Intersection of the ATF6 and XBP1 ER stress pathways in mouse islet cells

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Edited by Qi-Qun Tang

Success or failure of pancreatic beta cell adaptation to ER stress is a determinant of diabetes susceptibility. The ATF6 and IRE1/XBP1 pathways are separate ER stress-response effectors important to beta cell health and function. ATF6α and XBP1 direct overlapping transcriptional responses in some cell types. However, the signaling dynamics and interdependence of ATF6α and XBP1 in pancreatic beta cells have not been explored. To assess pathway-specific signal onset, we performed timed exposures of primary mouse islet cells to ER stressors and measured the early transcriptional response. Comparing the time course of induction of ATF6 and XBP1 targets suggested that the two pathways have similar response dynamics. The role of ATF6α in target induction was assessed by acute knockdown using islet cells from Atf6α flox/flox mice transduced with adenovirus expressing Cre recombinase. Surprisingly, given the mild impact of chronic deletion in mice, acute ATF6α knockdown markedly reduced ATF6-pathway target gene expression under both basal and stressed conditions. Intriguingly, although ATF6α knockdown did not alter Xbp1 splicing dynamics or intensity, it did reduce induction of XBP1 targets. Inhibition of Xbp1 splicing did not decrease induction of ATF6α targets. Taken together, these data suggest that the XBP1 and ATF6 pathways are simultaneously activated in islet cells in response to acute stress and that ATF6α is required for full activation of XBP1 targets, but XBP1 is not required for activation of ATF6α targets. These observations improve understanding of the ER stress transcriptional response in pancreatic islets.

Pancreatic beta cell dysfunction or loss leads to relative insulin deficiency and diabetes in many forms. Production and folding of the insulin polypeptide places significant demand on the beta cell endoplasmic reticulum (ER) (1). Adaptation to manageable ER stress can have a positive impact on beta cell mass and function (2–6), but failed beta cell adaptation to ER stress is a well-established contributor to lost beta cell function and survival (3, 4, 7–11). Understanding the normal beta cell response to stress is critical to learning more about how beta cells fail.

The unfolded protein response (UPR) is the primary mechanism of cellular adaptation to ER stress. The UPR is an elegant multipathway stress-response initiated when unfolded peptides recruit chaperone GRP78 away from ER transmembrane proteins PERK, IRE1, and ATF6, allowing them to activate downstream signals (7). PERK activation results in phosphorylation of eIF2α, which slows translation of certain proteins to decrease peptidome diversion into the ER (7). PERK and eIF2α have been extensively studied in pancreatic beta cells (11–18). The other two arms of the UPR, ATF6α and IRE1/XBP1, are less well-studied in beta cells. IRE1 activation initiates an atypical cytoplasmic RNA splicing event, converting unspliced uXbp1 to spliced sXbp1, which when translated generates a transcription factor, XBP1 (7). XBP1 is an important part of the beta cell ER stress response (4, 19–23) but can have negative effects if unregulated (24). ATF6α pathway members are bZip transcription factors, the most studied of which is ATF6α. When released from GRP78, ATF6α, a single-pass ER transmembrane protein, translocates to the Golgi where a cleavage event releases a cytoplasmic domain which is a potent but short-lived transcription factor (6). ATF6α, similar to XBP1, may have both positive (2, 25, 26) and negative (10, 27) effects in beta cells.

The relative timing of activation of UPR pathways has been reported to influence cellular outcome (28). In other cell types, ATF6α and XBP1 are known to co-regulate some genes (29–31). The dynamics of ATF6 and XBP1 pathway activation in beta cells following stress are currently unknown; similarly, whether these pathways cooperatively regulate genes in beta cells has not been tested.

The goals of this study were to define how pancreatic islet cells activate known ATF6α and XBP1 pathway transcriptional targets in response to ER stress, focusing on the time course of induction and any interdependence of the pathways. Primary mouse islet cells were tested using two parallel chemical stressor paradigms with subtly different onset of action to sensitively probe ATF6 and XBP1 stress-response dynamics. The results suggest that the transcriptional response to the ATF6 and XBP1 pathways occurs simultaneously in islet cells. XBP1 target induction depended upon ATF6α, whereas the converse was not true. Taken together, these results clarify the molecular dynamics and interdependence of the ATF6 and XBP1 pathways in mouse islet cells.

Results

Establishing a sensitive assay for the time course of ER stress activation

To assess the time frame of activation of the ATF6 and XBP1 pathways, we designed experiments to capture the early
response after acute stress induction. Building on our prior work (2), we isolated pancreatic islets from normal healthy adult male and female C57BL/6J mice, allowed them to recover from the stress of isolation, and then dispersed and plated the islet cells on plastic. Because we consistently observe an unexplained integrated stress response suggesting nutrient depletion when we plate dispersed islet cells in 5 mM glucose (Ref. 2 and data not shown), these experiments were performed in nutrient-replete conditions with 15 mM glucose for 72 h (Fig. 1a). In separate experiments, we determined that culture media glucose concentration does not meaningfully decline during 72 h of islet cell culture (Fig. S1). To assess whether islet dispersion impacts the stress response, we performed parallel experiments in whole islets. Two different stress paradigms were studied: thapsigargin, which causes ER calcium depletion stress, and tunicamycin, which causes ER glycosylation stress. Both chemicals were applied in relatively high doses to achieve robust acute activation. To confirm ER stress, we quantified splicing of the \( Xbp1 \) transcript, an early event in activation of the XBP1 pathway. Following thapsigargin exposure, spliced \( Xbp1 \) (\( sXbp1 \)) levels increased rapidly within 1 h, accompanied by an equally rapid decrease in unspliced \( Xbp1 \) (\( uXbp1 \)) (Fig. 1, b and c). However, following tunicamycin exposure, the increase in \( sXbp1 \) and decrease in \( uXbp1 \) levels were slower, not reaching maximal activation until 4–6 h (Fig. 1, d and e). Quantification of band intensity confirmed that thapsigargin exposure activated \( Xbp1 \) splicing earlier than tunicamycin (Fig. 1, f–i). The relative ratio of spliced/unspliced \( Xbp1 \) (\( s/uXbp1 \)) also demonstrated this pattern (Fig. 1, j and k). Perhaps surprisingly, the time course of \( Xbp1 \) splicing was quite similar in whole islets and dispersed islet cells. The abundance of \( Grp78 \) transcript, a sensitive readout of ER stress response that is induced by both ATF6 and XBP1 pathways (30), confirmed activation of a general ER stress response and a slightly accelerated time course with thapsigargin compared with tunicamycin in dispersed but not whole islets (Fig. 1, l and m). In sum, these conditions allowed a sensitive mapping of the early response to ER stress in mouse islet cells using two different stresses with onset ranging from 1 to 4 h.

**ATF6 and XBP1 transcriptional response occurs simultaneously in mouse islet cells**

Using this system, we first assessed the time course of activation of the ATF6 and XBP1 pathways, as determined by timing of increase of pathway-specific transcriptional targets, in both dispersed islet cells and whole islets. Pathway-selective targets were chosen based on a carefully performed unbiased analysis of the stress-independent transcriptional impact of ATF6 or XBP1 activation in HEK293 cells (30) and have been confirmed in other studies (20, 35–37). All ATF6 targets tested increased under both stress conditions (Fig. 2, a–h). Similar to \( Grp78 \), induction of ATF6 targets by tunicamycin was delayed relative to induction by thapsigargin. This difference was more evident in dispersed islet cells than in whole islets, but the results were statistically similar between the two, possibly related to subtle differences in drug responses in whole islets in the early time points (Fig. S2). One target, \( Herpud1 \), consistently rose earlier than the others, suggesting a different mechanism of activation (Fig. 2, c and d). All three XBP1 targets tested were also increased in islet cells and whole islets by ER stressors; induction of all three were slightly earlier for thapsigargin than tunicamycin (Fig. 2, i–n). Intriguingly, although some ATF6 target induction was slower (Fig. 2, a and b) or faster (Fig. 2, c and d), on the whole the timing of XBP1 target increase was very similar to the timing of ATF6 target increase. Taken together, the ATF6 and XBP1 pathways seem to be simultaneously activated during acute ER stress in mouse islet cells.

**ATF6α is required for activation of canonical ATF6 targets in islet cells**

ATF6 pathway members include a family of ER transmembrane proteins that are activated in SREBP-like fashion by cleavage in the Golgi, leading to release of a transcription factor (38). Deletion of ATF6α itself is relatively well-tolerated in mice (31, 39), but deletion of both ATF6α and ATF6β results in early lethality (31). To assess the specific role of ATF6α in the acute response to ER stress, we isolated islets from mice bearing genomic loxP sites flanking exons 8 and 9 such that expressing bacterial Cre recombines results in a null allele (26). Transducing \( Atf6^{Δ/Δ}\)/(\( Atf6^{Δ/Δ} \)) islet cells with an adenosivirus expressing Cre (Fig. 3a) markedly reduced \( Atf6α \) mRNA levels relative to control LacZ-transduced cultures (Fig. 3b), suggesting successful knockdown. ATF6α knockdown modestly reduced mRNA abundance of ATF6 pathway targets \( Grp78 \), \( Hspa5 \), and \( DnaJ \) under basal conditions (Fig. 3c), suggesting tonic ATF6 pathway activation, mediated by ATF6α, in glucose-replete culture conditions. Abundance of mRNA of PERK pathway members \( Atf4 \) and \( Chop \) were unchanged by \( Atf6α \) knockdown (Fig. 3d), confirming selectivity of the response. Intriguingly, contrary to our expectation that knockdown of \( Atf6α \) might induce a compensatory increase in expression of \( Atf6β \), levels actually decreased (Fig. 3e).

To assess the role of ATF6α in the islet cell response to acute stress, the early induction of ATF6 pathway targets was quantified after ATF6α knockdown. Although \( Atf6α \) itself was strongly induced by thapsigargin in control islet cells, it remained low in Cre-transduced cultures, confirming ongoing effective knockdown (Fig. 4a). Induction of ATF6 pathway targets over the first 6 h of ER stress was markedly blunted by \( Atf6α \) knockdown (Fig. 4, b–e). When the duration of stress was extended to 24 h, the impact of \( Atf6α \) knockdown was still evident, although most genes showed some continued increase for both thapsigargin and tunicamycin (Fig. 4, f–m). This was despite lack of meaningful recovery of \( Atf6α \) transcript abundance (Fig. 4, n and o). \( Atf6β \) levels were found to increase after ER stress, in a strongly \( Atf6α \)-dependent manner (Fig. 4, p and q). Taken together, these results show that \( Atf6α \) is critical for normal early response to stress in mouse islet cells but also suggest that either \( Atf6α \) protein levels were incompletely reduced or that other pathway members may also participate.

**ATF6α is not required for Xbp1 splicing in response to acute ER stress**

We hypothesized that \( Atf6α \) knockdown might lead to compensatory activation of the XBP1 pathway in islet cells. To
test for interaction between the ATF6 and XBP1 pathways, we first assessed whether Xbp1 splicing was increased by ATF6 knockdown. Transducing Atf6<sup>α<sup>lox/lox</sup></sup> islet cells with Ad-Cre did not significantly alter basal unspliced or spliced Xbp1 levels, nor the ratio of the two (Fig. 5, a and b). When thapsigargin or tunicamycin were applied, Xbp1 splicing increased similarly.
under control (Ad-LacZ) or ATF6α knockdown (Ad-Cre) conditions (Fig. 5, c–h). ATF6α knockdown caused a subtle reduction of the ratio of sXbp1/uXbp1 following thapsigargin but not tunicamycin exposure (Fig. 5, i and j), the significance of which was uncertain. On the whole, Xbp1 splicing in basal conditions or in response to acute ER stress in
**ATF6 and XBP1 pathways intersect in mouse islets**

**Figure 3.** ATF6α supports basal expression of canonical ATF6 pathway targets in mouse islet cells. a, ATF6α was knocked down by introducing adenovirus driving Cre under the cytomegalovirus promoter (Ad-Cre) into cultures of dispersed islet cells from ATF6α flox/flox mice. Control cultures were the same dispersed islets transduced with Ad-LacZ virus. RNA was quantified 72 h after transduction. b, Ad-Cre transduction markedly reduced ATF6α mRNA, suggesting effective knockdown. c and d, abundance of all four ATF6 pathway target genes were significantly reduced by ATF6α knockdown (c), but Atf4 or its downstream target Chop were not (d). e, abundance of Atf6b was reduced after ATF6α knockdown. Sample sizes were n = 10 in a–d and n = 6 in e. Statistical analysis was by Student's t test. *, p < 0.05; ***, p < 0.001; ****, p < 0.0001; ns, not significant.

Mouse islet cells was neither increased nor consistently impaired by ATF6α knockdown.

**Induction of ATF6 targets in islet cells does not require Xbp1 splicing**

To test whether ATF6 pathway target induction during ER stress requires Xbp1 splicing, we used a chemical inhibitor of IRE1, 4μ8c, which effectively inhibits IRE1-dependent Xbp1 splicing (40). Applied to mouse islet cells, the IRE1 inhibitor fully prevented the thapsigargin-induced increase in sXbp1 and reduction in uXbp1 (Fig. 6, a–e). As expected, inhibiting IRE1 reduced induction of XBP1 targets Sec24d, Erdj4, and Ssr3 (Fig. 6, f–h). Induction of XBP1 mRNA targets was only partially inhibited, possibly because the IRE1 inhibitor was added to the cultures at the same time as the thapsigargin, which may have allowed early Xbp1 splicing to proceed, leading to active nuclear XBP1 protein before the splicing was fully inhibited. In contrast to XBP1 targets, however, ATF6 pathway targets were not inhibited; in fact, two of three were slightly increased by IRE1 inhibitor (Fig. 6, i–k). These data show that ATF6 targets are not dependent upon Xbp1 splicing for full activation following ER stress in mouse islet cells; in fact, they suggest the converse possibility of a mild inhibition of ATF6 target induction by XBP1.

**Induction of XBP1 targets by ER stress in islet cells requires ATF6α**

ATF6α and XBP1 cooperatively regulate some genes during the ER stress response in other cell types (29, 31). To determine whether ATF6α is required for induction of XBP1 target genes in islet cells, we quantified expression of XBP1 targets in ATF6α flox/flox islet cells transduced with Ad-cre. All three XBP1 target genes tested showed reduced basal expression after ATF6α knockdown prior to ER stress induction (Fig. 7a),...
ATF6 and XBP1 pathways intersect in mouse islets

Acute stress induction (Thapsigargin)

- Control (LacZ) vs. Atf6α knockdown (Cre)

Longer term stress induction

- Control (LacZ) vs. Atf6α knockdown (Cre)
ATF6 and XBP1 pathways intersect in mouse islets

suggesting that ATF6α provides basal support for expression of these genes in the absence of stress or that the XBP1 pathway was slightly activated in basal nutrient-replete conditions. Remarkably, after induction of stress with thapsigargin, the early induction of all three XBP1 target genes was nearly completely eliminated, similar to canonical ATF6 target genes (Fig. 7, b–d; compare with Fig. 4, b–c). Extending the experiment to 24 h, and including both thapsigargin and tunicamycin as stressors, showed that longer duration induction of XBP1 target genes was also markedly reduced following ATF6α knockdown (Fig. 7, e–j). Taken together, these results show that in mouse islet cells ATF6α is not required for Xbp1 splicing but is absolutely required for activation of XBP1 target genes after an acute stress, with an effect size similar to the ATF6α requirement to induce ATF6-specific target genes.

Discussion

Here we established a sensitive assay for measuring early activation of ER stress-response pathways in dispersed islet cells and whole islets and applied this system to interrogate the timing and intensity of activation of the ATF6 and XBP1 pathways in response to two different chemical stressors. In mouse islets, ATF6 and XBP1 pathway activation occurred simultaneously. The pathways appeared to engage at similar intensity over multiple target genes, with the caveat that strength of activation is not directly comparable across genes using this methodology. Perhaps surprisingly, disrupting islet architecture by dispersing the islets to single cells did not meaningfully change the cellular stress response under these conditions. Intriguingly, for the transcripts tested, both basal and stress-induced activation of XBP1 pathway genes were strongly dependent upon ATF6α expression, whereas induction of the ATF6 pathway genes tested was not impaired by inhibition of XBP1 splicing.

Transcriptional regulation by ATF6α and XBP1 occurs via ER-responsive transcriptional elements ERSE, ERSE-II, and UPRE (6, 29, 31). Bipartite ERSE (CCAAT-Xy-CCACG) and ERSE-II (ATTGG-X-CCACG) regulatory sequences are activated by ATF6α in combination with NF-Y or other CCAAT box-binding proteins such as CCAAT/enhancer-binding factors (41, 42). The UPRE consensus TGACGTG(G/A) contains, in part, a complement CCACG sequence; UPRE is thought to be more responsive to XBP1 than ATF6 (29).

It is well-known in other cell types that the ATF6 and XBP1 pathways have overlapping gene signatures (29–31). Both ATF6α and XBP1 are basic leucine zipper domain (bZip) proteins, one of the largest family of transcription factors. Family members commonly heterodimerize to impart cell type specific gene regulation that is responsive to inputs from multiple signaling sources. Although dozens of bZip family members have been described, a FRET-based study suggested that ATF6α and XBP1 do not broadly heterodimerize with other factors (43). On the other hand, in beta cells ATF6α was reported to interact with CCAAT/enhancer-binding factor β, another stress-induced bZip factor that promotes cell death during unresolved stress (42).

In this study, acute ATF6α deletion did not impact Xbp1 splicing in primary mouse islet cells. These results differ from reported results in a neuroblastoma cell line, in which loss of ATF6α increased Xbp1 splicing related to increased IRE1 expression and activity (44). In that study, ATF6α was proposed to mediate an IRE1 signaling off switch. In another study, Xbp1 splicing was reported to be normal in mouse embryonic fibroblasts with chronic knockdown of ATF6α (45). Differences in results may be related to different cell types, use of primary versus transformed cells, methodology related to ATF6α knockdown, or other culture conditions. Our results suggest that in primary mouse islet cells ATF6α does not impact Xbp1 splicing but does play a key role in activating the downstream IRE1/XBP1 transcriptional response to stress.

The marked inhibition of basal and stress-induced target transcriptional response in the setting of ATF6α knockout observed in our study conflicts with some reports but is consistent with others. In mouse embryonic fibroblasts, in one study chronic knockdown of ATF6α, or even double knockdown of ATF6α and ATF6β, had little impact on expression of Grp78, Chop, or Grp94 (45), whereas another study showed significant impairment in stress gene induction (31). In mice, whole-body deletion of ATF6α or ATF6β was well-tolerated, whereas deletion of both ATF6α and ATF6β was embryonic lethal (31). These results suggest that ATF6β can compensate for loss of ATF6α, but in our current study a compensatory increase in Atf6β mRNA was not observed, with the caveat that this was only measured over the first 72 h of Atf6α knockdown. Taken together, given the strongly impaired islet cell ER stress response after acute ATF6α knockdown in this study, we propose either that ATF6α is uniquely required in islet cells or that ATF6α plays an important role in the usual stress response, but if ATF6α expression is reduced for an extended duration of time, other factors, possibly including ATF6β, can compensate.

Roles of ATF6α and XBP1 in beta cells are still under investigation. IRE1 and XBP1 are important for insulin processing and secretion; deletion of XBP1 using the rat insulin promoter caused glucose intolerance caused by both an impaired ER stress response and a compensatory overactivation of IRE1 (20). Inappropriate IRE1 activation decreased proinsulin and insulin levels through regulated RNA decay (46–48). On the
other hand, loss of IRE1 function also decreased insulin translation, and loss of XBP1 was responsible for impaired insulin secretion in a MODY model (22, 49). Our current observations confirming cooperativity of gene activation between ATF6α and XBP1 in islet cells suggest that ATF6 signaling may also be important for insulin production, but this has not yet been formally tested.

ATF6 and XBP1 signaling impact beta cell number as well. Under certain conditions ATF6α increases proliferation of beta cells as well as other cell types (2, 6, 50). Intriguingly, both

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**Figure 5. Xbp1 splicing is only minimally affected by ATF6α knockdown in basal or stress conditions.** a and b, dispersed mouse islet cells with ATF6α knockdown or control were tested for Xbp1 splicing after 72 h of culture using a gel-based assay. c–j, ER stress induction of Xbp1 splicing in control or ATF6α knockdown islet cells was assessed at 6 or 24 h after exposure to Tg (left panels) or Tm (right panels). Statistical analysis was by unpaired Student’s t test (b) one-way ANOVA (e–j), comparing each time point to time 0 (*) or control versus knockdown (#). Sample sizes were n = 10 for a and b; n = 4 for Tg (c, e, g, and i); and n = 3 for Tm (d, f, h, and j). #, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant.
ATF6 and XBP1 pathways intersect in mouse islets

Xbp1 splicing

![Diagram showing Xbp1 splicing](image)

XBP1 transcriptional targets

- **f**: Sec24d:Actin
- **g**: Erp57:Actin
- **h**: Ssa3:Actin

ATF6 transcriptional targets

- **i**: Hsou1:Actin
- **j**: Herpud1:Actin
- **k**: Pdia4:Actin
overexpression and inhibition of IRE1/XBP1 reduced glucose-induced beta cell proliferation (2). Global deletion of ATF6α resulted in beta cell failure, with dilated ER, in models of insulin resistance such as diet-induced obesity and the Agouti mutation; relative contribution of loss of beta cell mass and/or function was not assessed (25). Consistent with known roles in adaptation to ER stress, both ATF6α and XBP1 protect against beta cell apoptosis (3, 10, 19, 26, 27, 51, 52). However, overexpression of ATF6α can promote beta cell loss (53). Taken together, these studies, combined with our current observations, suggest a possible model in which ATF6α and XBP1, separately or in combination, both promote beta cell survival, but ATF6α acting alone may be responsible for increased beta cell proliferation during the adaptive stress response.

In dispersed islet cell cultures, both ATF6 and XBP1 pathways appeared to be modestly turned on under basal conditions, because pathway inhibition reduced gene target expression. This may suggest tonic activation of these stress-response pathways under basal conditions, which is a slightly different model than currently proposed. On the other hand, islet isolation, cell dispersion, growth on plastic, and culture in the presence of fetal bovine serum and an excess of glucose may induce a mild ER stress state (2). These current experiments put the mild degree of those stresses into context, because the stress-response pathways are induced to much higher levels with thapsigargin or tunicamycin treatment.

Our study has several weaknesses. We did not perform unbiased RNA sequencing to assess global transcription changes with these interventions but rather assessed pathway activation using a small number of known target genes. As such, the results observed on the genes tested may not be extrapolatable to all gene targets of these pathways. The role of the XBP1 pathway was interrogated using a chemical inhibitor, which may have off-target effects, although the inhibitor used has been well-characterized and broadly used (40). Our study was performed in mouse islets, which has the strong benefit of being a disease-relevant primary tissue but also caveats related to the mixed cell population included in islets. We have not yet performed this type of study in human tissue. Finally, the extent to which this acute ex vivo islet stress model is relevant to the chronic ER stress associated with the development of diabetes remains unknown.

Overall, this study advances knowledge of the time course, intensity, and interdependence of activation of the ATF6 and XBP1 pathways in primary islet cells. The ATF6 and XBP1 pathways were simultaneously and jointly activated in response to two different stressors, suggesting that it may be complicated to therapeutically intervene in a pathway-selective manner (54). Much work remains to be completed if we are to harness ER stress modulation as a therapeutic approach to preserve, or grow, pancreatic beta cell mass.

**Experimental procedures**

**Chemicals and reagents**

RPMI 1640 medium with glutamine without glucose (catalog number 11879020), Trypsin-EDTA 0.05% (catalog no. 25300054), and 1× Dulbecco PBS (DPBS, catalog no. 14190359) were obtained from Thermo Fisher Scientific. Glucose (catalog no. G-8270), thapsigargin (catalog no. T-9033), tunicamycin (catalog no. T-7765), and IRE1 inhibitor 4μ8c (catalog no. 412512) were obtained from Sigma–Aldrich. For knockdown of ATF6 in islet cell culture, adenoviruses expressing LacZ (control) and Cre recombinase were acquired from Vector Biolabs.

**Mouse approvals and husbandry**

All mouse procedures were approved by the University of Massachusetts Medical School and Well Cornell Medicine Institutional Animal Care and Use Committees. C57BL/6 WT (bred in-house) or Atf6a<sup>lox/lox</sup> mice on a C57BL/6 genetic background (harboring loxP sites flanking Atf6α exons 8 and 9, a generous gift from Dr. Gokhan Hotamisligil at the Harvard School of Public Health) were housed under specific pathogen-free conditions in a 12-h light/dark cycle with ad libitum access to food and water. For all experiments, male and female adult mice between the ages of 20–70 weeks were used to isolate islets.

**Pancreatic islet isolation**

The islets were isolated by pancreatic ductal collagenase (Roche) injection and Ficoll gradient (Histopaque-1077; Sigma) as described previously (32, 33). Whole islets were cultured at 37°C in 5% CO₂ in islet complete medium (RPMI 1640 medium containing 10% fetal bovine serum, 5 mM glucose, 2 mM glutamine, and 1% penicillin/streptomycin) for 24 h after isolation to allow recovery.

**Islet cell culture and adenoviral transduction**

Islets were counted manually using a 4× objective with ocular grid, washed once with PBS, and either cultured intact or dispersed to single cells using single use apportioned 0.05% trypsin. Whole islets (40–50 islet equivalents) or dispersed cells (70–100 islet equivalents) were plated directly on plastic in 24-well plates in islet complete medium. After 24 h, the wells were treated with 15 mM glucose for 72 h; thapsigargin (1000 nM) or tunicamycin (2000 ng/ml) was added to the culture medium during the last 1, 2, 3, 4, or 6 h of the experiment. To knock down ATF6α, islets were isolated from Atf6a<sup>lox/lox</sup> mice that did not contain any Cre allele. Atf6α<sup>lox/lox</sup> islets were dispersed

![Figure 6. Stress-induced activation of ATF6α targets does not require Xbp1 splicing in mouse islet cells.](image-url)

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**ATF6 and XBP1 pathways intersect in mouse islets**

The results observed on the genes tested may not be extrapolatable to all gene targets of these pathways. The role of the XBP1 pathway was interrogated using a chemical inhibitor, which may have off-target effects, although the inhibitor used has been well-characterized and broadly used (40). Our study was performed in mouse islets, which has the strong benefit of being a disease-relevant primary tissue but also caveats related to the mixed cell population included in islets. We have not yet performed this type of study in human tissue. Finally, the extent to which this acute ex vivo islet stress model is relevant to the chronic ER stress associated with the development of diabetes remains unknown.

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**Figure 6. Stress-induced activation of ATF6α targets does not require Xbp1 splicing in mouse islet cells.** a, IRE1 dimers splice Xbp1 transcript from unspliced (uXbp1) to spliced (sXbp1). The 4μBC IRE1 inhibitor reduces both kinase and splicing activities of IRE1. b–e, IRE1 inhibitor applied at the same time as 1000 nM Tg diminished spliced Xbp1 in mouse islet cells harvested 24 h later, restoring normal unspliced levels of uXbp1 (c) and keeping sXbp1 and s/u Xbp1 nearly at unstimulated levels (d and e). f–h, inhibition of IRE1 significantly decreased abundance of known XBP1 targets (f–h) but did not reduce abundance of ATF6 targets (i–k). Statistical analysis was by one-way ANOVA, comparing each time point to unstressed conditions (*) or IRE1 inhibitor (#). Sample sizes were n = 4 for all, #, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant.
**ATF6 and XBP1 pathways intersect in mouse islets**

**Figure 7. Stress activation of XBP1 targets requires ATF6α in mouse islet cells.**

**a.** Dispersed mouse islet cells with ATF6α knockdown harvested for RNA isolation after 72 h had lower abundance of XBP1 target transcripts. **b–d.** In the first 6 h following 1000 nM Tg exposure, induction of XBP1 targets was reduced in islet cell cultures with ATF6α knockdown. **e–j.** In experiments over a 24-h time frame, and comparing stress induced by Tg (left panels) or Tm (right panels), XBP1 target induction was consistently lower in islet cells with ATF6α knockdown. Statistical analysis was by Student’s t test: ***, p < 0.001; ****, p < 0.0001** for **a,** and one-way ANOVA comparing each time point to time 0 (*) or control versus knockdown (#). Sample sizes were n = 10 for **a;** n = 4 for **b–d;** n = 4 for Tg (e, g, and i); and n = 3 for Tm (f, h, and j). # or *, p < 0.05; ## or **, p < 0.01; ### or ***, p < 0.001; #### or ****, p < 0.0001; ns, not significant.
as described above, but at the time glucose was added, the cultures were treated with adenoviruses expressing LacZ (control virus) or Cre recombinase, both at a multiplicity of infection of 10. The duration from glucose addition to harvest was 72 h for all experiments. Glucose concentration in media after 72 h of culture with or without dispersed islet cells was quantified using the Amplex Red glucose oxidase assay kit (Invitrogen, catalog no. A22189) according to the manufacturer’s protocol.

**RNA isolation and cDNA synthesis**

Whole or dispersed islet cell cultures were harvested for RNA isolation at the end of the experiment by washing once with PBS and lysing using SKP lysis buffer included in the all-in-one RNA/DNA/protein purification kit (Norgen Biotek Corporation). RNA was isolated as per the manufacturer’s protocol. 100–500 ng of total RNA was used for cDNA synthesis using the SuperScript IV Vilo kit (Invitrogen) with a slight modification of the manufacturer’s protocol to increase the duration of the reverse transcriptase cDNA synthesis step from 10 to 30 min.

**Transcript quantification by qPCR**

qPCR was performed using PerfeCTa SYBR green Supermix (Quanta Bio) per the manufacturer’s protocol on a Realplex 2 (Eppendorf) or QuantStudio 5 (Thermo) PCR machine. All primers were described previously (2) except the primers directed to Atf6β and to exons 8 and 9 of Atf6α to quantify removal of the floxed region. These primers were as follows: Atf6β forward, 5’-actaaccacagttccggtgc-3’; Atf6β reverse, 5’-ggc-ataggggctggaacaat-3’; Atf6kd forward, 5’-acctacgtggtagaagtgtgctg-3’; and Atf6kd reverse, 5’-ttccttcgccgacgacaca-3’. qPCR data were normalized to Actin and expressed as fold change (ddCt).

**Gel assay to quantify Xbp1 splicing**

Splicing of Xbp1 was detected using a semiquantitative agarose gel-based assay (34). Xbp1 was first amplified by PCR using published primers (2) using one cycle of 98 °C for 2 min, 29 cycles of denaturation (98 °C), annealing (60 °C) and amplification (72 °C) each for 30 s, and a final extension at 72 °C for 5 min. The amplified product was run on a 3% agarose gel and imaged using an image-based gel documentation system (Bio-Rad). Bands were quantified using ImageJ (National Institutes of Health) software.

**Statistical analysis**

The data were analyzed and graphed using GraphPad Prism 8. The number of replicates for each experiment in which the data points are not presented individually is included in the figure legends. The data were compared using Student’s two-tailed t test when comparing two groups or one-way ANOVA with Sidak’s multiple comparisons test when comparing multiple groups; p < 0.05 was considered significant.

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**Data availability**

All data are contained within the article.

**Acknowledgments**—Atf6lox/flox mice were a generous gift from Gokhan Hotamisligil (Harvard University). We are grateful for many helpful conversations among the Beta Cell Biology Group at the University of Massachusetts Medical School, as well as with other Alonso lab members.

**Author contributions**—R. B. S. and L. C. A. conceptualization; R. B. S. data curation; R. B. S. and C. D. investigation; R. B. S. and C. D. methodology; R. B. S., C. D., and L. C. A. writing-review and editing; C. D. and L. C. A. project administration; L. C. A. supervision; L. C. A. funding acquisition; L. C. A. writing-original draft.

**Funding and additional information**—This work was supported by American Diabetes Association Grant 1-18-IBS-233 (to L. C. A.) in collaboration with the Order of the Amaranth; NIDDK, National Institutes of Health Grants R01DK114686 and R01DK113300 (to L. C. A.); and the George F. and Sybil H. Fuller Foundation. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: ER, endoplasmic reticulum; ANOVA, analysis of variance; UPR, unfolded protein response; bZip, basic leucine zipper domain; qPCR, quantitative PCR; Tg, thapsigargin; Tm, tunicamycin.

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