# Argonaute Slicing Is Required for Heterochromatic Silencing and Spreading

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# Argonaute Slicing Is Required for Heterochromatic Silencing and Spreading

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Small interfering RNA (siRNA) guides dimethylation of histone H3 lysine-9 (H3K9me2) via the Argonaute and RNA-dependent RNA polymerase complexes, as well as base-pairing with either RNA or DNA. We show that Argonaute requires the conserved aspartate-aspartate-histidine motif for heterochromatic silencing and for ribonuclease H–like cleavage (slicing) of target messages complementary to siRNA. In the fission yeast *Schizosaccharomyces pombe*, heterochromatic repeats are transcribed by polymerase II. We show that H3K9me2 spreads into silent reporter genes when they are embedded within these transcripts and that spreading requires read-through transcription, as well as slicing by Argonaute. Thus, siRNA guides histone modification by base-pairing interactions with RNA.

RNA interference (RNAi) results when double-stranded RNA (dsRNA) is processed into siRNA by the ribonuclease (RNase) III–type enzyme known as Dicer. These siRNAs then base-pair with complementary mRNA to target cleavage (and, in some cases, to repress translation). Argonaute proteins facilitate this process by binding the 3′ end of one siRNA strand via the conserved PAZ domain. Target messages complementary to the siRNA are then cleaved by the Argonaute PIWI domain, which is related to RNaseH and contains the highly conserved motif Asp-Asp-His (D-D-H), which is required for endonucleolytic cleavage (or slicing) (*1*). Argonaute proteins are required for transcriptional as well as posttranscriptional silencing in *Drosophila*, *Arabidopsis*, and *Schizosaccharomyces pombe*, which has only one Argonaute protein (Ago1) (*2*–*4*). Heterochromatic repeats, transposable elements (TEs), and some transgenes are associated with modified chromatin when they are transcriptionally silenced. Histone modification in *S. pombe* depends on Argonaute and RNAi (*5*) as well as on the Rik1 complex, which contains the histone methyltransferase Clr4 (*6*). Two models have been proposed for the role of siRNA in histone modification (*7*). First, siRNA might interact with DNA, recruiting modified histones via the RNA-induced initiation of transcriptional silencing (RITS) complex, which includes the chromodomain protein Chp1 as well as Ago1 (*8*). Alternatively, Argonaute might slice heterochromatic transcripts, recruiting RNA-dependent RNA polymerase (RdRP) via free 3′ OH ends, which promote polymerase activity (*9*). Association with nascent transcripts might then recruit the histone modification apparatus to the chromosome (*10*).

We tested the ability of fission yeast Ago1 to cleave target messages in a siRNA-dependent fashion. Recombinant glutathione *S*-transferase (GST)–SpAgo1 fusion proteins were purified from *Escherichia coli* (Fig. 1A) and incubated with two different 23-nucleotide (nt) RNA oligonucleotides complementary to a target message. When labeled message was added to the reaction, RNA fragments were detected, corresponding in size to products cleaved at each siRNA complementary site (Fig. 1B). These fragments were not observed in control reactions supplemented with EDTA, which chelates $Mg^{2+}$ and thereby inhibits cleavage. Thus, Ago1 from fission yeast has "slicing" activity and can direct site-specific cleavage of RNA substrates via siRNA.

In order to assess the role of slicing in heterochromatic silencing, we constructed *ago1*$^{-}$ mutants in the D-D-H motif (*11*–*13*). Alanine substitutions were introduced at each of the three conserved residues (fig. S1), resulting in much lower catalytic activity in vitro (Fig. 1C). The corresponding mutants in yeast were viable and grew normally but accumulated transcripts from both forward and reverse strands of heterochromatic repeats (Fig. 1D). Run-on transcription assays have previously revealed that the reverse strand is transcribed in wild-type cells, although RNA fails to accumulate, whereas the forward strand is not transcribed in detectable quantities and is silenced at the transcriptional level (*3*). The reverse-strand transcript [or pre-siRNA (*14*)] fails to accumulate because it is rapidly converted into siRNA. Thus, our results indicate that both posttranscriptional silencing (of the reverse strand) and transcriptional silencing (of the forward strand) require slicing via the D-D-H motif. In agreement with these results, siRNA from centromeric repeats was undetectable in the slicer mutants (Fig. 1E), as in strains in which the *ago1* gene has been deleted (*15*).

siRNA is derived from dsRNA. Only one strand of the repeats is transcribed in wild-type cells (*3*), so that generation of dsRNA depends on RdRP activity (*9*). DsRNA is then cleaved into 23-nt duplexes by Dicer. Ago1 promotes RdRP activity via interactions between the RITS and the RDRC complexes as well as template RNA (*9*). dsRNA synthesis begins at the 3′ OH end of single-stranded RNA (ssRNA) fragments (*9*), and such ends are generated by slicing (*1*). In slicer mutants, reduction in RdRP activity is expected to lead to loss of dsRNA and therefore loss of siRNA (*1*). Also, sliced transcripts are uncapped, which promotes RdRP activity (*16*). We therefore tested the association of both RdRP and Ago1 itself with centromeric heterochromatin in slicer mutant strains. We found that association of RdRP with the repeats was reduced (although not abolished) in *ago1*$^{-}$ slicer mutants (Fig. 2A). In contrast, association of slicer-defective Ago1 was slightly enriched (Fig. 2B), indicating that Ago1 catalytic activity, and not just localization to the repeats, is important for silencing. Histone H3 lysine-9 (H3K9me2) quantities were only partially reduced at the centromeric repeats

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themselves, as in *ago1*⁻ deletions (*3*) (Fig. 2C). This probably accounts for the retention of slicer-defective Ago1 at the repeats (Fig. 2B), because the RITS complex still binds to H3K9me2, even in the absence of siRNA (*6*, *8*).

The *ura4*⁺ reporter genes integrated into the pericentromeric outer repeats of centromere 1 are transcriptionally silenced in wild-type strains via H3K9me2 and Swi6 (*17*, *18*). Pericentromeric *ura4*⁺ was strongly derepressed in each of the three slicer mutants: H3K9me2 from the reporter gene was reduced to below the limit of detection, and H3K4me2 was increased fourfold (Fig. 2C). Slicer-defective Ago1 was also substantially lost from *ura4*⁺ (Fig. 2B), consistent with the loss of H3K9me2, but most Rdp1 was retained (Fig. 2A). Unlike RITS, whose association with the chromosome depends on H3K9me2, chromatin association of Rdp1 is thought to be dependent on nascent RNA, perhaps accounting for this distinction, although Rdp1 association with *ura4*⁺ is variable in replicate experiments (*9*). In the absence of RNAi, H3K9me2 is retained at pericentromeric repeats by the histone deacetylase Clr3, as previously reported (*19*). But H3K9me2 is lost from *ura4*⁺ reporter genes integrated into transcribed repeats in *ago1*⁻ slicer mutants (Fig. 2C), which resemble the RITS mutant *chp1*⁻ in this respect (*6*). Thus, spreading of H3K9me2 into neighboring reporter genes depends on slicing.

Several *ura4*⁺ reporter genes have been integrated into centromere 1 and differ in the extent to which they are silenced (*17*, *18*). We wondered whether this position effect depended on transcripts from the repeats, and so

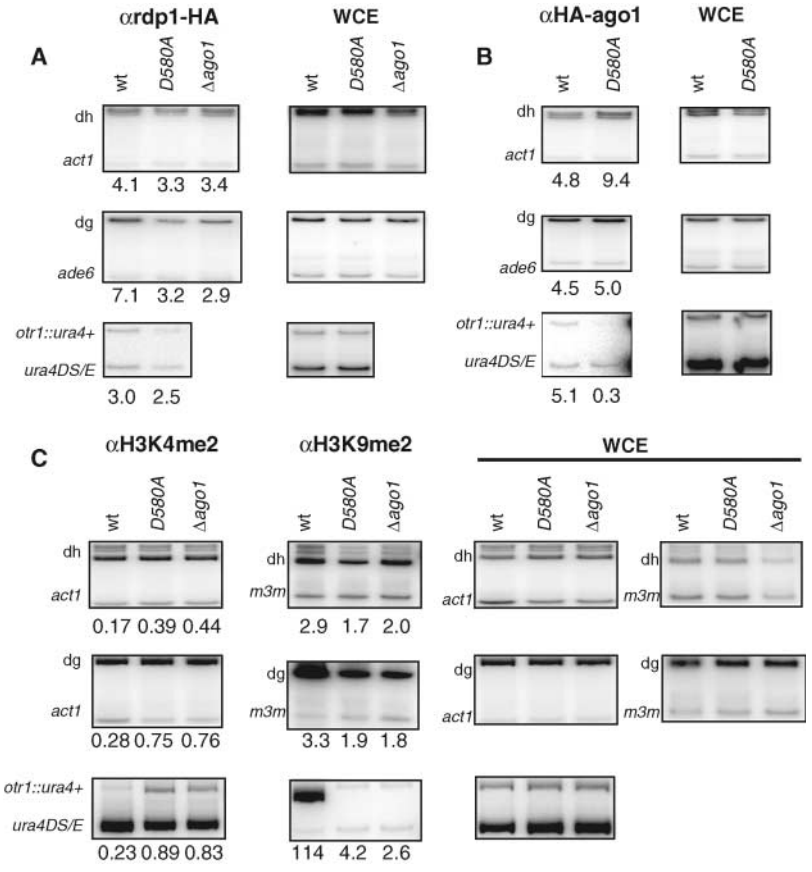


**Fig. 2.** Histone H3 methylation and localization of RITS and RDRC depend on slicer. Chromatin immunoprecipitation (ChIP) analysis of WT, *ago1D580A* mutant (*D580A*), and *ago1* knockout (Δ*ago1*) strains. In each sample, enrichment for each primer pair was measured relative to control primer pairs in whole cell extract (WCE) and is depicted under each lane. The regions assayed were the *dg* and *dh* repeats, together with regions unaffected by RNAi (*act1*, *ade6*, and *mat3M*), and a *ura4*⁺ insertion in the outer repeat of centromere 1 [*otr1R(SphI)::ura4*⁺], together with a *ura4* minigene (*ura4DS/E*) at the endogenous locus. (**A**) Hemagglutinin (HA)-tagged Rdp1. (**B**) WT and *ago1D580A* slicer-defective HA-tagged Ago1. (**C**) H3K9me2 and H3K4me2.

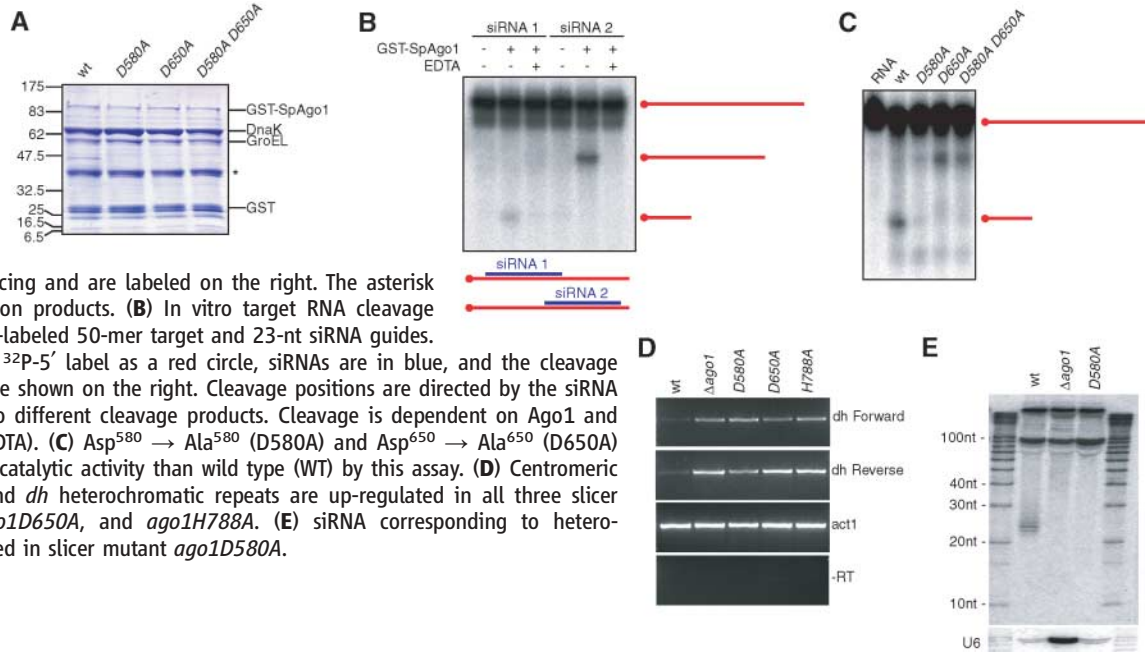


**Fig. 1.** Slicer mutants in *S. pombe*. (**A**) SDS–polyacrylamide gel electrophoresis (PAGE) after size exclusion chromatography of GST-SpAgo1. Positions for the molecular weight markers (ink D) are labeled on the left. Bands were identified by mass spectrometric sequencing and are labeled on the right. The asterisk denotes SpAgo1 degradation products. (**B**) In vitro target RNA cleavage (slicing) assay using $^{32}$P-5′-labeled 50-mer target and 23-nt siRNA guides. Target RNA is in red with $^{32}$P-5′ label as a red circle, siRNAs are in blue, and the cleavage products for each siRNA are shown on the right. Cleavage positions are directed by the siRNA sequences, resulting in two different cleavage products. Cleavage is dependent on Ago1 and magnesium (absence of EDTA). (**C**) Asp$^{580}$ → Ala$^{580}$ (D580A) and Asp$^{650}$ → Ala$^{650}$ (D650A) mutants have much lower catalytic activity than wild type (WT) by this assay. (**D**) Centromeric transcripts from the *dg* and *dh* heterochromatic repeats are up-regulated in all three slicer mutants, *ago1D580A*, *ago1D650A*, and *ago1H788A*. (**E**) siRNA corresponding to heterochromatic repeats is reduced in slicer mutant *ago1D580A*.

we sequenced the ends of cDNA clones isolated from *dcr1*⁻ strains by using the *dg* centromeric repeat as a probe. Most of these cDNA clones mapped to the 16 *dg* repeats from centromeres 2 and 3, but one clone matched the *dg* repeat from centromere 1 (fig. S2). Although it was truncated at the 3′ end, the 5′ end of the clone mapped precisely to the promoter of the reverse transcript identified previously (*14*). By comparing these sequences with the *ura4*⁺ insertion sites, we found that reporter genes inserted downstream of the promoter, within the transcription unit of the *dg* repeat, were silenced at least fourfold more efficiently than those inserted upstream of the transcription unit (fig. S2), indicating that transcription itself might play a role in spreading.

Read-through transcripts from *ura4*⁺ insertions in the *dg* repeat downstream of the reverse-strand promoter were present in low quantities in wild-type cells but in high quantities in *ago1*⁻ slicer mutants, indicating they were the targets of slicer activity (Fig. 3, A and B). By using rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR), we could detect full-length cotranscripts in mutant cells ($^{17}/_{17}$ cloned products), but we could not detect either full-length or cleaved transcripts in wild-type cells (Fig. 3C). Cleavage products could be recovered, however, from the exosome mutant *rrp6*⁻ (Fig. 3C), in which an exonuclease gene had been disrupted. The exosome degrades uncapped and nonpolyadenylated (or cleaved) transcripts (*20*, *21*), such as those cleaved by Ago1 slicer activity on either side of the *ura4*⁺ insertion. In *rrp6*⁻ cells, different cleavage sites were sequenced in the region that matched siRNA ($^{20}/_{27}$ cloned products), but none were detected in the *ura4*⁺ gene itself (Fig. 3D). *ura4*⁺ insertions located within the transcribed *dh* repeat (FY988 and FY939) also gave rise to read-through transcripts in *ago1*⁻ slicer mutants (fig. S2) and were strongly silenced (*17*, *18*). But read-through transcripts corresponding to *ura4*⁺ genes located upstream of the reverse strand promoter (FY496 and FY501) could only just be detected, consistent with reduced silencing in these strains (fig. S2). This reduced silencing is still sensitive to RNAi (*3*) but, unlike downstream *ura4*⁺ insertions silencing, also depends on the histone deacetylase Clr3 (*17*, *18*), which is independent of RNAi (*19*). Similar read-through transcripts were observed when repeats were fused to reporter genes elsewhere in the genome and were also targets of RNAi (*22*). Thus, reporter gene silencing depends, at least in part, on slicing of heterochromatic cotranscripts by Ago1 (Fig. 3C).

The siRNA from the *ura4*⁺ reporter gene was undetectable (fig. S3), suggesting that interactions between siRNA and DNA are unlikely to account for *ura4*⁺ silencing. Instead, recruitment of H3K9me2 and RITS to *ura4*⁺ depends on siRNA from the pericentromeric repeats (*6*, *8*), which are required for slicing of read-through transcripts. Sliced nascent transcripts might recruit the silencing apparatus to reporter genes by virtue of cotranscription (Fig. 3E). A heterochromatic role for polymerase (Pol) II in spreading H3K9me2 is reminiscent of its euchromatic role in spreading H3K4me2 (*23*). In plants, TE insertions bring genes under their control when they integrate within the transcription unit but not when they integrate further away (*5*, *24*). In animals, cotranscription of heterochromatic repeats may also play a role in some forms of position effect variegation, but genes are silenced at much greater distances from heterochromatin and intervening genes can retain activity (*25*), so that other spreading or antispreading mechanisms are likely to be involved (*26*), such as those involving Swi6 (*27*).

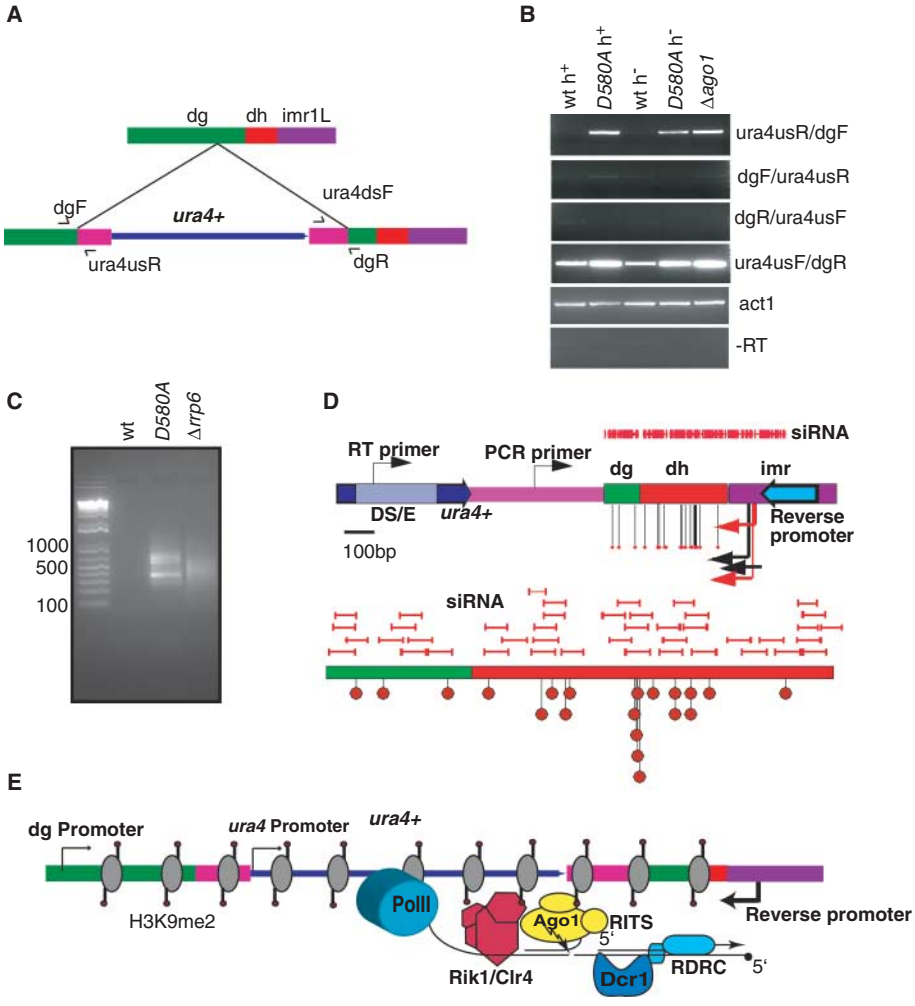


**Fig. 3.** Spreading of transcriptional silencing requires slicing. (**A**) The FY648 *otr1R(SphI)::ura4*⁺ insertion and the primers used for cotranscript detection are shown. (**B**) *dg*::*ura4*⁺ read-through transcripts were detected by strand-specific reverse transcription (RT) PCR. The first primer listed was used in RT to determine strand specificity. Forward strand transcripts are up-regulated in *ago1D580A* slicer mutants. Reverse strand transcripts are also up-regulated in *ago1D580A* slicer mutants but detectable in WT cells. (**C**) 5′ RACE PCR products were detected from slicer mutant *ago1D580A* and exosome mutant *rrp6*⁻. (**D**) 5′ RACE PCR products from slicer mutant *ago1D580A* (black) and exosome mutant *rrp6*⁻ (red) are shown. Putative transcript start sites [within 20 base pairs of the promoter (*14*)] are shown as arrows, whereas cleavage products are shown as lollipops. (**E**) Pol II reads through the *ura4*⁺ reporter gene from the centromeric repeat promoter. RITS is bound to the repeat via H3K9me2, and Ago1 slices transcripts guided by repeat siRNA. Slicing recruits the Rik1-Clr4 histone modification apparatus and provides templates for RdRP. Dcr1 processes RdRP products into siRNA after they are cleaved and released from the chromosome and is not necessarily associated with nascent transcripts.

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**Supporting Online Material**

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# Chemical Chaperones Reduce ER Stress and Restore Glucose Homeostasis in a Mouse Model of Type 2 Diabetes

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**Endoplasmic reticulum (ER) stress is a key link between obesity, insulin resistance, and type 2 diabetes. Here, we provide evidence that this mechanistic link can be exploited for therapeutic purposes with orally active chemical chaperones. 4-Phenyl butyric acid and taurine-conjugated ursodeoxycholic acid alleviated ER stress in cells and whole animals. Treatment of obese and diabetic mice with these compounds resulted in normalization of hyperglycemia, restoration of systemic insulin sensitivity, resolution of fatty liver disease, and enhancement of insulin action in liver, muscle, and adipose tissues. Our results demonstrate that chemical chaperones enhance the adaptive capacity of the ER and act as potent antidiabetic modalities with potential application in the treatment of type 2 diabetes.**

Insulin resistance is a common feature of obesity and predisposes the affected individuals to a variety of pathologies, including hypertension, dyslipidemias, cardiovascular disease, and type 2 diabetes mellitus (*1*). Although considerable progress has been made in understanding the molecular mechanisms underlying the insulin resistance and type 2 diabetes, satisfactory treatment modalities remain limited.

Studies in the past decade have demonstrated that obesity is associated with inflammation and established a link between inflammatory responses, particularly through the c-Jun N-terminal kinase (JNK) and inhibitory kappa B kinase (IKK) signaling pathways, and abnormal insulin action (*2*). We have recently shown that obesity also induces ER stress, and this, in turn, plays a central role in the development of insulin resistance and diabetes by triggering JNK activity via inositol-requiring enzyme–1 (IRE-1) and inhibition of insulin receptor signaling (*3*). Subsequent independent studies have also verified the role of ER stress in insulin resistance in several experimental systems (*4*, *5*). Taken together, in vitro and in vivo genetic evidence demonstrate a strong and causal relation between the functional capacity of the ER and insulin action, suggesting the possibility of exploiting this mechanism for therapeutic application.

Chemical or pharmaceutical chaperones, such as 4-phenyl butyric acid (PBA), trimethylamine *N*-oxide dihydrate (TMAO), and dimethyl sulfoxide, are a group of low molecular weight compounds known to stabilize protein conformation, improve ER folding capacity, and facilitate the trafficking of mutant proteins (*6*). Likewise, endogenous bile acids and derivatives such as ursodeoxycholic acid and its taurine-conjugated derivative (TUDCA) can also modulate ER function (*7*). In this study, we investigated whether pharmacologically active, small-molecule chemical chaperones could alleviate the increased ER stress seen in obesity and reverse insulin resistance and type 2 diabetes in experimental models.

To investigate the action of putative chemical chaperones, we first tested whether PBA and TUDCA protected against experimental ER stress in cultured cells. Pretreatment of Fao rat hepatoma cells with PBA suppressed tunicamycin-induced phosphorylation of double-stranded RNA-activated protein kinase–like endoplasmic reticulum kinase (PERK) (Thr-980) and eukaryotic initiation factor 2 alpha (eIF2α) (Ser-51) and JNK activation (fig. S1A). TUDCA pretreatment showed similar effects on tunicamycin-induced ER stress (fig. S1B). Pretreatment of liver cells with TUDCA reduced PERK and eIF2α phosphorylation and JNK activation upon exposure to tunicamycin (fig. S1B). Under these conditions, ER stress–induced splicing of X-box binding protein 1 (XBP-1) mRNA was also markedly reduced by both PBA and TUDCA (fig. S1, C and D). To exclude the possibility that PBA and TUDCA block general stress signaling without specificity for ER stress, we treated Fao cells with anisomycin, which activates JNK independent of ER stress. Neither PBA nor TUDCA prevented anisomycin-induced JNK activation (fig. S1, E and F).

XBP-$1^{-/-}$ mouse embryonic fibroblasts (MEFs) are hypersensitive to ER stress (*3*) because of the decreased ER folding capacity (*8*). Treatment of XBP-$1^{-/-}$ MEFs with PBA (fig. S2) also suppressed low-dose tunicamycin-induced phosphorylation of PERK and eIF2α, and activation of JNK, indicating that chemical chaperone treatment can reduce ER stress in multiple cell types and in a XBP-1–independent manner.

To investigate the in vivo effects of the chemical chaperones, we studied leptin-deficient (ob/ob) mice, a model of severe obesity and insulin resistance. Oral administration of PBA to *ob/ob* mice reduced ambient blood glucose to normoglycemic levels seen in the lean wild-type (WT) controls (434.2 ± 34.7 mg/dl versus 125.8 ± 12.6 mg/dl in vehicle versus PBA-treated *ob/ob* mice at 20 days, $P < 0.001$) (Fig. 1A). Normoglycemia in *ob/ob* mice was established within 4 days of PBA treatment, was maintained for up to 3 weeks, and was not associated with changes in body weight (Fig. 1B). PBA-treated *ob/ob* mice showed a more than twofold reduction ($P < 0.001$) in hyperinsulinemia (Fig. 1C), suggesting that the blood glucose–lowering effect of PBA is due to

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