

EVOLUTION OF PROTEINS INVOLVED IN RESPONSE TO REACTIVE OXYGEN SPECIES (=ROS)

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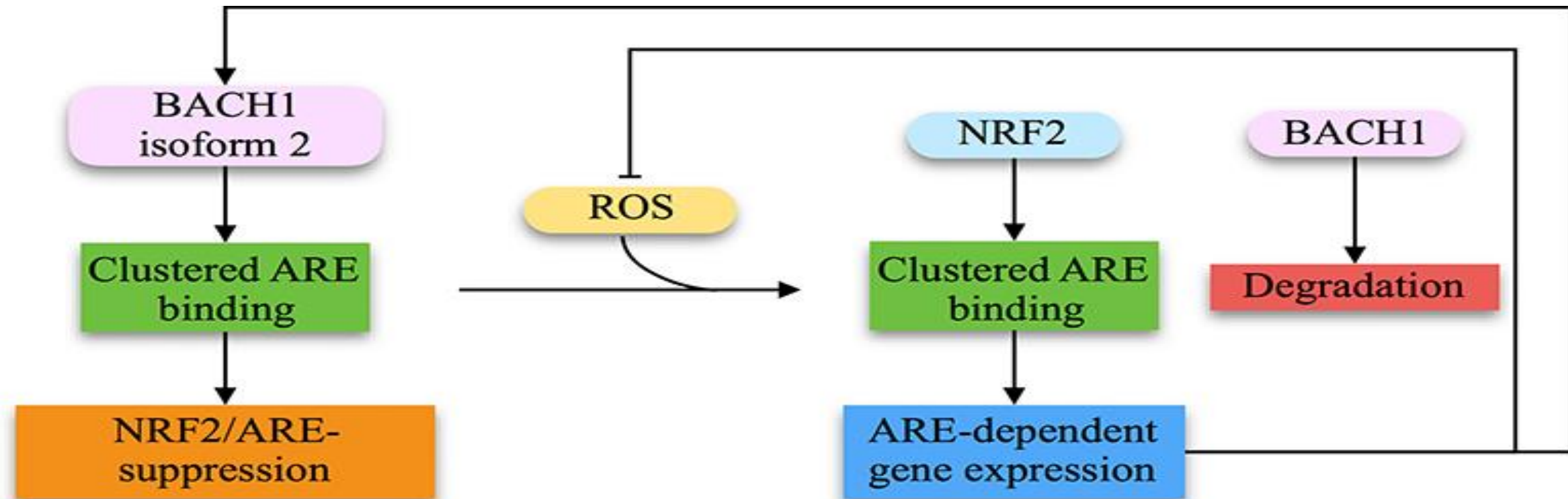
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In many vertebrates, Nrf2 (Nfe2l2) and Bach proteins (Bach1 and Bach2), are components of the regulatory network (e.g., in *Mus musculus*) that is, among other things, associated with **regulation of the level of reactive oxygen species (=ROS)**.

The **functioning** of this network is related to species-specific LIFESPAN.



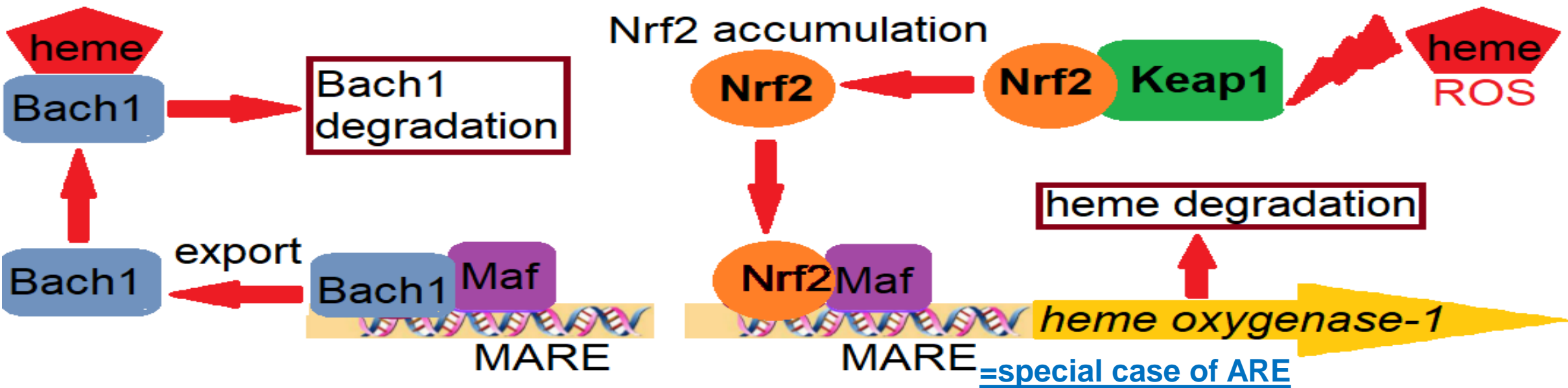
These proteins usually function by **binding with other proteins** (typically **Maf**) and **DNA**.

Nrf2 and **Bach1** are **antagonistic** in **controlling ROS levels**, which is also due to the **competition for MARE** sites.

(Analogously we investigated **Keap1**, **β -TrCP**, **c-Myc**, **GSK3b**, but now only concentrate on **Nrf2** and **Bach**.)

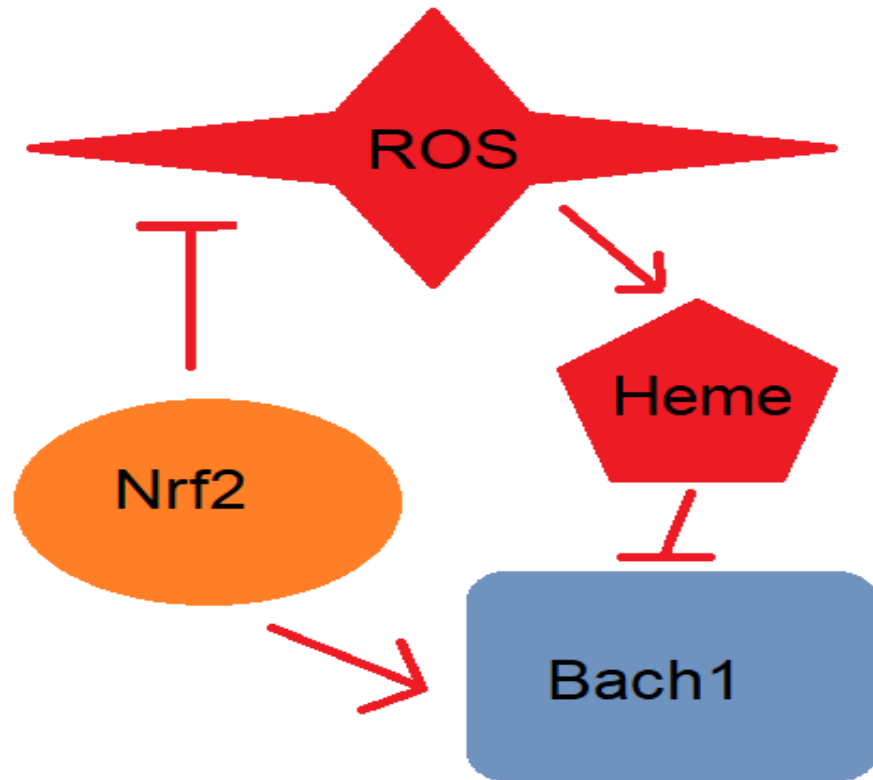
Let us **EXEMPLIFY** this function in detail.

Competition between Nrf2 and Bach1 on MARE in response to oxidative stimuli



In oxidative stress, Nrf2 **dissociates** from Keap1 & Nrf2 degradation is **inhibited**; so Nrf2 accumulates in the cytoplasm and **translocates** into the nucleus. Then, Nrf2 **binds** to MAREs as a heterodimer with small Mafs, which activates the **expression** of heme oxygenase-1 (and 200 other genes), thus **degrading heme**. At the same time, Bach1 is **displaced** from MAREs and **exported** out the nucleus. **IF insufficient heme** is available, Bach1 is **released** from heme and **again binds** Mafs and DNA.

Thus, the relationship between Nrf2 and Bach1 is critical for the **regulation** of heme oxygenase-1 expression, which **provides for heme degradation** according to this schematic diagram:



Now the small topic:

the heme-binding regions in Bach1 and Bach2 are **dissimilar**; in particular, they **differ** in the NUMBER of **regulatory cysteine-proline motifs critical for heme binding** (also called **CP-motifs**).

The **heme-binding motifs** (including those CP-motifs) have been identified by us in Bach in chordates;

as well as the **sites of Bach homodimerization**.

The **functioning** of this network is **related to aging** as well as to many **human diseases**.

E.g., mice homozygous for a **knock-out *Nrf2*** exhibit increased sensitivity to **oxidative stress** in a variety of tissues and cells (including the **brain, liver, erythrocytes, and spleen**), abnormal tooth **enamel**, and abnormal response to various '**injuries**, chemical **treatments**, and induced **inflammatory diseases**.

Single nucleotide variants in *Bach2* have been linked to a number of **autoimmune diseases** in humans. Mendelian *Bach2*-related immunodeficiency and autoimmunity (**BRIDA**) **syndrome** in humans is caused by haploinsufficiency of **this transcription factor** resulting from germline mutations.

We present the results of the computer-aided search for Nrf2 and Bach in deuterostomes. In other words, we found true orthologs of human proteins

NFE2L2 (NP_006155.2), **BACH1** (NP_001177.1), **BACH2** (NP_068585.1).

There is a well-known problem of **similarity thresholds between two sequences as a function of the evolutionary distance between species.**

Accordingly, we used criteria to **determine homologs with “the same molecular function”** in species **evolutionary distant from human.**

“The same function” means here the **ANALOGOUS RESPONSE TO OXIDATIVE STIMULI.**

Remember that:

bZIP domain, **typical of both Nrf2 and Bach** proteins, is determined **even in Choanoflagellates;**

both bZIP and BTB domains are widespread **even in plant proteins.**

This shown how hard can be **determine Nrf2 and Bach** in **distance species**. For that we used the following criteria.

Apart from the similarity extracted from the **local** and **global alignments** with the human genes, these **criteria include**: the presence or absence of the **BTB** and **bZIP-Maf domains**, satisfactory **alignment of the secondary structure**, high similarity of the **3D structures** in the N- and C-terminal regions of the genes; the **absence of “wrong” domains** like kelch-type beta-propeller in kelch-like proteins, BTB in Bach (for Nrf2 case), bZIP-Jun in Jun, and other specific domains from bZIP proteins; **as well as** high 3D structure similarity with NFE2L2 at the C-terminus coupled with a significant **difference from “wrong” bZIP proteins at the N-terminus.**

Also the method **takes into consideration**: **CP-motifs**, **N-hooks** and presence of **specific amino acids**.

Additionally **the method** includes the **SPLITTING** of the unrooted tree **into the clades of Bach and Nrf2 separated with a nearly 100% support** (for dividing all found proteins into the **Nrf2** and **Bach1–2 groups**). The tree is shown below. Similar splitting has been done for other **protein groups**.

This exemplifies our METHOD to the problem of **mutual evolution of the Nrf2 and Bach1–2 genes**.

Among invertebrate deuterostomes, Bach proteins have been found
only in *Ciona intestinalis* and *C. savignyi*

(tunicates diverged early from other chordates). Shown below.

At least one Bach protein has been found in each vertebrate.

On these grounds, the **Bach** and **Nrf2 protein tree** was generated:

The **Bach** and **Nrf2** protein tree

(blue indicates MYA)

Thus, the Bach gene emerges in the

Olfactores (a

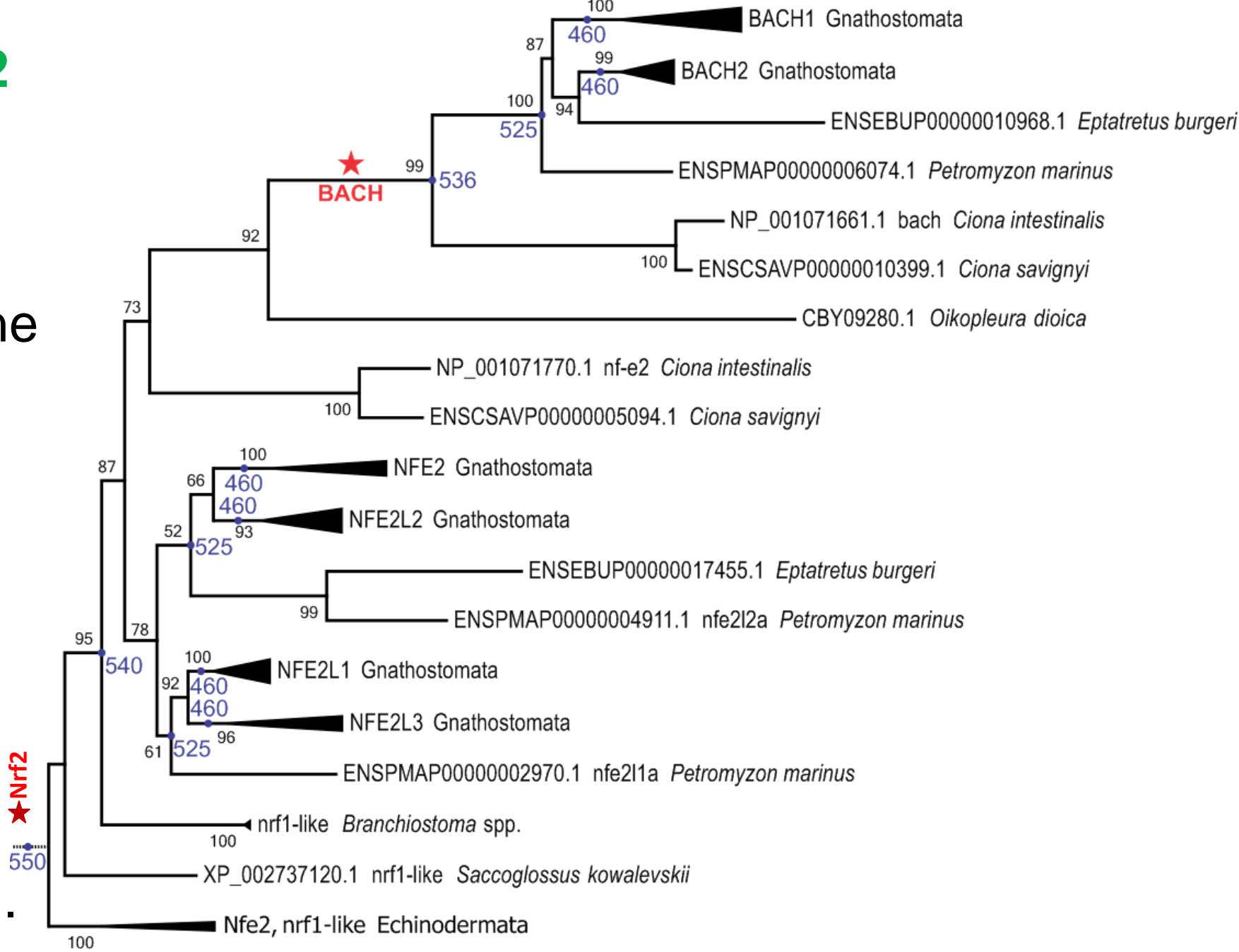
subphylum within

the Chordata that

comprises the

Tunicata

and the Vertebrata).



Nevertheless, the **genomic rearrangement** that gave rise to the Bach genes remains unclear.

The *Bach* is most similar to *Nrf2* in the early diverging deuterostomes (by bZIP), and the BTB exists in dozens of ancient proteins (e.g. in the BTB-ZF family).

Therefore we think that ***Bach* emerged by duplication of *Nrf2***, an ortholog of *Nfe2l2*, in the **chordate ancestor**. The **ancestral Bach was provided by BTB from those ancient proteins**.

(Bach has to contain both BTB и bZIP.)

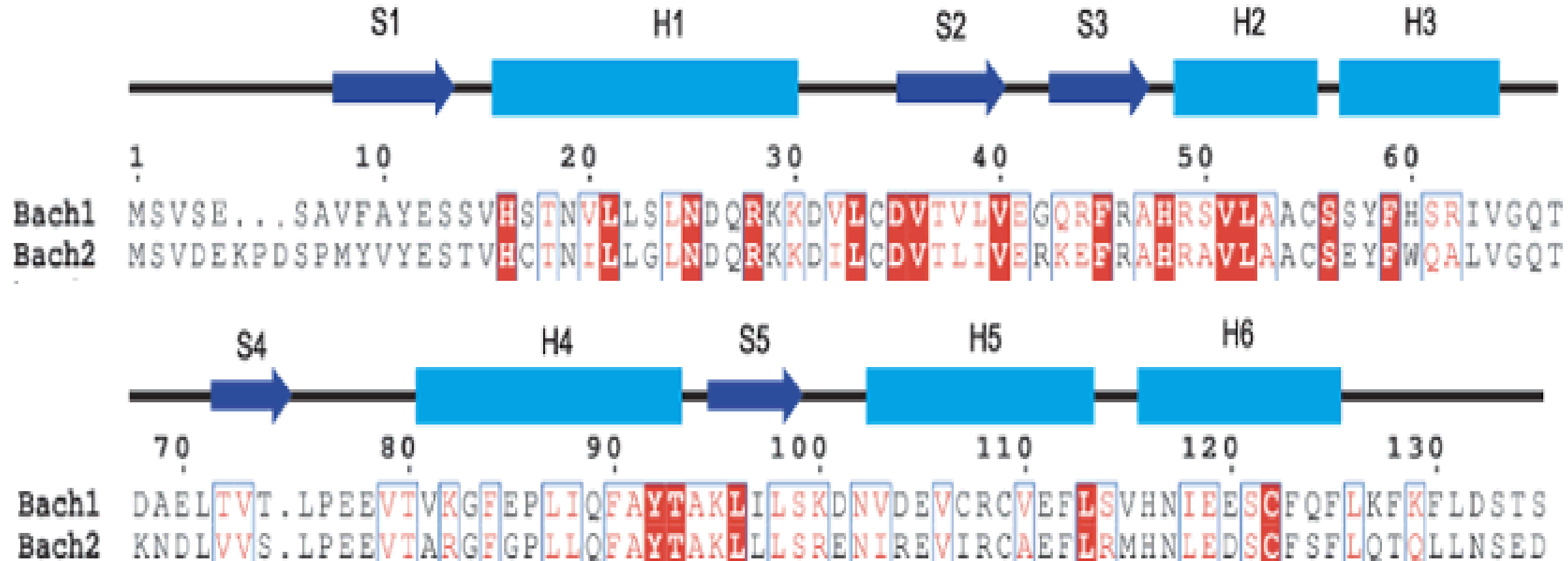
To follow our **METHOD**,

we enumerates characteristics of the **Nrf2** and **Bach** proteins

that we have used:

The primary and secondary structures of the BTB domain
in mouse (in Bach1) and human (in Bach2);

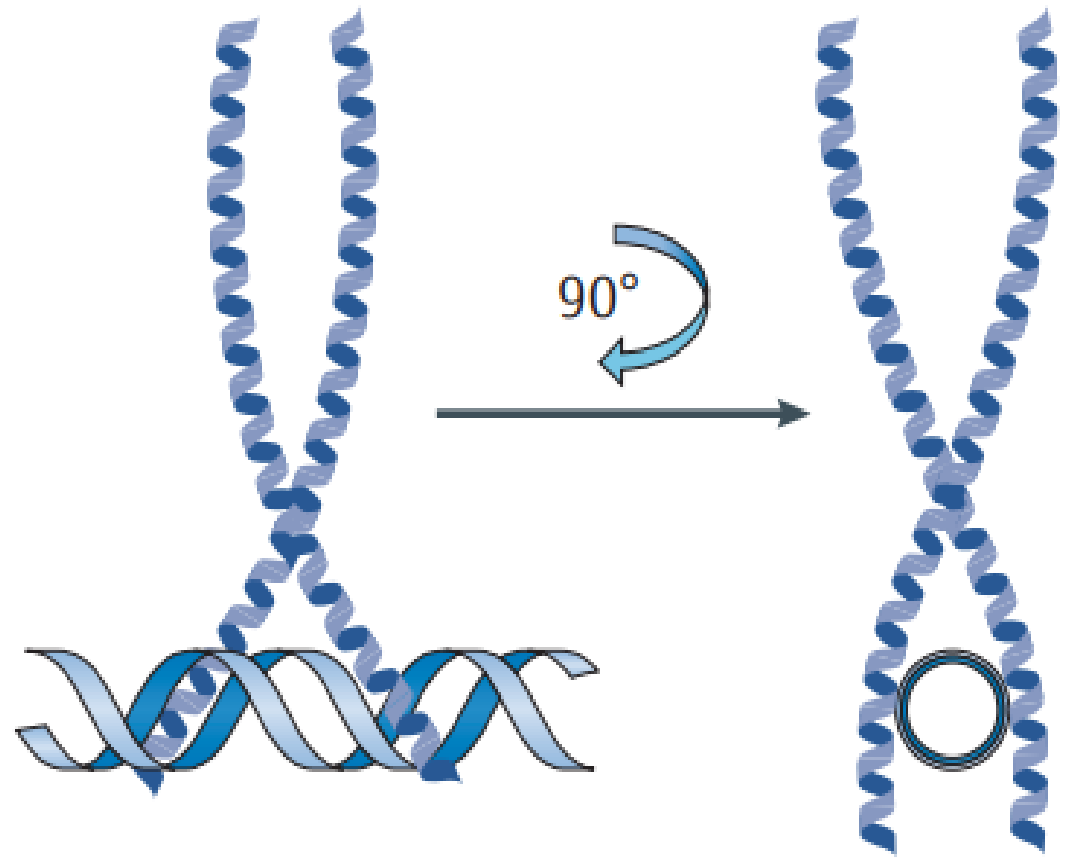
S, beta-sheets; H, alpha-helices:



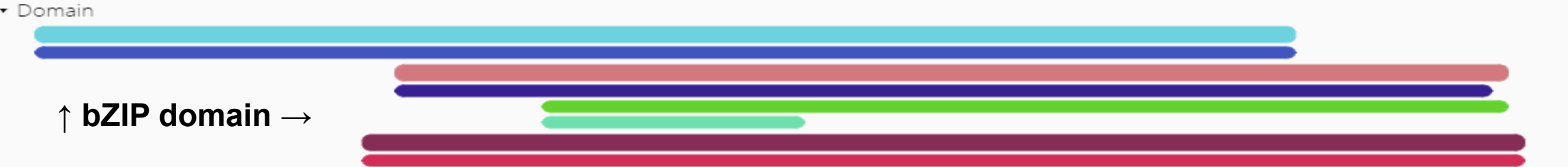
Dimeric interaction of bZIP at DNA in

Bach1 and **Nrf2** – both with Mafs:

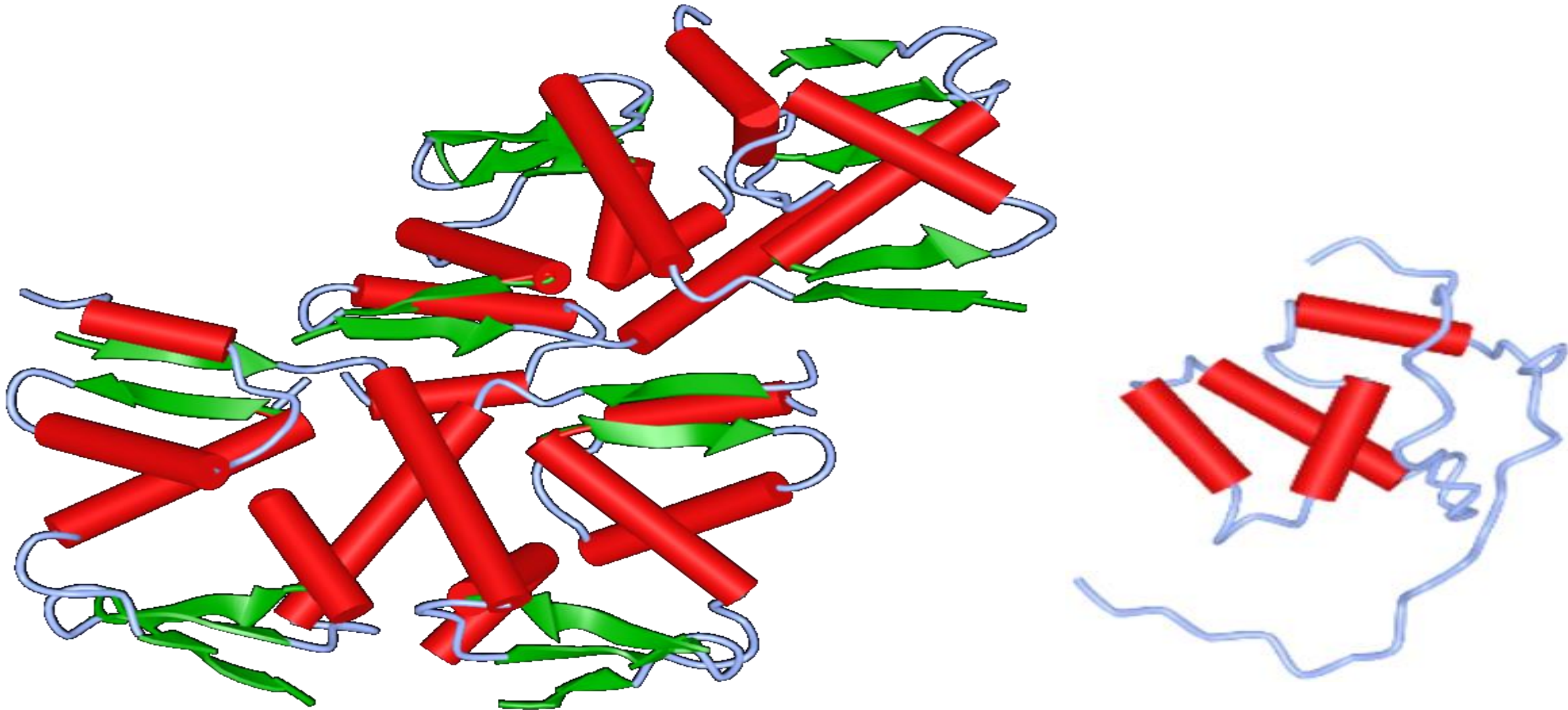
Their primary structure is shown underneath:



QR I I S L S R N D F Q S L L K M H K L T P E Q L D C I H D I R R R S K N R I A A Q R C R K R K L D C I Q N L E S E I E K L Q S E K E S L L K E R D H I L S T L G E T K Q N L T G L C

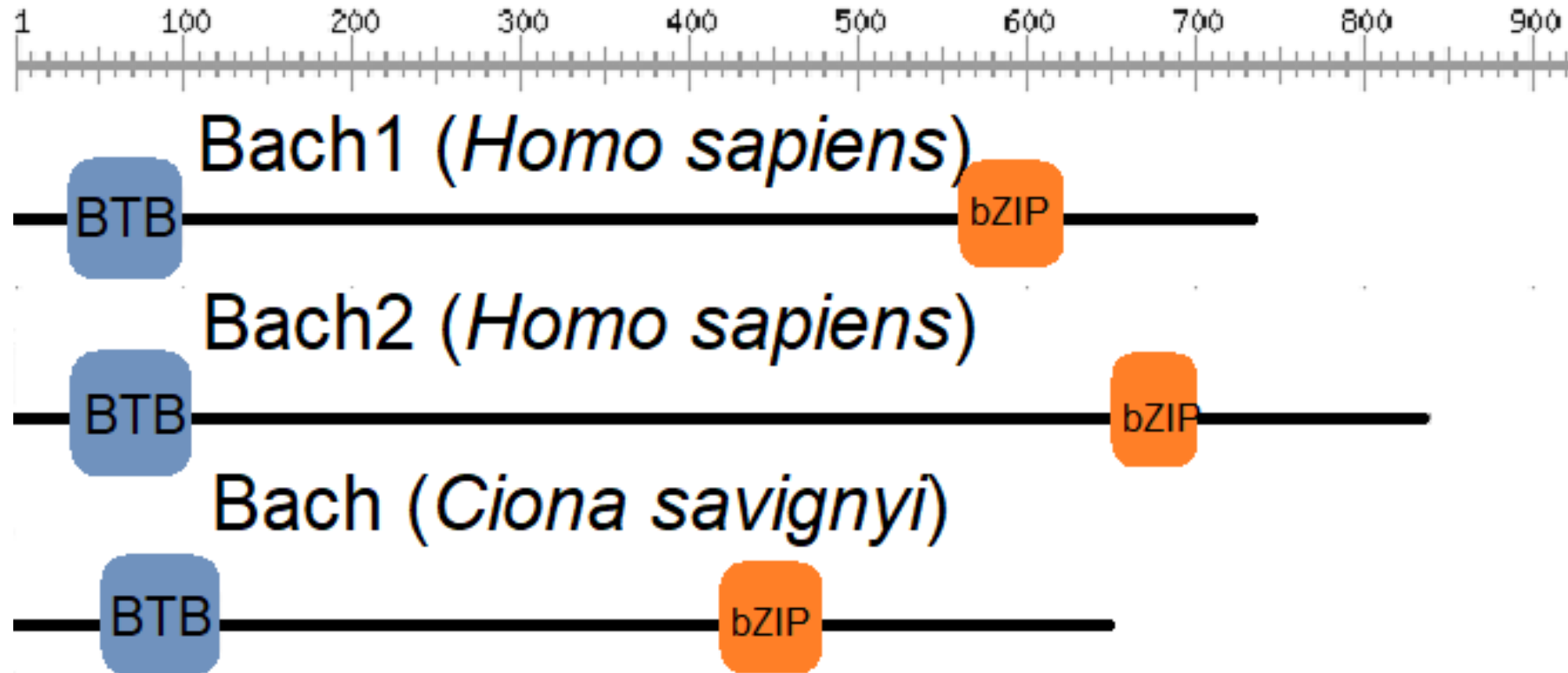


The tertiary structure of BTB domain (4 BTB-chains) and the bZIP domain (DNA binding fragment) – in human:

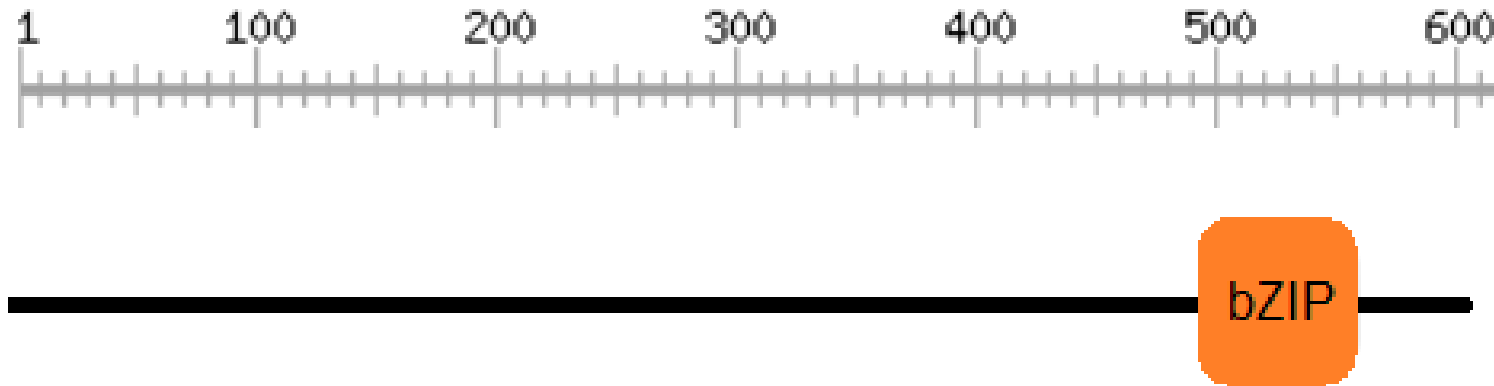


Conserved domains **BTB** and **bZIP** in Bach1–2:

BTB domain at the N-terminus and the bZIP domain at the C-terminus:



Conserved domain **bZIP** at the C-terminus in Nrf2:



(Nrf2 includes no BTB domain.)

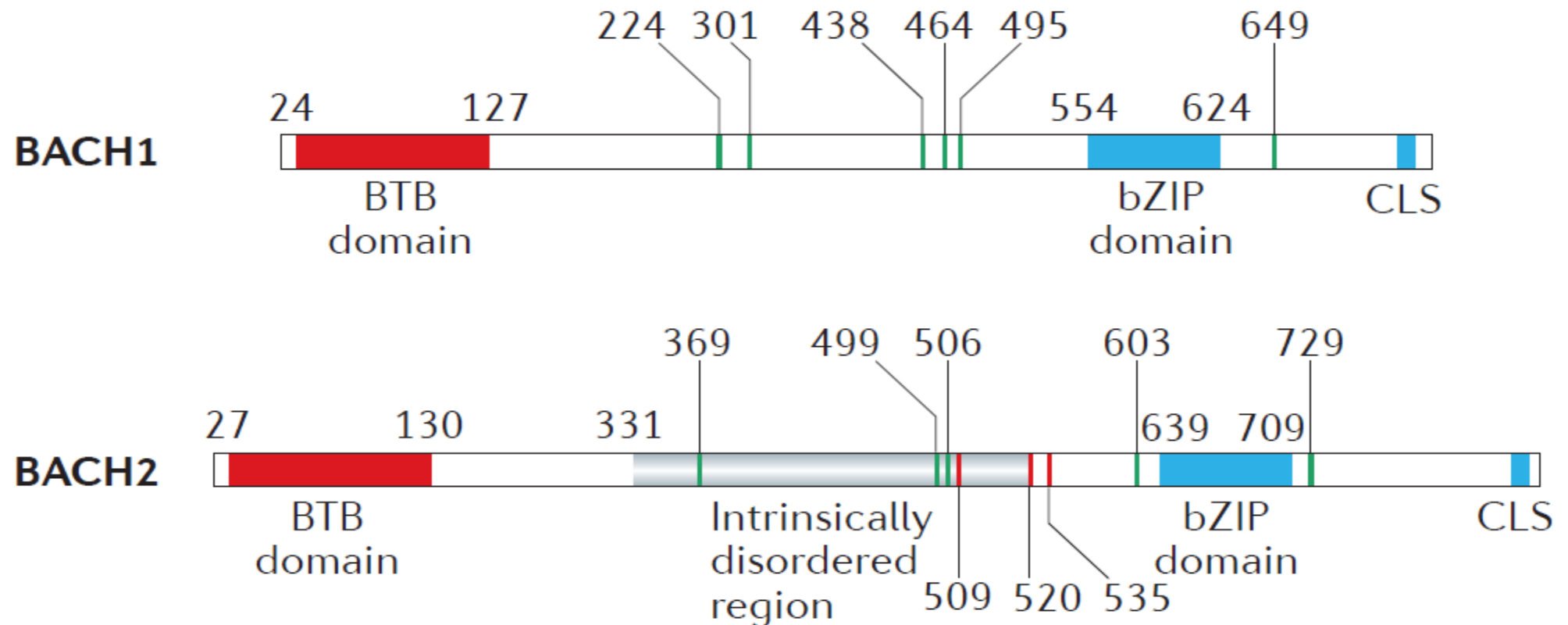
Thus, the **BTB domain** is 'present near the N-terminus in proteins that contain the **Kelch motif**.

(The BTB domain mediates **homodimerization** and in some instances **heterodimerisation** as well as it mediates **transcriptional repression**.)

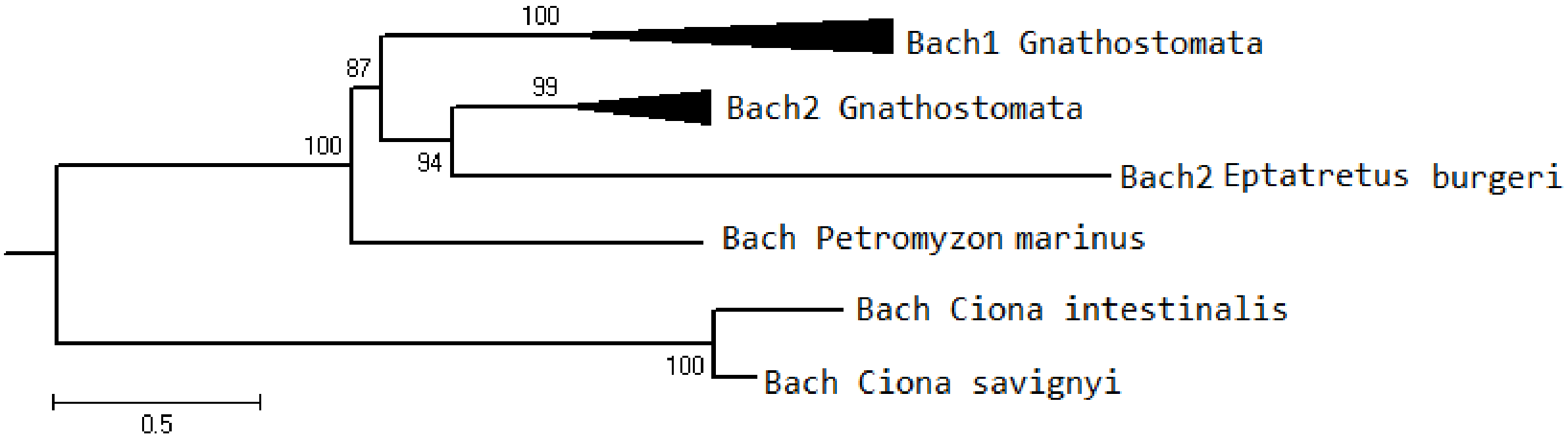
The Basic Leucine Zipper Domain-Maf-type (**bZIP-Maf domain**) is found in many DNA-binding proteins. <One part of the domain mediates sequence specific DNA binding, and the leucine zipper is required to hold together (dimerize) two DNA-binding regions. The DNA-binding region comprises a number of basic amino acids such as arginine and lysine.>

The motifs are termed the **Maf-recognition element (MARE)**.

Detailed domain structures and post-translational modifications of the **mouse Bach1–2**. Green bars indicate **cysteine–proline residues**; numbers indicate the positions of **cysteine**. Red lines indicate selected identified **phosphoresidues**. CLS is the cytoplasmic localization signal:



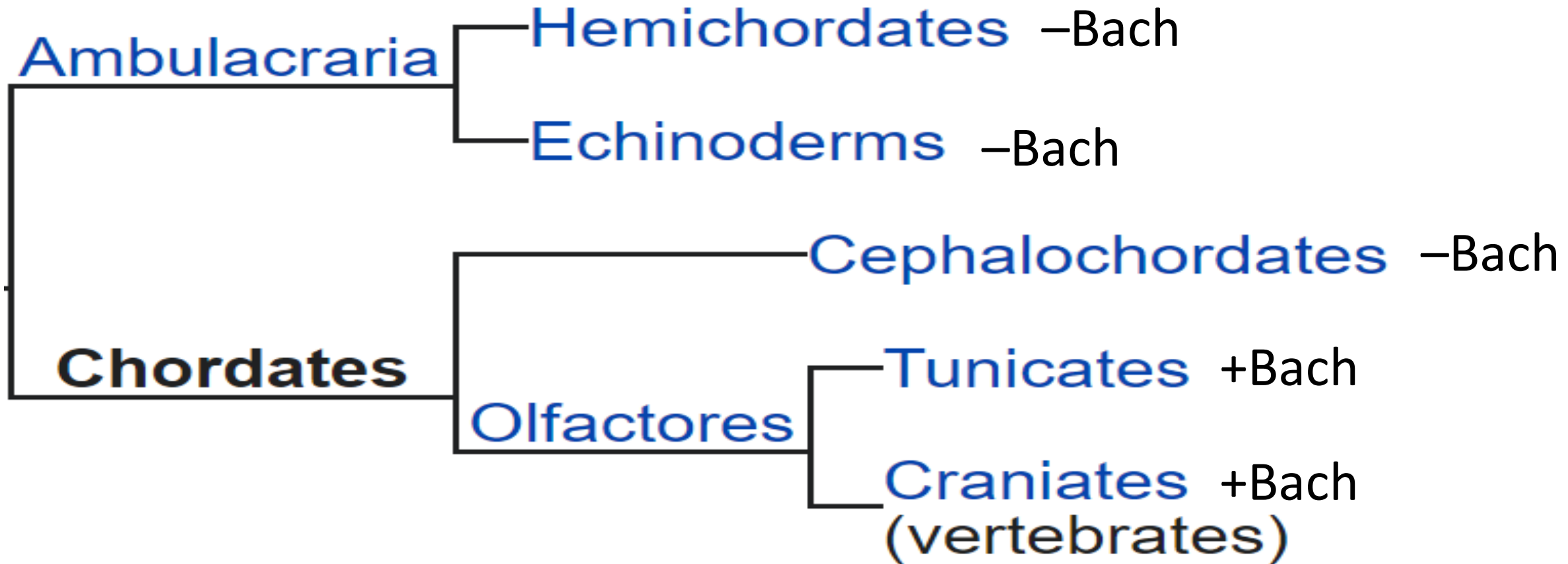
Now we continue: Bach1 and Bach2 proteins in Human are more similar to each other **than to any protein in *Ciona* spp.**, and **we got the tree:**



The following evolutionary assumptions have been proposed:

- the common ancestor of the *BACH* genes **existed in chordates before the divergence of vertebrates** since it exists in *Ciona* spp.;
- two *BACH* genes **emerged in gnathostomatous**.

The *Bach* genes are missing in lancelets, hemichordates, and echinoderms. The absence of *Bach* in *Branchiostoma* spp. (Cephalochordates) agrees with the proposed closer phylogenetic relation between **vertebrates and tunicates** rather than between **vertebrates and lancelets**.



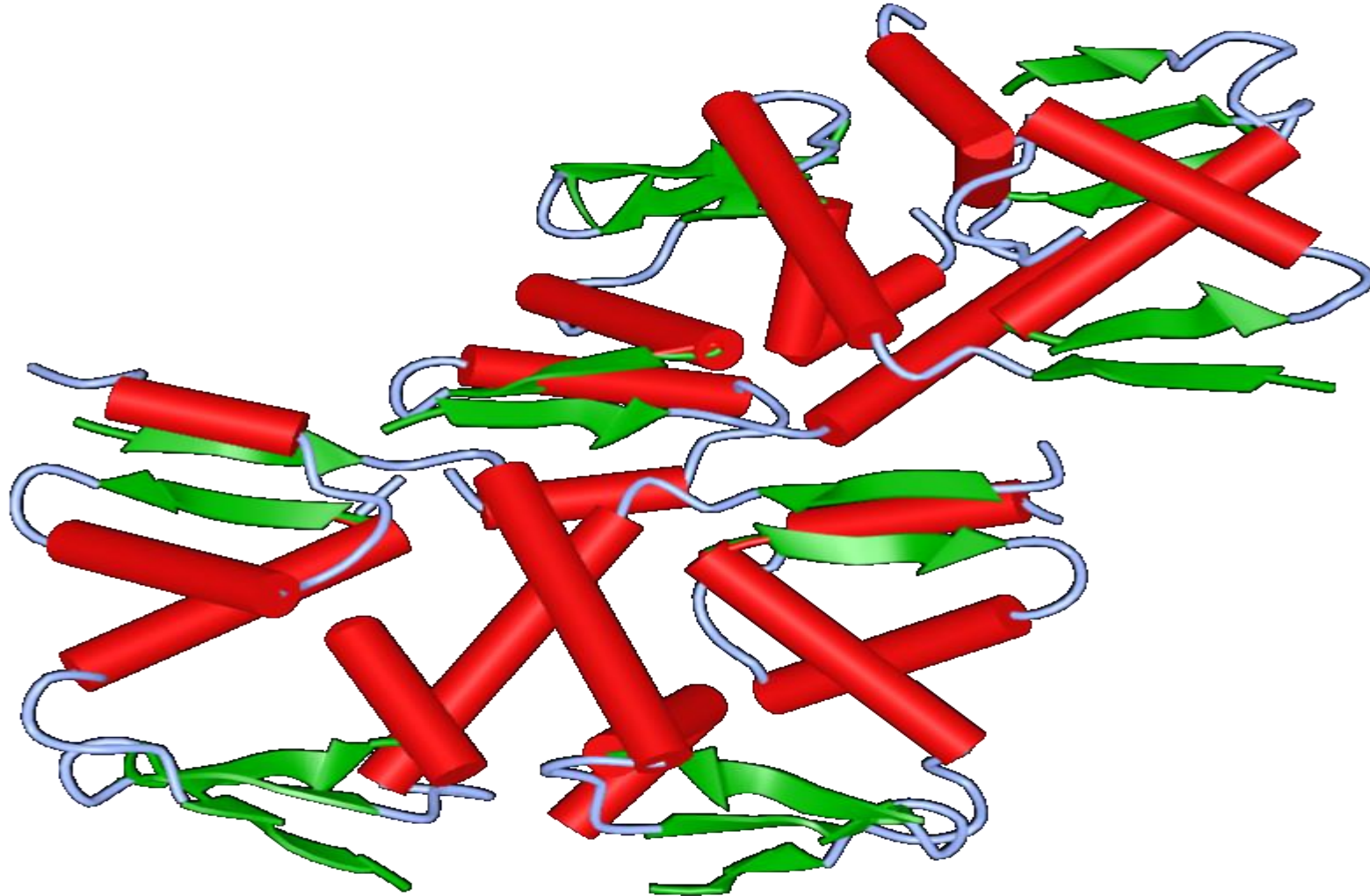
Now let us sum up the method as applied to the Bach protein: it has been identified in tetrapods, cartilaginous and bony fishes, cyclostomes, and ascidians using a local alignment with the human Bach1, the presence of the BTB and bZIP-Maf domains, satisfactory alignment of the secondary structure, and high similarity of the 3D structure at the N- and C-terminal regions with the human Bach1.



The possible absence of Bach in *Oikopleura dioica*, which is relatively close to *Ciona* spp., **can be attributed to its neoteny**. Indeed, like other appendicularians, adult *O. dioica* has a discrete body and tail and preserves the notochord throughout its life, while the body structure of *Ciona* spp. substantially changes in development.

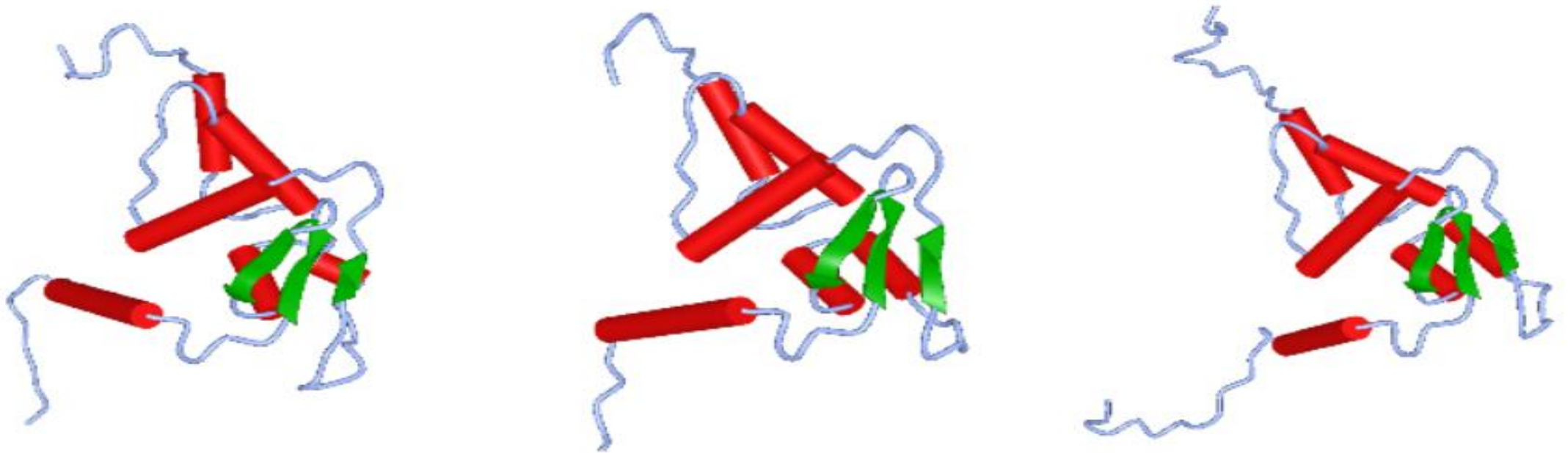
We recall **the tertiary structure of the BTB domain of human BACH1**

(4 chains):



The tertiary structures of the BTB domain in **Bach1** (single chain)

in *Ciona intestinalis*, *Ciona savignyi*, *Eptatretus burgeri*



No significant differences in Bach1 have been revealed in primates.

Beyond Euarchontoglires, the Bach1 sequences of the sloth *Choloepus hoffmanni*, tenrec *Echinops telfairi*, shrew *Sorex araneus*, dolphin *Tursiops truncatus*, and flying fox *Pteropus vampyrus* are similar to those in primates and most rodents.

Conversely, other representatives of Laurasiatheria as well as the nine-banded armadillo *Dasypus novemcinctus* have insertions or deletions in the same region of Bach1 as in NMR *Heterocephalus glaber*.

The Bach1 of the hedgehog *Erinaceus europaeus*, elephant *Loxodonta africana*, and hyrax *Procavia capensis* has a very long C-terminal deletion covering both conserved and variable regions.

Let us continue comparing the **Bach1** and **Bach2** proteins.

In jawed vertebrates (Gnathostomata), the homodimer formation was found to involve interactions of the kinked N - terminus (**N - hook**) and the partner's **C - terminal residues in Bach1**:

mouse	Bach1	1	MSVSE	...	SAVFA	10
human	Bach1	1	MSLSE	...	NSVFA	10
mouse	Bach2	1	MSVDE	KPG	SPVFA	13
human	Bach2	1	MSVDE	KPD	SPMYV	13

The sequence logo of
N-termini in Bach2:



The CP-motif **223-LCPKYR-228** inside the **Bach1** protein contains substitution in marsupials as well as in some lizards (essential for heme binding):

<i>Mus musculus</i>	LCPKYR	
<i>Monodelphis domestica</i>	LGPKYR	Marsupials
<i>Sarcophilus harrisi</i>	LGPKYR	
<i>Phascolarctos cinereus</i>	LGPKYR	
<i>Vombatus ursinus</i>	LGPKYR	
<i>Ornithorhynchus anatinus</i>	LCPKYR	
<i>Pogona vitticeps</i>	QCPKYR	Lizards
<i>Podarcis muralis</i>	LYPKYR	
<i>Lacerta agilis</i>	LYPKYR	
<i>Anolis carolinensis</i>	QCPKYR	
<i>Gekko japonicus</i>	LCPKYR	
<i>Thamnophis elegans</i>	LCPKYR	
<i>Thamnophis sirtalis</i>	LCPKYR	
<i>Notechis scutatus</i>	LCPKYR	
<i>Pseudonaja textilis</i>	LCPKYR	
<i>Python bivittatus</i>	LCPKYR	

Thus, the predicted heme-binding motifs in Bach1 of tetrapods insignificantly differ from those in mouse **excluding the species specified** (≥ 5 CP-motifs):

223-LCPKYR-228 (C→G in marsupials, although it is the same in the mouse and platypus *Ornithorhynchus anatinus*; or C→Y in the common wall lizard *Podarcis muralis*);

300-QCPAEQ-305, which considerably changed or disappeared in most mammals;

435-ECPWLG-340 (conserved in all tetrapods);

463-NCPFIS-468 (the cysteine is conserved in tetrapods; and I→M, in placentals including DMR, NMR and the common degu *Octodon degus*);

494-PCPYAC-499 (conserved in all tetrapods except the bearded dragon *Pogona vitticeps* and platypus);

648-DCPLSF-653 (conserved in almost all tetrapods).

Now about CP motifs for heme-dependent regulation.

The **Bach** proteins in *Ciona spp.* have ONLY two conserved CP motifs with each other. They DIFFER from those involved in the heme-dependent regulation in human and mouse that corresponds early diverging of Ciona:

Ciona savignyi CKNSKGD**CP**LMAKLSL

Ciona intestinalis VKNSKGD**CP**LMAKLSL

Ciona savignyi CSVMSQAC**CP**MMSQACT

Ciona intestinalis CSVMSQS**CP**MMSSPCS

In human *Homo sapiens* and elephant shark *Callorhinchus milii* (**chimera**) the **Bach1 proteins have three conserved** (for these species) **CP motifs involved in the heme-dependent regulation**. But in hagfish *Eptatretus burgeri* (**Myxini**) there are only two such motifs common with humans. Thus, the quantity of heme-binding motifs decreases but stay sufficient (**6 > 5 > 3 > 2**).

<i>Homo sapiens</i>	ALALPSL CP KYRKFQKAFG
<i>Callorhinchus milii</i>	QSRWASL CP KYRKFQLACG
<i>Eptatretus burgeri</i>	SCTSPLK CP TTRSLCLETQ

<i>Homo sapiens</i>	TTLSSVN CP FISTLSTEGC
<i>Callorhinchus milii</i>	SSLSSSK CP FGYTAGSSVC
<i>Eptatretus burgeri</i>	HVGDSPS CP INLSLACKDP

<i>Homo sapiens</i>	AKYSAAD CP LSFLISEKDK
<i>Callorhinchus milii</i>	AKYSSPE CP LSVLNIQRST
<i>Eptatretus burgeri</i>	-----

The **Bach2** proteins have **CP motifs that are conserved in most vertebrates**. They holds for the West Indian Ocean coelacanth *Latimeria chalumnae* (kindred of tetrapods), **cartilaginous fishes**: the elephant shark *Callorhynchus milii*, the whale shark *Rhincodon typus*, the thorny skate *Amblyraja radiata* (**jawed vertebrates**). In *Latimeria* **unusual C→Y**.

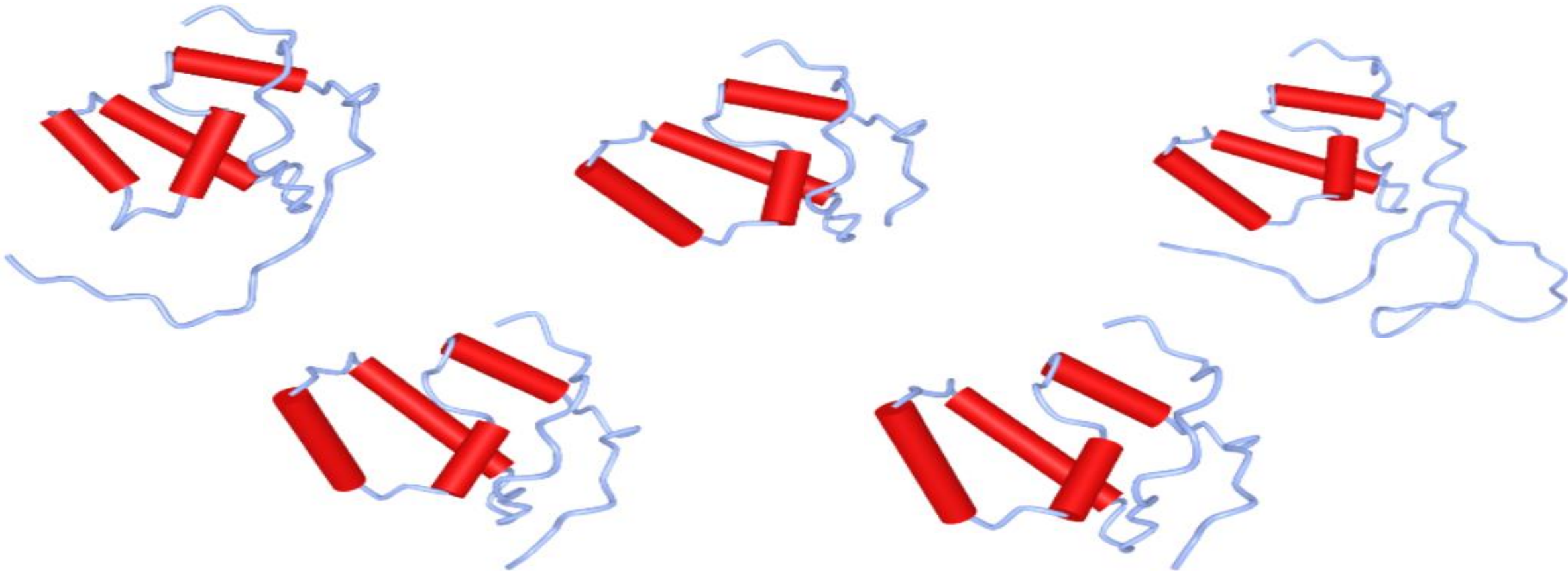
<i>Homo sapiens</i>	NTSC CP VPIK VC PRSPPLETRTRTSSSSCSSSYAEDGSG ...
<i>Mus musculus</i>	NTSC CP VPIK VC PRSPPLETRTRTSSSSCSSSYAEDGSG ...
<i>Latimeria chalumnae</i>	NTSC CP VPIKVYPRSPPLETRTRTSSSSCSSSYAEDGSG ...
<i>Callorhynchus milii</i>	NTSC CP VPIK VC PRSPPE-ETRTRTSSSSCSSSYFPEDGSG ...
<i>Rhincodon typus</i>	KTSC CP VPIK VC PRSPPE-ETRTRTSSSSCSSSYFPEDGSG ...
<i>Amblyraja radiata</i>	NTSC CP VPIK VC PRSPPE-ETRTRTSSSSCSSSYFPEDGSG ...

<i>Homo sapiens</i>	SPEQIQALHRY CP VLRPMDLPTASSINPAPL-GAEQNI ...
<i>Mus musculus</i>	SPEQIQALHRY CP VLIIPMDLPGAS-VNPPPV-GVEQSL ...
<i>Latimeria chalumnae</i>	SPEQIQALHRY CP VLRPMEQPVTASIDPSLS-LLEQSL ...
<i>Callorhynchus milii</i>	SSEQIQSLHRY CP ALRPLDQAATAGSDTSPSAEFEQKP ...
<i>Rhincodon typus</i>	NPEQIQSLHK F CPVFRPMDESAASKASTTLPLAGLEQKI ...
<i>Amblyraja radiata</i>	SPEQMQT--- F CPVFRPVDEPAATGSTISPPDGLERKF ...

Now let us sum up the method as applied to Nrf2:

the **Nrf2** protein has been **identified in deuterostomes** **considering** a local alignment with the human NFE2L2 (NP_006155.2), the presence of the bZIP-Maf domain, a satisfactory alignment of the secondary structure, the absence of some “wrong” domains (kelch-type beta-propeller in kelch-like proteins, BTB in Bach, bZIP-Jun in Jun and similarly for other bZIP proteins), and a high 3D structure similarity with NFE2L2 at the C-terminus coupled with a significant difference from other human bZIP proteins at the N-terminus.

As well as the tertiary structures of DNA-binding domain in Nrf2 in *Homo sapiens*, *Branchiostoma belcheri* (Chordata), *Saccoglossus kowalevskii* (Hemichordata), *Strongylocentrotus purpuratus* (Echinodermata), *Drosophila melanogaster* (Protostomia) are quite similar:



Thank You

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