 activation on these two ATF6 isoforms remains to be defined. Despite the dependence on ATF6, administration of BiX did not significantly induce expression of other well-studied ATF6 target



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Figure 3. Screening Strategies to Identify Pharmacological Activating Transcription Factor 6 (ATF6) Activators. (A) Illustration showing the ATF6-selective luciferase reporter used to identify compounds that preferentially activate the ATF6 unfolded protein response (UPR) signaling pathways. This reporter was prepared using a promoter fragment from the ATF6-regulated gene *BiP* including three endoplasmic reticulum (ER) stress element (ERSE)-binding sites. (B) Illustration showing the strategies used to identify the ATF6-activating compounds BIX [(i) [68]] and 147 [(ii) [26]]. (C) Illustration showing the mechanism by which compound 147 selectively activates ATF6. Compound 147 is metabolically activated by oxidases to yield a reactive quinone-imine (QI) and/or quinone-methide (QM). These electrophilic compounds selectively label free cysteines within ER protein disulfide isomerases (PDIs) involved in regulating ATF6 redox status. This modification increases ER populations of reduced, monomeric ATF6 that can traffic to the Golgi for proteolytic activation (see Figure 1B). Adapted from [73].

genes, such as *GRP94*, indicating that this compound is selective for ATF6-dependent induction of *BiP* and does not robustly activate the entire ATF6 gene program [68]. While the mechanism by which BiX preferentially induces ATF6-dependent expression of BiP remains to be delineated, the identification of this compound provided the first indication that ATF6 could be pharmacologically targeted, motivating efforts to identify compounds with improved potential for global activation of the ATF6 transcriptional program.

A more recent HTS of >640 000 compounds that used the same luciferase reporter utilized to identify BiX identified numerous new compounds found to selectively, and more globally, activate the ATF6 arm of the UPR (Figure 3Bii) [26]. An important addition used in this screening strategy was the incorporation of multiplexed transcriptional profiling as part of the screening pipeline to increase the potential for identifying compounds that globally activate the ATF6 transcriptional program. Prioritized hits from this screen included compound 147, which was further shown using both RNA-seq transcriptional profiling and whole-cell quantitative proteomics to preferentially activate the ATF6 transcriptional program independent of other arms of the UPR or other stress-responsive signaling pathways [26]. Compound 147 selectively activated the ATF6 arm of the UPR in multiple cell culture models and embryonic stem cells [26,38,72]. Furthermore, intravenous (IV) injection of compound 147 in mice selectively induced ATF6 target genes, including *BiP* and *CAT*, in multiple tissues, such as the heart, liver, kidney, and brain [38], the latter indicating that this compound can cross the blood–brain barrier. Importantly, genes that are downstream of other UPR signaling arms were not activated in mice injected with compound 147 [38]. The maximal level of ATF6 activation that can be achieved by compound 147 is ~40% of that observed for the chemical ER stressor thapsigargin (Tg) [26,72]. This 40% level of activation is similar to the level of ATF6 activation observed in patients with achromatopsia expressing constitutively active class 2 ATF6 mutants [63], suggesting that compound 147-dependent ATF6 activation to these levels will not induce systemic or neurological defects in adults. Consistent with this, administration of compound 147 to mice for 2 weeks did not show any adverse outcomes in the heart, brain, kidney, or liver [38], reflecting the potential safety of this compound in mammals.

Genetic disruption of *ATF6*, or pharmacological inhibition of site 1 protease blocked compound 147-dependent activation of ATF6 target genes [26], indicating that this compound activates ATF6 through a mechanism involving the canonical ATF6 activation mechanism. Using a combination of medicinal chemistry and genetics, it was shown that compound 147 promoted ATF6 activation through a mechanism involving both metabolic activation and selective modification of a subset of ER PDIs [73] (Figure 3C). This selective modification of PDIs appeared to disrupt PDI-dependent maintenance of ATF6 disulfide bonds within the ER luminal domain, which increases the levels of reduced ATF6 monomers that can traffic to the Golgi (Figure 1B). Increasing populations of reduced ATF6 monomers, on their own, are not predicted to fully activate signaling through this pathway [23]. However, increased populations of reduced ATF6 monomers could sensitize this pathway to homeostatic changes in the ER to allow low levels of activation, providing a potential mechanism to explain the moderate ATF6 activation observed upon treatment with compound 147. While the complete mechanism by which compound 147 activates ATF6 remains to be fully established, these data indicate that it involves the disruption of PDI-dependent maintenance of ATF6 disulfide bonds. Importantly, despite the fact that compound 147 appears to influence ATF6 activation by targeting PDI activity, administration of this compound did not impact the secretion of wild-type proteins or the endogenous secreted proteome [26], indicating that compound 147-dependent alterations in PDI activity do not cause global impairment of secretory proteostasis.

Apart from ATF6-activating compounds, highly selective ATF6 inhibitors have also recently been reported. These compounds, referred to as ‘ceapins’, were identified through a HTS screen focused on identifying compounds that prevented the ER stress-dependent activation of an ATF6-selective luciferase reporter, similar to that shown in Figure 3A [74]. These compounds inhibited ATF6 activation by stabilizing ATF6 oligomers in the ER, preventing the oligomer dissociation required for ATF6 trafficking to the Golgi [74,75]. Importantly, these

Clinician's Corner

The UPR is an endogenous stress-responsive signaling pathway activated during ischemia. The UPR comprises three integrated signaling pathways activated downstream of the stress-sensing proteins PERK, IRE1, and ATF6. These pathways are activated in response to ER stress and function to both adapt cellular physiology and dictate cell survival in response to diverse physiological and pathological insults. The ATF6 arm of the UPR has been shown to contribute to tissue survival in response to I/R in preclinical models. Genetic depletion of *Atf6* in the hearts or brains of mice increases I/R-associated damage in these tissues, while increasing ATF6 activity protects these tissues against I/R.

In response to ER stress, ATF6 activation leads to increased expression of multiple, protective stress-responsive genes involved in diverse biological functions. The induction of ATF6 during I/R leads to increases in proteins known to reduce I/R damage. These include ER chaperones that can alleviate I/R-associated ER stress, Ca^{2+} pumps that can normalize imbalances in cellular Ca^{2+} homeostasis associated with I/R, and antioxidants that can attenuate oxidative stress-associated damage that accumulates during I/R.

Clinical and preclinical evidence suggests that ATF6 is an attractive therapeutic target that could correct pathological defects associated with diverse diseases, including ischemic diseases. Genetic activation of ATF6 in different mouse tissues does not induce any overt phenotype, but instead protects against numerous types of tissue damage and cellular stress. Furthermore, mutations in ATF6 that render this pathway constitutively active have been identified in patients with the congenital disorder achromatopsia, caused by defects in retinal development. However, no systemic or neurological defects have been reported in these patients to date. Although the number of patients with achromatopsia is low, the lack of systemic or neurological phenotypes does suggest that ATF6 activation could be well tolerated in adult humans.

compounds did not influence the regulated trafficking and proteolysis of other proteins regulated through a mechanism similar to ATF6, such as ATF6 β or SREBP [74], highlighting the selectivity of ceapins for ATF6 α . While the specific underlying molecular mechanisms by which ceapins stabilize ATF6 oligomers within the ER remain to be elucidated, it is clear that these compounds block ATF6 activation at an early step in its activation mechanism. Consistent with this, administration of an active ceapin compound blocked compound 147-dependent ATF6 activation [73]. While genetic evidence indicates that inhibiting ATF6 signaling will be detrimental for ischemic disease, these compounds provide a powerful, highly selective tool to pharmacologically inhibit ATF6 signaling that can be used to further define the specific involvement of ATF6 signaling at various times after I/R, a clinically relevant issue that had only been previously accessible using inhibitors of S1P that disrupt signaling through multiple other stress-responsive transcription factors that use a similar regulated proteolysis activation mechanism, such as SREBP [21].

Ameliorating I/R Injury *In Vivo* through Pharmacological ATF6 Activation

The establishment of compounds that selectively activate ATF6 (e.g., BiX and compound 147) provides a unique opportunity to further define both the importance of ATF6 activation in protecting tissues against I/R and the potential for pharmacological ATF6 activation to ameliorate pathological phenotypes in preclinical models of ischemic disease. BiX protected neuronal cells in culture against ER stress, suggesting that this compound could protect neurons during ER stress induced by ischemic stroke [68]. Consistent with this, intracerebroventricular (ICV) injection of BiX protected hippocampal neurons against degeneration in a MCAO surgical model of stroke in both mice and gerbils [68,69]. BiX was protective in this model even when administered 3 h post ischemia, resulting in both reduced infarct size and improved neurological function [71]. This indicates that protection against stroke using pharmacological compounds, such as BiX, does not require pretreatment or immediate administration after the ischemic event. Apart from animal models of stroke, intraperitoneal or subrenal capsule injection of BiX also reduced renal cell death and improved kidney function in mouse models of kidney I/R, demonstrating that BiX protects multiple tissues from I/R-associated damage [70]. While the mechanistic basis and dependence on ATF6 activation for BiX-dependent protection in these models remain undefined, this compound likely functions through ATF6-dependent regulation of the ER chaperone BiP, mirroring the benefits for increased BiP activity in protecting tissues against I/R injury [41–44].

Similar to BiX, compound 147 has also been shown to protect against I/R-associated insults. Treatment with compound 147 improved the survival of primary cardiomyocytes treated with either ER stress or *in vitro* simulated I/R [38]. RNAi depletion of ATF6 blocked this protection, confirming its dependence on compound 147-dependent ATF6 activation. Interestingly, genetic depletion of ATF6-regulated genes, such as *BiP* or *Cat*, differentially influenced compound 147-dependent protection against ER stress or simulated I/R [38]. This demonstrates that pharmacological ATF6 activation can protect against different types of stress through mechanisms dependent on distinct subsets of protective ATF6-regulated genes (Figure 2).

IV administration of compound 147 to mice increased expression of multiple protective, ATF6-regulated genes in the heart, including *BiP*, *Serca2a*, and *Cat*, highlighting the potential for this compound to protect the heart against I/R through multiple mechanisms (Figure 2). Consistent with this, IV injection of compound 147 improved cardiac recovery in an *ex vivo* model of I/R [38]. Furthermore, IV administration of compound 147, either 24 h before ischemic insult or following ischemic insult during reperfusion, reduced cardiac infarct size, decreased cardiac

Small molecules that selectively activate ATF6 alleviate I/R-associated damage in preclinical mouse models of ischemic diseases, such as stroke and MI. Administration of such pharmacological ATF6 activators either before or following ischemia reduces tissue damage and improves tissue function in these models. This suggests that pharmacological ATF6 activation is a strategy that could be used to mitigate I/R damage in diverse tissues. However, significant work in disease models that more closely recapitulate ischemic disease in humans is required to determine whether pharmacological ATF6 activation has translational potential to intervene in ischemic disease.

damage, reduced heart hypertrophy, and improved cardiac function for at least 1 week [38]. Importantly, IV administration of compound 147 did not show cardioprotection from I/R in cardiac-specific *Atf6*-knockout mice [38], indicating that the protection afforded by this treatment required ATF6. Apart from the heart, IV administration of compound 147 to mice also protected the kidney and brain against I/R damage [38]. While the specific dependence of this protection in other tissues on ATF6 and specific ATF6-regulated genes remains to be defined, these results, combined with those discussed earlier using BiX, clearly demonstrate that pharmacological ATF6 activation is broadly protective against I/R damage in multiple preclinical models of tissue-specific ischemic disease.

Concluding Remarks

The central importance of ATF6 in protecting diverse tissues against I/R damage in the context of ischemic diseases, such as MI and stroke, has just begun to be defined and many new questions remain (see Outstanding Questions). However, it is now clear that ATF6 activation integrates the expression of numerous protective genes, beyond those involved in the canonical ER stress response, to mitigate damage associated with I/R in multiple tissues. The establishment of new pharmacological approaches to both activate and inhibit ATF6 signaling provides new opportunities to carefully dissect the timing and extent of ATF6 signaling involved in protecting different tissues against I/R. As these and new experimental approaches are applied, the central importance of ATF6 signaling in tissue protection during ischemia and I/R will continue to be defined, revealing new mechanisms by which this pathway functions and integrates with other stress-responsive signaling pathways to influence tissue damage and survival in response to ischemia-associated disorders (Box 2). In addition, the establishment of highly specific ATF6-activating compounds will facilitate further preclinical studies aimed at determining the potential for pharmacological ATF6 activation to improve tissue outcomes in rodent and larger animal models of ischemic disease. Although many previous pharmacological strategies aimed at ameliorating I/R have failed [76,77], the apparent safety of constitutive ATF6 activity in mice and humans, combined with the ability of ATF6 to provide protection through multiple mechanisms in various cellular locations (Figure 2), suggest that compounds that activate ATF6 should be tested in further preclinical models of I/R damage. However, a significant amount of work remains to define the translational potential for this approach to mitigate I/R-associated pathology in humans. As researchers continue to develop new ATF6-activating compounds with increased potency and *in vivo* activity, new opportunities will emerge to continue testing this potential in larger animal models (e.g., pigs) that better reflect disease pathology in patients and further define the therapeutic potential for targeting ATF6 in ischemia-related and other diseases.

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Outstanding Questions

Does ATF6 activation integrate with other stress-responsive signaling pathways to ameliorate I/R damage in models of ischemic disease?

Does ATF6 activation induce tissue-specific remodeling to selectively protect different tissues against I/R damage?

Can ATF6-activating compounds be used in combination with compounds that influence other aspects of UPR signaling (e.g., IRE1 activating compounds; see Box 1) to synergistically promote recovery following ischemic or I/R?

What is the translational potential for pharmacological ATF6-activating compounds, such as BIX and compound 147, for ischemic disease?

Can ATF6 activation be used to mitigate ER stress and/or oxidative damage associated with the pathogenesis of other diseases, such as Alzheimer's disease and amyloid lateral sclerosis?

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