The proteolytic cleavage of therapeutic antibody hinge region inhibits Fc-mediated effector functions

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Context and aims

The hinge region of IgG, which is necessary for FcγR and C1q binding, is susceptible to a proteolytic cleavage downstream the disulfide bridges. The cleavage of one of the heavy chain, generating single-cleaved IgG (scIgG), is associated with impaired Fc-mediated functions.

In this study we aimed to compare uncleaved and single-cleaved monoclonal antibodies (mAbs) in terms of :

1) **Binding to membrane FcγRIIIa and FcγRIIIa-dependent functional responses of NK cells**
2) **C1q binding and complement activation**

Materials & Methods

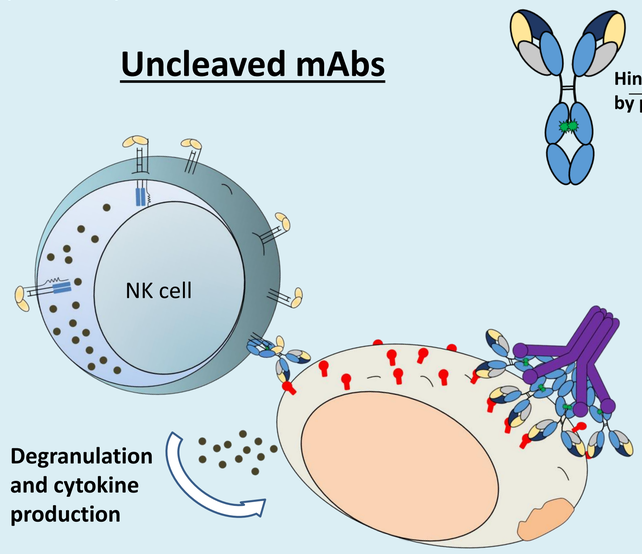
-Single-cleaved forms of 4 allotypes of IgG1 mAbs : cetuximab (3;-1), ipilimumab (3;1), trastuzumab (17;-1), rituximab and infliximab (17;1) ; panitumumab (IgG2) and nivolumab (IgG4) were generated using IdeS. (Purity of 80 to 100% scIgG)

-Binding to CD16-expressing NK92 cells as well as degranulation and/or IFNγ production by NK cells was evaluated by flow cytometry

-C1q binding to mAbs and cleaved mAbs was assessed by an ELISA method

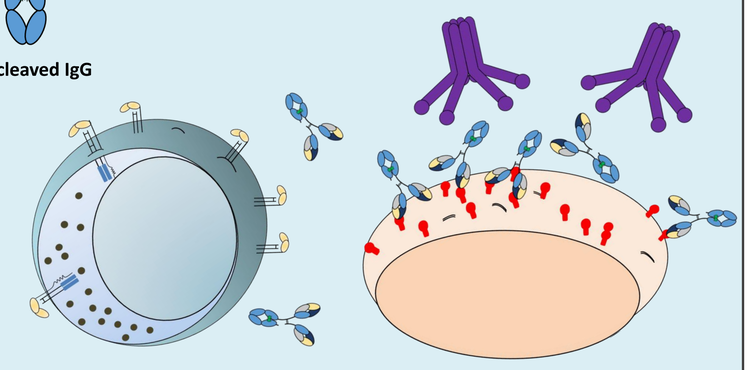
-Complement activation by rituximab was evaluated by monitoring C1q binding, C4c deposition on Daudi cells ans lysis of Daudi cells by flow cytometry.

Uncleaved mAbs



IgG1 bind to NK cells and trigger functional responses
IgG1 can bind C1q and activate the complement cascade

Effects of cleaved mAbs ?



1) Interaction with NK cells

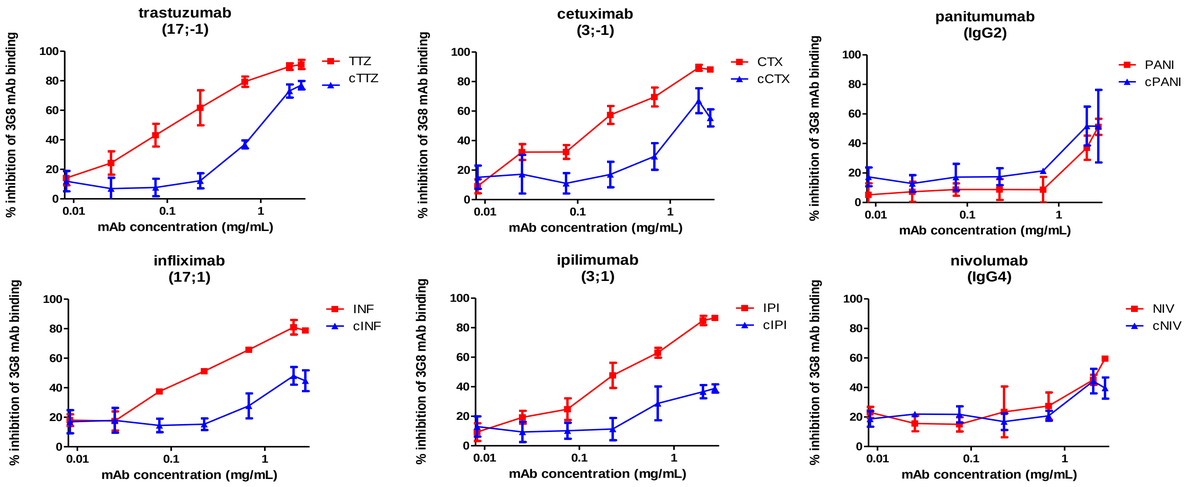
- **Binding to CD16-expressing NK cells**
- **Influence of CD16 polymorphism**
- **Functional consequences**

-degranulation
-cytokine production

2) C1q binding and complement activation

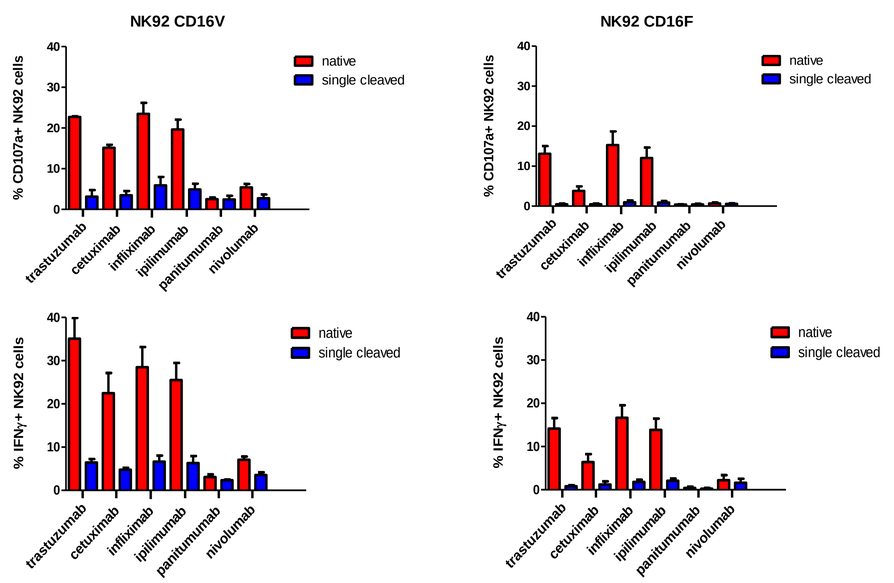
Binding to FcγRIIIa and FcγRIIIa-mediated functional responses of NK cells

Reduced binding of cleaved IgG1 mAbs to NK cell FcγRIIIa compared to their native forms



Binding of cleaved mAbs to human CD16V-transduced NK92 cells. CD16-transduced NK92 cells were incubated with increasing concentrations of native (red) or cleaved (blue) therapeutic mAbs for 30 min at 4 °C followed by FITC-conjugated anti-CD16 3G8 mAb and analyzed by flow cytometry. Results are indicated as a percentage of inhibition of 3G8 mAb binding to membrane FcγRIIIa.

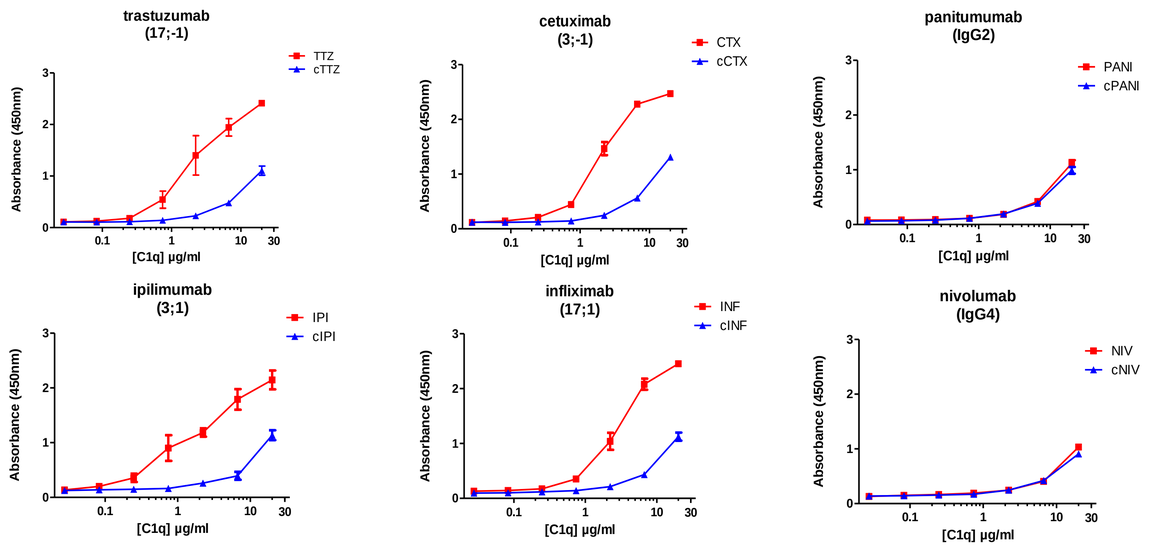
Reduced degranulation and IFN-γ production by NK92 cells after stimulation with plate-bound cleaved IgG1 mAbs



FcγRIIIa-dependent degranulation and interferon-γ production following stimulation by native mAbs (red) or cleaved mAbs (blue). NK92 cells expressing either V158 or F158 polymorphism of FcγRIIIa were stimulated 4hrs by plate bound native mAbs or cleaved mAbs. The percentage of CD107a+ or IFNγ+ cells are illustrated.

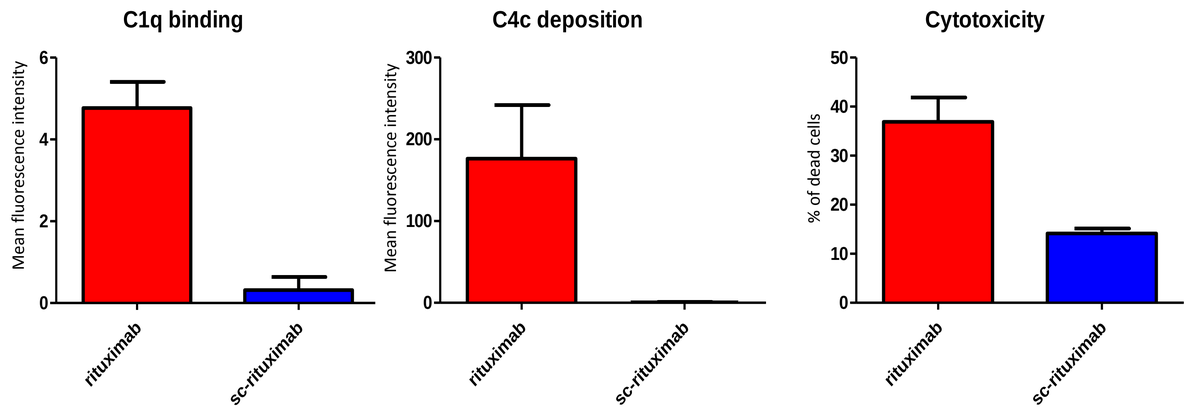
Binding of C1q and complement activation

Reduced binding of C1q to cleaved IgG1 mAbs compared to their native forms



Binding of C1q to native (red) and cleaved mAbs (blue). Increasing concentration of C1q were incubated on plate sensitized with 10µg/mL of mAbs or cleaved mAbs. Apparent C1q binding was revealed using TMB after incubation with an HRP-conjugated α-C1q antibody.

Reduced binding of C1q on Daudi cells incubated with cleaved rituximab and decreased C4c deposition and complement mediated cytotoxicity



Effect of rituximab on Daudi cells. Complement C1q and C4c were detected after incubation of CD20-expressing Daudi cells with uncleaved or cleaved rituximab for 30 min followed by staining with FITC-conjugated α-C1q or α-C4c. Lysis of Daudi cells induced by uncleaved or cleaved rituximab is also shown.

Single-cleaved IgG1 mAbs exhibit reduced FcγRIIIa and C1q binding and decreased Fc-mediated effector functions, whatever their allotype.

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EXPLOITING RHO HELICASE ACTIVITY FOR THE PURPOSE OF SYNTHETIC REGULATION

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BACKGROUND

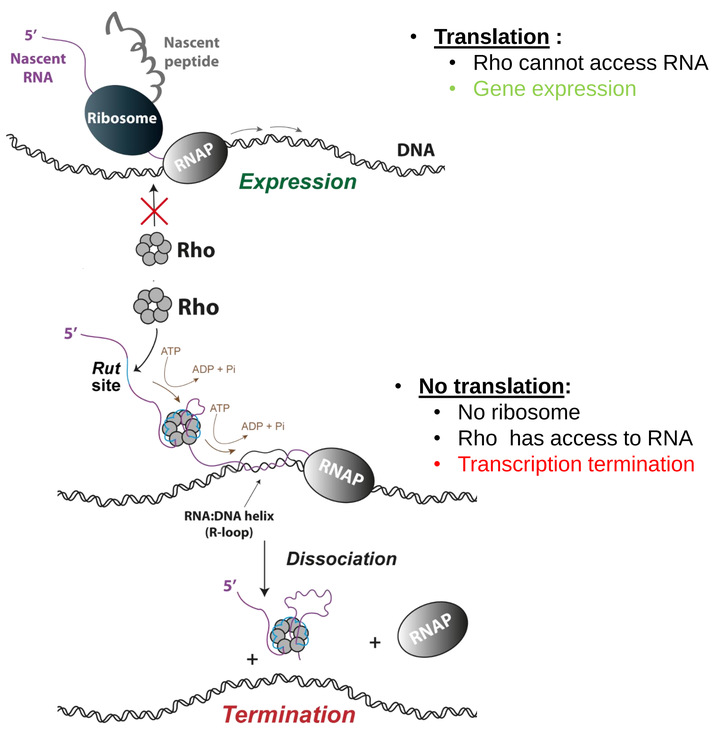
Synthetic biology : engineering new biological systems or functions

A major challenge in synthetic biology is to finely and tightly control gene expression. Switchable RNA sequences able to control transcription or translation upon binding to a cognate ligand have been engineered to this end. These regulatory devices called **riboswitches** are RNA structures adopting an alternative conformation upon binding to a cognate ligand. Riboswitches are made of two allosterically connected domains: i) aptamer (ligand sensing) and ii) expression platform (biological effect). Although these systems are promising, a low dynamic range limits their applicability.

We aim to design composite systems wherein riboswitches operating by distinct mechanisms are combined to control gene expression synergistically. To this end, we aim to develop a new class of synthetic riboswitches able to govern the biological activity of the bacterial Rho helicase.

The Rho factor as a new regulatory tool

This bacterial ring-shaped, ATP-dependent motor triggers transcription termination by disrupting transcription elongation complexes in a manner that is often coupled to other regulatory signals.

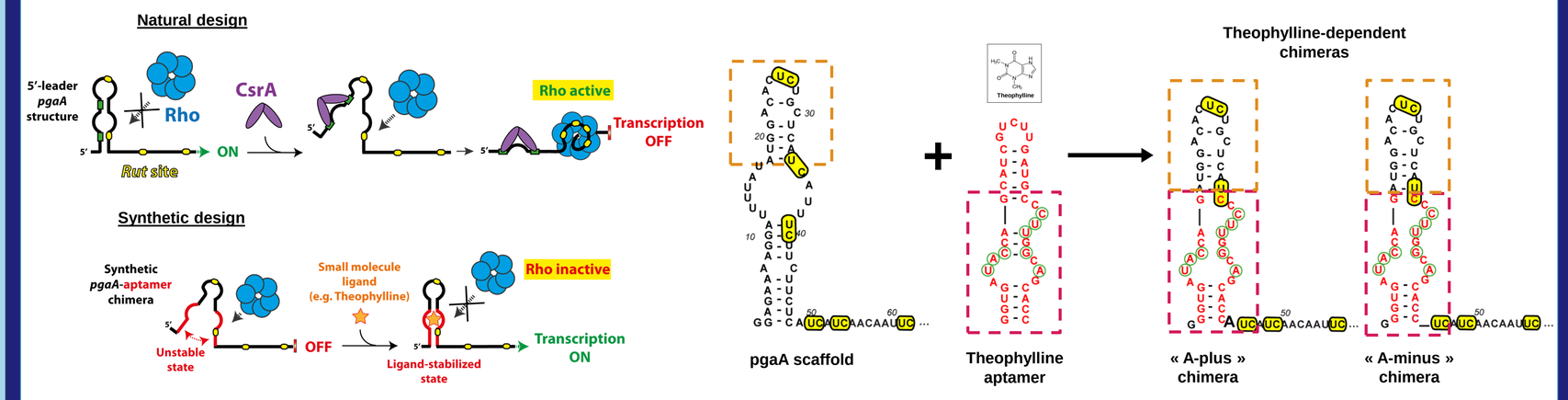


- **Translation :**
  - Rho cannot access RNA
  - Gene expression
- **No translation:**
  - No ribosome
  - Rho has access to RNA
  - Transcription termination

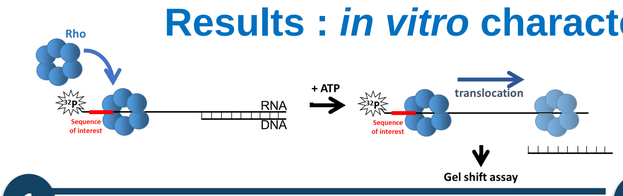
This mechanism is widely used in bacteria to regulate gene expression. However, **Rho activity has never been exploited for synthetic regulation purposes.**

Goal 1: development of a synthetic riboswitch controlling Rho activity

- A RNA structure present in the 5'-leader of the *pgaABCD* operon of *Escherichia Coli* mediates Rho access to RNA by masking a *Rho utilization* site (*Rut* site). In this case, a dimeric protein, **CsrA**, causes changes in the mRNA secondary structure and unshields the *Rut* site.
- We used this as a scaffold to design first-generation chimeras with a well characterized aptamer binding theophylline. This aptamer has already been engineered into various riboswitch-like constructs.

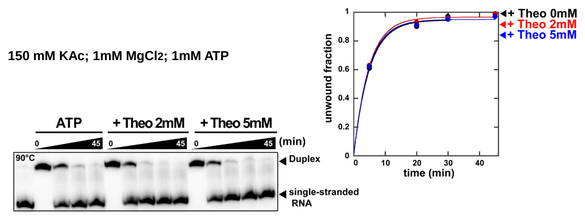


Results : *in vitro* characterization of the new Rho-dependent riboswitch



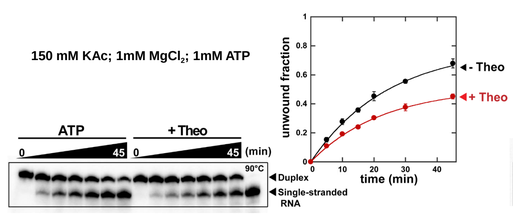
We performed bio-chemical assays based on radio-labelled RNA to monitor Rho helicase activity *in vitro* for each chimera sequence in different experimental conditions.

1 Positive control : unstructured *Rut* site

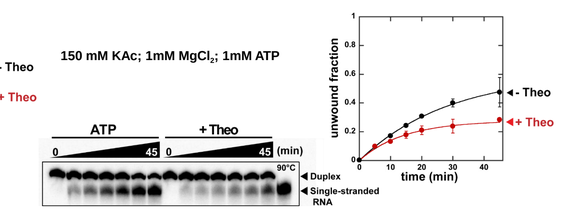


Theophylline has no effect on Rho helicase activity with a standard substrate.

2 « A-plus » chimera

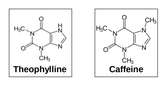


« A-minus » chimera

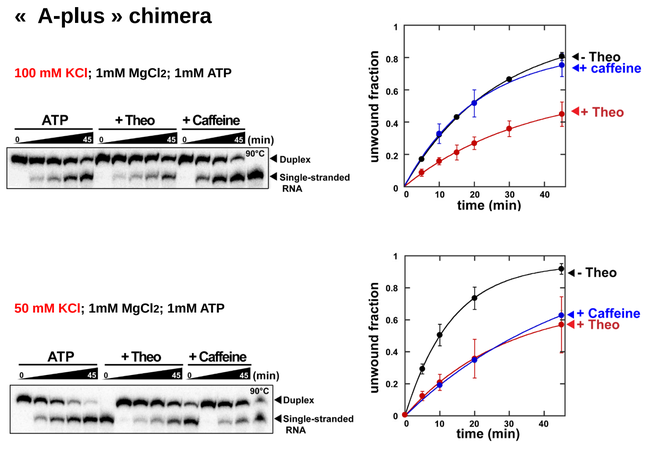


- **Theophylline modulates Rho helicase activity with the chimera substrates.**
- **Low dynamic range ➜ Optimization of experimental conditions**

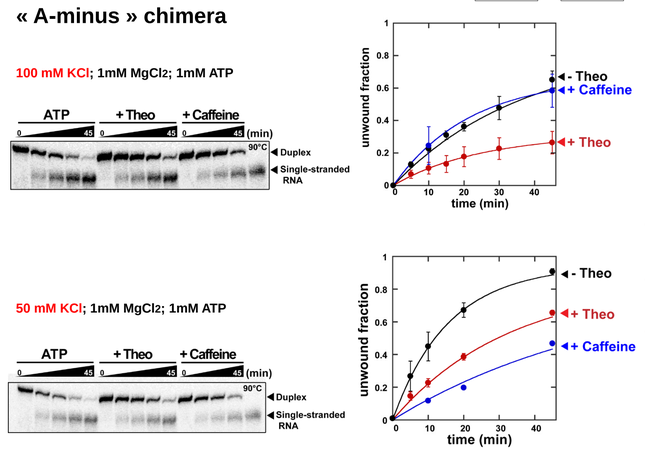
3 We searched for experimental conditions providing an optimal response of the chimeras to theophylline. We also tested the specificity of our constructs with the caffeine analogue.



« A-plus » chimera



« A-minus » chimera



- **Chimera constructs govern Rho activity in a theophylline-dependent manner.**

These 1ST-generation sequences need to be optimized to increase the dynamic range and to improve the specificity to theophylline.

Modulation of Rho helicase activity is specific for theophylline at high KCl concentration. This specificity is lost as the KCl concentration is decreased.

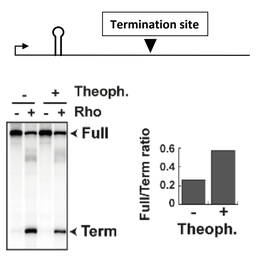
37°C, 5 nM duplex, 20 nM Rho, 2mM theophylline or caffeine in helicase buffer (20 mM Hepes pH 7.5, 0.1 mM EDTA supplemented with indicated concentration of salt). Reaction initiated by addition of $MgCl_2$-ATP (1 mM, final concentration) and an oligo trap (200 nM, final concentration; to trap DNA strands released). Quench buffer (6% ficoll, 0,75% SDS, 30 mM EDTA, 0.15 M sodium acetate). All substrates contain a top RNA strand (Rut site or chimeras) labelled at its 5'-end with ^{32}P, and a complementary DNA.

Goal 2: test of the synthetic Rho-dependent switches in more complex contexts

WORK IN PROGRESS

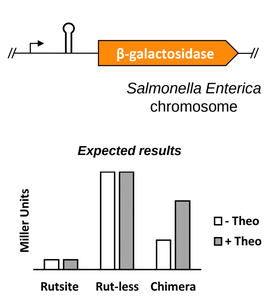
In vitro transcription termination and *in vivo* (or cell-free extract) reporter assays

Transcription termination assay

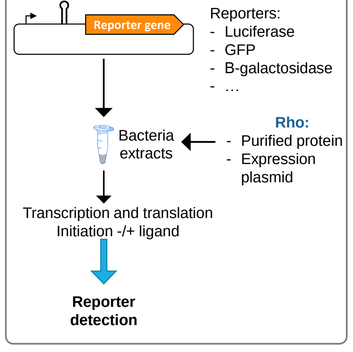


Theophylline modulates Rho biological activity with the chimera substrate.

In vivo reporter assay



Cell-free extract reporter assay



Controls:
Rut site → strong Rho-dependent termination → **No gene expression**
Rut-less (no *Rut* site) → no Rho-dependent termination → **gene expression**

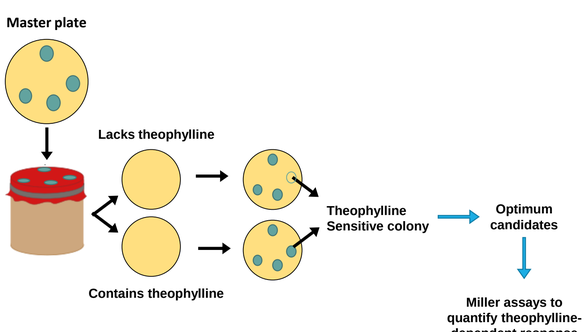
Goal 3: optimization of the Rho-dependent switch response

1 Randomization of the chimera sequences by error-prone PCR

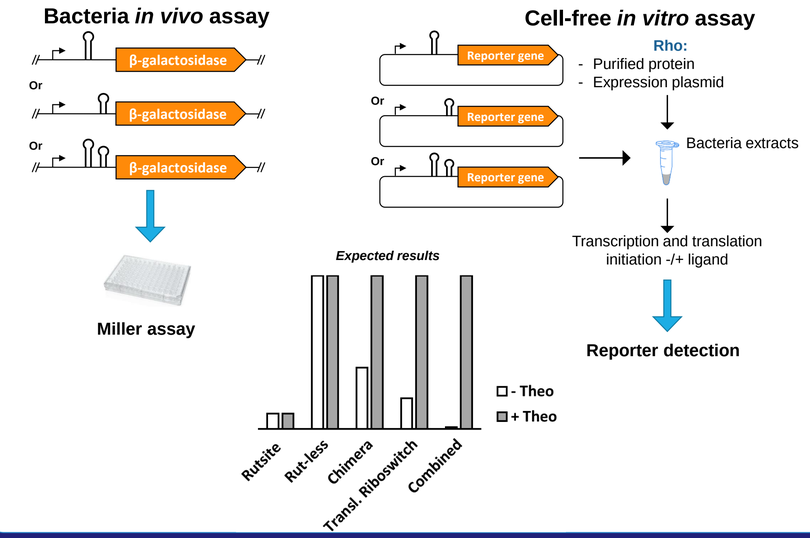
2 Insertion of the bank of sequences in the bacterial chromosome

3 Selection on a lactose minimum medium -/+ theophylline

- No theophylline ➜ transcription termination ➜ β-gal not expressed ➜ no growth
- Theophylline ➜ no transcription termination ➜ β-gal expressed ➜ growth



Goal 4: combination of the optimized Rho-dependent switch with a translational riboswitch



It is POSSIBLE to develop a synthetic riboswitch able to govern the helicase activity of the bacterial Rho factor.

- **Additional studies are in progress to optimize this synthetic riboswitch and to demonstrate its efficiency *in vivo*.**
- **Combining this Rho-dependent riboswitch with other devices will be the next step to test if higher dynamic responses can be obtained with tandem riboswitch systems.**

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The role of the auxiliary $Na_V\beta4$ subunit in maintaining epithelial phenotype

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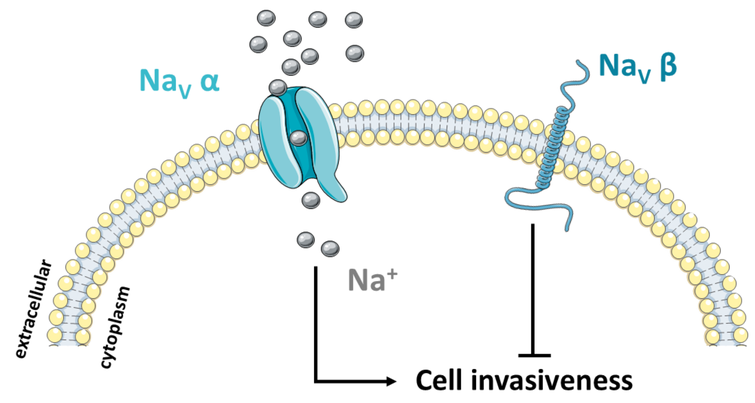
Background

Cancer metastasis is the main cause for the high mortality attributed to carcinomas. As such, understanding the cellular mechanisms leading to metastases development is crucial. Our team has described the *SCN4B* gene, and its expression product, the $Na_V\beta4$ auxiliary subunit of voltage-gated sodium channels (Na_V), to be implicated in the invasive progression of breast cancer, independently of $Na_V\alpha$ pore-forming subunits. *SCN4B* is highly expressed in epithelial cells of normal breast, but is reduced in breast cancer tissues and cells. *SCN4B* is reduced in grades II and III compared to grade I, which is correlated with cancer aggressiveness and a poor patient survival. *In vitro*, the loss of *SCN4B* expression promotes breast cancer cell invasiveness by increasing the RhoGTPase RhoA activity, but the link between $Na_V\beta4$ and RhoA remains unclear. In contrast, the overexpression of $Na_V\beta4$ reduces breast cancer cell invasiveness and mammary tumour progression in animal models (Bon et al., Nat Commun 2016).

In this study, we want to address the possible role of the loss of $Na_V\beta4$ in early steps of carcinogenesis. Therefore, we have generated stables $Na_V\beta4$ down-expressed clones from the non-cancerous MCF10A cell line, using CRISPR/Cas9 technology (MCF10A CRISPR $Na_V\beta4$), and performed 2D and 3D, transcriptomic and proteomic experiments, to assess the effects on epithelial morphology. We describe here characteristics of this cell line compared to the Wild Type cells (MCF10A WT).



1. Loss of the epithelial phenotype in 2D cell culture.

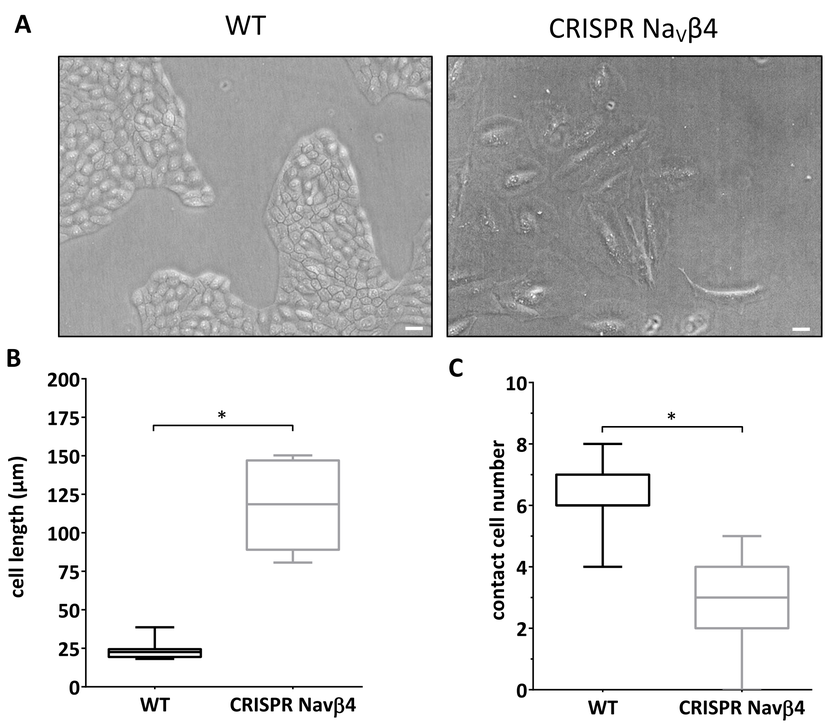


Figure 1 : **A.** Representative micrographs (phase-contrast) of MCF10A WT and MCF10A CRISPR $Na_V\beta4$. Scale bar 20 µm. **B.** Quantification of the main axis length of cells. **C.** Quantification of the number of cells in contact with one. Mann-Whitney rank-sum tests were used (N=20, $p < 0.0001$).

*** The loss of $Na_V\beta4$ induces important morphology changes in 2D cell culture, particularly on the size of the cells and on cell-cell interactions.**

2. Loss of the epithelial phenotype in 3D cell culture.

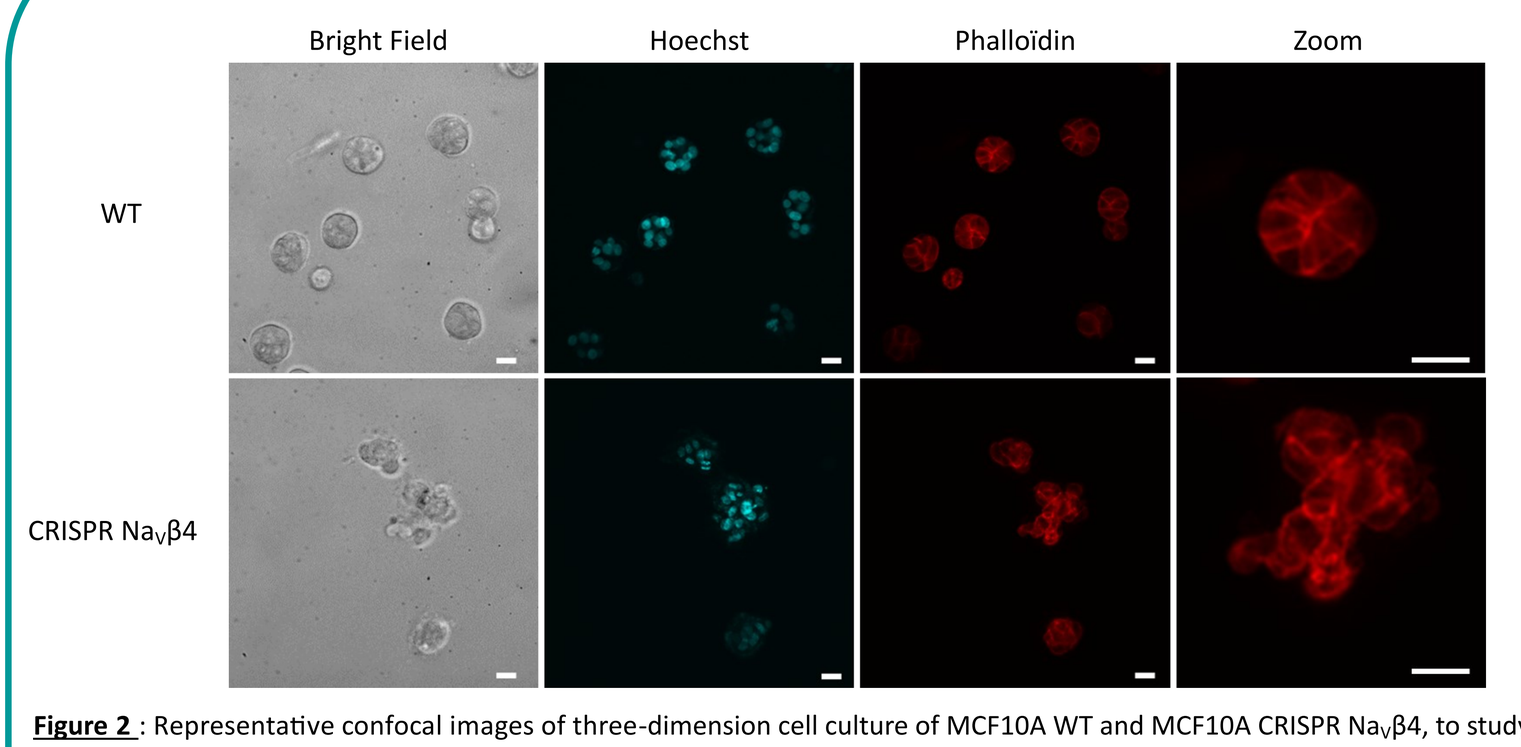


Figure 2 : Representative confocal images of three-dimension cell culture of MCF10A WT and MCF10A CRISPR $Na_V\beta4$, to study steps of "cysts" formation, cell polarity and cell organisation, in a matrix composed of Geltrex (#A1413202). Nucleus are stained with Hoechst and actin filaments with Phalloïdin AF594. Scale bar 20 µm.

*** The loss of $Na_V\beta4$ induces a complete disorganisation of 3D cysts. This suggests a loss of epithelial and cell polarity, and adhesion.**

3. Down-regulation of $Na_V\beta4$ decreases the protein expression of β-catenin, a key cell-cell adhesion protein.

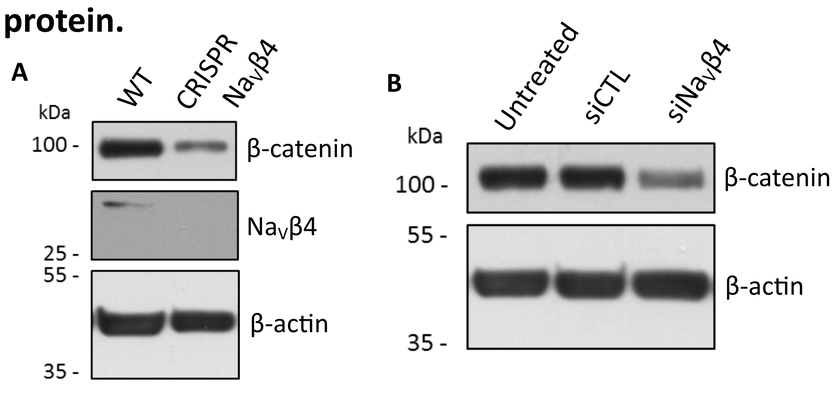


Figure 3 : **A.** Western blots showing a reduction in levels of β-catenin in the MCF10A CRISPR $Na_V\beta4$ compared to MCF10A WT (N=4, independent experiments). **B.** Western blots showing a similar decrease of β-catenin protein content with a transient knock-down of $Na_V\beta4$ ($siNa_V\beta4$) compared to siCTL (N=5, independent experiments).

*** β-catenin is involved in many pathways leading to cancer aggressiveness, in particular in cell adherens junctions and invasion/migration. Therefore, we wonder if $Na_V\beta4$ could modulate this protein. Indeed, a decrease at protein level of the β-catenin occurs with a loss of $Na_V\beta4$ in non-cancerous breast cells.**

4. But no change at transcriptional level.

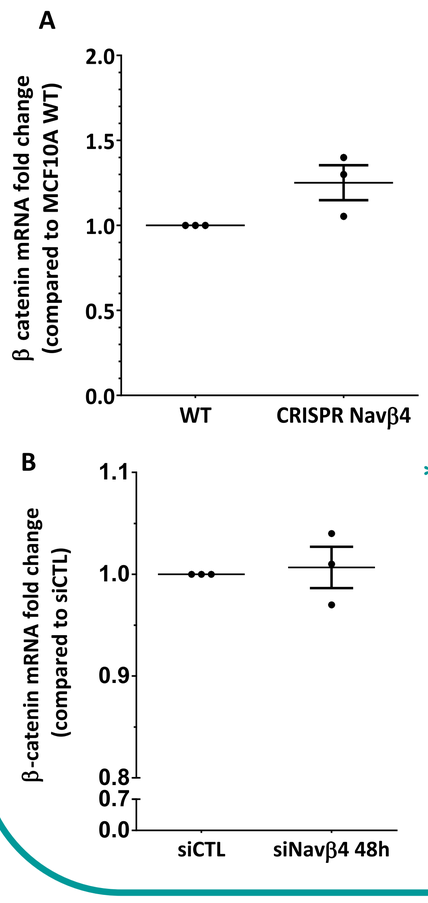


Figure 4 : **A.** RT-qPCR showing no significant difference between MCF10A WT and MCF10A CRISPR $Na_V\beta4$ (N=3, independent experiments). **B.** RT-qPCR showing that β-catenin mRNA seems to not be regulated with a transitory knock-down of $Na_V\beta4$ ($siNa_V\beta4$ 48h) (N=3, independent experiments).

*** The loss of $Na_V\beta4$ has no effect on β-catenin at transcriptional level. But it induces a protein decrease (Figure 3). Our main hypothesis is that the β-catenin degradation is increased by the proteasome (see in figure 5).**

5. Down-regulation of $Na_V\beta4$ induces β-catenin degradation.

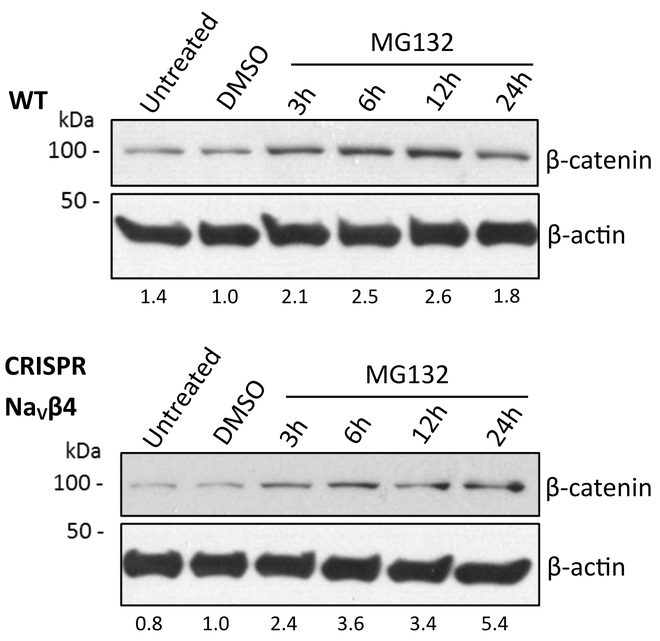


Figure 5 : Western blots showing protein expression of β-catenin, using MG132 (10 µM), a proteasome inhibitor, on MCF10A WT and MCF10A CRISPR $Na_V\beta4$.

*** $Na_V\beta4$ could prevent β-catenin degradation by the proteasome**

Conclusions & Perspectives

* In this work, we have developed stable $Na_V\beta4$ down-expressed MCF10A clones, using CRISPR/Cas9 technology, the MCF10A CRISPR $Na_V\beta4$. This developed cell line shows important cell morphology and organisation changes in 2D and 3D cell cultures. These cells seem to loose their epithelial phenotype, suggesting a regulation of cell polarity, and cell-cell adhesion. Our purposes are to understand mechanisms leading to these phenotypical changes in epithelial cells when *SCN4B* expression is reduced, and to study consequences on the epithelial function and carcinogenesis.

* β-catenin protein is involved in many pathways (adherens junctions, and invasion/migration by Wnt/Frizzled pathway), and highly regulated in cancers. The loss of $Na_V\beta4$ induces a decrease of β-catenin protein but no change at transcriptional level. **Our results show that $Na_V\beta4$ could prevent β-catenin degradation by the proteasome, and maintain epithelial phenotype.**

* Our ongoing work is to understand and describe the functional links between $Na_V\beta4$ and β-catenin (direct interaction, other proteins involved, ubiquitination, etc.).

Identification of more closely related PiggyBac-like elements of genes coding PiggyBac derived transposases among Vertebrates, using an evolutionary *in silico* approach

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Background

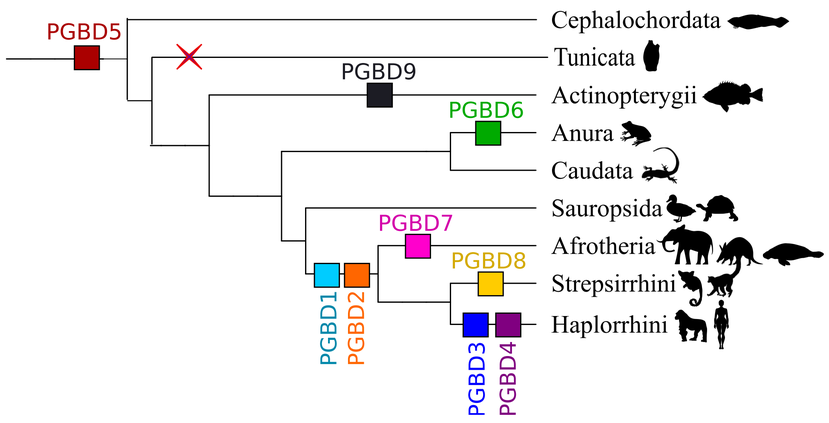
- Transposable elements (TEs) are mobile genetic elements present in almost all organisms.
- *IFP2* is an autonomous mobile element of 2475bp in length commonly called *piggyBac*[1].
- *IFP2* is the reference element of the Terminal Inverted Repeats (TIR) transposon family *piggyBac*. It was originally isolated in a clonal mutant baculovirus on a culture of Cabbage Looper (*Trichoplusia ni*) cells.
- *PiggyBac* and *piggyBac-like* elements (*pble*) are transposons that insert into the tetranucleotide TTAA which they are duplicated upon insertion[2,3]. They are flanked at their extremities by TIRs. In the inner region between outer ends of the element can be present an open reading frame (ORF) of about 1.8kb that encodes a piggyBac transposase of about 600 aa.
- TEs have contributed to the evolution of genes by being a source of cis-regulating factors for host gene expression. TEs can also be responsible for the emergence of new genes derived from their ORFs that bring new functions to the host genome. This process is called molecular domestication or exaptation.
- Despite the sequence conservation between piggyBac-derived transposases (PGBDs), the issue of their DNA binding specificity arises. Indeed, the ability of PGBD5 to mediate transposition was demonstrated in hopping assays using as a transposon source distantly related, *IFP2*[2,4]. This suggests that PGBD5 can mobilize distantly related *pble* and that this domesticated transposase is able to bind their ends and transpose them (or at least excise them) while we observed that piggyBac is unable to do it.

Objective: Locate the closest "*pble* species" related to each vertebrate *pgbd* gene to investigate the sequence organization and conservation of TIRs of these *pbles*.

1. Inventory of *pgbd* in tetrapodes

Evidence that a piggyBac transposase can be domesticated and become a "piggyBac-derived" sequences (*pgbd*):

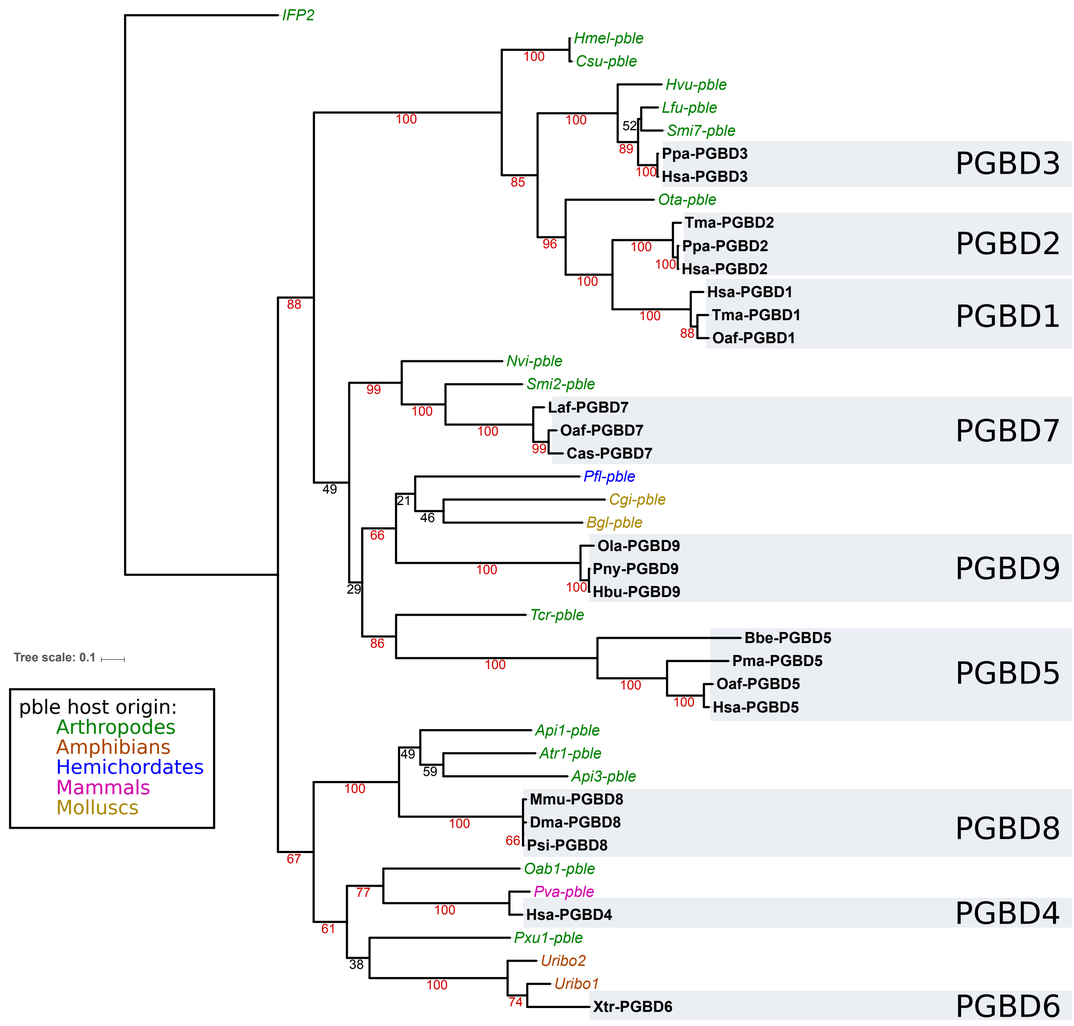
- Synteny of its genomic environment
- dN/dS ratio (if < 1, negative selection)
- Other features: introns and loss of TIRs (by age of domestication)



Three new *pgbd* genes were validated :

- *Pgbd7* was specific to afrotherian, its encoded protein was characterized by a SCAN domain at its N-terminal end and a CRD at its C-terminal end.
- *Pgbd8* was specific to strepsirrhinian species and encoded a PGBD that displayed a zF-FCS domain at its N-terminal end and a partial CRD at its C-terminal end
- *Pgbd9* was specific to actinopterygian species

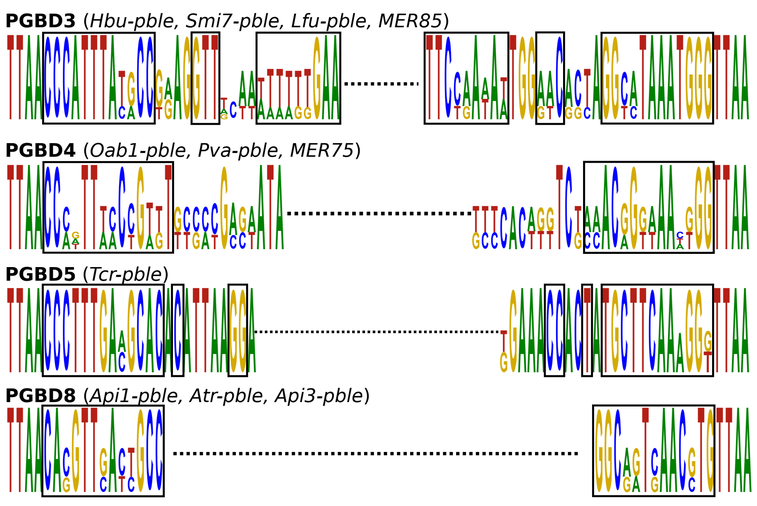
2. Finding the closest *pble* relatives of each *pgbd* gene in databases



- *Ota-pble* is located at the root of both *pgbd1* and *pgbd2* suggesting one single domestication event or two highly related *pbles* would be at the origin of both genes through two domestication events occurred at close times.
- *Tcr-pble* was the closest known relative of *pgbd5* genes. Because *pgbd5* is the most ancient domestication, the evolutionary distance between *Tcr-pble* and *pgbd5* is greater than for any other couple of *pble/pgbd*.

3. Sequence conservation of *pble* ends

Gapped local alignment of the 5' and 3' *pble* extremities



Beyond the conservation of the motif "CNNNTN" at the outer ends, we did not found strongly conserved nucleotide positions between TIRs of nine sets of *pbles*.

Extremities 5' of *pble* ends

The common point may be their secondary (SII) and tertiary (SIII) structures that would play a role in the transposase binding.
The search for SII and SIII in the ends of *pble* with non-B Database revealed the presence of inverted repetitions that can form cruciform structures close to at least one TIR for *Tcr-pble* and *IFP2*.

```
>Tni-pble
TTAACCCTAGAAAGATAGTCTGCGTAAAATTGACGCATGCATTCTTGAAATAT ---
>Tcr-pble
TTAACCCTTTGAAGCATAGTGGTTTCACTTGGTAACCACCTTTTATTTAATTA ---
```

Conclusion

- PiggyBac elements were domesticated at least at 8 occurrences during vertebrate evolution.
- We propose that *pgbd5* was transmitted vertically and then domesticated through hemichordate and vertebrate evolution as RAG1/2 did. Its roles are not defined for the moment.
- TIRs are different depending on the *pble*. This suggests that unrelated *pgbd* and *pble* cannot cross-recognize TIR.
- We show that PGBD5 can move *IFP2* and *Tcr-pble*. If they can cross-recognize the same target, how can they survive together in a genome?
- Our hypothesis is that TIR recognition consists of the « CCCTTT » cleavage site and a cruciform structural binding domain.

References :
1. Fraser et al., 1983 (PMID: 16789244; PMCID: PMC255260); 2. Mitra et al., 2008 (DOI: 10.1038/emboj.2008.41); 3. Mitra et al., 2013 (DOI: 10.1073/pnas.1217548110);
4. Morellet et al., 2018 (DOI: 10.1093/nar/gky044)