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Why endogenous TRPV6 currents are not detectable-what can we learn from bats?

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ABSTRACT

TRPV6 is a calcium selective TRP channel and is expressed in many species. TRPV6 transcripts are abundantly expressed in few tissues but strangely channel properties are only accessible to electrophysiological recordings after overexpression whereas in native tissue functional channel currents seem not to be detectable. Another exceptional property of human and mouse TRPV6 proteins is that the initiation of translation starts from a non-canonical ACG triplet which is translated as methionine. This triplet is located 120 bp upstream of the first in-frame AUG codon of the human/mouse TRPV6 mRNA. In contrast, the TRPV6 gene of bats is initiated from an AUG triplet at the corresponding position of the human ACG. On the basis of these structural nucleotide differences between human and bats we studied the role of the absolute N-Terminus for the regulation of translation by developing chimera and mutants of human/bat TRPV6 channels. The human sequence which is located downstream of the initiation codon slows down ribosomal scanning in 3′ direction. We suggest that the mechanism involves most likely the deceleration of ribosome scanning by stem-loop formation and the use of the common initiator tRNA, $tRNA_i^{Met}$, which is placed onto the inappropriate ACG codon resulting in low protein synthesis. The reduced translation efficiency is important to protect TRPV6 expressing cells from toxic calcium overload. The regulation of the TRPV6 translation in bats may be an adaptation to low calcium amounts present in the natural nutrition. In addition, we show that also the GFP protein can be controlled using the translational mechanism of human TRPV6.

1. Introduction

TRPV6 is a calcium selective ion channel which is expressed in humans in the placental trophoblast layer, in pancreatic acini and in several glands including lacrimal and salivary gland ([1], refer also to protein atlas, https://www.proteinatlas.org/ ENSG00000165125 -TRPV6/summary/rna). TRPV6 channels consist of 4 identical subunits [2] which are detectable in the plasma membrane of overexpressing cells. In overexpressing cells using a divalent free solution an inward rectifying current with a single channel conductance of ~50 pS is measurable [3]. Although TRPV6 transcripts are abundantly expressed in placental and pancreatic tissues nevertheless native TRPV6 currents have never been shown in these tissues [4]. We published that the TRPV6 protein starts at a non-canonical start triplet, ACG, which is translated into methionine, (UniProtKB: Q9H1D0, accession number: NM_018646.6 [4]). Initiation from non-canonical triplets were thought to be rare events in mammals but there is growing evidence that non canonical triplets are relatively frequently used [5–7]. The mentioned ACG triplet of the human TRPV6 cDNA is located 120 bp upstream of the first AUG triplet in frame. The expression of TRPV6 as full length protein starting from the ACG codon or from the first AUG codon did not reveal significant differences in terms of amplitude, activation and inactivation behaviour of the corresponding TRPV6 currents. In the current work we try to find an explanation why the initiation of the translation of TRPV6 is different compared to most other translated genes. In fact we want to answer three questions: 1. Is there a biological reason to translate the

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ACG codon into methionine? 2. Is there a specific function of the 120 bp sequence which is located upstream of the first AUG triplet? 3. Why are TRPV6 currents undetectable in tissues which express TRPV6 transcripts?

2. Material and methods

2.1. Cloning of TRPV6 expression constructs

The cDNA of human TRPV6 including the 5′UTR (NCBI reference sequence NM_018646.6) was subcloned into the bicistronic expression vector pCAGGS-IRES-GFP as described earlier [1]. The vector allows simultaneous expression of the TRPV6 and the GFP cDNAs. In addition the cDNA of TRPV6 was also cloned in pCDNA3 vector in which mutations/deletions were introduced by PCR using the phusion polymerase (NEB, Ipswich, USA) and subcloned in the appropriate vectors. The cDNA including the 5′ UTR of the bat *Myotis brandtii* was humanized using the JCat codon adaptation tool software (http://www.jcat.de/). The humanized *Myotis brandtii* cDNA was commercially synthesized (BioCat, Heidelberg, Germany) and cloned in pUC18 vector. The cDNA was subcloned in the pCAGGS-IRES-GFP vector. Mutation/deletions were introduced in the pUC18 clone and subcloned in the appropriate vectors. The TRPV6 N-terminus of the vampire bat *Desmodus rotundus* was cloned from genomic DNA using the primer 5′ccagctctgccaagagtaac 3′and 5′cgtcttcttgggcagtggc 3′. The N-terminus of TRPV6 from *Myotis brandtii* (not humanized) was synthesized using the complementary primers which were annealed and subcloned in a deletion clone of *Myotis brandtii* (humanized) TRPV6-puc18. The chimeric constructs of Myotis/human, Myotis/Desmodus, Myotis (humanized)/Myotis (not humanized) were subcloned as SacI fragments in the SacI sides of the Myotis TRPV6 cDNA constructs.

2.2. Cell culture and transfection

HEK293 cells were grown in 3,5 cm diameter culture dishes with and without polylysine-coated glass coverslips (diameter 2.5 cm) until 80 % confluence and then transiently transfected with 2,5 µg of the respective cDNA constructs described above in 4 µl of Lipofectamin 3000 (ThermoFisher, Waltham, USA). For Fura-2AM measurements, cells were transfected with the pcAGGS-IRES-GFP vector were used as control. Coverslips with transfected cells were used for Ca^{2+} imaging experiments 24–48 h after transfection.

2.3. Western blot

Protein lysates of HEK293 cells expressing the appropriate TRPV6 constructs were eluted in 100 µL of 2-times denaturing electrophoresis sample buffer and incubated at 60 °C for 30 min before SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 4–12 % Bis-Tris gels (NuPAGE®-Novex, Invitrogen) in a MOPS buffer system (50 mM MOPS, 50 mM Tris, 0.1 % SDS, 1 mM EDTA, pH 7.7). The proteins were separated by electrophoresis, blotted, and probed with the antibodies 429, 1271 and GFP, respectively. The endoplasmic reticulum protein calnexin was used as a control.

2.4. Antibodies

The following in-house-generated anti-TRPV6 antibodies were used: Polyclonal antibody 429 directed to the C terminus of TRPV6 [8,9], the polyclonal antibody 1271, directed against the elongated N-terminus of human TRPV6 ([4], amino acids 1–40) and monoclonal YFP/GFP antibody (Roche, Basel, Swiss). All antibodies were affinity-purified before use. Antibodies for Calnexin were from Enzo (Lausen, Swiss).

2.5. Fluorescence microscopy

Fluorescence pictures were taken using an Axio Observer Z1 microscope equipped with a 10× objective, a HXP 120 C lamp and an Axiocam color CCD camera (Zeiss, Oberkochen, Germany) as described earlier [10]. The following wavelength were adjusted: GFP (excitation 470/40 nm, dichroic mirror 495 nm, emission >500 nm, AHF Analysetechnik AG, Tübingen, Germany). Fluorescence pictures were analysed with the AxioVision software (Zeiss) and the ImageJ software (NIH).

2.6. Prediction of secondary structure

The TRPV6 mRNA structure was predicted with RNAfold program from the ViennaRNA services (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). The MFE, minimum free energy, was calculated using RNAfold and bases 20–500 of the TRPV6 mRNA of several organisms.

2.7. Ca^{2+} imaging

Intracellular live cell Ca^{2+} imaging experiments were performed using a Polychrome V and CCD camera (TILL Imago)-based imaging system from T.I.L.L. Photonics (Martinsried, Germany) with a Zeiss Axiovert S100 fluorescence microscope equipped with a Zeiss Fluar 20×/0.75 objective. Data acquisition was accomplished with the imaging software Live Aquisition software (T.I.L.L. Photonics, Martinsried, Germany). Before the experiments, cells were incubated in Ca^{2+} free solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, pH adjusted to 7.2 with NaOH) supplemented with 4 µM Ca^{2+}-sensitive fluorescent dye Fura-2AM (Molecular probes, Eugene, USA) for 30 min in the dark at RT followed by 4 times washing with nominally Ca^{2+}-free external solution to remove excess Fura-2AM. The Fura-2-loaded cells, growing on 2,5 cm glass coverslips, were transferred to a bath chamber containing nominally Ca^{2+}-free solution, and Fura-2 fluorescence emission was monitored at >510 nm after excitation at 340 and 380 nm for 30 msec each at a rate of 1 Hz over 600 s. Cells were marked, and the ratio of the background-corrected Fura-2 fluorescence at 340 and 380 nm (F340/F380) were plotted versus time. After reaching a stable F340/F380 base line, 2 mM Na-EGTA and 2.0 mM $CaCl_2$ were added to the bath solution and cytosolic Ca^{2+} signals were measured, respectively.

3. Results and discussion

To find an explanation why the translation of TRPV6 is initiated at a non-canonical start triplet, namely ACG, we used a TRPV6 construct in which the translated part and the 5′ UTR of TRPV6 were cloned in a dicistronic vector which allows the independent expression of TRPV6 and the GFP protein. The ACG triplet is located 120 bp upstream of the first AUG codon of the TRPV6 cDNA. We used the construct to mutate the ACG triplet to ATG and analysed the protein amount of TRPV6 that is translated from these plasmids. We used the GFP protein as an internal control. The GFP protein is expressed from the same plasmid and is useful to a make the results independent from the transfection efficiency. It has to be noted that the overexpression of TRPV6 is toxic for any cell type because of cytosolic calcium overload of expressing cells [11–14]. To overcome the toxic properties we introduced a mutation, D582A, within the pore of the channel. The D582A mutation leads to a TRPV6 protein without a conductance for any ion. The D582A corresponds to the D581A mutation in murine TRPV6. [15–17]. In addition we analysed a construct in which 117 bp of the above mentioned 120 bp sequence were deleted and in which the initiation codon was switched to AUG. We transiently transfected cells and performed a Western blot using the TRPV6 specific antibody 429, which is directed to the C-terminus, and a GFP antibody (Fig. 1A, B). It can be seen that on the Western blot that two TRPV6 proteins of different molecular mass are

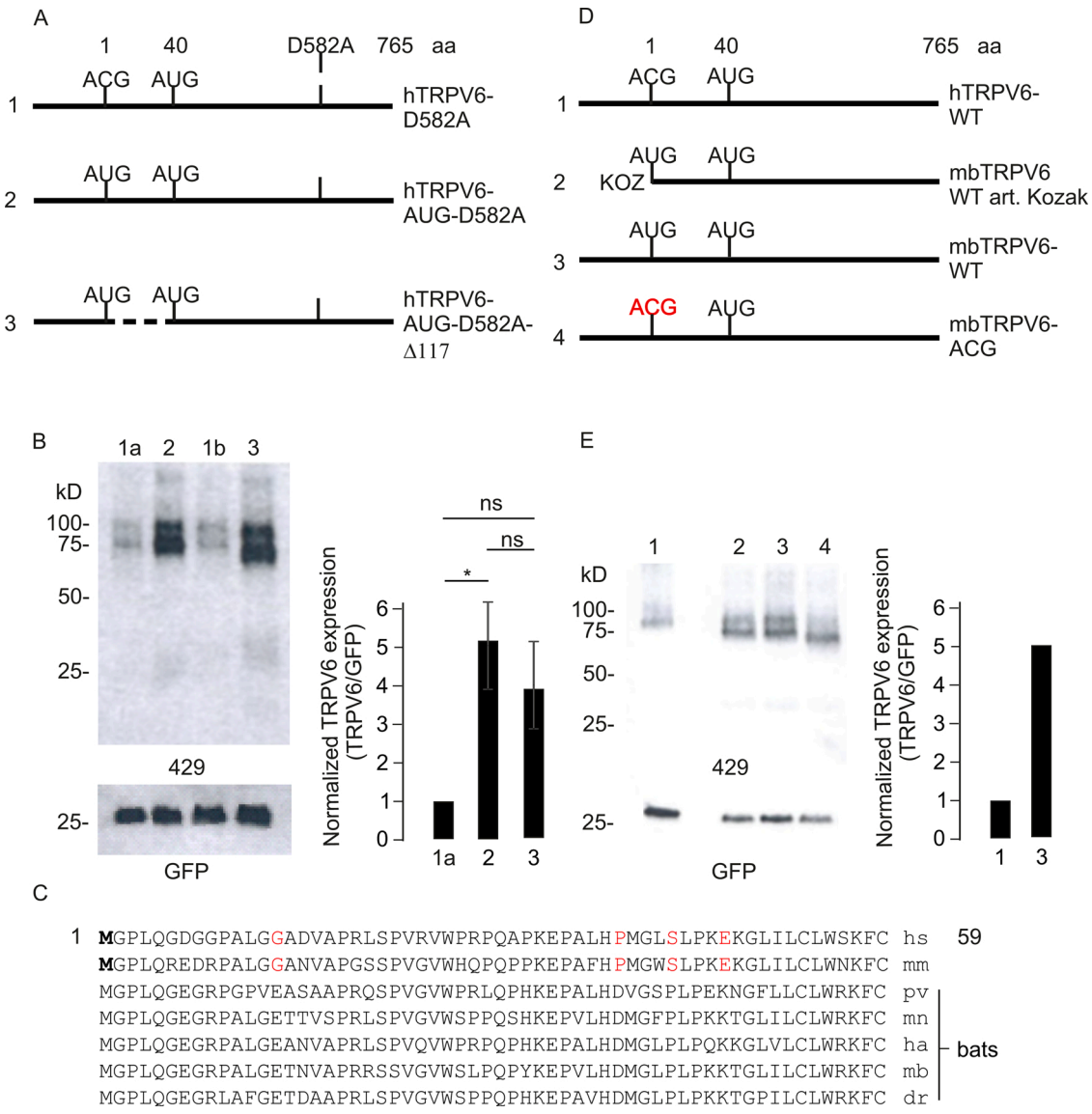

Fig. 1. The amount of the TRPV6 protein depends on the initiation triplet. A, TRPV6 constructs were expressed in HEK293 cells. 1, human TRPV6; 2, human TRPV6 with a mutation in the initiation triplet (ACG to AUG); 3, human TRPV6 with the deletion of 117 bp as indicated (dotted line). Numbering of amino acids (aa) on top. All three constructs contain a mutation, D582A which leads to a TRPV6 protein without Ca^{2+} conductance. B, Same constructs as in A analysed by Western blots (n = 3) with an antibody directed against the C-terminus of TRPV6 (429) and GFP. In the first (1a) and third lane (1b) two different human TRPV6 clones were analysed. Densitometric analysis is shown at the right. The TRPV6 signals were normalized to GFP and the TRPV6 signal of construct 1a was defined as 100 %*. C, Alignment of the very N-terminus of several TRPV6 proteins of bats with human (hs)/mouse (mm) TRPV6, pv, *Pteropus vampyrus* (Large flying fox); mn, *Miniopterus natalensis* (Natal long-fingered bat); ha, *Hipposideros armiger* (Great roundleaf bat); mb, *Myotis brandtii* (Brandts bat); dr, *Desmodus rotundus* (Common vampire bat); hs, *Homo sapiens*; mm, *Mus musculus*. The first amino acid of human and mouse TRPV6 is encoded by an ACG triplet (M, fat). Amino acids different between human/mouse TRPV6 and bats are indicated (red). D, Similar experiment as in A and B. 1, expression of human TRPV6; 2, mbTRPV6 (*Myotis brandtii*) with artificial Kozak sequence; 3, mbTRPV6 and 4, mbTRPV6 in which the initiation triplet was changed to ACG. E, Western blot of the constructs shown in D incubated with the antibodies 429 (upper blot), GFP (lower blot) and densitometric analysis (right, n = 1). All TRPV6 constructs were cloned in IRES-GFP vectors. *Data shown as mean ± S.E.M. with P values calculated using one-way-ANOVA followed by Bonferroni post-hoc test.

detectable. The upper band (~95 kDa) corresponds to glycosylated TRPV6 whereas the lower band (~80 kDa) corresponds to the non-glycosylated TRPV6 protein [8,12,18]. A densitometric analysis shows that the protein amount increases ~5 times in cells which express the TRPV6 construct and in which the naturally occurring ACG codon was switched to an AUG codon indicating that the naturally occurring ACG codon leads to a reduced synthesis of the amount of the TRPV6 protein (Fig.1B). As mentioned the expression of TRPV6 is toxic to most cell types because the cytosolic Ca^{2+} concentration is significantly increased in TRPV6 overexpressing cells. The reduction of the TRPV6 amount as consequence of the low translation efficiency may be a mechanism to avoid toxicity of the TRPV6 protein.

We deleted in another construct a 117 bp fragment which is located in between the initiation codon ACG and the first AUG triplet of the TRPV6 mRNA and in addition we changed the initiation codon to AUG and expressed the construct (Fig. 1A). It can be seen that the protein amount also increases compared to constructs in which the ACG triplet is used to initiate the translation (Fig. 1B). It is also visible that the resulting TRPV6 protein is smaller as consequence of the deletion of the 117 bp. The result shows that the translation of the TRPV6 protein starts upstream of the first AUG of the TRPV6 cDNA and that the translated 117 bp fragment is a part of the TRPV6 protein. Furthermore it demonstrates that the translation efficiency is independent from the presence of the N-terminal sequence of 117 bp. But the sense behind the 117

bp sequence is still unanswered.

Interestingly the ACG codon of the human TRPV6 mRNA at which the translation is initiated as well as the 117 bp sequence upstream of the first AUG codon are conserved among most mammals with the exception of the group of bats and fruit bats. Bats exhibit at the corresponding position of the ACG a classical AUG codon (Fig. 1C). Thus the TRPV6 protein of bats is most likely translated from an AUG codon as usual. To test this we made several constructs which contain the humanized TRPV6 sequence of the bat *Myotis brandtii* including the 5′ UTR. First we compared expression of human and bat constructs and found that the Myotis TRPV6 protein amount is ~5x higher than the amount of the human TRPV6 as concluded from densitometric analysis (Fig. 1 D, E lanes 1, 3). It should be noted that the antibody 429 is directed against the C-terminus of the human TRPV6 sequence and also detects the Myotis TRPV6 protein although the epitope is not complete identical (10/13 identical amino acids). Thus the amount of the Myotis TRPV6 protein could be underestimated and the real difference of the protein amount may be larger.

The efficiency of the translation depends not only on the initiation codon itself and involves also the sequence immediately up- and downstream of the initiating codon [19]. The sequence of human and myotis TRPV6 are identical from the -4 to +9 position with exception of the initiating codon itself at the +2 position. Therefor the different amount of the TRPV6 proteins of the two species depends on the triplet which initiates the translation.

Next we tested if the first AUG triplet of the Myotis sequence is indeed used for the initiation of the translation. Therefore we made a construct in which the 5′ UTR of the Myotis TRPV6 cDNA was replaced by a Kozak sequence (GCCGCCACC) [20,21]. Thus the translation initiation AUG triplet of the Myotis TRPV6 is placed in an optimal context and ensures that the translation of the protein starts at this particular AUG codon. The protein size shown in Fig. 1E, lanes 2 and 3 seems to be identical which indicates that the first AUG codon of TRPV6 of Myotis (and most likely also in other bats) serves as initiation codon for the translation. In addition we made the inverse experiment shown in Fig. 1A. We switched the initiation triplet AUG of the Myotis TRPV6 cDNA to ACG (Fig. 1E lane 4). It can be seen that not the opposite effect occurs, namely the reduction of the protein amount of TRPV6. Instead a protein of a lower molecular weight is detectable. Obviously in the context of the Myotis sequence an ACG codon is not accepted as initiation codon. Most likely a downstream located AUG codon is used for the initiation of the translation. We propose that in case of bats the scanning mechanism of the ribosomes takes place as it was described earlier [20,22]. The scanning model suggests if the first AUG codon is replaced by another triplet the ribosomes scan in 3′ direction to start translation at another AUG codon which potentially results in a shorter gene product.

Next we asked why the ACG codon of human TRPV6 is used as initiation triplet in humans but not if placed at the corresponding position in the Myotis mRNA. To answer this question we made a series of chimeric constructs in which parts of the Myotis TRPV6 sequence were replaced by the corresponding human sequence. The constructs were expressed and analysed by Western blot (Fig. 2A, human parts are shown in red). The comparison of lanes 1 with lanes 2, 3 (Fig. 2B, suppl. Fig. 1A) again shows that if an ACG codon replaces the AUG codon in the Myotis cDNA, the translated gene product has a smaller size. The gene product of the Myotis cDNA becomes larger when the human 5′ UTR and in addition the first 146 bp including the ACG initiation codon of the human TRPV6 replaced the original Myotis sequence (lanes 4, 5). We used the 146 bp sequence because around the first AUG codon (of human TRPV6) there are several nucleotide exchanges between human and Myotis TRPV6 mRNA which also affect the proteins sequence of TRPV6.

We found that it is sufficient to replace only the 146 bp of the Myotis TRPV6 by the human sequence, including the ACG initiating codon, to get a larger Myotis TRPV6 protein again (lanes 8, 9) and the gene products have virtual identical mass if an AUG codon was used as initiating codon (lanes 10, 11). Also a gene product of similar size was detectable if the human 5′ UTR including an AUG and the 146 bp sequence replaced the corresponding Myotis part of the cDNA (lanes 6, 7). The data illuminate the role of the146 bp sequence of the human TRPV6 cDNA: The 146 bp sequence of the human TRPV6 cDNA prevents scanning of the ribosomes in 3′ direction and brings the translation machinery to start the translation at the non-canonical ACG. However in bats the mechanism is different, because the TRPV6 cDNA has a canonical AUG triplet as initiating codon and therefore there is no need to prevent the ribosomes from scanning.

As mentioned we used for expression of the Myotis TRPV6 an artificial cDNA sequence which was adapted to the human codon usage to avoid troubles by expressing the Myotis protein in a human cell line. Next we tested if the naturally occurring Myotis sequence of 146 bp does the same as the humanized Myotis one. That is the case: If the 146 bp of the original Myotis sequence was expressed in the context with an ACG as initiation codon, this sequence was not able to prevent scanning of the ribosomes to the 3′ direction (Fig. 2C, D, compare lanes 1–3, suppl. Fig. 1B). Lane 1 shows the humanized Myotis construct including an AUG initiating triplet, the gene product has a larger size as a similar construct which includes an ACG as initiating triplet (lane 2 and 5). The gene product has a larger size if the 146 bp of the human sequence including ACG was introduced (lane 3) but the gene product is smaller again if the 146 bp of the original Myotis was used (lane 4) or the corresponding sequence of the vampire bat Desmodus rotundus (lane 6).

The data indicate that the 146 bp of the humanized Myotis-, the naturally ocurring Myotis- and the Desmodus-sequence do not affect ribosomal scanning, only the corresponding human sequence has a different effect on ribosomal behaviour. What are the special features of this sequence?

The human sequence contains a number of GC nucleotides that can form a stable stem-loop (Fig. 2E, left). In Fig. 2E the stem-loop is located between the initiation triplet ACG (red) and the next AUG codon which is located downstream. We suggest that this stem-loop prevents ribosomal scanning to the 3′ direction. It is known that hairpin structures which are located downstream of a non AUG initiation side can stabilize or even enhance translation [23,24]. In addition the translation machinery uses the ACG triplet to start the translation of the human TRPV6 cDNA. However the folding of the stem-loop has been done manually, a RNA folding program (RNAfold) does the folding in a very similar way (not shown). The corresponding sequence of Myotis was also manually folded and the putative stem-loop does exhibit less GC base pairs which can potentially stabilize the stem-loop structure (Fig. 2E, different nucleotides compared with the human sequence are shown in green). Most likely the Myotis sequence does not form a stable stem-loop and does not prevent the ribosomes from scanning to the 3′ direction. Thus if one replaces the initiating AUG codon within the TRPV6 cDNA of the Myotis sequence a protein of smaller size is translated. We also calculated the free energy of the folded stem-loop of human TRPV6 (-71 kCal/mol), the free energy of the human stem-loop is lower indicating stable folding compared with the stem-loops from several different bats (average -54 kCal/mol). It is known that the corresponding sequence of the TRPV6 cDNA of mice starts at the ACG triplet and also prevents ribosomal scanning similar to humans [4]. We analysed the free energy of the murine stem-loop and found no significant difference to bats (-58 kCal/mol, suppl. Fig. 2). In addition we calculated the free energy of the 5′ UTR and the first 300 bp of the translated region of TRPV6, which includes the stem loop. The free energy of human, murine and Myotis mRNA sequences were 140.8, 145.2 and 133.6 kCal/mol, respectively. Also non-canonical base pairing like G–U can potentially stabilize a stem loop structure. However as suggested from Fig. 2E only a few additional G–U wobbles were found, one in human and Myotis and 3 in mouse. The latter analysis indicates that the function of the stem-loop cannot be explained solely by the free energy. Thus the structure of the stem-loop may be stabilized by yet unknown interacting molecules.

The regulation of the translation of TRPV6 could be regulated also on the protein level. In principle it is possible that the 146 bp sequence is

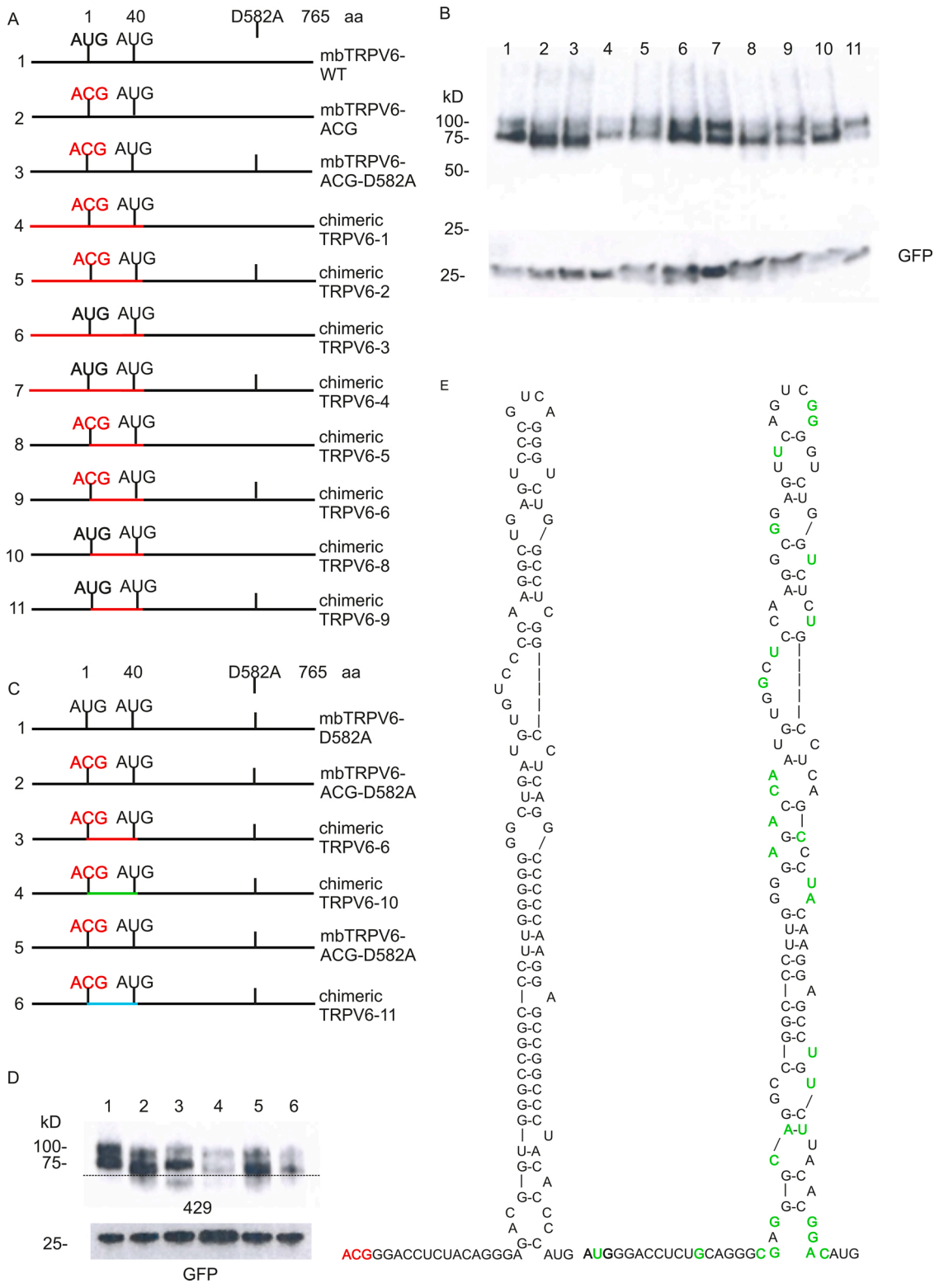

Fig. 2. Expression of chimeric TRPV6 constructs in HEK293 cells. A, B, Chimeric constructs of human and humanized *Myotis brandtii* of TRPV6 were expressed and analysed by Western blot with TRPV6 antibody 429 (upper blot) and GFP antibody (lower blot). 1, mbTRPV6-WT; 2 mbTRPV6 initiation triplet was changed to ACG; 3 similar as clone as 2 in addition D582A mutation was introduced; 4-11, chimeric constructs of human (red) and *Myotis brandtii* (black) TRPV6. C, D, Similar experiment as in A, B, 1, mbTRPV6 with D582A mutation; 2, mbTRPV6 with initiation codon mutated to ACG; 3 chimeric construct of human (red) and Myotis (black, humanized) TRPV6; chimeric; 4, chimeric construct of Myotis (green, not humanized) and Myotis (black, humanized) TRPV6; 5 same construct as 2; 6, chimeric construct of *Desmodus rotundus* (blue) and Myotis TRPV6. E, putative structure of the human TRPV6 mRNA nucleotides 110-232 (left, NM_018646.6) and the corresponding sequence of *Myotis brandtii* TRPV6 (right, XM_005862779.2). Note: The translation of human TRPV6 starts at an ACG triplet (red). Nucleotides within the Myotis sequence different to the human TRPV6 are indicated (green).

translated but the ongoing translation is blocked by unknown molecules which bind to the N-terminus of TRPV6 protein. To test this hypothesis we compared the cDNA of the N-terminus of human/murine TRPV6 with the several sequences derived from bats and found only few differences in which the human and mouse protein sequences differ from all bats (Fig. 3A). These differences lead only to three amino exchanges of the translated sequence. We introduced 5 bp of the human sequence, which are responsible for the three amino acid substitutions, into the humanized Myotis sequence (indicated by arrows), used as translational start an ACG codon and tested if the modified sequence prevents scanning of the ribosomes (Fig. 3A, B, C, suppl. Fig. 3). It is obvious that the introduction of the 5 bp mutations does not lead to a TRPV6 protein of larger size which shows that the mutations does not prevent ribosomal scanning (compare lanes 1–3 with lanes 4, 5). We suggest that the mRNA structure is responsible for the low efficacy of the translation and not the N-terminus of the TRPV6 protein itself.

Next we tested if the regulation of the translation of the TRPV6 protein can be used also to down-regulate another protein. To answer this we fused the human TRPV6 UTR and in addition the first 120 bp of the translated TRPV6 cDNA to the cDNA of GFP. We made another GFP construct without the TRPV6 UTR/120 bp which uses a canonical AUG codon (Fig. 4A). As it can be seen the green fluorescence of the GFP is dramatically decreased in cells expressing the construct with ACG as translation initiator (Fig. 4B). We also analysed the proteins on a Western blot (Fig. 4C). We used a GFP antibody (left), a TRPV6 specific antibody, (1271), which is directed to the translated sequence of the 120 bp sequence of the TRPV6 cDNA([4], middle) and as control a calnexin antibody (right). The GFP antibody detects two protein masses one

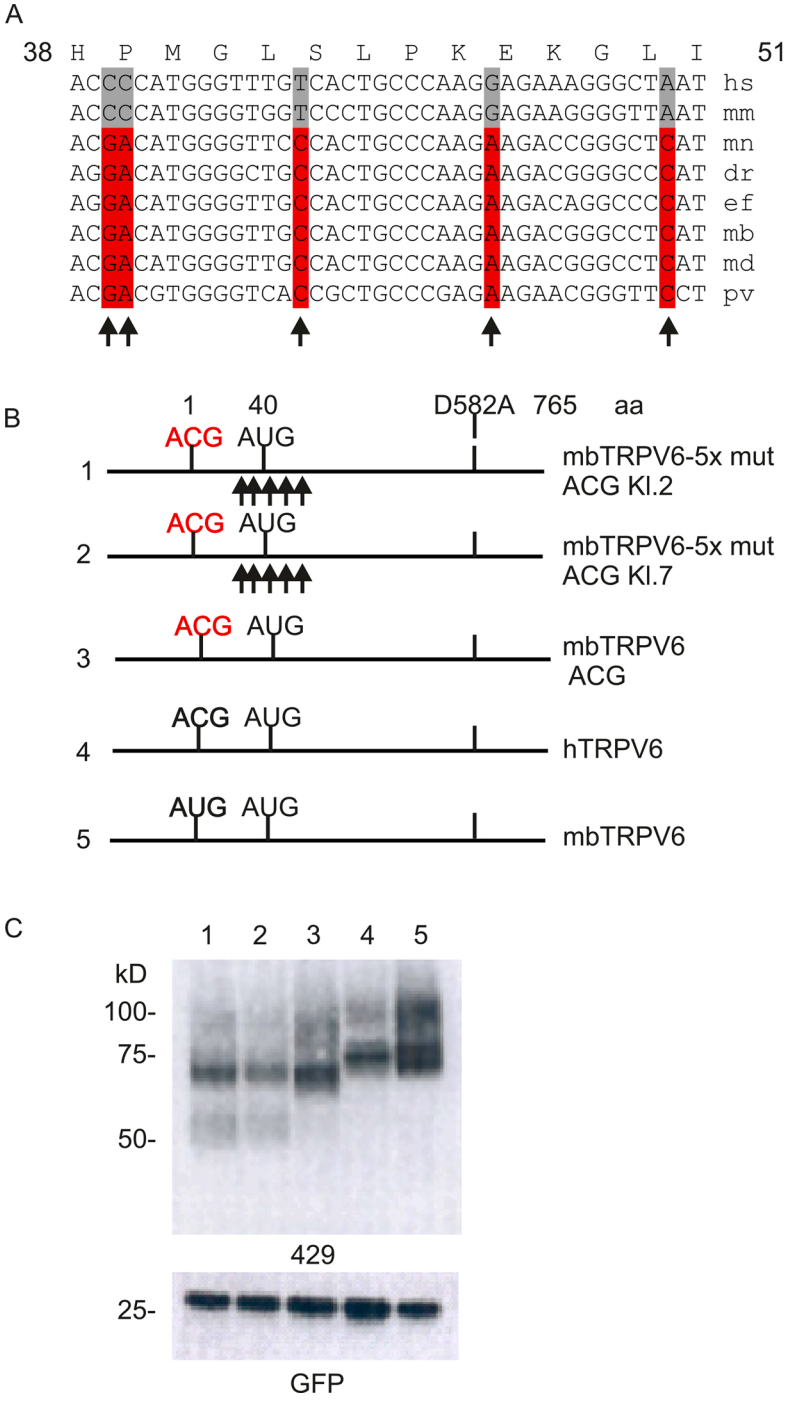

Fig. 3. Mutations which affect the protein sequence of TRPV6 do not influence ribosomal scanning. A, Nucleotide alignment corresponding to amino acids 38-51 (aa) of human TRPV6 compared to murine TRPV6 and the orthologues sequences of bats. A part of the human TRPV6 protein sequence is shown on top. The arrows indicate base pair substitutions in bats which lead to different amino acids compared to the human and mouse TRPV6 protein sequences. hs, Homo sapiens; mm, *Mus musculus*; mn, *Miniopterus natalensis* (Natal long-fingered bat); dr, *Desmodus rotundus* (Common vampire bat); ef, *Eptesicus fuscus* (Big brown bat); mb, *Myotis brandtii* (Brandts bat); md, *Myotis davidii* (Davids myotis); pv, *Pteropus vampyrus* (Large flying fox). B, expression of mbTRPV6 constructs including base pair substitutions from human TRPV6 shown in A (arrows). 1, 2 mbTRPV6 including the following mutations: Initiation codon switched to ACG and 5 base pair substitutions described above (arrows). Two different clones are shown. 3, mbTRPV6 with ACG mutation. 4, hTRPV6. 5, mbTRPV6. All constructs contain in addition a mutation, D582A, which leads to channels without a conductance. C, The constructs shown in B were expressed in HEK293 cells and analysed by Western blot using antibodies 429 and GFP.

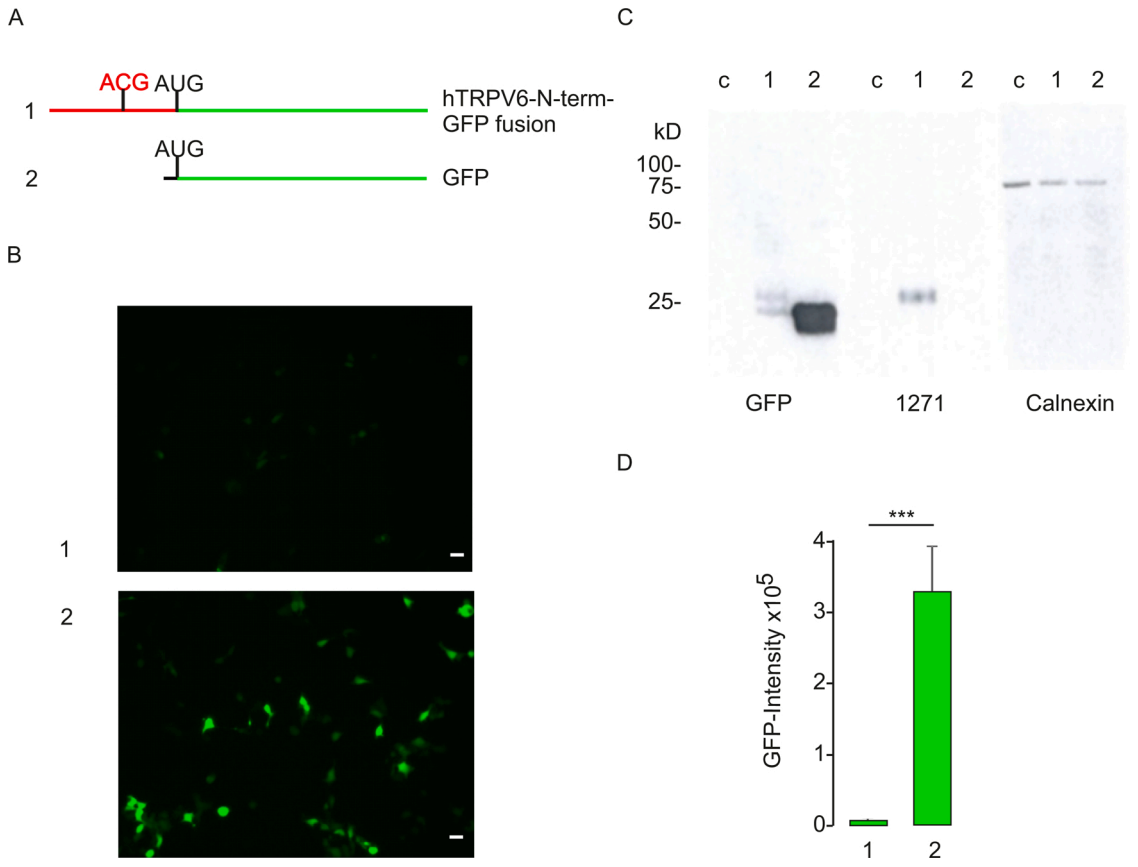

Fig. 4. The N-terminus of TRPV6 controls the efficiency of the translation of the green fluorescent protein. A, 1, Fusion construct of the human TRPV6 N-terminus (red) and the eGFP protein (green); 2, eGFP. B, Microscopic images showing green fluorescence of HEK293 cells transfected with the constructs shown in A, excitation wave length 480 nm, 10x objective, scale 20 µm. C, Western blot of the constructs shown in A probed with antibodies directed to GFP (left), TRPV6 (1271, middle) and calnexin (right, c = non transfected HEK293 cells); D, ***Bar graphs show the densitometric analysis of GFP signal intensities from 3 independent Western blots. Data are shown as mean S.E.M. and *** $P < 0001$ calculated using unpaired two-tailed Student's *t*-test.

starting at the ACG codon and the other most likely from a downstream located AUG of the GFP cDNA (lane 1, left). Also in the GFP transfected cells two distinct masses of GFP are visible (lane 2, left). The Western blot with the TRPV6 specific antibody shows that the protein with the higher molecular weight contains the N-terminus of the TRPV6 protein (lane 1, middle) but does not detect a protein in non-transfected cells (c, middle) or in cells that were transfected with the GFP construct (lane 2, middle). Obviously the GFP protein (lane 1) is under the control of the translational mechanism that is used to down-regulate the TRPV6 protein level. Densitometric analysis of three independent Western blots probed with a GFP antibody show that the protein amount of GFP is ~50 times lower if a hTRPV6-*N*-term-GFP fusion construct was used (Fig. 4A, D). Thus, it is likely that this mechanism can be used to regulate the translation of any arbitrary protein. The data present the rational basic why in native tissues which express TRPV6 transcripts, the TRPV6 protein is hardly detectable using Western blot, mass spectrometry or even patch clamp analysis.

The translation mechanism of the TRPV6 protein shows a relatively rare phenomenon: The translational start triplet is an ACG codon which is decoded by methionine. The ACG codon is located 120 bp upstream of the first in frame AUG codon of the TRPV6 cDNA. Surprisingly if TRPV6 is overexpressed using neither a construct which includes the ACG triplet and the 120 bp sequence nor a shorter construct in which the first AUG codon is used as translation initiation, the resulting TRPV6 protein is very similar in terms of channel behaviour (activation, selectivity, Ca^{2+} dependent inactivation and membrane localization) [4]. In the current work we try to find an explanation why TRPV6 expressing cells use this sophisticated translation mechanism. In detail we want to answer three questions: What is the sense behind the ACG triplet and is there any particular function which is associated with the 120 bp sequence which is located upstream of the first AUG codon. Can we explain the finding that endogenous TRPV6 currents are not detectable in tissues which express the corresponding transcripts? First, the ACG codon is used to down-regulate the protein amount of TRPV6. This may be a reasonable mechanism to prevent TRPV6 expressing cells from toxic Ca^{2+} overload. The experiment presented in Fig. 1 shows that the amount of the TRPV6 protein increases about ~5 times if an AUG codon serves as initiation triplet. The translation mechanism of human TRPV6 probably involves the classical initiator $tRNA_i^{Met}$ to initiate the protein translation. This works much better if the tRNA fits to the correct anticodon (AUG) and may explain why the TRPV6 protein amount increases if AUG is used as initiating triplet.

An insertion of poly-CGG repeats in the cDNA of the FMR1 protein (fragile X mental retardation 1) leads to an N-terminal extension of the protein [25]. The resulting protein FMRpolyG is involved in the Fragile X-associated tremor/ataxia syndrome (FXTAS). The FMRpolyG protein is initiated from an ACG triplet but decoded as methionine. However this occurs only in a pathological context.

Second, the mentioned 120 bp sequence of the TRPV6 mRNA can form a stable stem-loop which is important to prevent the ribosomes from scanning in 3′ direction and to start translation from a downstream located AUG codon present in the TRPV6 mRNA. In addition, the 120 bp sequence may be necessary that the $tRNA_i^{Met}$ is forced to interact with ACG triplet to initiate only from this triplet the protein translation.

In most mammals, the ACG codon and the 120 bp sequence of the TRPV6 mRNA are conserved which indicates that in mammals in general the ACG codon serves as translational start triplet. However the ACG codon is not present in the sequences of the family of bats. Bats exhibit at

the corresponding position of the ACG codon an AUG triplet. Bats underwent a divergent evolution that leads to the consequence that the translational regulation of TRPV6 equates to a normal translation mechanism. Thus, if in bats the AUG codon is mutated to ACG, the corresponding 120 bp sequence of bats does not prevent the ribosomes to scan to a downstream located AUG codon present in the TRPV6 sequence. We show that the 120 bp sequence of humans placed in the bat context can prevent ribosomal scanning and can similarly to the human TRPV6 induce translation from a non-canonical ACG codon in the bat context.

Although the identity of the mRNA sequences of the stem loops of human and mouse TRPV6 compared to bats is high (~80 %), the stem loops of bats and mammalians are clearly placed in separate clades as indicated by the phylogenetic relation (suppl. Fig. 4).

Is there a reason why bats use the standard mechanism? Most bats are insectivores and consume a low Ca^{2+} diet [26,27]. Similarly bats which consume mostly fruits ingest also a low Ca^{2+} source [28] although the Ca^{2+} concentration of the blood is in the mM range [29–31]. Thus bats have to ensure to take up enough Ca^{2+} from the nutrition. In this context one should mention that the expression pattern of TRPV6 varies between species. For example, TRPV6 transcripts are highly expressed in the small intestine of rats but nearly undetectable in the small intestine of humans. One could speculate that TRPV6 transcripts and the TRPV6 protein is expressed in the small intestine of bats as ecological adaptation to the low Ca^{2+} amount which is present in the naturally nutrition.

We cloned a part from the TRPV6 gene of the vampire and found that it contained on the genomic level an ATG codon which may serve as initiating triplet of the TRPV6 protein. Also the 120 bp sequence of the vampire behaves as the one of Myotis. As it can be seen in Fig. 2D the 120 bp sequence does not prevent ribosomes from scanning if an ACG codon was used instead of the naturally occurring AUG.

We expressed the Myotis and human TRPV6 in HEK293 cells and measured Ca^{2+} influx over time (Fig. 5A). It is apparent that the Myotis protein shows a smaller Ca^{2+} peak after Ca^{2+} re-addition (Fig. 5B, 2, red) compared with the human TRPV6 (blue). In addition it seems that the Myotis TRPV6 protein tends to a slower kinetic (Fig. 5B, 1, 3). However the kinetic parameters were not statistically significant different. Thus bats can potentially use two mechanism to enhance Ca^{2+} uptake from nutrition. First the non-canonical ACG codon present in other mammals was changed to AUG which results in a more efficient protein synthesis and second the bat TRPV6 protein shows a tendency to a slower inactivation kinetic.

Human TRPV6 is involved in the transplacental calcium transport from mother to foetus. This is underlined by the finding that infants with dysfunctional TRPV6 alleles possess under-mineralized bones and accelerated level of the parathyroide hormone [32–35]. However after birth the phenotype normalizes if the nutrition is supplemented with adequate amounts of Ca^{2+} which argues that in humans the placenta among other organs is responsible for the underlying disease [32].

We aligned the TRPV6 sequences of several reptiles and mammalian TRPV6 sequences and found that the 120 bp sequence (Fig. 5C, shown as translated sequence, Fig. 6A, B) is not conserved within reptiles and organisms from much older lineages. From the alignment one can conclude that in organisms of older lineages than placental animals the first AUG triplet of the TRPV6 mRNA serves as initiation codon for the translation. This might explain that the TRPV6 protein of humans behaves very similar if the N-terminus of the protein -the translated 120 bp sequence- is present or not. Furthermore it shows that the N-terminal extension of the TRPV6 protein is only present in placental animals. Most notable, the genome of the duckbill Ornithorhynchus anatinus which offers properties of reptiles and mammals, contains only a part of the mentioned N-terminal extension (Fig. 5C). The sequence data indicate that the precursor of the 120 bp N-terminal extension of placental animals evolved already on the level of the monotremes.

The present work shows that the translation of the human TRPV6 protein is controlled by the initiating codon ACG which decreases the

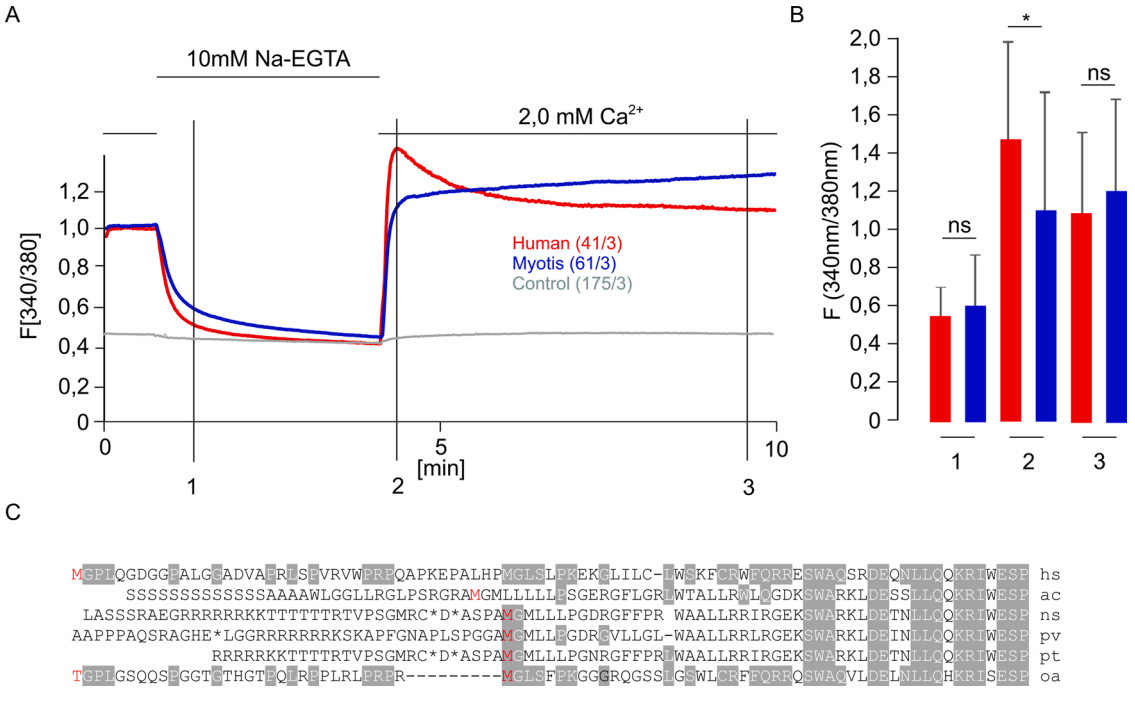

Fig. 5. Calcium imaging of human and Myotis TRPV6. A, Ca^{2+} imaging of HEK293 cells expressing human (red, n = 41/3), Myotis (blue, n = 61/3) TRPV6 and mock control (grey, n = 175/3). Cells were incubated in buffer containing 2.0 mM Ca^{2+}, followed by buffering with 10 mM Na-EGTA and re-addition of Ca^{2+} n = cells/number of experiments. B, Statistical analysis at the indicated time points (1, 2, 3), Data are shown as mean + S.E.M. and *, $p < 005$ calculated using unpaired two-tailed Student's *t*-test. C, Alignment of human TRPV6 compared to orthologous proteins from reptilians and the duckbill. Amino acids which show putative translational starts of the TRPV6 proteins of reptilians and the duckbill are indicated (red). Identical amino acids are shaded, hs, *Homo sapiens*; ac, *Anolis carolensis* (American chameleon); ns, *Notechis scutatus* (Tiger snake); pv, *Pogona vitticeps* (Central bearded dragon); pt, *Pseudonaja textilis* (Eastern brown snake); oa, *Ornithorhynchus anatinus* (duckbill).

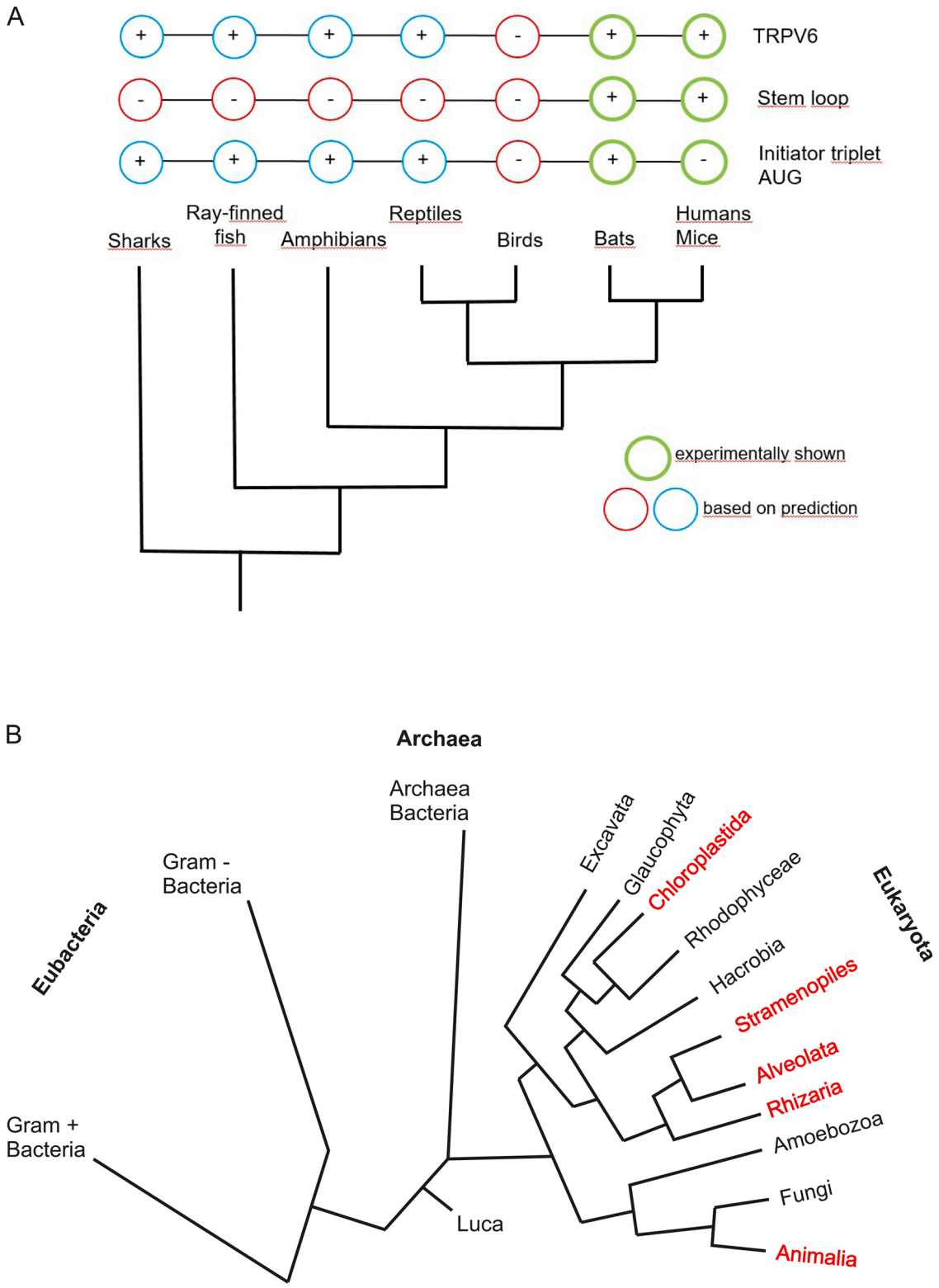

Fig. 6. Phylogenetic tree of vertebrates. A, Based on predictions from GeneBank data TRPV6 is present in all groups of vertebrates (blue circles) with the exception of birds (red circle). Experimental results show that the N-terminal extension of TRPV6, the so called stem loop, is present in bats, in humans, in mice (green circles) and probably in all other placental animals (blue). The stem loop is not conserved in non-placental animals (red). Murine and human TRPV6 are initiated from an ACG codon whereas the TRPV6 of bats is initiated at an AUG codon (green). Initiation of TRPV6 from an ACG codon seems to be a typical feature of placental animals. Non-placental animals most likely use an AUG triplet to initiate the translation of TRPV6. The AUG triplet of non-placental animals corresponds to an AUG triplet of placental animals which is located downstream of the stem loop (see also Fig.5C). B, TRPV6-like sequences are found in in several eukaryotic clades (red) but not in eubacteria and archaebacteria.

efficiency of the translation. A sequence of 117 bp which is immediately located downstream of the ACG codon controls that the initiation of the translation starts at this ACG codon. The 117 bp also prevents that the ribosomes scan in 3′ direction and induce the translation of TRPV6 from a downstream located AUG codon. The 117 bp sequence can also be used to control the translation efficiency of the GFP protein and most likely from any other protein.

4. Conclusion

The translation of the human TRPV6 protein is initiated at a non-classical ACG codon. Immediately downstream of the initiation codon a GC rich sequence located which prevents scanning of the ribosomes and enables TRPV6 expressing cells to start the translation at the respective ACG codon. This leads to a reduced synthesis of the human TRPV6 protein and is most likely the reason that TRPV6 currents cannot be measured in cells which endogenously express TRPV6 transcripts. Due to a massive calcium influx, TRPV6 expression is potentially toxic for cells. The described translation mechanism may protect cells from calcium overload. In addition, we show that the translation mechanism of the human TRPV6 protein is also useful to control the translation efficiency of the GFP protein and could be a general mechanism.

Author contributions

K.W. cloning strategies and design of the study, C.F.T. and C.W. performed Western blots and purified antibodies, H.L. responsible for cell culture and transfection. A.B. and S.P. critical reading of manuscript and design of the study. G.P. isolation of genomic DNA, U.W. cloning, calcium imaging, microscopy, designed the study, wrote manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ceca.2020.102302.

References

[1] U. Wissenbach, B.A. Niemeyer, T. Fixemer, A. Schneidewind, C. Trost, A. Cavalie, K. Reus, E. Meese, H. Bonkhoff, V. Flockerzi, Expression of CaT-like, a novel calcium-selective channel, correlates with the malignancy of prostate cancer, J. Biol. Chem. 276 (2001) 19461–19468.
[2] K. Saotome, A.K. Singh, A.I. Sobolevsky, Determining the crystal structure of TRPV6, in: J.A. Kozak, J.W. Putney Jr (Eds.), Calcium Entry Channels in Non-Excitable Cells, Boca Raton (FL), 2018, pp. 275–292.
[3] L. Yue, J.B. Peng, M.A. Hediger, D.E. Clapham, CaT1 manifests the pore properties of the calcium-release-activated calcium channel, Nature 410 (2001) 705–709.
[4] C. Fecher-Trost, U. Wissenbach, A. Beck, P. Schalkowsky, C. Stoerger, J. Doerr, A. Dembek, M. Simon-Thomas, A. Weber, P. Wollenberg, T. Ruppert, R. Middendorff, H.H. Maurer, V. Flockerzi, The in vivo TRPV6 protein starts at a non-AUG triplet, decoded as methionine, upstream of canonical initiation at AUG, J. Biol. Chem. 288 (2013) 16629–16644.
[5] C. Touriol, S. Bornes, S. Bonnal, S. Audigier, H. Prats, A.C. Prats, S. Vagner, Generation of protein isoform diversity by alternative initiation of translation at non-AUG codons, Biol. Cell 95 (2003) 169–178.
[6] S. Tikole, R. Sankararamakrishnan, A survey of mRNA sequences with a non-AUG start codon in RefSeq database, J. Biomol. Struct. Dyn. 24 (2006) 33–42.
[7] J.L. Wegrzyn, T.M. Drudge, F. Valafar, V. Hook, Bioinformatic analyses of mammalian 5′-UTR sequence properties of mRNAs predicts alternative translation initiation sites, BMC Bioinformatics 9 (2008) 232.
[8] D. Hirnet, J. Olausson, C. Fecher-Trost, M. Bodding, W. Nastainczyk, U. Wissenbach, V. Flockerzi, M. Freichel, The TRPV6 gene, cDNA and protein, Cell Calcium 33 (2003) 509–518.
[9] P. Weissgerber, U. Kriebs, V. Tsvilovskyy, J. Olausson, O. Kretz, C. Stoerger, R. Vennekens, U. Wissenbach, R. Middendorff, V. Flockerzi, M. Freichel, Male fertility depends on Ca(2)+ absorption by TRPV6 in epididymal epithelia, Sci. Signal. 4 (2011) ra27.
[10] A. Beck, C. Fecher-Trost, K. Wolske, S.E. Philipp, V. Flockerzi, U. Wissenbach, Identification of Sidt2 as a lysosomal cation-conducting protein, FEBS Lett. 591 (2017) 76–87.
[11] C. Cao, E. Zakharian, I. Borbiro, T. Rohacs, Interplay between calmodulin and phosphatidylinositol 4,5-bisphosphate in Ca2+-induced inactivation of transient receptor potential vanilloid 6 channels, J. Biol. Chem. 288 (2013) 5278–5290.
[12] (a) J.B. Peng, Y. Suzuki, G. Gyimesi, M.A. Hediger, TRPV5 and TRPV6 calcium-selective channels, in: J.A. Kozak, J.W. Putney Jr (Eds.), Calcium Entry Channels in Non-Excitable Cells, CRC Press/Taylor & Francis, 2020;
(b) 2017 Taylor & Francis Group, LLC., Boca Raton (FL), 2018, pp. 241–274.
[13] A.K. Singh, L.L. McGoldrick, E.C. Twomey, A.I. Sobolevsky, Mechanism of calmodulin inactivation of the calcium-selective TRP channel TRPV6, Sci. Adv. 4 (2018) eaau6088.
[14] J.M. Stewart, TRPV6 as a target for Cancer therapy, J. Cancer 11 (2020) 374–387.
[15] T. Voets, A. Janssens, G. Droogmans, B. Nilius, Outer pore architecture of a Ca2+-selective TRP channel, J. Biol. Chem. 279 (2004) 15223–15230.
[16] M. Bodding, V. Flockerzi, Ca2+ dependence of the Ca2+-selective TRPV6 channel, J. Biol. Chem. 279 (2004) 36546–36552.
[17] P. Weissgerber, U. Kriebs, V. Tsvilovskyy, J. Olausson, O. Kretz, C. Stoerger, S. Mannebach, U. Wissenbach, R. Vennekens, R. Middendorff, V. Flockerzi, M. Freichel, Excision of Trpv6 gene leads to severe defects in epididymal Ca2+ absorption and male fertility much like single D541A pore mutation, J. Biol. Chem. 287 (2012) 17930–17941.
[18] J.G. Hoenderop, T. Voets, S. Hoefs, F. Weidema, J. Prenen, B. Nilius, R.J. Bindels, Homo- and heterotetrameric architecture of the epithelial Ca2+ channels TRPV5 and TRPV6, EMBO J. 22 (2003) 776–785.
[19] M. Kozak, Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs, Nucleic Acids Res. 12 (1984) 857–872.
[20] M. Kozak, An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs, Nucleic Acids Res. 15 (1987) 8125–8148.
[21] M. Kozak, At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells, J. Mol. Biol. 196 (1987) 947–950.
[22] M. Kozak, The scanning model for translation: an update, J. Cell Biol. 108 (1989) 229–241.
[23] M. Kozak, Downstream secondary structure facilitates recognition of initiator codons by eukaryotic ribosomes, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 8301–8305.
[24] M.G. Kearse, J.E. Wilusz, Non-AUG translation: a new start for protein synthesis in eukaryotes, Genes Dev. 31 (2017) 1717–1731.
[25] C. Sellier, R.A.M. Buijsen, F. He, S. Natla, L. Jung, P. Tropel, A. Gaucherot, H. Jacobs, H. Meziane, A. Vincent, M.F. Champy, T. Sorg, G. Pavlovic, M. Wattenhofer-Donze, M.C. Birling, M. Oulad-Abdelghani, P. Eberling, F. Ruffenach, M. Joint, M. Anheim, V. Martinez-Cerdeno, F. Tassone, R. Willemsen, R.K. Hukema, S. Viville, C. Martinat, P.K. Todd, N. Charlet-Berguerand, Translation of expanded CGG repeats into FMRpolyG is pathogenic and may contribute to fragile X tremor Ataxia syndrome, Neuron 93 (2017) 331–347.
[26] J.O.S. Keeler, E. H, Nutrition in pregnant big brown bats (Eptesicus fuscus), Feeding on June Beetles Journal of Mammalogy 73 (1992) 426–430.
[27] C.M.H. Booher, W.R, Calcium utilization during reproduction in big brown bats (Eptesicus fuscus), J. Mammal. 91 (2010) 952–959.
[28] S.L.M. Nelson, D.V. Humphrey, S.R. Kunz, T.H, Fruit choice and calcium block use by tongan fruit bats in american Samoa, J. Mammal. 86 (2005) 1205–1209.
[29] A.H. Doherty, C.K. Ghalambor, S.W. Donahue, Evolutionary physiology of bone: bone metabolism in changing environments, Physiology Bethesda (Bethesda) 30 (2015) 17–29.
[30] V. Jekl, Reproductive Medicine, An Issue of Veterinary Clinics of North America: Exotic Animal Practice, Vol. 20-22, 2017 st Edition.
[31] D.A. Torres, M.B. Freitas, R.V. Goncalves, Changes in bone turnover and calcium homeostasis during pregnancy and lactation in mammals: a meta-analysis, Reprod. Fertil. Dev. 30 (2018) 681–688.
[32] C.P. Burren, R. Caswell, B. Castle, C.R. Welch, T.N. Hilliard, S.F. Smithson, S. Ellard, TRPV6 compound heterozygous variants result in impaired placental calcium transport and severe undermineralization and dysplasia of the fetal skeleton, Am. J. Med. Genet. A 176 (2018) 1950–1955.
[33] Y. Suzuki, D. Chitayat, H. Sawada, M.A. Deardorff, H.M. McLaughlin, A. Begtrup, K. Millar, J. Harrington, K. Chong, M. Roifman, K. Grand, M. Tominaga, F. Takada, S. Shuster, M. Obara, H. Mutoh, R. Kushima, G. Nishimura, TRPV6 variants interfere with maternal-fetal calcium transport through the Placenta and cause transient neonatal hyperparathyroidism, Am. J. Hum. Genet. 102 (2018) 1104–1114.
[34] S. Yamashita, H. Mizumoto, H. Sawada, Y. Suzuki, D. Hata, TRPV6 gene mutation in a dizygous twin with transient neonatal hyperparathyroidism, J Endocr Soc 3 (2019) 602–606.
[35] A.E. Mason, D. Grier, S.F. Smithson, C.P. Burren, E. Gradhand, Post-mortem histology in transient receptor potential cation channel subfamily V member 6 (TRPV6) under-mineralising skeletal dysplasia suggests postnatal skeletal recovery: a case report, BMC Med. Genet. 21 (2020) 64.