

## RESEARCH ARTICLE

# Translational autoregulation of *BZW1* and *BZW2* expression by modulating the stringency of start codon selection

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## Abstract

The efficiency of start codon selection during ribosomal scanning in eukaryotic translation initiation is influenced by the context or flanking nucleotides surrounding the AUG codon. The levels of eukaryotic translation initiation factors 1 (eIF1) and 5 (eIF5) play critical roles in controlling the stringency of translation start site selection. The basic leucine zipper and W2 domain-containing proteins 1 and 2 (BZW1 and BZW2), also known as eIF5-mimic proteins, are paralogous human proteins containing C-terminal HEAT domains that resemble the HEAT domain of eIF5. We show that translation of mRNAs encoding BZW1 and BZW2 homologs in fungi, plants and metazoans is initiated by AUG codons in conserved unfavorable initiation contexts. This conservation is reminiscent of the conserved unfavorable initiation context that enables autoregulation of *EIF1*. We show that overexpression of BZW1 and BZW2 proteins enhances the stringency of start site selection, and that their poor initiation codons confer autoregulation on *BZW1* and *BZW2* mRNA translation. We also show that overexpression of these two proteins significantly diminishes the effect of overexpressing eIF5 on stringency of start codon selection, suggesting they antagonize this function of eIF5. These results reveal a surprising role for BZW1 and BZW2 in maintaining homeostatic stringency of start codon selection, and taking into account recent biochemical, genetic and structural insights into eukaryotic initiation, suggest a model for BZW1 and BZW2 function.

## Introduction

Eukaryotic translation initiation is complex and requires the activities of many factors [1, 2]. In eukaryotic translation initiation the small ribosome subunit, including the tRNA<sub>i</sub><sup>Met</sup> and assorted initiation factors constituting the 43S pre-initiation complex (PIC), binds to the mRNA 5' cap and scans in the 3' direction for an initiation codon. In most cases, initiation occurs at an AUG codon with favorable flanking nucleotides [3, 4]. The consensus initiation context in mammals is GCC(A/G)CCAUGG. The underlined nucleotides at positions -3 and

+4 (relative to the +1 A of AUG, shown in italics) play the most important role [4] although the slight possibility that the +4 position effect could be an amino acid N-end rule effect [5] has yet to be definitively excluded. Although the consensus sequence varies to some extent from organism to organism and from one eukaryotic kingdom to another, the preference for purine at position -3 is nearly universal, as is selection against (and presumably unfavorability) of U at positions -6 to -1. Even though G at position +4 is preferred only in vertebrates and plants, selection against A at the same position is conserved in metazoa, fungi and plants [6, 7]

*In vitro* and *in vivo* work has shown that the PIC component, eIF1, is the critical player in discrimination between poor and optimal initiation contexts [8, 9]. eIF1 binds near the P-site on the 40S ribosomal subunit [10–12]. This results in the formation and maintenance of an ‘open’ conformation PIC favoring scanning [13]. Start codon recognition is followed by release of eIF1 from the PIC leading to a ‘closed’ conformation of the small subunit which favors initiation and prevents scanning [14, 15].

In *Saccharomyces cerevisiae*, mutations in *EIF1*, *EIF1A*, *EIF2*, *EIF3*, *EIF4G* and *EIF5*, which each encode factors associated with the PIC, affect start codon selection [14,15]. Until recently it was thought that the sole role of eIF5 is to promote hydrolysis of eIF2-bound GTP in response to start codon recognition [16,17]. It is now known that eIF5 also stabilizes the binding of GDP to eIF2 and acts to inhibit the GDP-GTP exchange function of eIF2 $\beta$ , i.e. it works as a guanine nucleotide dissociation inhibitor, or GDI [18]. There is additional evidence for a distinct role for eIF5 as a functional competitor to eIF1 for binding at a critical site in the small ribosomal subunit such that successful competition leads to ejection of eIF1 [14,19–21]. eIF1 dissociation would then stimulate the formation of the PIC in its closed conformation that favors initiation [22].

We previously showed that elevated levels of eIF1 in mammalian cells reduces utilization of poor context AUG codons and of non-AUG start codons [9]. Interestingly, the AUG initiation codon of eIF1 is flanked by poor context nucleotides in most eukaryotes for which sequence data is available [9,23]. In *S. cerevisiae*, overexpression of eIF1 led to reduced utilization of its own poor context AUG and also of non-AUG codons [24]. These and other experimental data are consistent with a model in which eIF1 levels are controlled by an autoregulatory mechanism where high eIF1 levels reduce translation initiation from its own mRNA’s start codon.

In another study we also showed that elevated eIF5 levels result in increased initiation at poor-context AUG codons and at non-AUG start codons [25]. Many eukaryotic mRNAs encoding eIF5 contain one or more inhibitory upstream open reading frames (uORFs) whose start codons are in conserved poor contexts. This suggested a model for autoregulation in which elevated eIF5 levels can increase initiation at these uORF start codons and consequently decrease translation from the *EIF5* main ORF start codon. Using a series of reporters initiated by either AUGs in different contexts or by non-AUG start codons, eIF5 and eIF1 overexpression were observed to have opposite and additive effects on the stringency of start codon selection. This suggests that eIF5 and eIF1 positively cross-regulate each other’s expression at the level of translation initiation, providing additional means for a homeostatic cellular control mechanism to maintain stringency in start codon selection [25].

Vertebrate BZW1 and BZW2 proteins (the latter is also known as 5MP1) contain C-terminal W2-type HEAT domains. Other proteins with this domain are the translation initiation factors eIF4GI (also known as EIF4G1), DAP5 (also known as EIF4G2), eIF4GII (also known as EIF4G3), eIF2Be (EIF2B5) and eIF5. Of these, the similarity between the W2 HEAT domains of BZW and eIF5 is most notable [26], which has prompted some to refer to BZW2 as eIF5-mimic protein 1, 5MP1 [27]. It has been reported that BZW2 interacts with eIF2 and eIF3 and has weak GDI activity on eIF2 recycling [28]. Furthermore, strong interaction between BZW2 (5MP1) and eIF2 is responsible for the activation of ATF4 translation in

response to *BZW1* and *BZW2* overexpression by sequestering the latter away from the ribosome [28]. Despite their homology to initiation factors and experiments implicating *BZW* orthologs in translation [27–31], the exact role of *BZW1* and *BZW2* in initiation is still under investigation.

Here we analyze sequences of *BZW* homologs throughout eukaryotes and demonstrate that, similar to *EIF1*, their start codons are in conserved poor context. We demonstrate that the poor initiation context, when present on luciferase reporters, directs lower initiation if *BZW* levels are high, implying autoregulation and implicating these proteins in the selection step of translation initiation.

## Materials and methods

### Sequence analysis

*BZW* homolog sequences were obtained from GenBank by tBLASTn with human *BZW1* and *BZW2* protein sequences as query. The sequences were derived from the Expressed Sequence Tag (EST), Transcriptome Shotgun Assembly (TSA), Whole-Genome Shotgun contigs (WGS) or the RefSeq nucleotide databases. WGS sequences were processed manually to predict intron/exon junctions for the mRNA sequence. All alignments in this study were performed with the ClustalX algorithm [32]. Sequences used in this study are available upon request.

### Plasmids

Sense and antisense oligonucleotide pairs 1–2 and 3–4 (see [S1 Table](#)) were annealed and ligated into *Pst*I / *Bam*HI-digested dual luciferase vector p2-Luc [33] to make pSV40-firefly reporter constructs with the *BZW1* and *BZW2* initiation contexts respectively. The GUG initiated reporter was described previously [9]. *BZW1*-native and *BZW1*-optimal\* were amplified by RT-PCR from RNA isolated from HEK-293T cells using sense primers 5 (native) and 6 (optimal\*) and separately with antisense primer 7. *BZW2*-native and *BZW2*-optimal\* were amplified by RT-PCR from RNA isolated from HEK-293T cells using sense primers 8 (native) and 9 (optimal\*) and separately with antisense primer 10. Amplicons were cloned into *Nhe*I / *Xba*I-digested phRL-CMV (Promega). All constructs were verified by sequencing. The plasmid used to overexpress deregulated eIF1 (eIF1g\*), and deregulated eIF5 (eIF5-AAA), have been described previously [9,25]. The plasmid used for control transfections was pcDNA3 (Invitrogen).

### Cell culture and transfections

HEK-293T cells (ATCC) were maintained in DMEM supplemented with 10% FBS, 1 mM L-glutamine and antibiotics. HEK-293T cells were transfected in quadruplicate with Lipofectamine 2000 reagent (Invitrogen), using the 1-day protocol in which suspended cells are added directly to the DNA complexes in half-area 96-well plates. For each transfection the following were added to each well: 50 ng of protein overexpressing vector (or 25 ng each for mixing experiments), 5 ng pSV40-firefly vector (with initiation contexts and/or codons as indicated in the figures), 0.2 ng pSV40-*Renilla* vector and 0.2  $\mu$ l Lipofectamine 2000 in 25  $\mu$ l Opti-Mem (Gibco). The transfecting DNA complexes in each well were incubated with  $3 \times 10^4$  cells suspended in 50  $\mu$ l DMEM + 10% FBS. Transfected cells were incubated at 37°C in 5% CO<sub>2</sub> for 16hr.

### Dual luciferase assay

Firefly and *Renilla* luciferase activities were determined using the Dual Luciferase Stop & Glo<sup>®</sup> Reporter Assay System (Promega). Relative light units were measured on a Veritas

Microplate Luminometer with two injectors (Turner Biosystems). Transfected cells were washed once with  $1 \times$  PBS and then lysed in  $12.6 \mu\text{l}$  of  $1 \times$  passive lysis buffer (PLB) and light emission was measured following injection of  $25 \mu\text{l}$  of either *Renilla* or firefly luciferase substrate. Firefly luciferase activity was calculated relative to the activity of the co-transfected control plasmid expressing *Renilla* luciferase (pSV40-*Renilla*).

### Western analysis

Cells were transfected in 6-well plates using Lipofectamine 2000 reagent, again using the 1-day protocol described above, with  $1 \mu\text{g}$  of each indicated plasmid. The transfecting DNA complexes in each well were incubated with  $8 \times 10^5$  HEK-293T cells suspended in  $3000 \mu\text{l}$  DMEM + 10% FBS and incubated overnight at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . Transfected cells were lysed in  $100 \mu\text{l}$   $1 \times$  PLB. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Protran), which were incubated at  $4^\circ\text{C}$  overnight with primary antibodies. Immunoreactive bands were detected on membranes after incubation with appropriate fluorescently labelled secondary antibody using a LI-COR Odyssey<sup>®</sup> Infrared Imaging Scanner.

### Antibodies

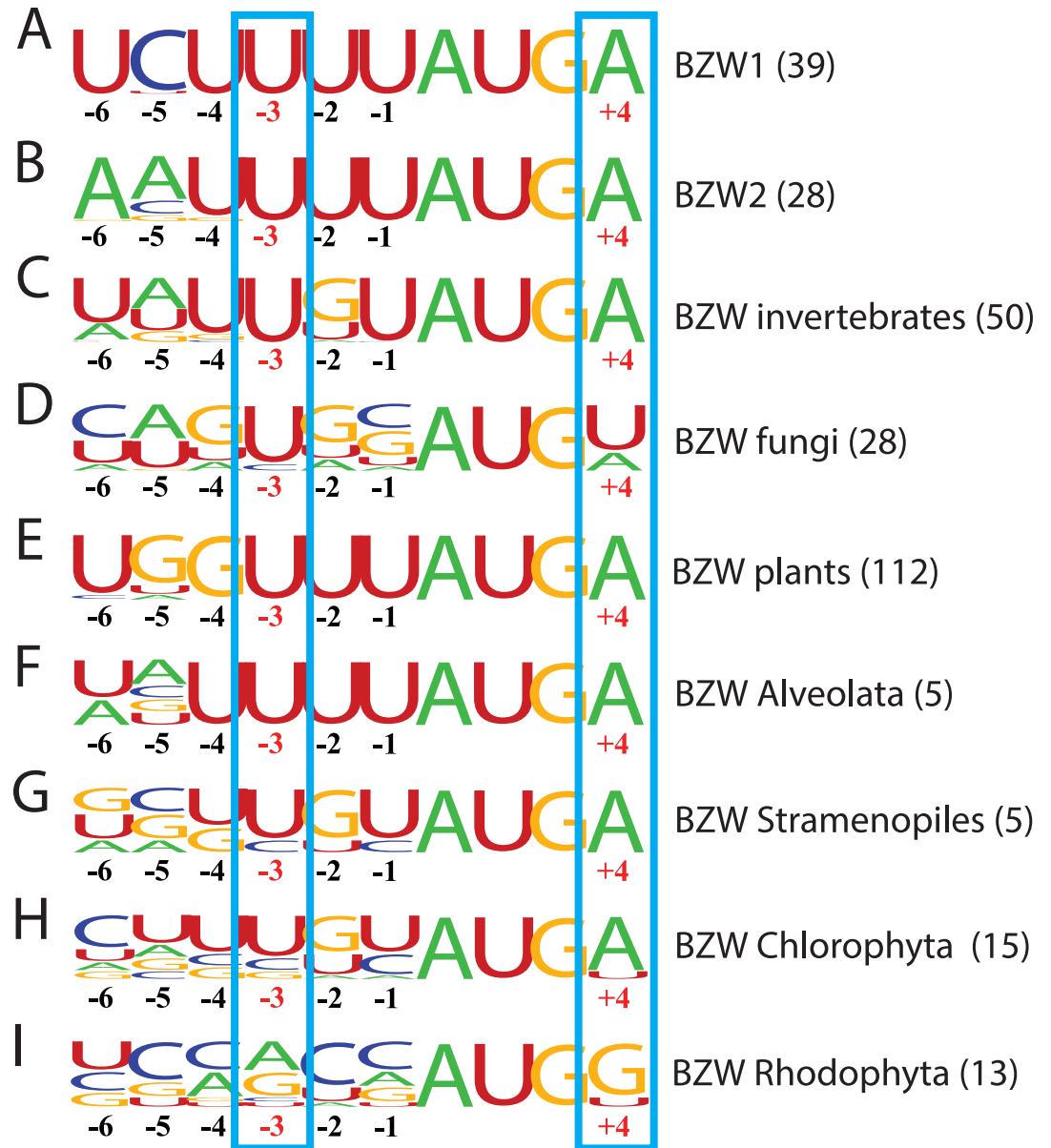
The following commercially available antibodies were used: Rabbit anti-BZW1 (Abcam ab85090) diluted 1:500, rabbit anti-BZW2 (Abcam ab96682) diluted 1:1000, goat anti-eIF1 (Santa Cruz D-15) diluted 1:100, rabbit anti-eIF5 (Abcam ab85913) diluted 1:1000 and mouse anti- $\beta$ -actin (Sigma A3853) diluted 1:5000.

## Results

### *BZW1* and *BZW2* display deeply conserved poor translation initiation context

Following up on the translational autoregulation observed with *EIF1* [9] and *EIF5* [25], which both exploit conserved poor translation initiation context, we decided to perform a search for other human genes with clear patterns of conserved poor context surrounding their initiation codons. This search identified a pair of human paralogs called *BZW1* and *BZW2*, with poor initiation context displaying deep evolutionary conservation (Fig 1, S1 Fig). Previously, the amino acid sequences of 97 eukaryotic *BZW* homologs were examined [30] and here we extend this analysis by comparing partial or complete nucleotide sequences of 250 eukaryotic *BZW* homologs. *BZW* homologs are present in metazoa, some fungi (though *BZW* orthologs are not apparent in Ascomycota, including *S. cerevisiae*), plants, green algae (Chlorophyta), red algae (Rhodophyta), brown algae (Stramenopiles), and also in some protists (e.g. Alveolata). The initiation context of the first in-frame AUG of *BZW* homologs from animals, plants, fungi, Alveolata, and brown algae is present in conserved poor context (Fig 1). A purine is never observed at position  $-3$ , but rather the least favorable nucleotide, U, and on rare occasions the almost equally unfavorable C are found at that position (Fig 1A–1G). Furthermore, there is never a G at position  $+4$  and there is a preponderance of unfavorable U-s at positions  $-6$ ,  $-4$ ,  $-2$  and  $-1$  in many evolutionary branches. In green algae the  $-3$  position is predominantly unfavorable U or C. Although there is no apparent selection for poor initiation context in red algae, overall the selection for poor context at positions  $-3$  and  $+4$  is comparable to that observed in *EIF1*, where the poor context has been shown to be essential for its autoregulation [9].

In humans, *BZW1* and *BZW2* contain 3 and 4 out-of-frame AUG codons, respectively, between the first and second in-frame AUG codons of the main ORF. In each case at least two



**Fig 1. The AUG start codon of BZW homologs in eukaryotes is in conserved poor initiation context.** The sequences surrounding the start codon of BZW homologs, from position -6 to +4, were aligned for the evolutionary branch indicated and the alignment represented as a frequency logo [34], with the height of each letter proportional to the frequency with which the corresponding nucleotide occurs at a given position. Alignment of: (A) *BZW1* orthologs in vertebrates; (B) *BZW2* orthologs in vertebrates; (C) *BZW* homologs in invertebrates; (D) *BZW* homologs in fungi; (E) *BZW* homologs in land plants; (F) *BZW* homologs in Alveolata; (G) *BZW* homologs in brown algae; (H) *BZW* homologs in green algae; and (I) *BZW* homologs in red algae. The critical positions -3 and +4 are indicated with red numbers and also boxed. Numbers in parentheses indicate the number of sequences in each alignment.

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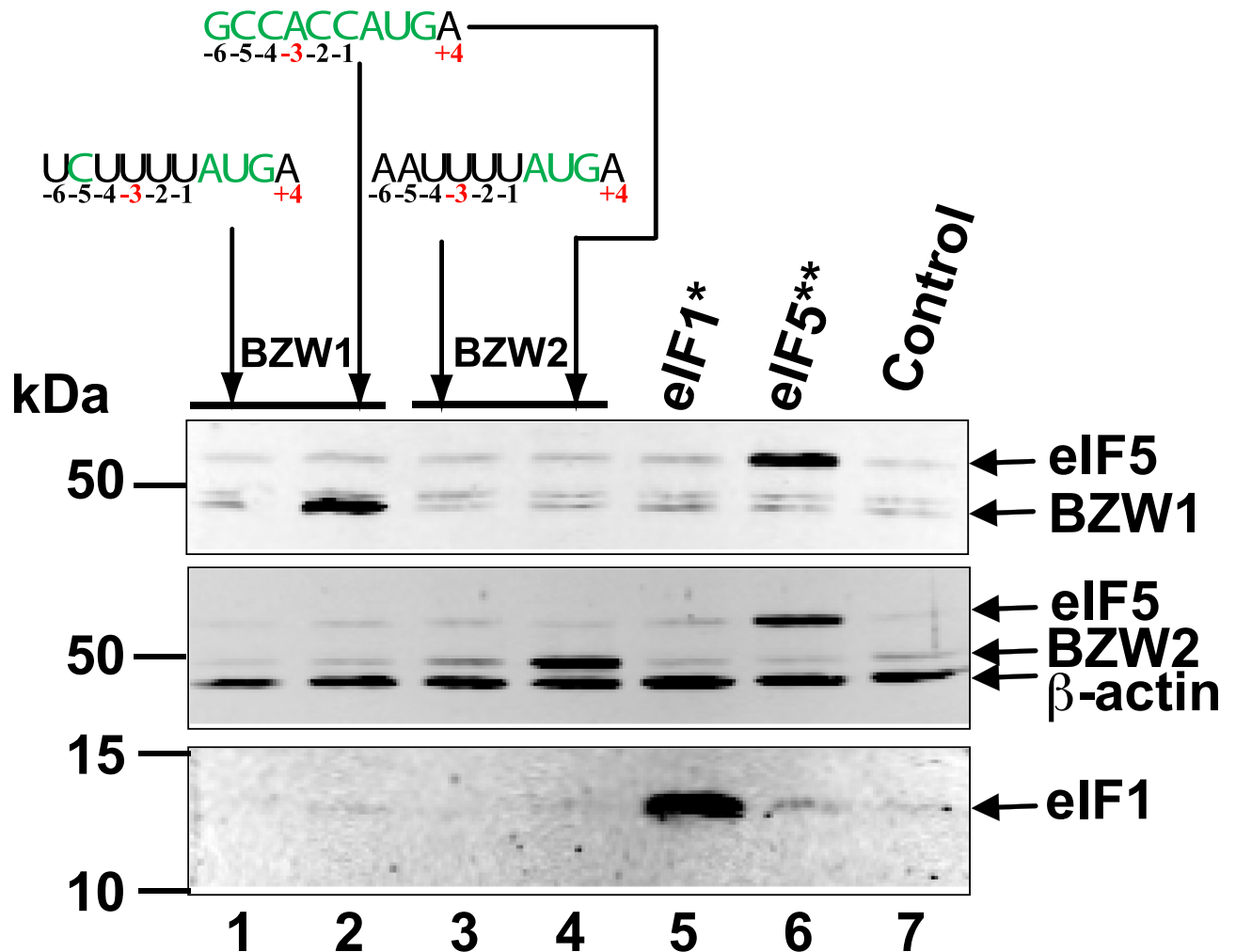
of these out-of-frame AUG codons are present in intermediate or better initiation context (S2 Fig). Following the principles of leaky scanning it would be expected that any 43S subunits which scan past the first in-frame AUG codon would likely be “captured” by one of the out-of-frame AUG codons, so that only an insignificant fraction of 43S subunits would ever reach the second in-frame AUG. This ORF architecture, which is also common in *BZW* homologs in

other eukaryotes, suggests that the first in-frame AUG is the sole initiation codon in both *BZW1* and *BZW2* mRNAs, and that the purpose of the conserved weak initiator codon in *BZW1* and *BZW2* is not to generate one of several isoforms by leaky scanning, as is observed in some other mRNAs in which the first in-frame AUG is in poor context [35,36].

### Overexpression of *BZW1* leads to higher initiation stringency, which is autoregulatory

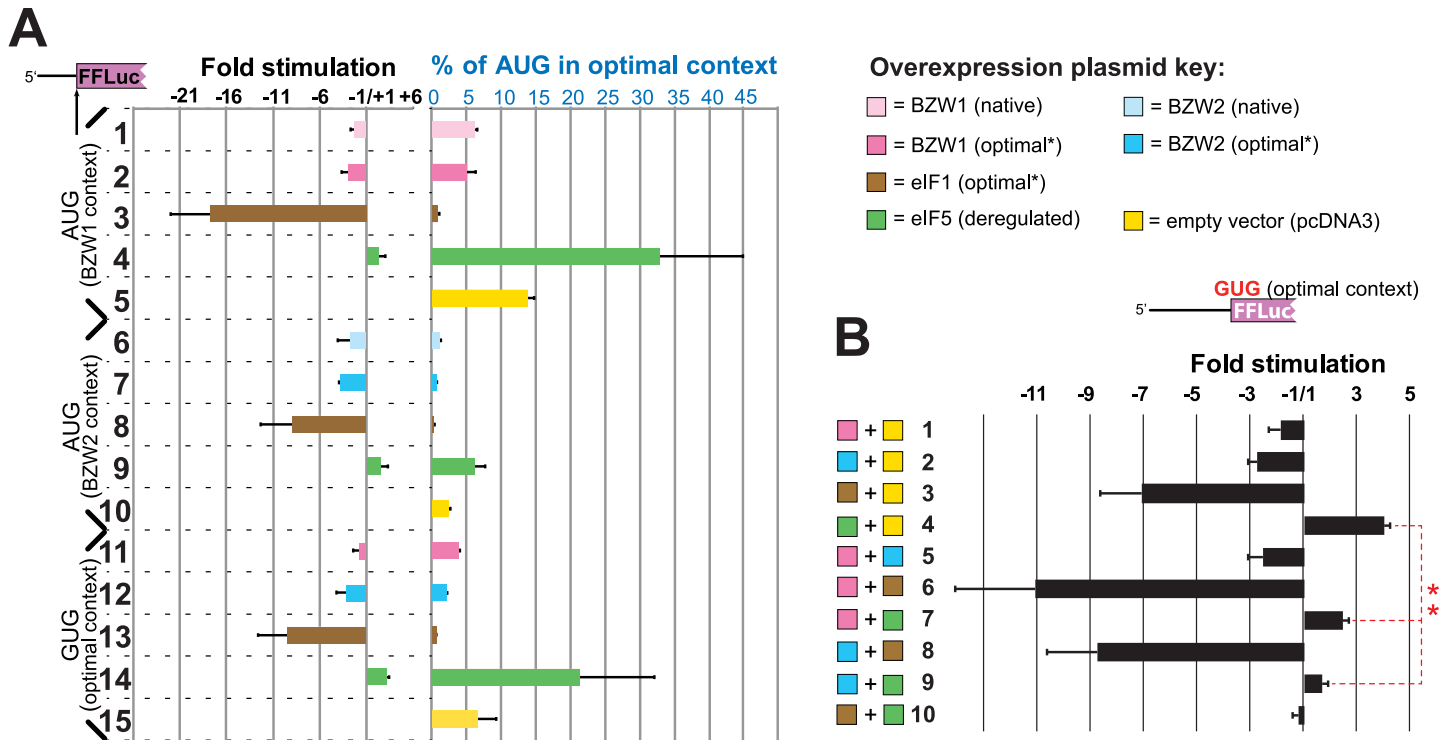
If the conserved *BZW1* and *BZW2* initiation sites are sensors for negative feedback regulation, then initiation from these sites should be inefficient under control conditions and should become increasingly so as the intracellular concentration of *BZW1* and *BZW2* increase.

The prediction that *BZW1* and *BZW2* contexts are poor were tested by cotransfecting HEK-293T cells with plasmids expressing human *BZW1* or *BZW2* initiated by AUG in either native (predicted to be poor) or optimal initiation context (as defined by Kozak) (Fig 2). Western blots from lysates of these transfected cells confirm that *BZW1* and *BZW2* proteins



**Fig 2. *BZW1* and *BZW2* are expressed poorly from their native initiation context.** Anti-BZW immunoblots of lysates prepared from HEK-293T cells transfected with BZW-expressing plasmids with start codons in different contexts as indicated. *BZW1* in native context (lane 1), *BZW1* in optimal context (lane 2), *BZW2* in native context (lane 3), *BZW2* in optimal context (lane 4). Overexpression of deregulated stringency factors eIF1 and eIF5 are shown in lanes 5 and 6 respectively. The control lane shows lysates from cells transfected with pcDNA3.

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**Fig 3. Overexpression of BZW1 and BZW2 leads to higher stringency of start codon selection and cancels the effect of eIF5 overexpression.** (A) Dual luciferase assays showing fold stimulation (left-hand side) or percentage initiation relative to AUG in optimal context (right-hand side) of firefly luciferase reporters initiated with start codons in native BZW contexts or with GUG as indicated (left-hand margin) in response to overexpression of the indicated initiation factors. (B) Dual luciferase assays showing fold stimulation of GUG-initiated firefly luciferase reporters in response to overexpression of combinations of the indicated initiation factors. For both (A) and (B), firefly luciferase measurements were normalized to those from a co-transfected *Renilla* luciferase expressing construct. For the fold stimulation calculations, the ratios in test cells were compared to the luciferase ratio in control cells transfected with pcDNA3. Negative "stimulation" values indicate repression. "Optimal\*" indicates initiation context which is optimized at nucleotide positions -6 to -1 relative to the start codon, but not at position +4 which is kept the same as the native sequence to prevent changes in the encoded amino acid. \*\*p<0.001 (Student's two-tailed t-test, n = 6).

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overexpressed from plasmids with *BZW1* and *BZW2* native initiation contexts are inefficiently expressed compared to the same proteins initiated by AUG in context optimized at positions -6 to -1 (compare lane 1 to lane 2 for *BZW1* and lane 3 to lane 4 for *BZW2* in Fig 2).

Initiation of reporters starting with the native *BZW1* and *BZW2* AUG contexts was measured using firefly luciferase reporters relative to reporters starting with AUG in optimal context. The level of initiation at the *BZW1* and *BZW2* start codons when fused to firefly luciferase is ~13% and ~2.5% of an optimal context AUG, respectively (see 5 and 10 in Fig 3A). Although both AUGs are in poor context, the much lower level of expression from the *BZW2* AUG likely underlies the importance of minor context determinants when there is neither a purine at -3 nor a G at +4 (see Fig 1).

Overexpression of *BZW1* (with native AUG context) results in a 2.3 fold decrease in *BZW1*-reporter expression compared to cells transfected with empty vector (compare 1 and 5 Fig 3A). A further slight, but not statistically significant, decrease in firefly luciferase activity is observed when cells overexpress *BZW1* that has been deregulated by optimizing its AUG context at positions -6 to -1 (compare 1, 2 and 5 in Fig 3A). Overexpression of *BZW2* has a similar effect on reporters starting with AUG in the native *BZW2* context (compare lanes 6, 7 and 10 in Fig 3A). Together, these data indicate that *BZW1* and *BZW2* can suppress initiation of their own start codons, and by implication they can autoregulate their own expression.

To assess the effect of *BZW1* and *BZW2* levels on overall stringency we also tested reporter constructs initiated by GUG in the presence of overexpressed *BZW1* or *BZW2* (with optimized context at positions –6 to –1) (compare lanes 11, 12 and 15 in Fig 3A). Since we know that both eIF1 and eIF5 can regulate initiation at suboptimal translation start codons we also separately overexpressed each of these initiation factors (see 3, 4, 8, 9, 13 and 14 in Fig 3A). These results indicate that the effect of *BZW1* or *BZW2* overexpression is not restricted to their native initiation context but leads to an overall increase in stringency of start codon selection on both AUGs in poor context and on near-cognate start codons.

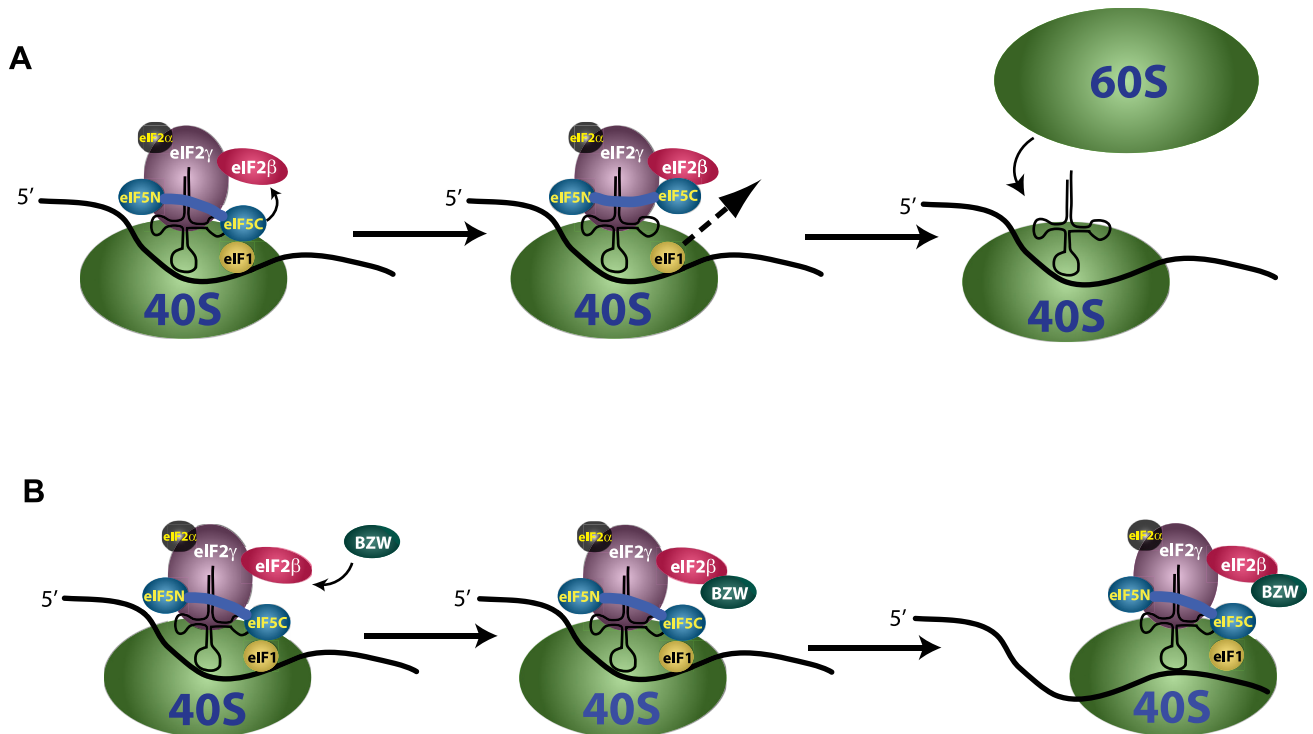
### Overexpression of BZW inhibits the effect of overexpressing eIF5, suggesting the two have an antagonistic relationship in setting the stringency of start codon selection

As discussed above, *BZW2* and also *BZW1* are thought to act as eIF5-mimic proteins by interfering with ternary complex (TC) recruitment to the 40S subunit. At the same time, high concentrations of eIF5 lead to ejection of eIF1 from the PIC and induce a closed conformation of the 40S subunit. If *BZW1* or *BZW2* can block this latter activity of eIF5, their overexpression would be expected to increase stringency by preventing ejection of eIF1 from the PIC even in the presence of high levels of eIF5, irrespective of their earlier role in PIC recruitment. To test this hypothesis we transfected constructs expressing *BZW1* or *BZW2* together with either eIF1 or eIF5 along with firefly luciferase reporters initiated with GUG (Fig 3B). Overexpression of *BZW1*, *BZW2* or eIF1 alone (plus equivalent amounts of empty vector) causes ~1.5, ~3 and ~7 fold decreases in initiation of reporters starting with GUG, respectively (1–3 in Fig 3B). Elevated levels of eIF5, by contrast, leads to ~4 fold increase in expression of the same reporter (4 in Fig 3B). When *BZW1* or *BZW2* are co-transfected with eIF5, the reduction of stringency seen with overexpression of eIF5 alone is significantly diminished, from ~4 fold with eIF5 alone to either ~2.5 fold (with co-overexpression of *BZW1*) or ~0.75 fold (with co-overexpression of *BZW2*) (7 and 9 in Fig 3B). An additive effect for co-transfection of *BZW1* or *BZW2* along with eIF1 is less clear (6 and 8 in Fig 3B). These results indicate that *BZW1* and *BZW2* can affect the stringency of start codon selection by reducing the actions of eIF5. Given the homology of *BZW1* and *BZW2* with eIF5 and the findings of Nanda and colleagues [14,19], we suggest that *BZW1* and *BZW2* act as dominant negative inhibitors of eIF5 as shown in the model (Fig 4).

## Discussion

The results presented above provide strong evidence that elevated levels of *BZW1* and *BZW2* increase the stringency of start codon selection in mammalian cells. They also suggest that this is used in autoregulation of *BZW1* and *BZW2* expression in most if not all eukaryotes that possess orthologs of these genes. A central feature of this autoregulation is the presence of an obligatory AUG start codon in unfavorable initiation context. The suboptimal initiation contexts of human *BZW1* and *BZW2* was established by western analysis of *BZW1* and *BZW2* levels synthesised from plasmids where they are initiated by their native or by optimal context (Fig 2). High levels of *BZW1* and *BZW2* were shown to result in greater discrimination against these start codons lowering the synthesis of firefly luciferase reporters starting with *BZW1* and *BZW2* initiation contexts (Fig 3A). The results also show that high levels of *BZW1* and *BZW2* by themselves increase the stringency of start codon selection and significantly neutralize the effect of overexpressing eIF5, which by itself lowers the stringency of start codon selection (Fig 3).





**Fig 4. A model for the molecular interactions likely to account for the effect of BZW1 and BZW2 on stringency of start codon selection.** During scanning, when the 40S subunit is in the open conformation, eIF1 is bound near the P-site and monitors the interaction between  $tRNA_i^{Met}$  and nucleotide triplets in the mRNA. The eIF5 C-terminal domain is loosely bound to eIF1 working as a lid to prevent the premature release of eIF1 from the PIC. (A) Under low BZW levels, the eIF2β N-terminal domain and eIF5 C-terminal domain occasionally interact pulling eIF5 away from eIF1, allowing its looser association with the PIC. The lower affinity of eIF1 for the PIC facilitates more frequent initiation at near-cognate or suboptimal AUG codons. (B) BZW, perhaps through its C-terminal HEAT domain, binds to the eIF2β N-terminal domain. When levels of BZW protein are high this interaction is favored, and this prevents interaction between eIF2β and eIF5. This leaves the “lid” on eIF1 closed, essentially increasing its effective concentration on the PIC and this enhances stringency of start codon selection, resulting in lower frequency of initiation at near-cognate start codons or poor context AUG triplets, including the start codons of BZW genes, with ribosome continuing to scan in the 3' direction for a better start codon.

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The precise molecular mechanism of start codon selection is gradually coming into focus. Several initiation factors have been identified genetically and biochemically that provide interactions implicated in this process. These include eIF1, eIF1A, eIF5, eIF2, eIF3 and eIF4G [2,37]. With two of them, eIF1 and eIF5, it has been shown that their protein levels in the cell directly affect stringency of start codon selection, with high levels of eIF1 increasing stringency and high levels of eIF5 relaxing it [9,24,25,38,39]. In both of these cases, as is shown here for BZW1 and BZW2, this is used for autoregulation [9,24,25]. eIF1 promotes high stringency by binding 40S ribosomes to facilitate the open scanning complex [12, 13]. Its release, upon  $tRNA_i^{Met}$  encountering a favorable start codon, leads to a closed PIC, which precludes further scanning [14,40]. The exact molecular mechanism by which high levels of eIF5 reduce stringency are not known, however, high concentrations of eIF5 help eject eIF1 from a PIC which has encountered a suboptimal start codon *in vitro* [14,19]. It has been proposed that eIF5 has a lower affinity binding site on the 40S subunit and that this site overlaps the binding site of eIF1 [14]. In addition, the eIF5 C-terminal domain (CTD), encoding its HEAT domain, interacts with eIF1 and this interaction is involved in start codon recognition [20], and it is the CTD domain of eIF5 that antagonizes the binding of eIF1 to the PIC [19]. Separately, the eIF5 CTD interacts with the N-terminal domain (NTD) of eIF2β [20], and also with eIF3c and eIF4G

[41]. The surfaces on the eIF5 CTD that bind to eIF1 and eIF2 $\beta$  partially overlap, implying mutually exclusive interactions. It is believed that the interaction of the eIF5 CTD with eIF1 impedes the premature release of the latter during scanning. Conversely, the interaction between the NTD of eIF2 $\beta$  and the CTD of eIF5 is believed to separate eIF5 from eIF1, thus facilitating the release of eIF1 [20]. Immunoprecipitation experiments suggest interaction between BZW and eIF2 $\beta$  [27]. The precise locations of the eIF5 CTD and the eIF2 $\beta$  NTD are not defined in any of the published structures for the PIC, implying that they are afforded considerable flexibility. Here we show that overexpression of either *BZW1* or *BZW2* increases stringency of start codon selection and this might be synergistic with the activity of eIF1 and is antagonistic with the activity of eIF5. Based on these data, and the published results outlined above, we propose the following model for the role of BZW proteins in start codon selection (Fig 4): During scanning, when the 40S subunit is in the open conformation, eIF1 is bound near the P-site and monitors the interaction between tRNA<sup>Met</sup> and nucleotide triplets in the mRNA. The eIF5 CTD is loosely bound to eIF1 working as a lid to prevent the premature release of eIF1 from the PIC. Under low BZW levels, the eIF2 $\beta$  NTD and eIF5 CTD occasionally interact pulling eIF5 away from eIF1, allowing its looser association with the PIC and facilitating more frequent initiation at near-cognate or suboptimal AUG codons. When BZW levels are high the protein, perhaps through its CTD HEAT domain, binds to the eIF2 $\beta$  NTD and prevents its interaction with eIF5. This leaves the “lid” on eIF1 closed and enhances stringency of start codon selection.

Given the known interactions of eIF2 $\beta$ , eIF1, eIF5, *BZW1* and *BZW2* with the 40S subunit we prefer a model where these competitive interactions involve the PIC but competition in solution cannot be ruled out.

## Supporting information

**S1 Fig. Conservation of the initiation context of *BZW* homologs.** Sequences used for generating the logograms in Fig 1. Species identifiers of the sequences used are indicated on the left. Start codons are highlighted in green. Numbers in parentheses indicate the number of sequences in each phylogenetic branch.

(PDF)

**S2 Fig. The nucleotide sequences of human *BZW1* and *BZW2* precludes the likelihood of leaky scanning producing N-terminally truncated proteins.** (A) The sequence of human *BZW1* mRNA. (B) The sequence of human *BZW2* mRNA. The main open reading frame is highlighted in yellow. The stop codon is highlighted in red. The first and second in-frame AUG codons are highlighted in light green. The out-of-frame AUG codons between the first and second in-frame AUG codons are highlighted in dark green. -3 and +4 nucleotides matching favorable initiation context are highlighted in gray, while those that are unfavorable are highlighted in magenta.

(PDF)

**S1 Table. DNA oligonucleotides used in this study.**

(PDF)

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