5° MEETING F.O.P. 5th F.O.P. CONFERENCE

FIBRODISPLASIA OSSIFICANTE PROGRESSIVA
FIBRODYSPLASIA OSSIFICANS PROGRESSIVA

Study of the regulation of *ACVR1* expression

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ROMA - ITALY MARZO 2011 MARCH
GIOVEDI 24, VENERDI 25, SABATO 26
THURSDAY 24, FRIDAY 25, SATURDAY 26

➢Study of the regulation of ACVR1 expression

▪Characterization of the structure and composition of the *ACVR1* transcripts.

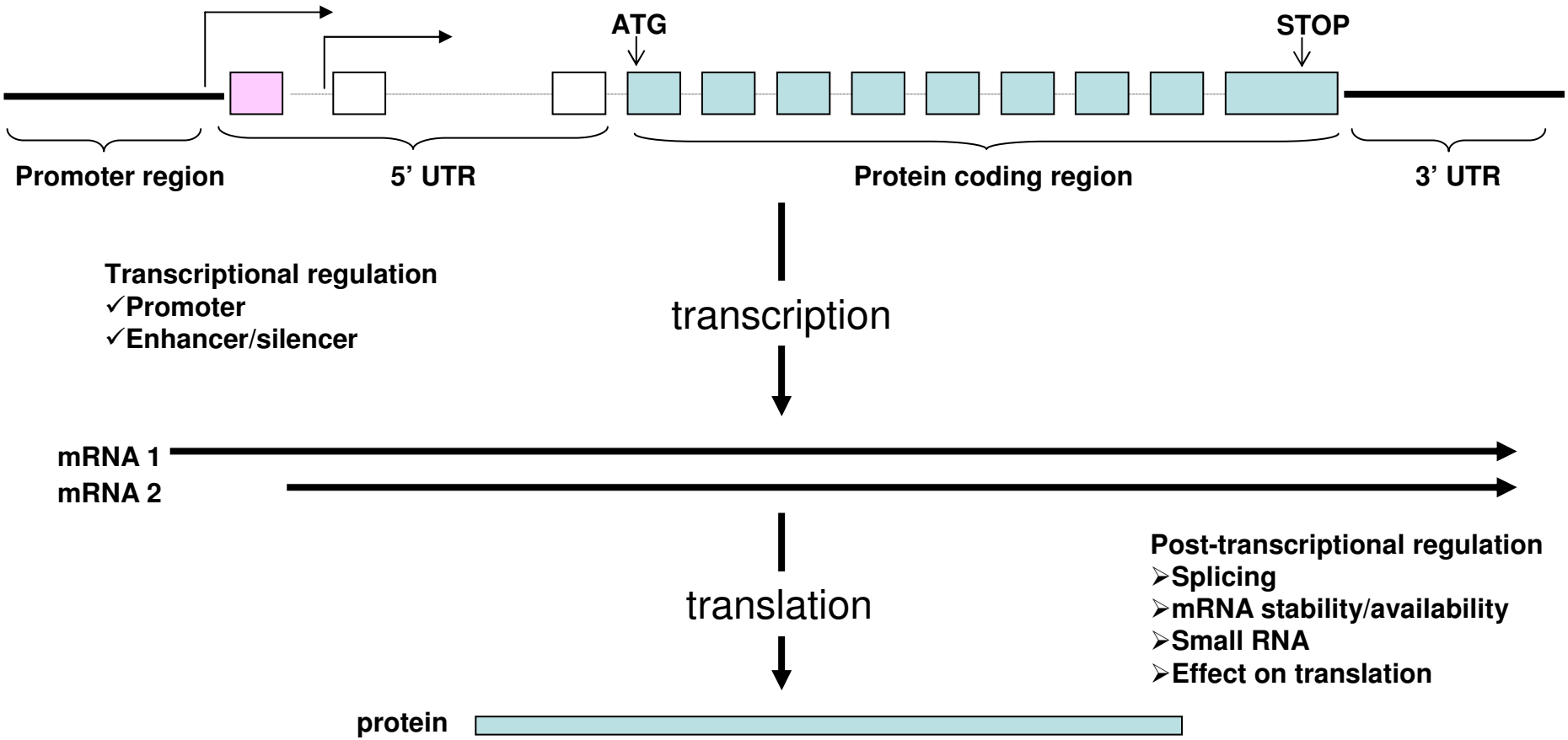
▪Definition and characterization of sequences playing a role in the regulation of *ACVR1* expression.

- **5'*UTR* sequences**
- **Promoter region**
- **3'*UTR* region**

Study of the regulation of *ACVR1* expression

Characterization of the structure and composition of the *ACVR1* transcripts.

Definition and characterization of genomic regions playing a role in the regulation of *ACVR1* expression, such as gene promoter/s and/or other sequences with regulatory activity.



Study of the regulation of ACVR1 expression

- Characterization of the structure and composition of the *ACVR1* transcripts.

- Definition and characterization of sequences playing a role in the regulation of *ACVR1* expression.

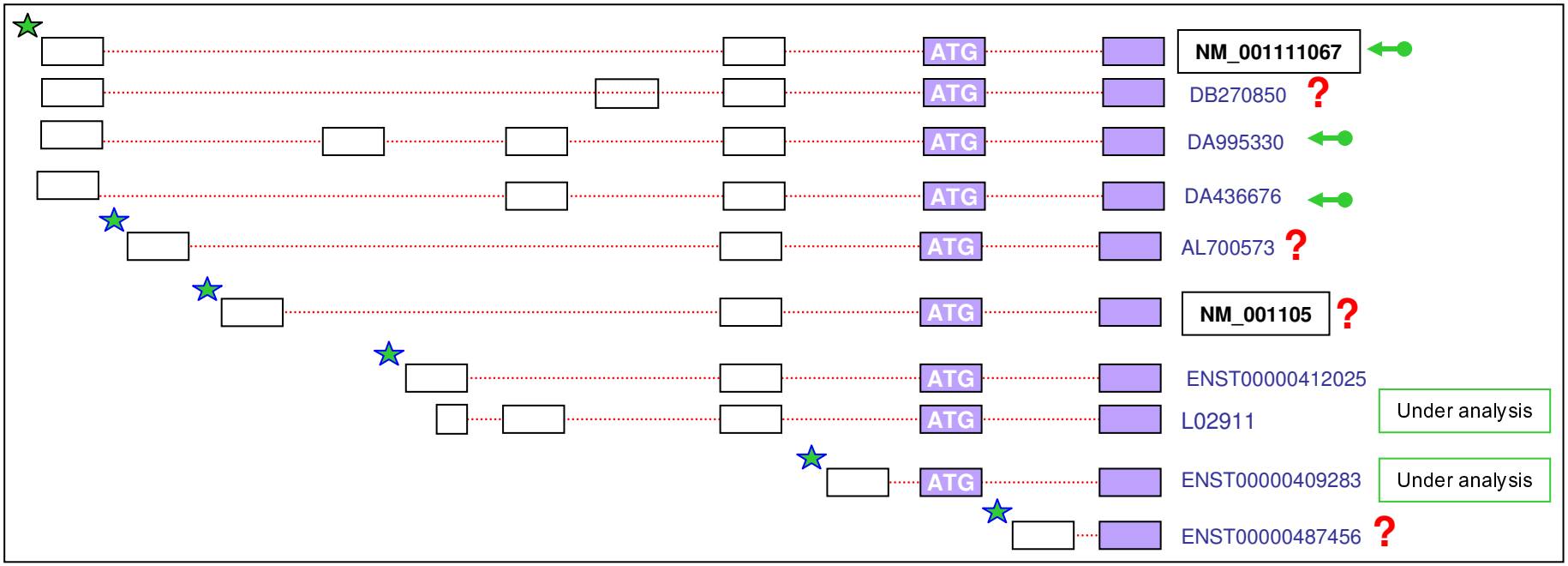
✓ **5'*UTR* sequences**

Promoter region

3'*UTR* region

Characterization of the structure and composition of the *ACVR1* transcripts.

How many are *ACVR1* transcripts?

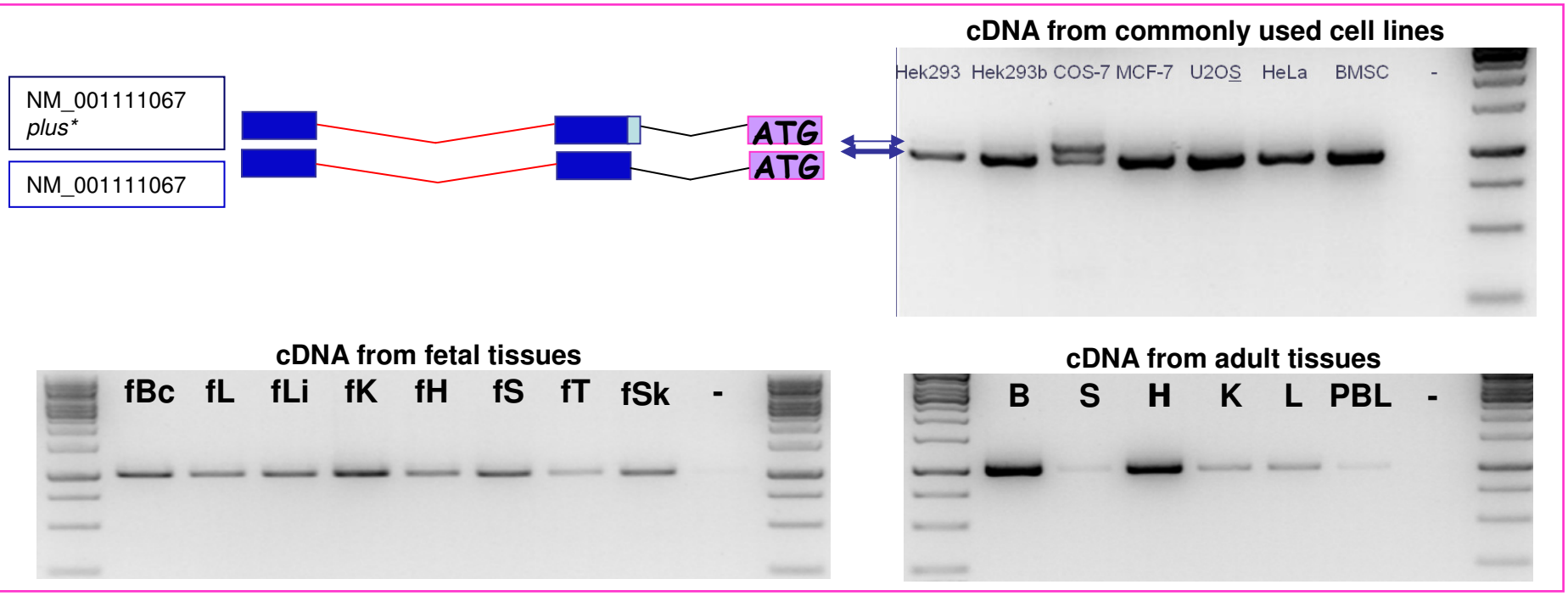


✓ **Our bioinformatic analysis suggests the putative presence of different transcription start sites (green and blue stars) and of different transcripts showing the alternative combination of several 5'*UTR* exons (white rectangles) with common protein coding sequences (violet rectangles, only the first two out 9 protein coding exons are indicated).**

✓ **The 5'UTR region of the *ACVR1* gene that undergoes alternative splicing spans around 57 kb of genomic DNA. Untranslated exons are flanked by canonical splicing sites and show a different degree of conservation.**

Characterization of the structure, composition and expression profile of the *ACVR1* transcripts (I)

- ***ACVR1*** **is widely expressed.**
- **The most common cDNA isoform is that defined by the NM_001111067 RefSeq.**
- **We have identified a new NM_001111067 isoform carrying an insertion at the 3' end of the second UTR exon (NM_001111067*plus*).**

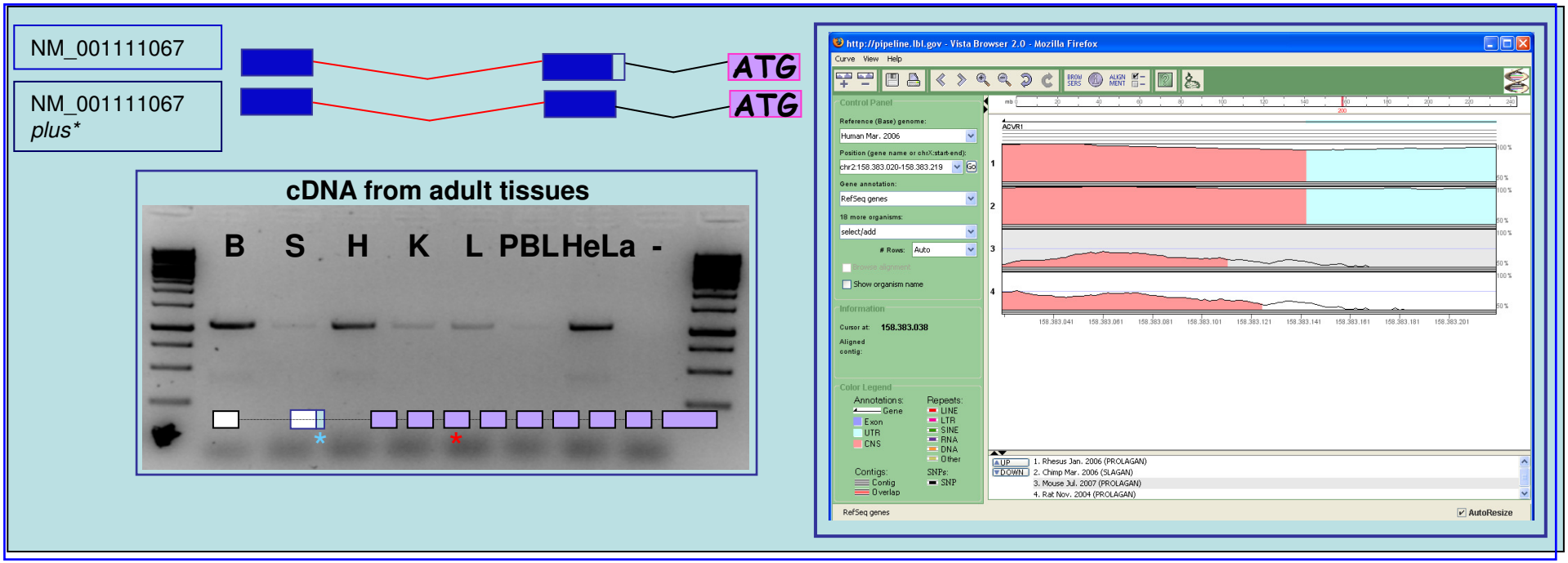


Characterization of the structure, composition and expression profile of the *ACVR1* transcripts (II)

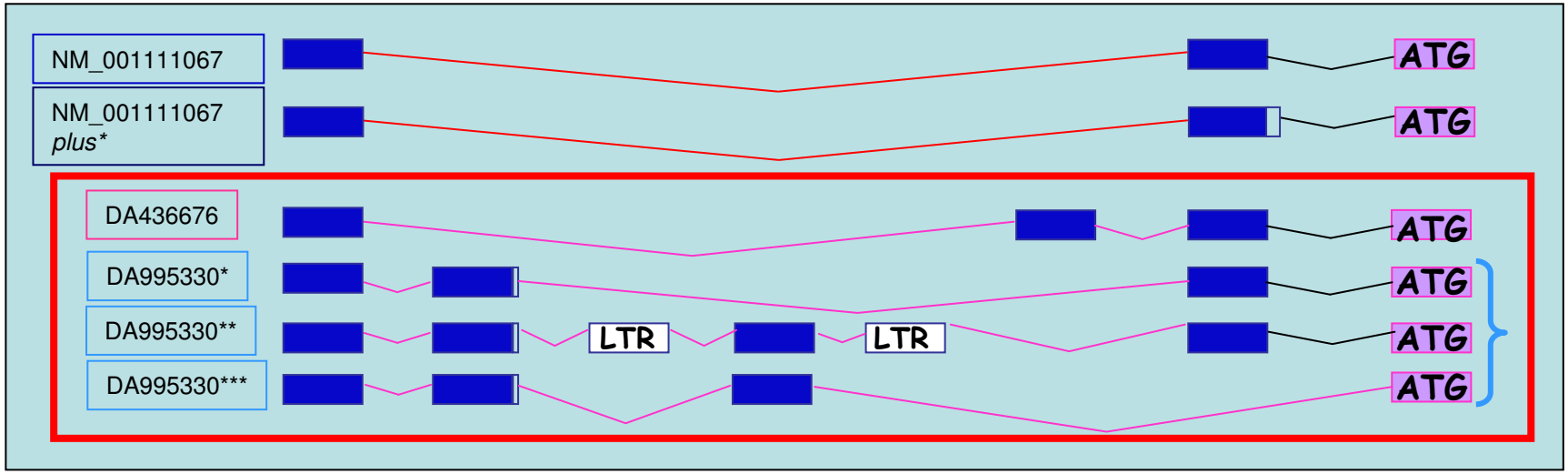
✓ **The insertion is due to an alternative splicing that leads to the incorporation of 116 bp of the 5'end of the adjacent intron in the mRNA.**

✓ **Although quantitatively less represented, also this isoform is widely expressed as assessed by RT-PCR on different cDNA with a forward oligonucleotide specific for the insertion (*) combined to the reverse mapping in the coding sequence (*).**

✓ **The inserted sequence is highly conserved among species and is even more conserved than the adjacent exon.**

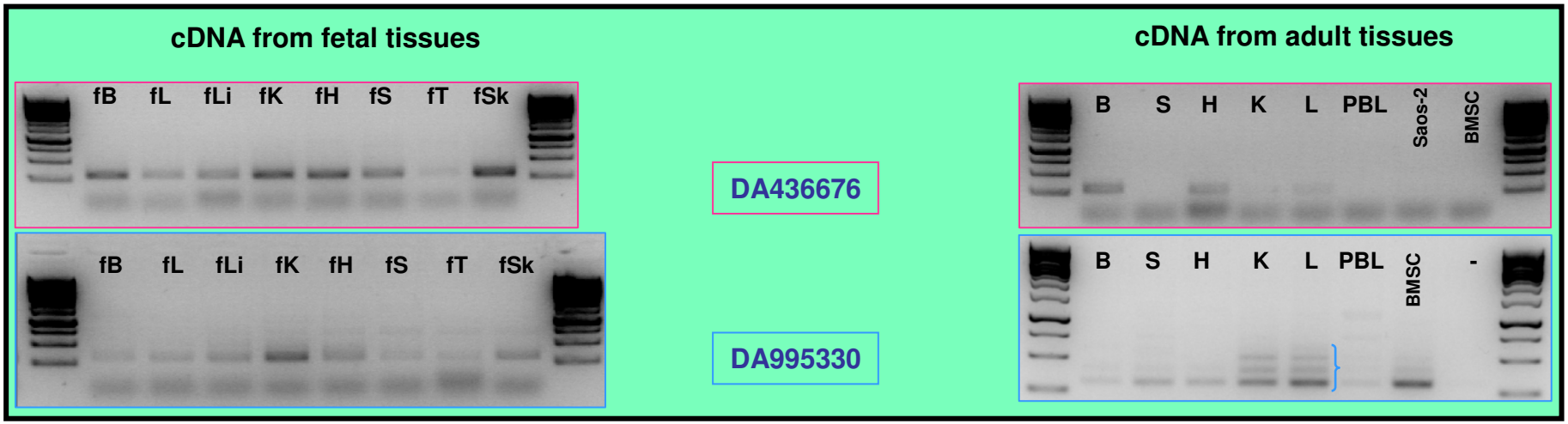


Characterization of the structure, composition and expression profile of the *ACVR1* transcripts (III)



✓ We could obtain specific RT-PCR products also for isoforms corresponding to DA436676 and DA995330 ESTs.

✓ We found that DA995330 EST is also subjected to alternative splicing



ACVR1 gene shows several transcripts due to alternative splicing combining different 5'UTR sequences to the common protein coding region.

These alternative isoforms are apparently less expressed compared to the main transcript.

What is the role of these different 5'UTR sequences?

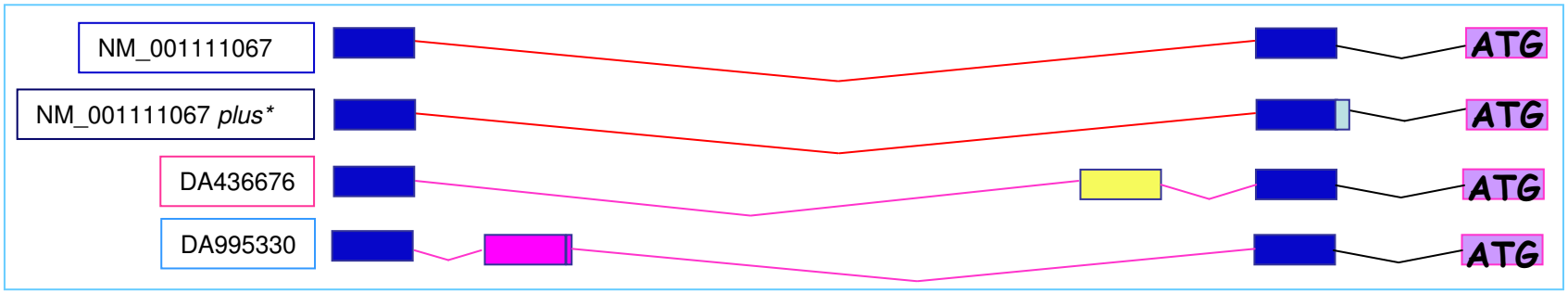
✓5'UTR regions can be involved in regulating gene expression at post transcriptional level by different mechanisms, for example, by forcing mRNA in a stable stem-loop secondary structure that may prevent an efficient scanning of the ribosome along the 5'UTR in search for the primary ATG start codon.

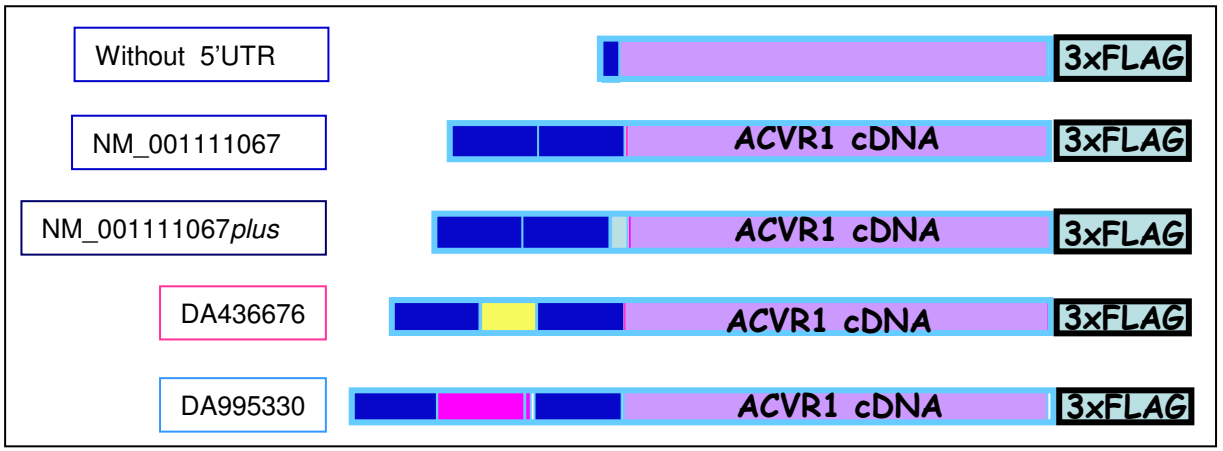
✓5'UTRs may affect the mRNA stability.

Full-lenght ACVR1 cDNA carrying the different 5'UTR sequences were subcloned in the pCMV3-Tag8 expression vector in frame with a 3xFLAg epitope.

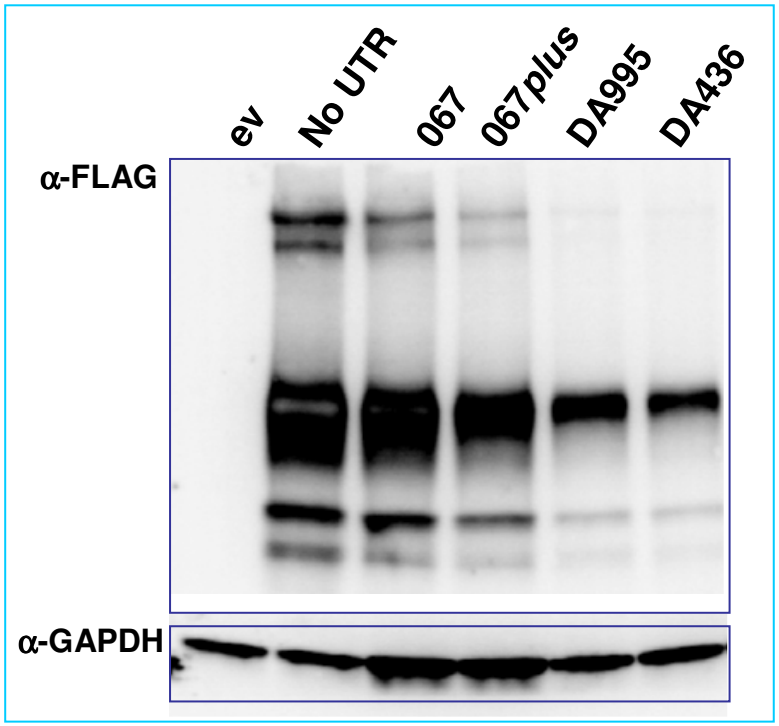
Constructs were transiently transfected in U2OS cells together with a pRL expression vector for Renilla luciferase as transfection control. After 48 h cells were lysed and analysed by western blot.

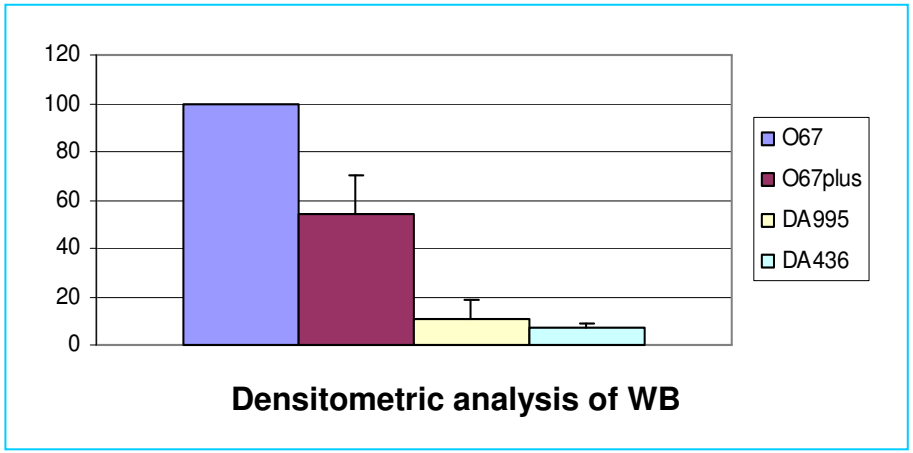
ACVR1 protein expression was normalized to GAPDH levels (as loading control) and to Renilla firefly activity (as transfection efficiency control).

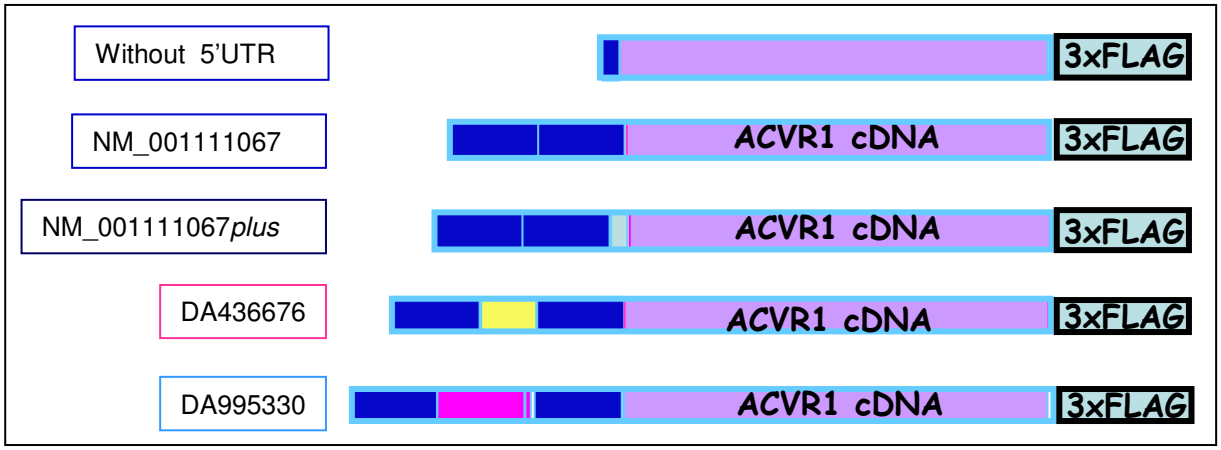




ACVR1 encoded by cDNAs carrying the different 5'UTR sequences are differently expressed







✓We have characterized the structure and composition of *ACVR1* transcripts that show a complex organization at the 5'UTR end of the gene.

✓We have identified a transcription start site common to all isoforms experimentally verified

✓The role of these sequences has to be clarified, they may intervene in negative modulation of *ACVR1* expression at post transcriptional level (upon specific stimuli?) as suggested by the different expression of the alternative constructs in transfection experiments.

✓Identification of this transcription start site gave us the opportunity to characterize the ACVR1 promoter region.

Study of the regulation of ACVR1 expression

▪Characterization of the structure and composition of the *ACVR1* transcripts.

▪Definition and characterization of sequences playing a role in the regulation of *ACVR1* expression.

✓ **5'*UTR* sequences**

✓ **Promoter region**

3'*UTR* region

Generation of a cellular model suitable for the high through-put screening of small molecules with potential pharmacological effect on the BMP/ACVR1 mediated pathway.

Identification of the *ACVR1* promoter

- **The *ACVR1* promoter has not been identified yet.**

- **To this aim we have selected and isolated a genomic region of around 2.8 kb mapping upstream the verified transcription start site.**

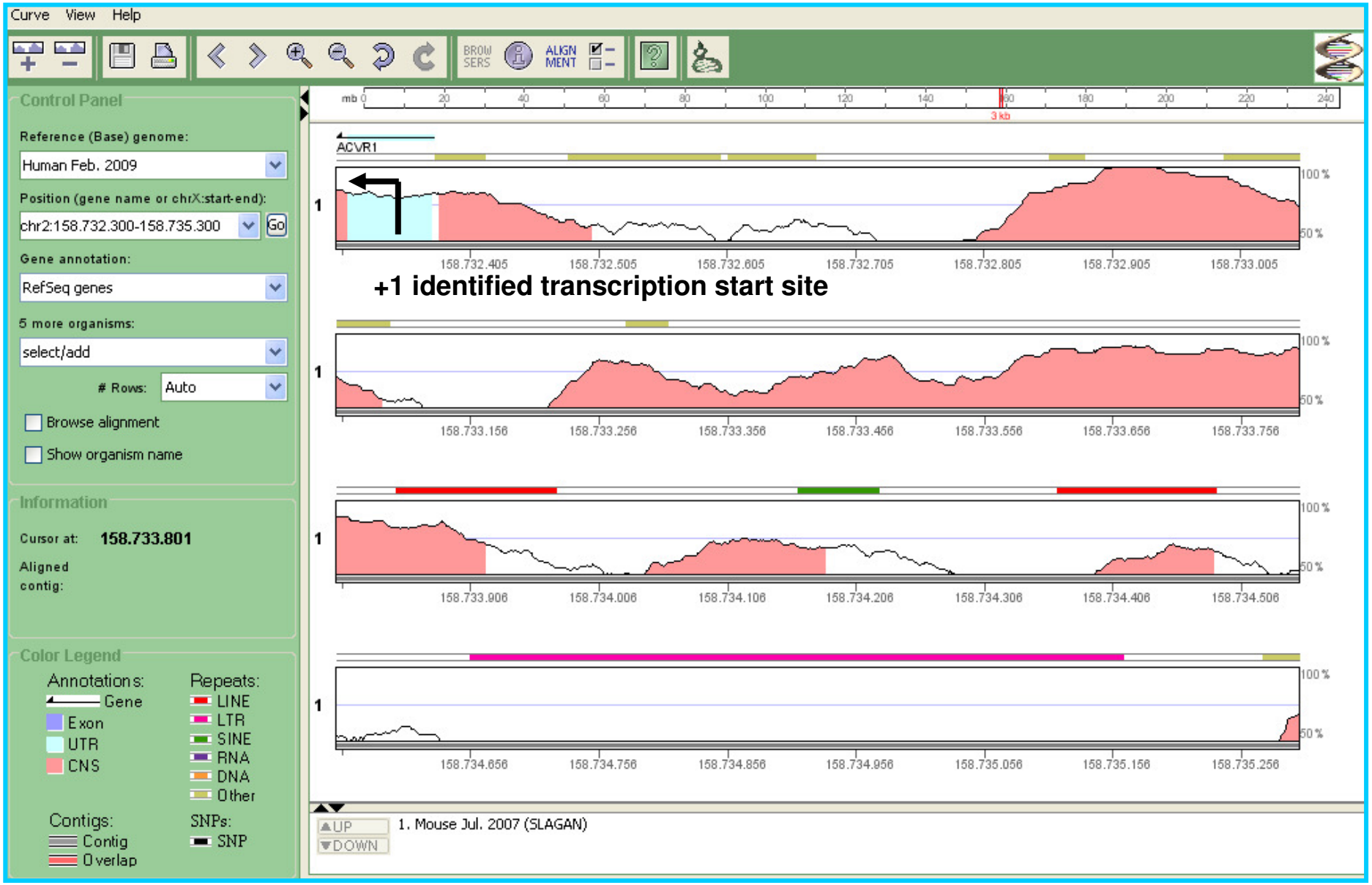
- **This region has 55% of GC nucleotides clustered in long stretches and predominantly mapping in the proximal 1 kb segment.**

- **No TATA –boxes are present.**

- **Several binding sites for different transcription factors are predicted by the *in silico* analysis (Sp1, SMAD, KLF...).**

- **The region appeared to be well conserved in mouse.**

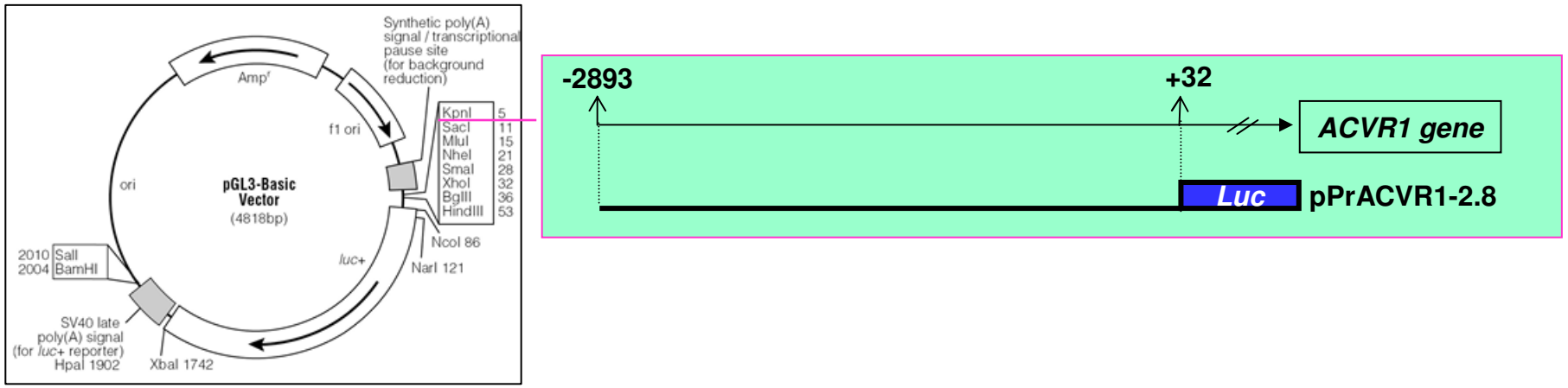
Vista Genome browser tracks comparing human _versus_ mouse genome of the selected 2.8 kb upstream the _ACVR1_ transcription start site and harbouring different well conserved sequences (red peaks)



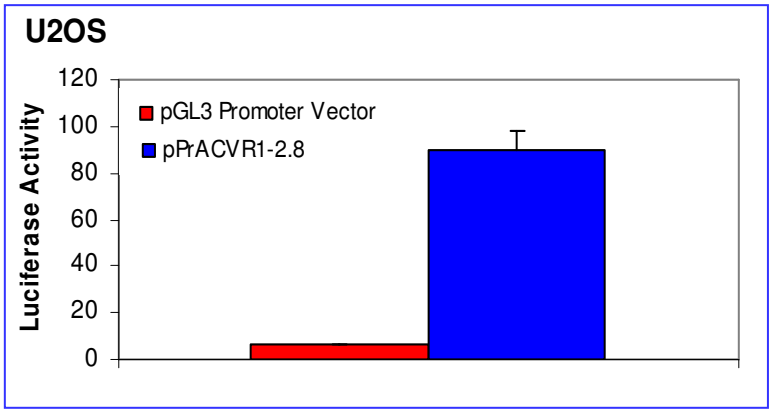
Functional study of *ACVR1* promoter

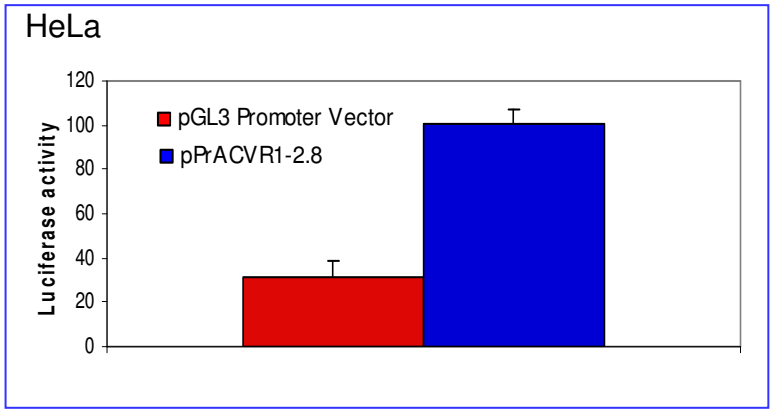
✓**Subcloning of the 2.8 kb fragment in a luciferase expression vector (pGL3- basic vector).**

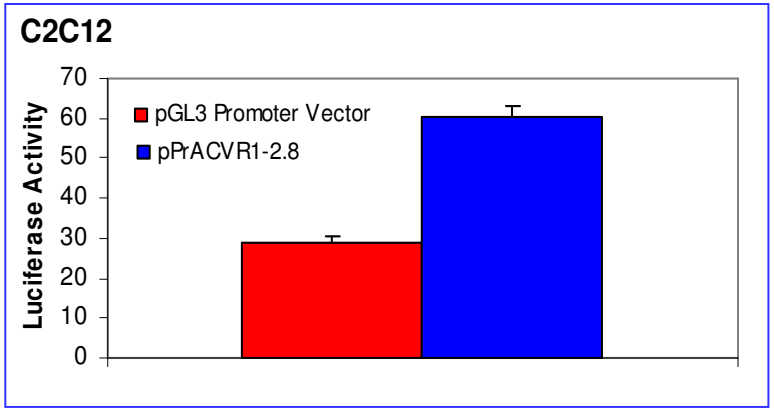
✓**Transfection in different cell lines and evaluation of the Luciferase activity.**



The selected fragment showed a strong promoter activity in different cell lines.

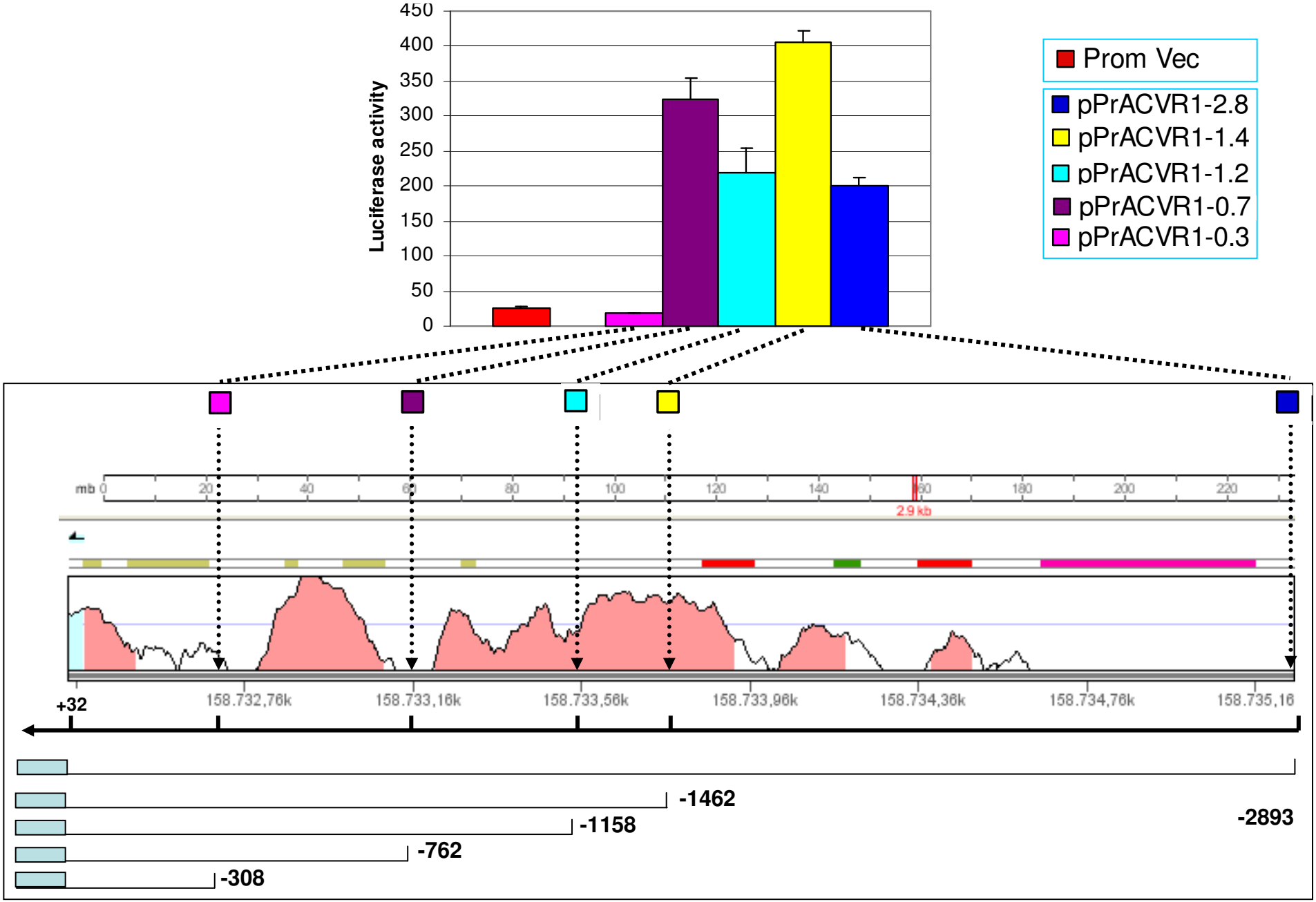




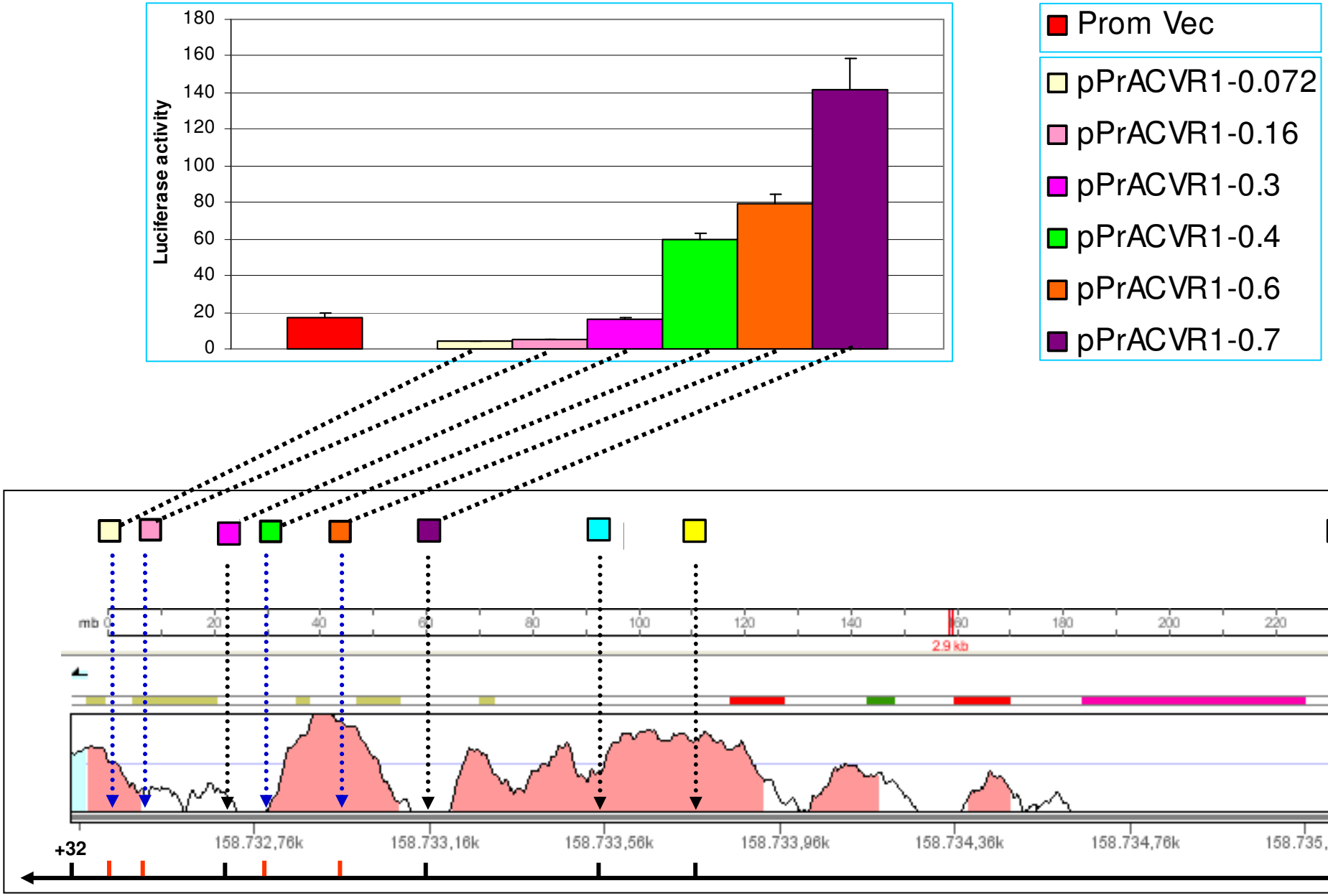


We decided to continue the analysis by generating different deletion constructs to be used in further transfection experiments.

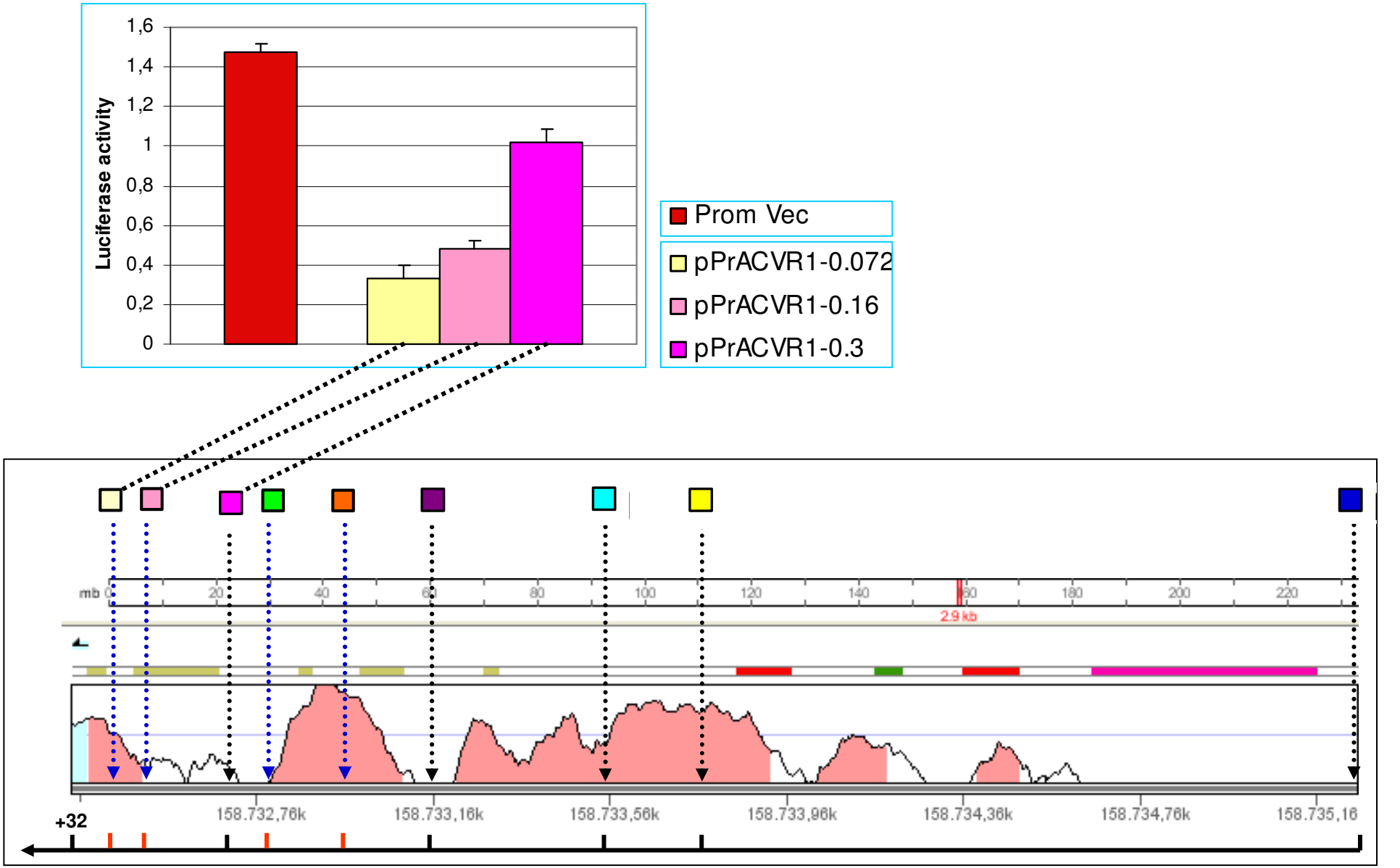
Functional analysis of ACVR1 promoter deletion constructs (I)



Functional analysis of ACVR1 promoter deletion constructs (II)



Functional analysis of ACVR1 promoter deletion constructs (III)

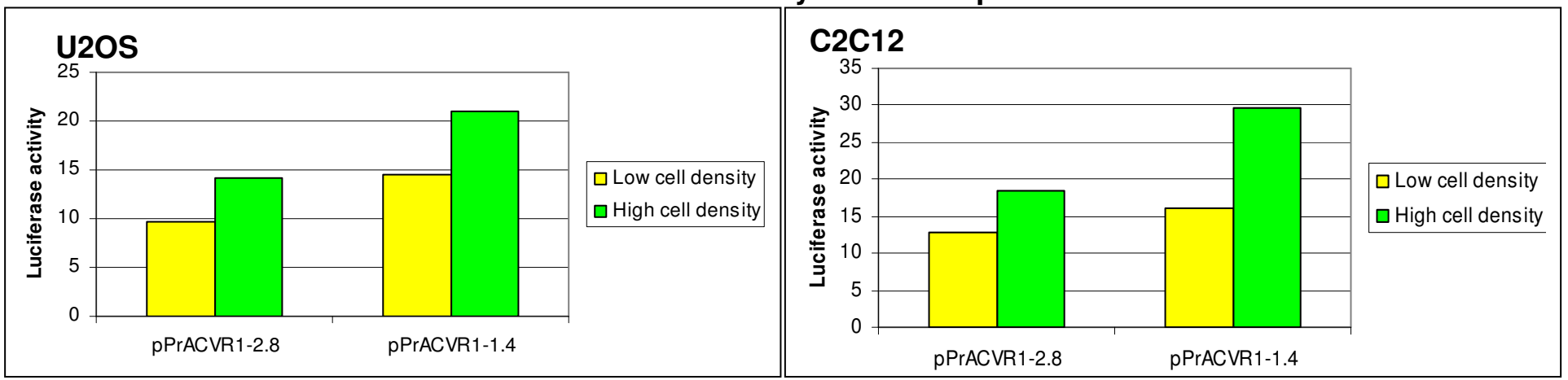


✓**We have characterized a 2.8 kb genomic region with strong promoter activity upstream the identified transcription start site.**

✓**Sequence from -308 to +1 still has a detectable promoter activity ranging from 20% to 60%, depending on the cell lines used, of the activity of a strong viral promoter (SV40) used as control**

✓The ACVR1 promoter activity controlled by the two longest fragments seems to be sensitive to cell culture conditions such as cell densitiy

Effect of the cell density on ACVR1 promoter



Rosi and FOP

Journal of Bone and Mineral Research
JBMR

Case Report

Rosiglitazone therapy is associated with major clinical improvements in a patient with fibrodysplasia ossificans progressiva

Davide Gatti, Ombretta Viapiana, Maurizio Rossini, Adami Silvano

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Issue

Journal of Bone and Mineral Research

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Rosiglitazone

Rosiglitazone (Rosi) belongs to the class of Thiazolidinediones (TZDs) and is an orally active hypoglycemic agent for the treatment of non-insulin-dependent diabetes mellitus or Type 2 Diabetes (T2D).

Rosi, as the other TZDs, is a known agonist of the Peroxisome Proliferator Activated Receptor γ (PPARγ) .

PPARγ is a ligand dependent transcription factor that belongs to the family of nuclear hormone receptors.

It is involved in several different physiological and pathological processes such as differentiation, inflammation, aging, obesity, infertility and cancer.

In particular, it is a key molecule for adipogenesis, and is important for glucose homeostasis and control of inflammation.

Is there a biological basis for treatment of Fibrodysplasia Ossificans Progressiva (FOP) with Rosiglitazone?

PPARγ shows two relevant features

(I) PPARγ is a potent anti-inflammatory molecule.

(II) PPARγ affects bone metabolism, inhibits osteogenesis by switching cell differentiation towards adipogenesis.

Rosi and ACVR1

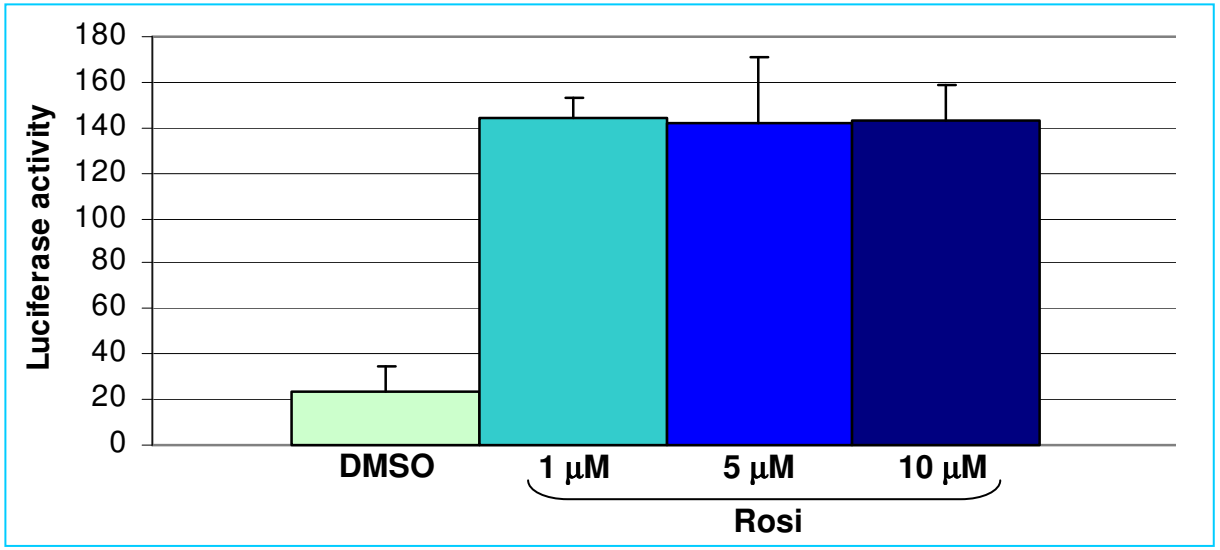
1) Does Rosi affect *ACVR1* gene espression?

2) Does it affect the ACVR1 mediated BMP pathway?

Set up of cell treatment with Rosi

•Transient transfection of an expression construct for PPARg (pSG5-PPARg) together with a Luciferase expression vector driven by a PPARγ responsive element (PPRE-Luc).

•Treatment of cells with different doses of Rosi for 24 h.



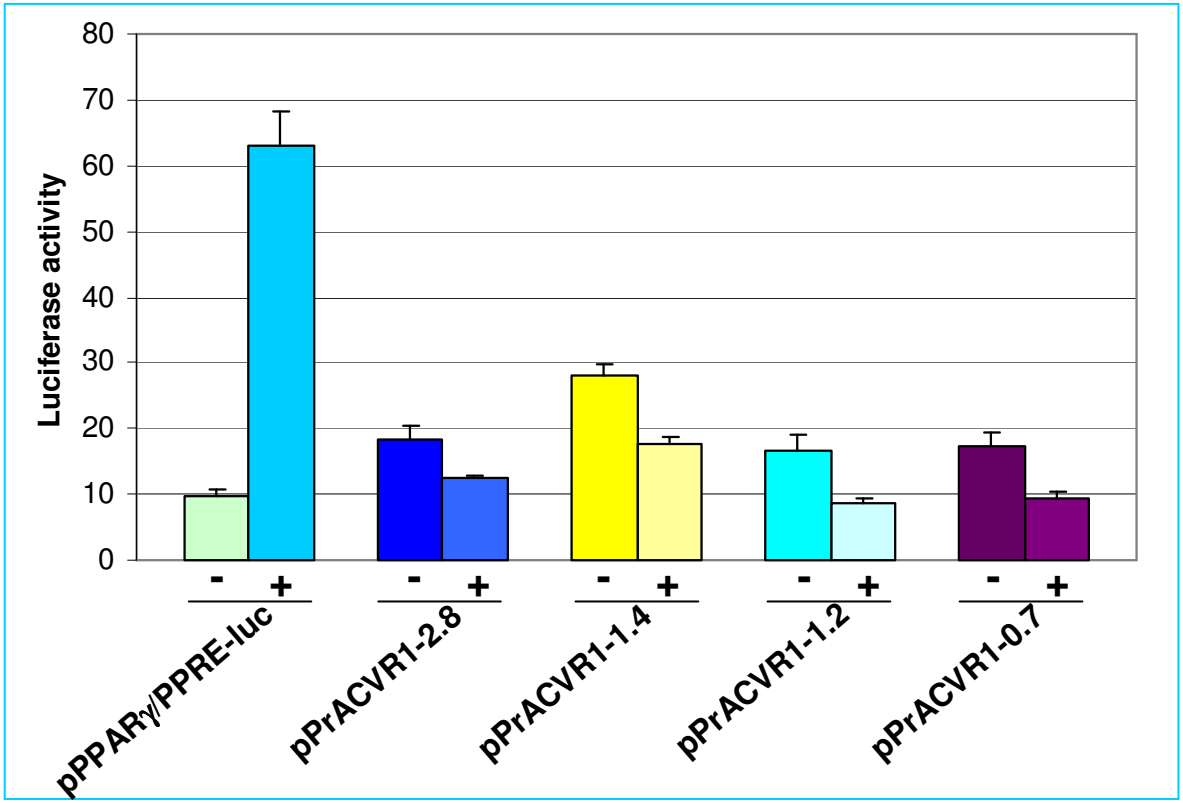
Rosi and ACVR1

✓Transient transfection of PPARg (pSG5-PPARg) expression vector with the Luciferase reporter construct carrying the different ACVR1 promoter fragments.

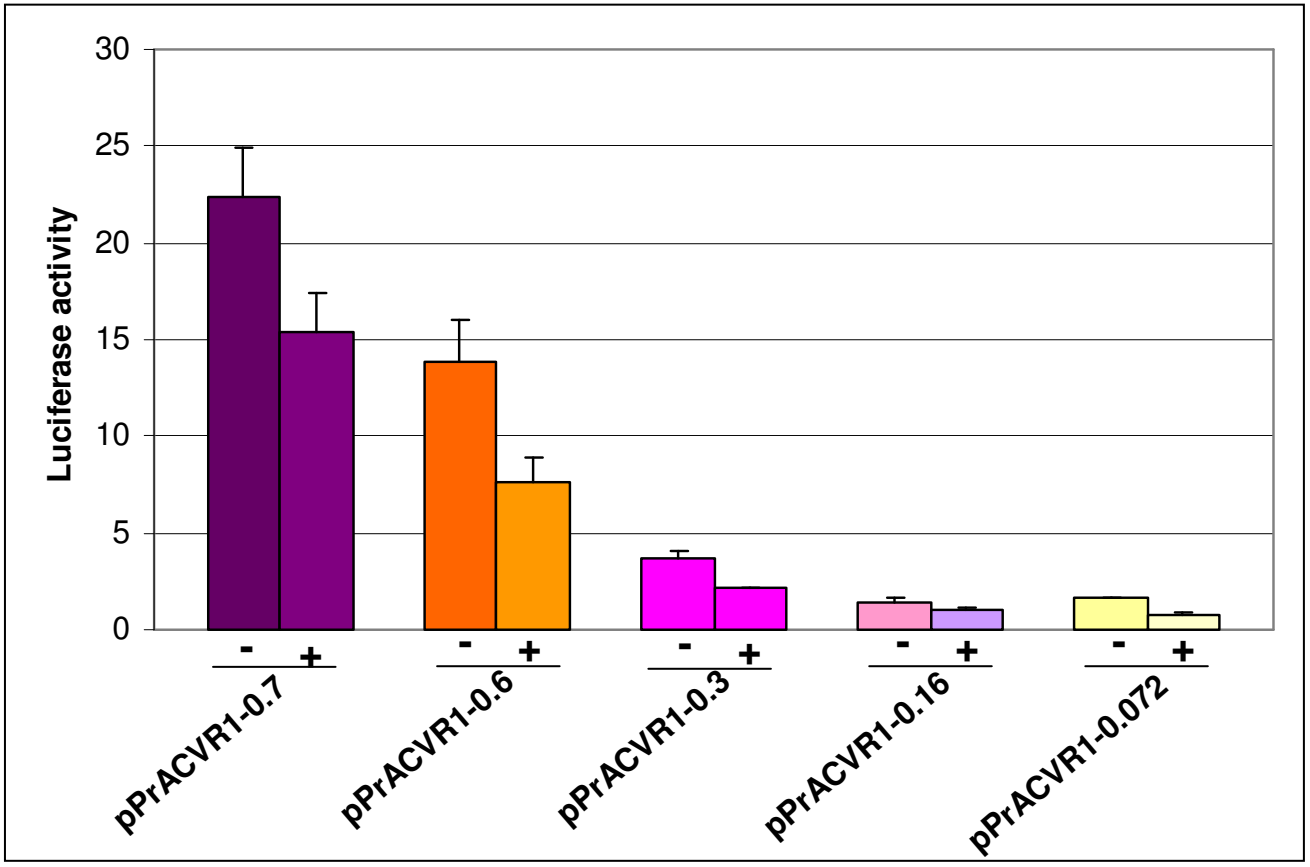
✓Treatment of transfected cells with 1 μM Rosi (+) or DMSO (-) for 24h.

✓Detection of the Luciferase activity.

Effect of Rosi treatment on ACVR1 promoter: fragment from – 2800 bp to -700 bp



Effect of Rosi treatment on ACVR1 promoter: fragment from – 700 bp to -72 bp



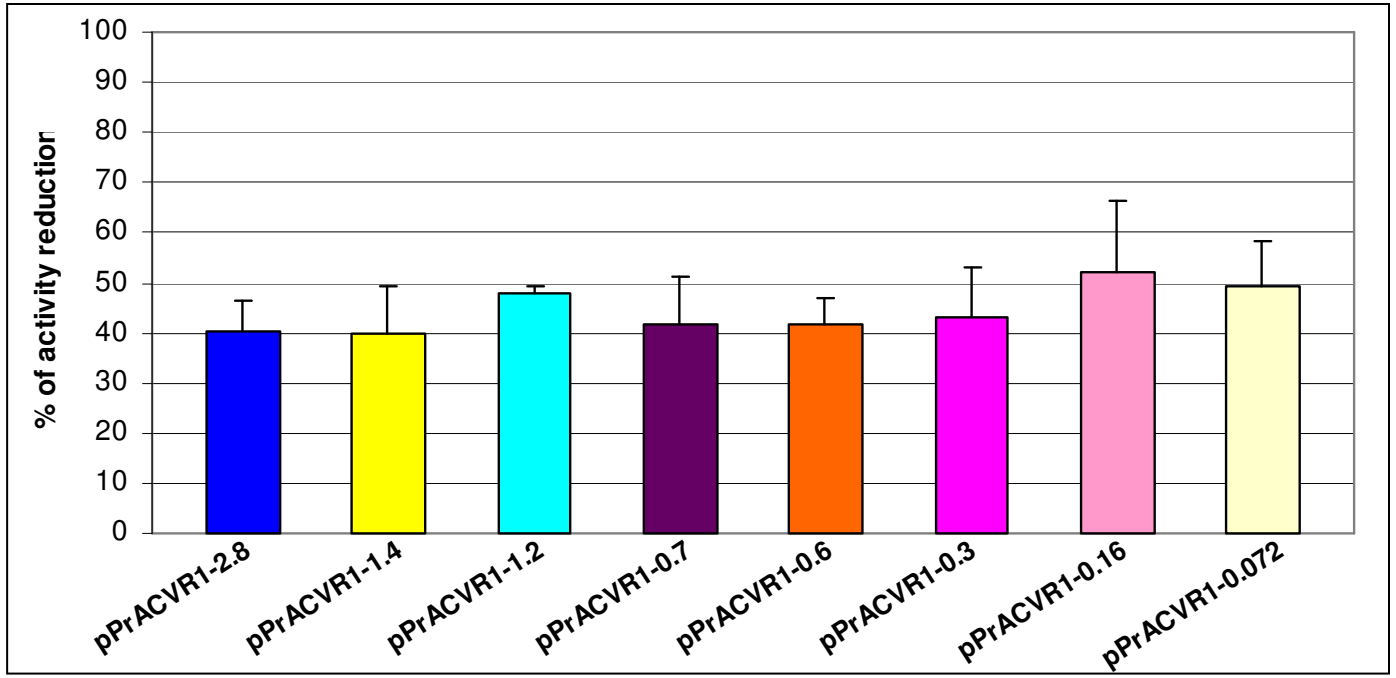
Rosi treatment induces a reduction in ACVR1 promoter activity.

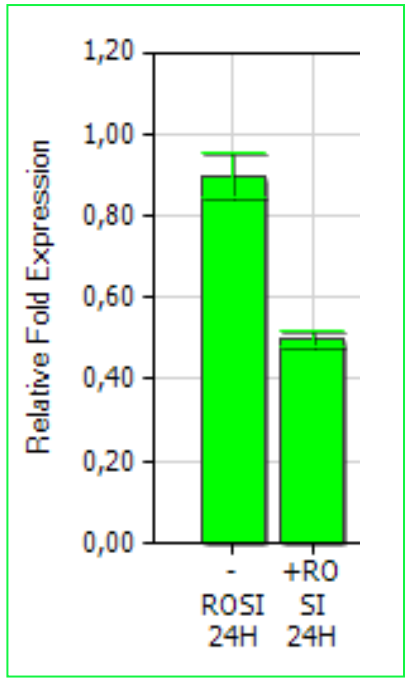
➢**Activity of all deletion fragments is inhibited of around 40% upon Rosi treatment. Inhibitory effect is maintained up to a region as short as 72 bp upstream of the transcription start site.**

(% of reduction is calculated from different independent experiments, respect to the activity obtained for each fragment in untreated cells considered as 100%).

➢**In accordance, cells transfected with PPARγ expression vector and treated with Rosi showed a reduction in the endogenous expression of ACVR1 gene**

(as assessed by qRT-PCR experiments).





PPARγ transcriptional activity, either positive (transactivating) or negative (transrepressing), is modulated both by the action of a ligand/agonist and by post-translational modifications.

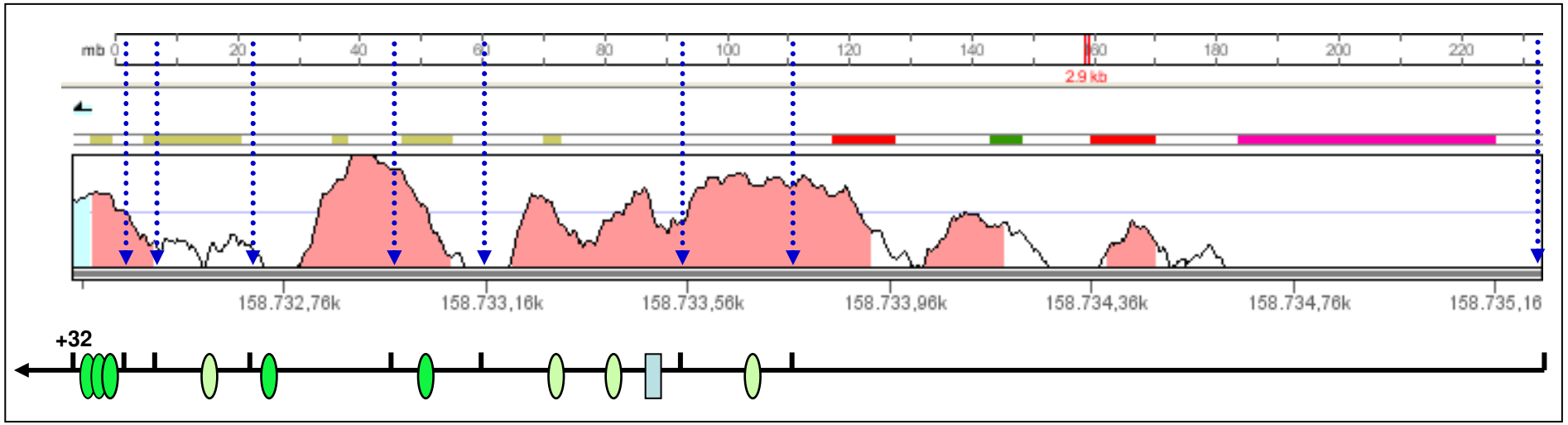
Direct transcriptional regulation of target genes is usually dependent on the obligate dimerization with RXR factors and DNA binding at specific sites.

However, *in silico* analysis of the ACVR1 promoter sequence has revealed the presence of a unique PPARγ/RXR binding site probably not involved in the ACVR1 responsiveness to Rosi.

Therefore, we postulated that the transcriptional regulation operated by Rosi/PPARγ might be mediated by interaction with others transactivating factor(s) recruited to the ACVR1 promoter and/or cis-regulatory element(s).

- ACVR1 promoter sequence has 55% of GC nucleotides clustered in long stretches and “GC- boxes”.

- *In silico* analysis predicted the presence of several binding sites for **Sp1** transcription factor.



Sp1 binding sites (1/1, core/matrix score)

Sp1 binding sites (1/>0.9, core/matrix score)

PPARg/RXR binding site

❖ Sp1 is a well known, ubiquitous nuclear transcription factor involved in multiple biological processes (control of cell growth, differentiation, apoptosis etc.).

❖ Sp1 binds to GC-rich sequences and can regulate the transcription of housekeeping and of TATA-less promoter genes.

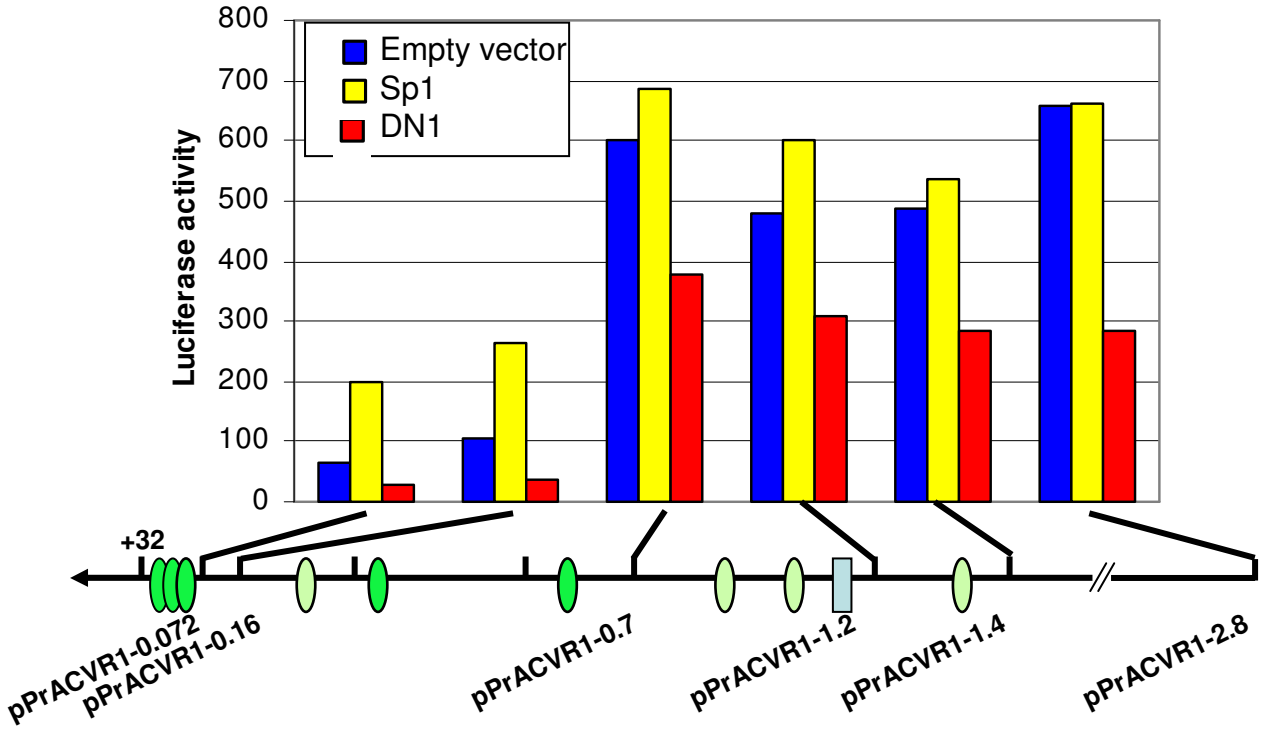
❖ Recent studies have shown that DNA-binding and transcription activity of Sp1 change in response to post-translational modifications induced by extracellular stimuli through a variety of signal transduction pathways.

❖ Moreover, several studies indicate that Sp1 can be involved in the transcriptional repression of target genes (resistin, follistatin, α1(I) collagen...) induced by TZDs.

❖ Indeed, TZD-activated PPARγ can be recruited to the promoter region of target genes through the interaction with Sp1 and thus can exert its repression activity also on those promoters lacking specific PPARγ/RXR binding sites.

Does Sp1 regulate the transcription of ACVR1 gene?

Co-tranfection of U2OS cells with reporter contructs for the ACVR1 promoter fragments with expression vector for wild-type Sp1 transcription factor (SP1) or an Sp1 dominant negative form with impaired transactivating function (DN1).



- **With longest constructs Sp1 overexpression shows small effect on ACVR1 transcription, while the effect of the DN Sp1 is well detectable.**
- **With shorter constructs both effects are more evident suggesting that Sp1 binding elements predicted in the proximal promoter sequence play a relevant role in ACVR1 transcriptional regulation.**

Conclusions

✓**We have characterized the structure and composition of *ACVR1* transcripts that show a complex organization at the 5'UTR end of the gene.**
These sequences may intervene in modulation of *ACVR1* expression at post transcriptional level and we are currently investigating in this direction.

✓**We have identified a transcription start site common to all isoforms experimentally verified**

✓**We have characterized a 2.8 kb genomic region with strong promoter activity upstream the identified transcription start site. Deletion constructs containing different fragments of the region show a strong activity in the proximal 700 bp. We have also defined a basal promoter region of 72 bp upstream the tss.**

✓**We have demonstrated that ACVR1 promoter activity is inhibited by Rosi treatment. Our preliminary results suggest that Sp1 expression can be involved in ACVR1 promoter regulation.**

✓**Data from the literature indicate that TZD-activated PPARγ can mediate gene repression through cross-coupling with SP1 and...we are currently investigation also in this direction...**

Acknowledgements

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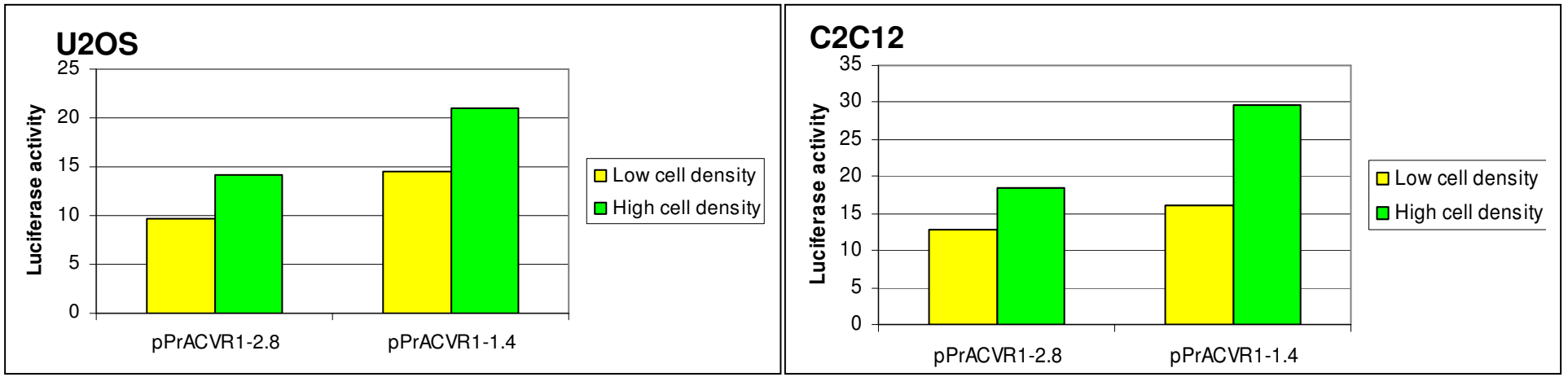
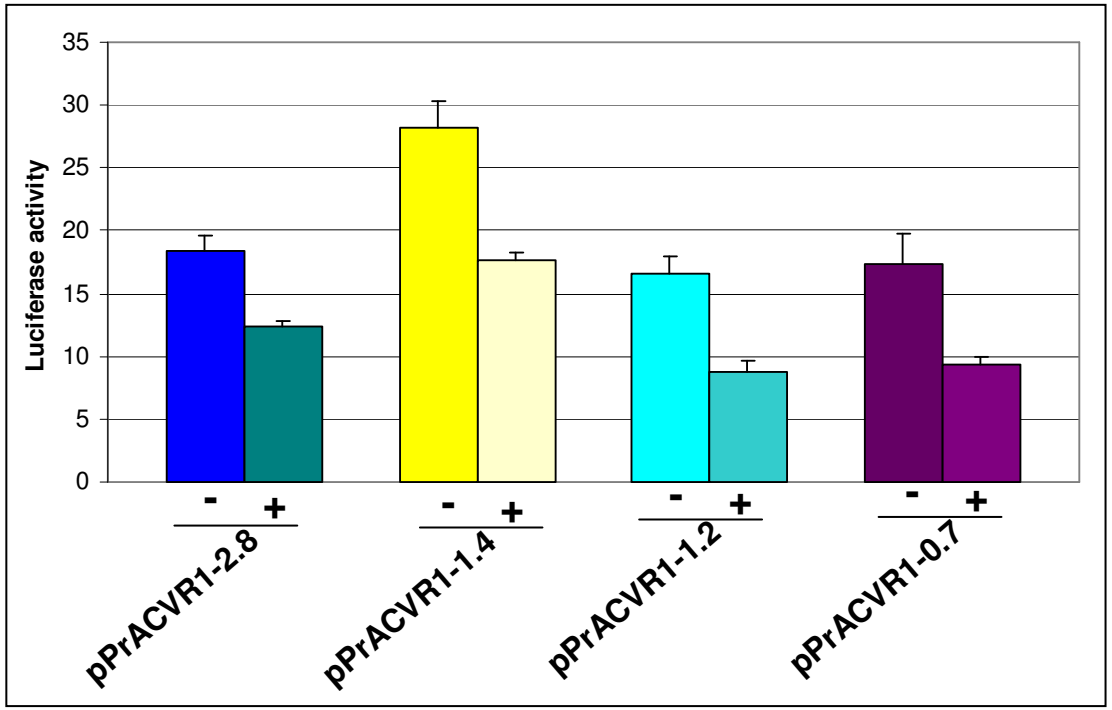
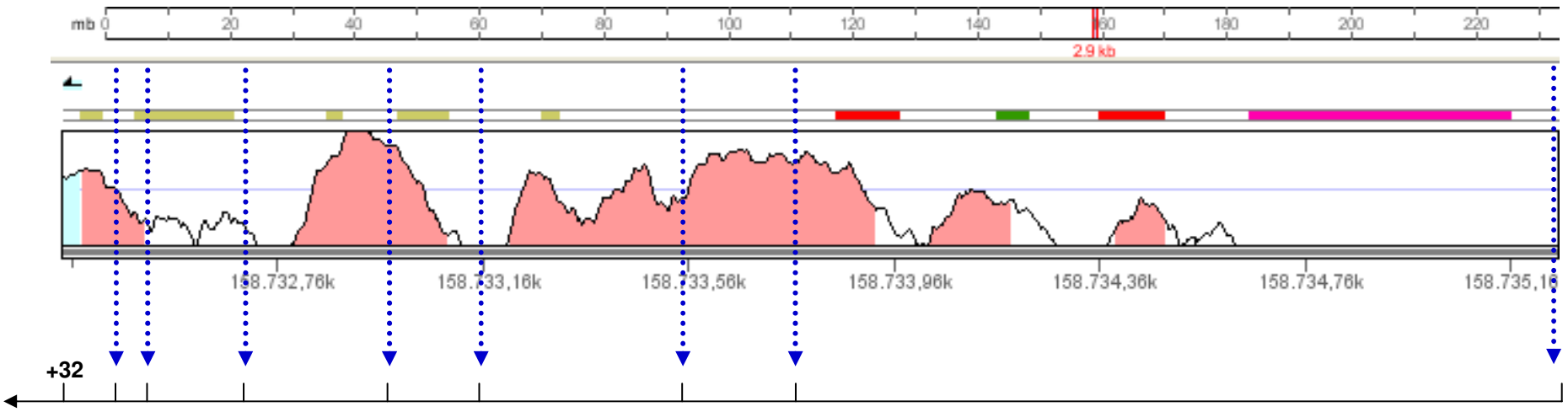
ACVR1 promoter activity is sensitive to cell culture conditions and to cell densitiy

Effect of the cell density on ACVR1 promoter




35
30
25
20
15
10
5
0
Luciferase activity
-
+
-
+
-
+
-
+
pPrACVR1-2.8
pPrACVR1-1.4
pPrACVR1-1.2
pPrACVR1-0.7



-2893 pPrACVR1-2.8

-1462 pPrACVR1-1.4

-1158 pPrACVR1-1.2

-762 pPrACVR1-0.7

-581 pPrACVR1-0.6

-308 pPrACVR1-0.3

-131 pPrACVR1-0.16

-72 pPrACVR1-0.72

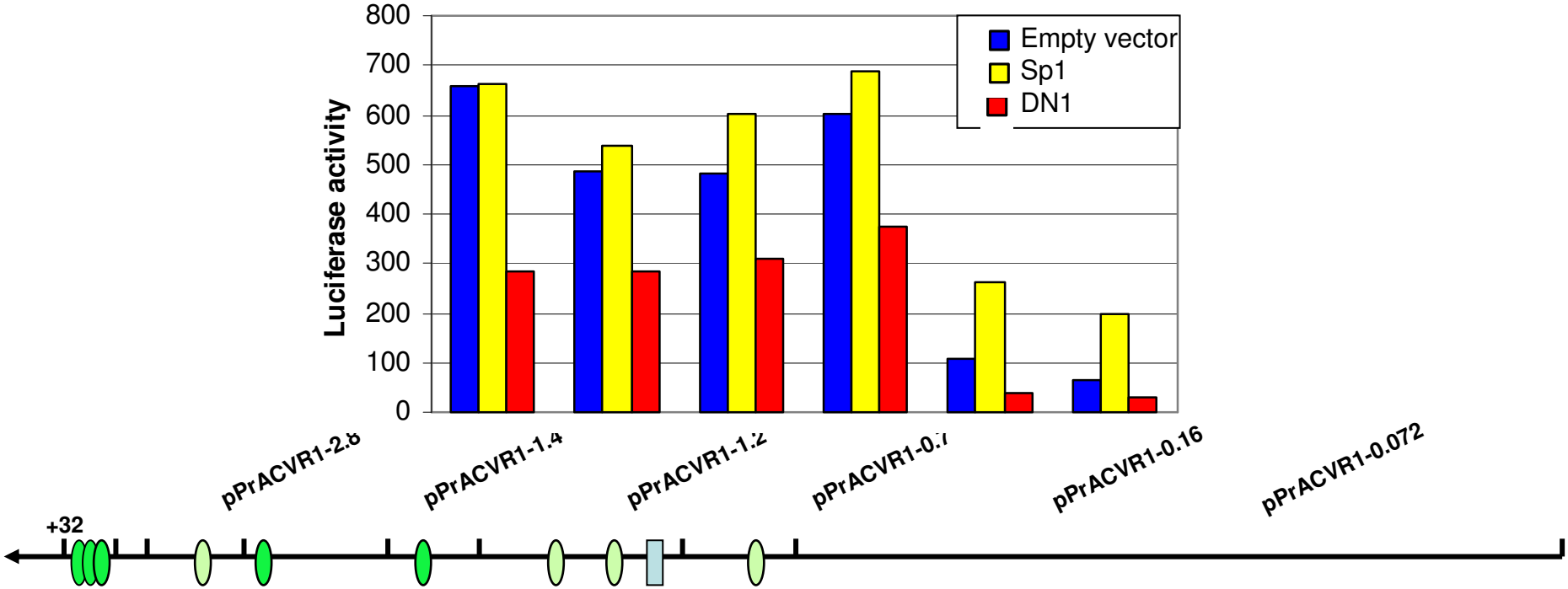
RXR

PPARg

SP1

Does Sp1 regulate the transcription of ACVR1 gene?

Co-tranfection of U2OS cells with reporter contructs for the ACVR1 promoter fragments with expression vector for wild-type Sp1 transcription factor (SP1) or an Sp1 dominant negative form with impaired transactivating function (DN1).



Our preliminary results suggest that Sp1 can regulate ACVR1 promoter transcription