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Ministero dell'Università e della Ricerca

Italiadomani
PIANO NAZIONALE DI RIPRESA E RESILIENZA

THE NEW AGE OF RECOMBINANT MINI PROTEINS: the example of ED-sRGN

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Ref. Scientifico: Prof.ssa Marzia Bianchi

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CRISPR-Cas System

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) – associated Cas is guided towards the target DNA sequence by a sgRNA generating a DSB (Double Strand Break)

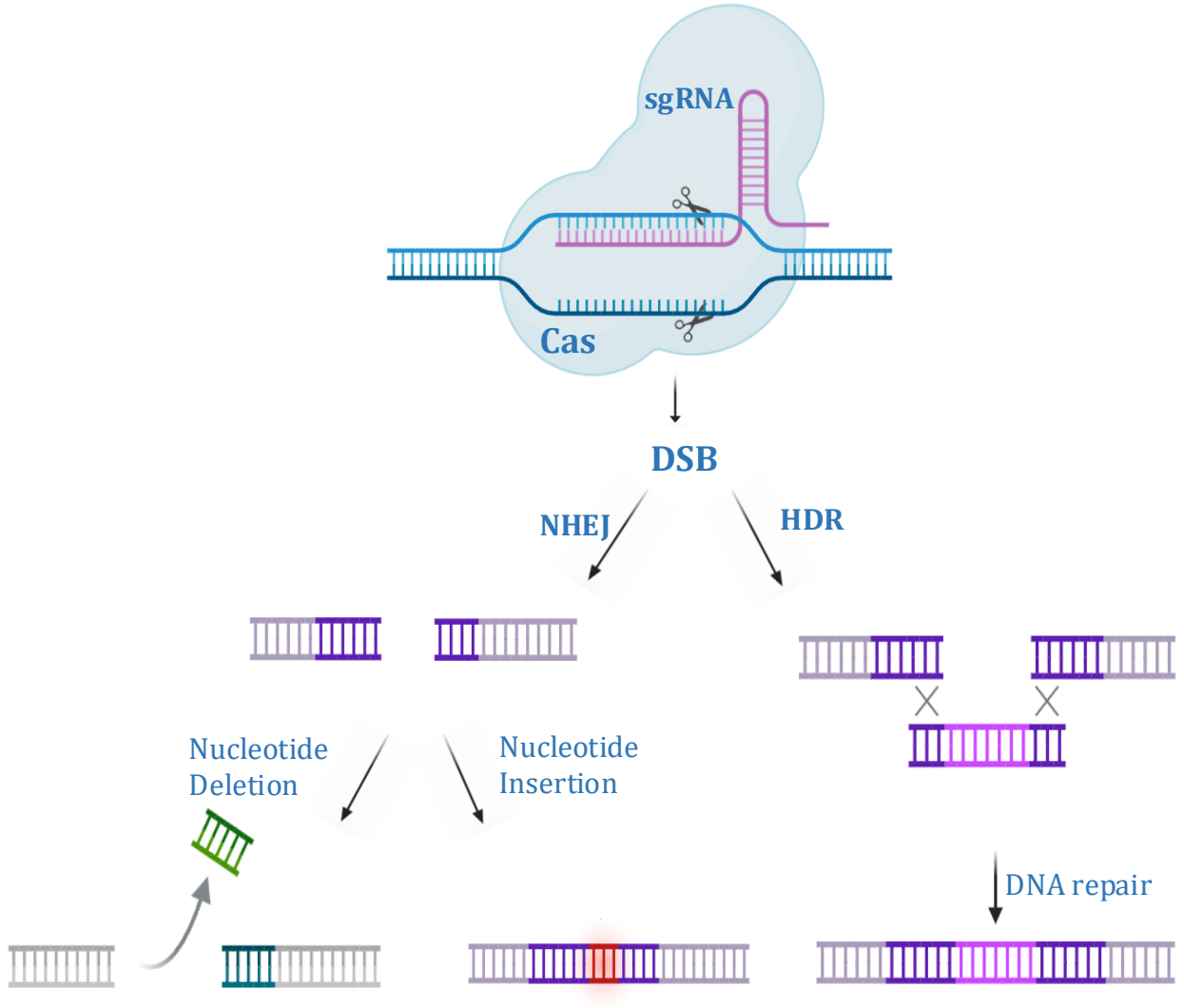
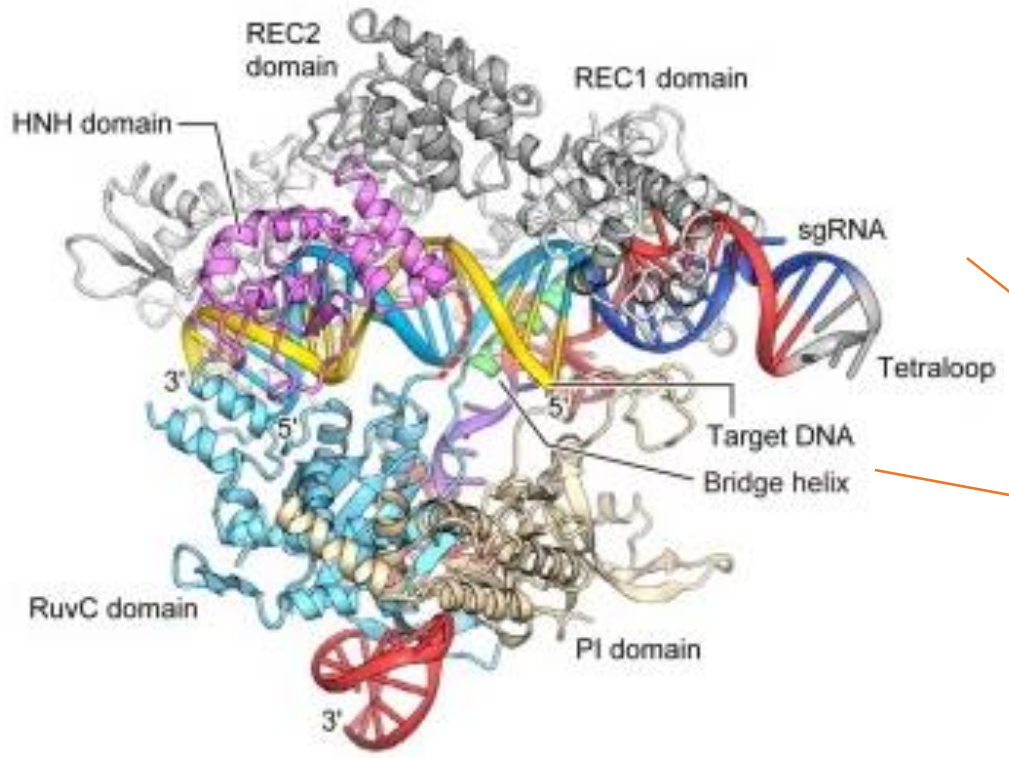


Fig 1. Representative image of the CRISPR-Cas system mechanism of action

SpCas9 *(Streptococcus pyogenes)* *[1368aa-160kDa]*



SpCas9 presents a bilobed structure:

- **Recognition lobe (REC)**
- **Nuclease lobe (NUC)**

The sgRNA-target DNA heteroduplex is placed in a positively charged groove at lobes interface

Fig 2. Structure representation of Cas9-sgRNA-DNA complex at 2.5 A resolution (Nishirnasu H. et al., 2014)

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ED-sRGN *(Enhanced Deletion-single RNA Guided Nuclease)*

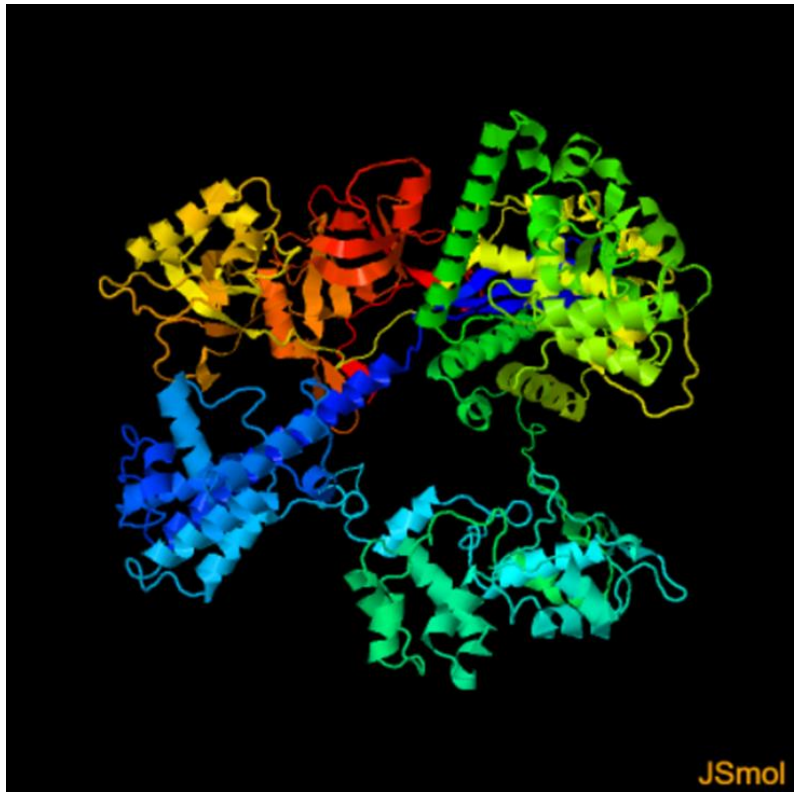

Fig 3. Predicted structure of ED-sRGN by Phyre2

sRGN
Mini-Cas9 [124.3kDa]

+

TREX2
Exonuclease protein [25.8kDa]

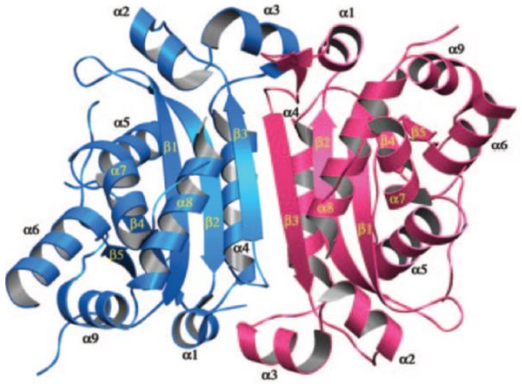

Fig 4. TREX2 structure (Perrino et al., 2005)

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In vitro Digestion Assay verified on agarose electrophoresis gel shows nuclease activity of bacterial ED-sRGN

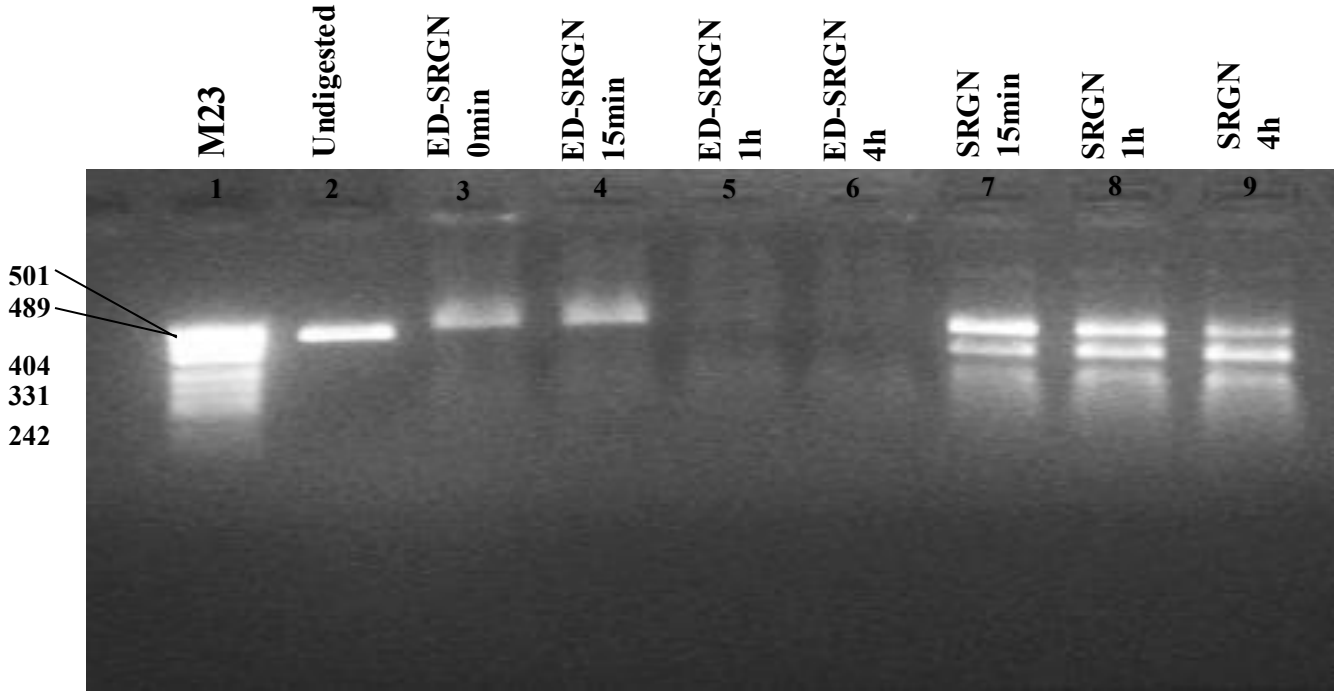

Fig 5. 2% (w/v) Agarose electrophoresis gel shows clear catalytic activity of ED-SRNG/gRNA and SRGN/gRNA

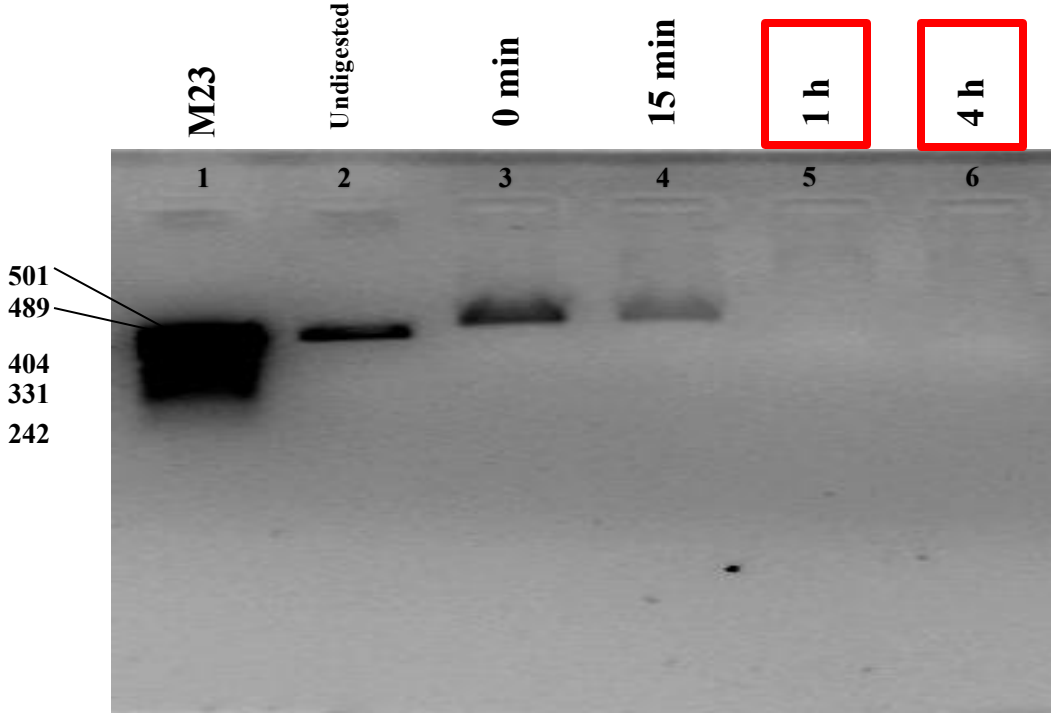

Fig 6. 2% (w/v) Agarose electrophoresis gel shows degradation product of target sequence by ED-SRGN when not complexed with specific gRNA

NO gRNA: unspecific bacterial nucleases!

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pKLV2 construct designed for protein expression in Eukaryotic cells

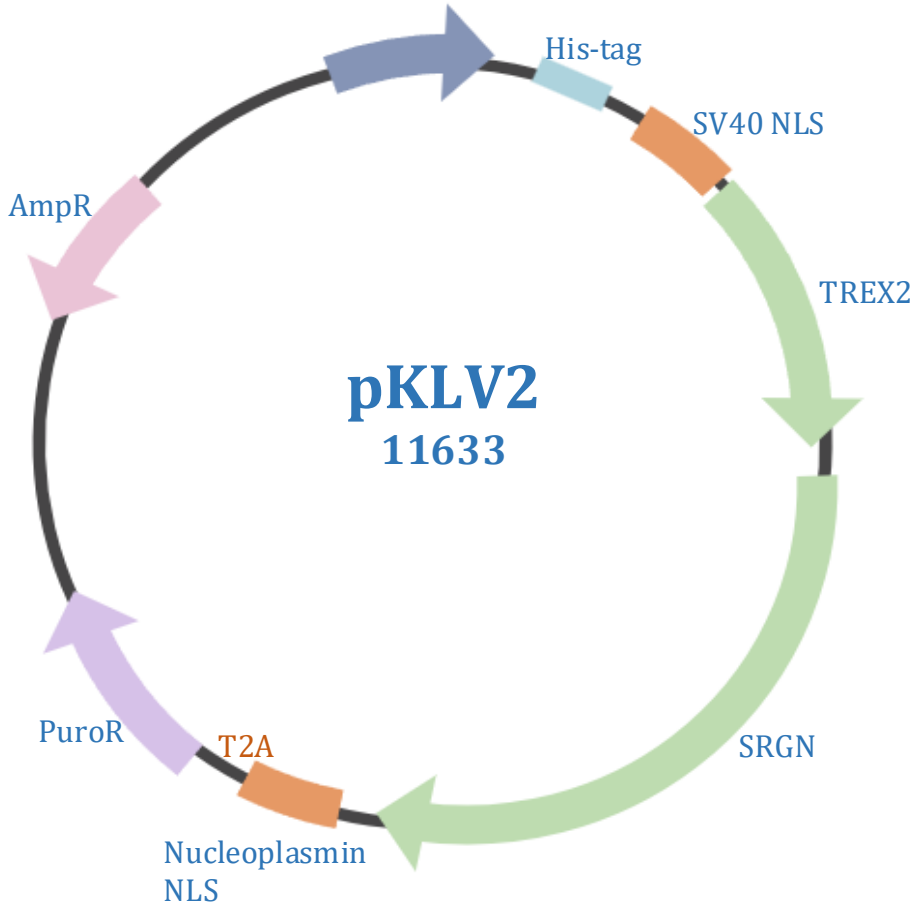

Fig 7. Representative image of the pKLV2 construct

pKLV2 Legend

SV40 NLS: 21bp

TREX2: 705bp

Linker 32aa: 96bp

SRGN: 3171bp

Nucleoplasmin NLS: 48bp

T2A: 54bp

PuroR: 597bp

AmpR: 861bp

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Thanks to:
Dr. Pietro de Angeli, Ph.D.

Stable and transient transfection procedure of the 3 Lentiviral construct

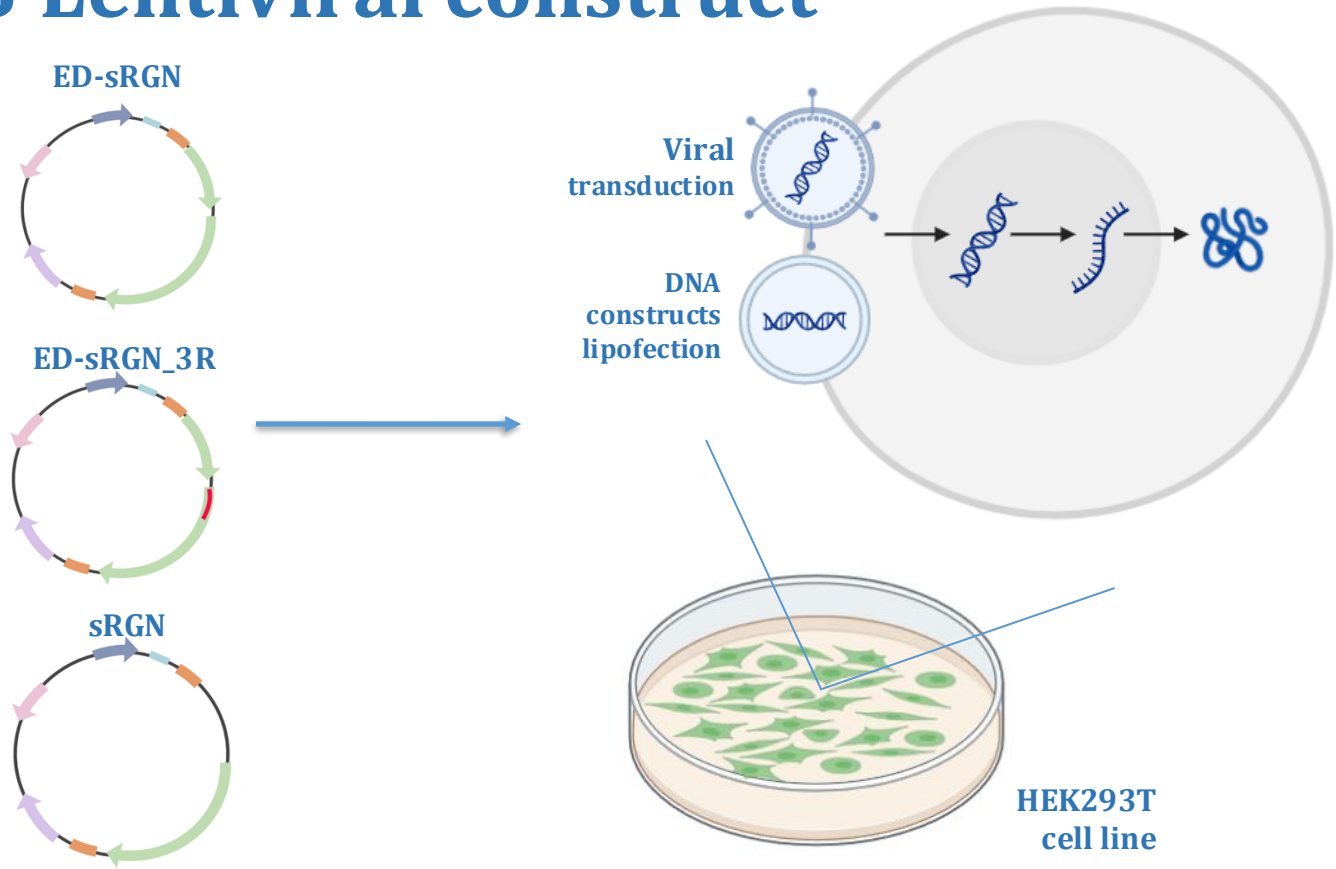

Fig 8. Workflow of the transfection procedure

GFP construct expression indicates a **positive** transfection outcome

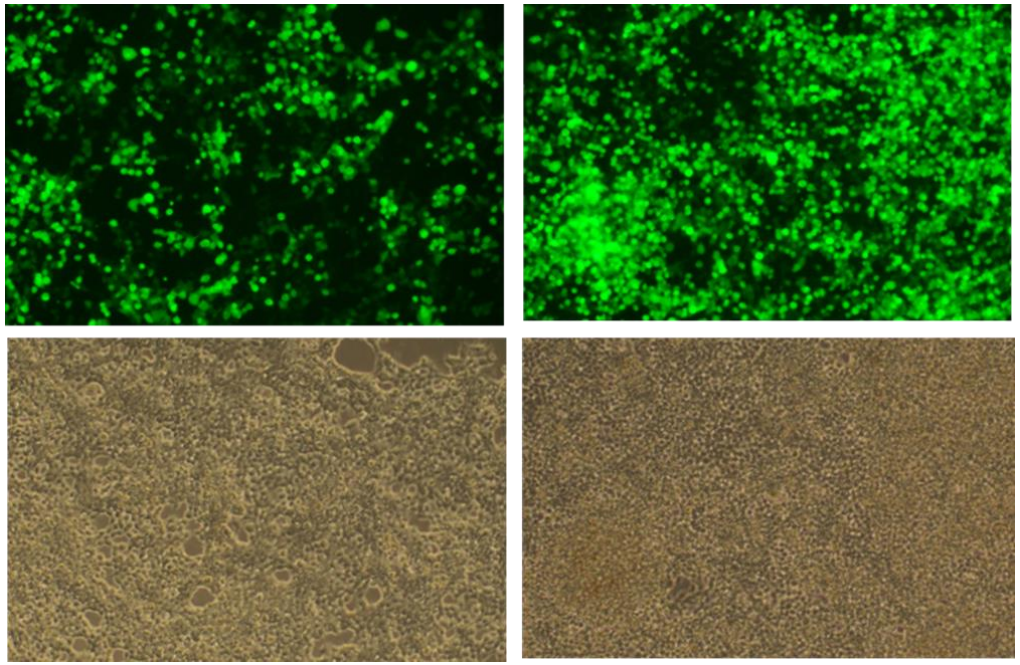

Fig 9. Acquired image with fluorescence microscopy of GFP-HEK293T *(10x)*

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WB analysis do not detect the presence of the recombinant protein

ED-SRGN
mw: **157,05 kDa** (1363aa)

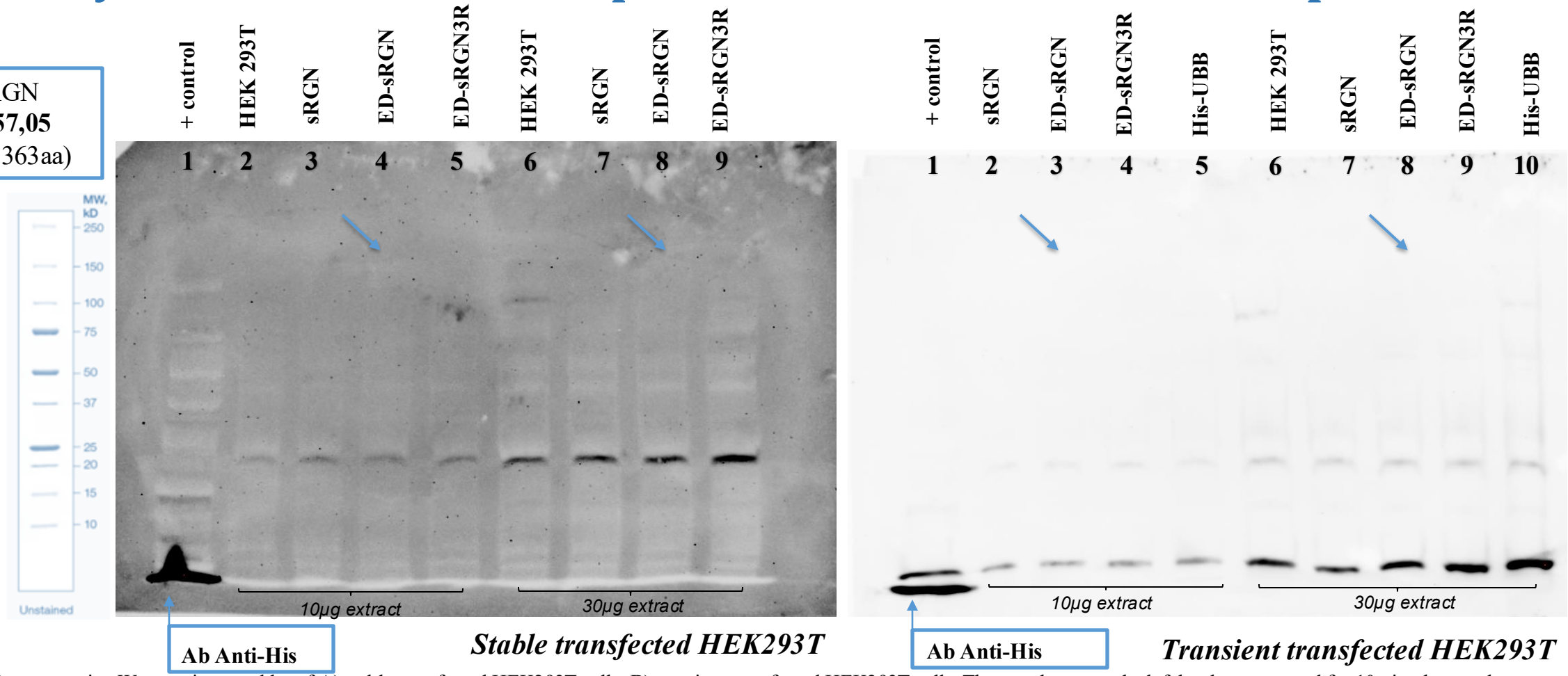

Fig 10. Representative Western-immunoblot of **A)** stable transfected HEK293T cells; **B)** transient transfected HEK293T cells. The membrane on the left has been exposed for 10min; the membrane on the right for 10sec. The blue arrows indicate the expected product band. *Marker: BioRad Precision Plus Unstained Protein Standard.*

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RT-qPCR Analysis validate the presence of ED-sRGN transcript

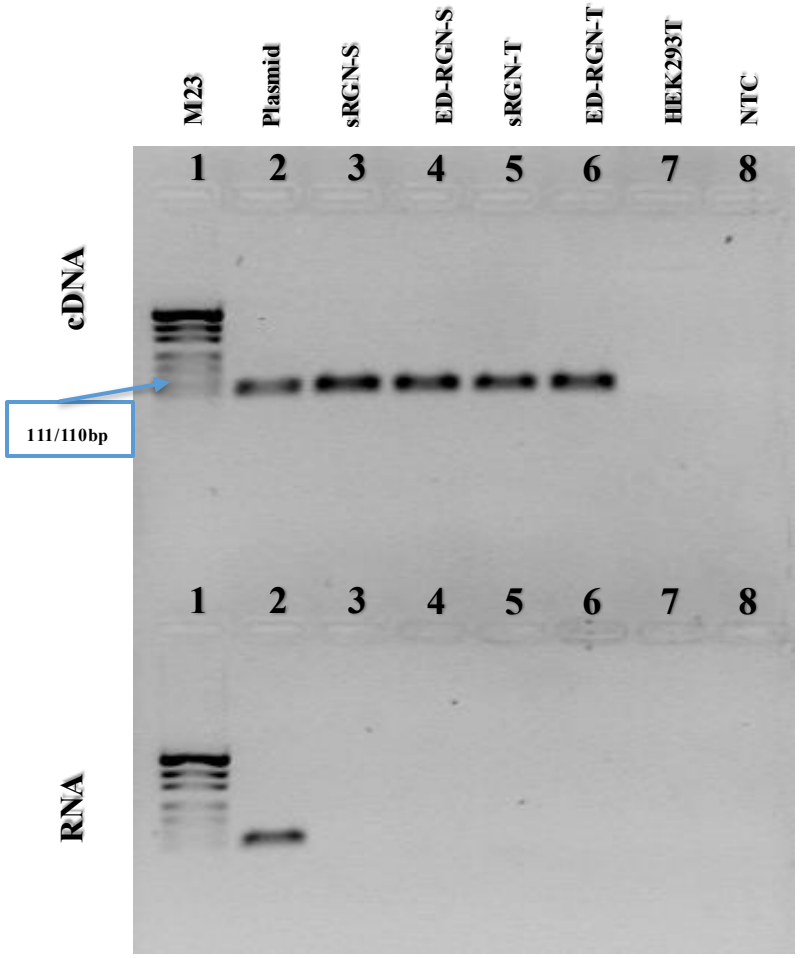

Fig 11. 2% (w/v) Agarose electrophoresis gel of RT-qPCR products

Sample name	Ct Value
HEK293T	**Undetermined**
sRGN-Stable	**27,24**
ED-sRGN-Stable	**23,13**
sRGN-Transient	**27,14**
ED-sRGN-Transient	**18,13**
ED-sRGN Plasmid	**29,34**

Tabella 1. RT-qPCR of post-DNase transfected sample

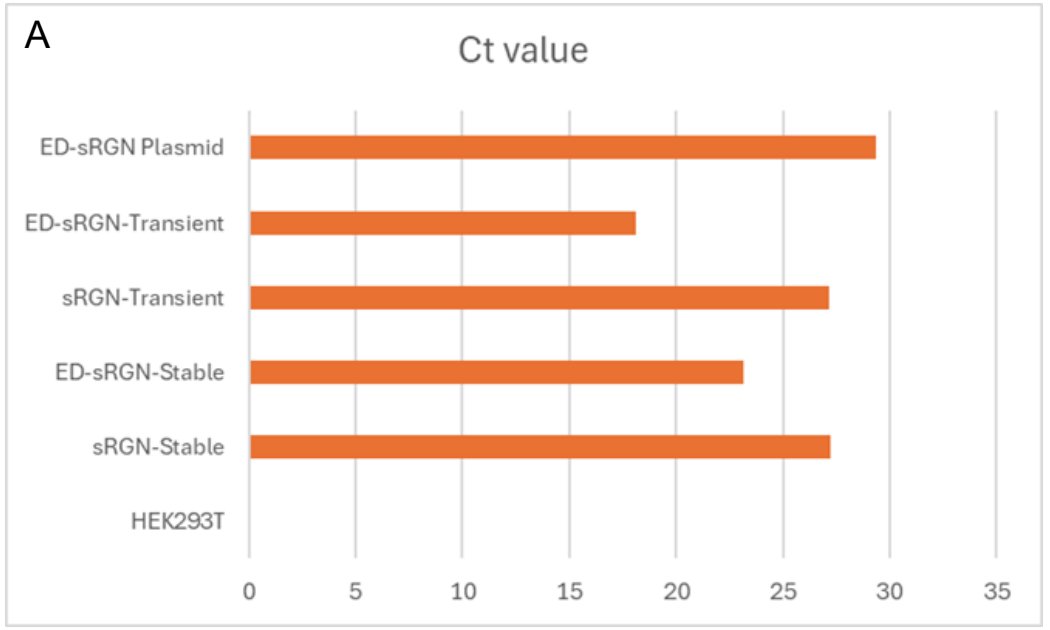

Fig 11. Ct value of post-DNase cDNA samples of transfected HEK293T cells

In-silico analysis

- Construct evaluation → Possible donor splicing site
- AA sequence study → Glycosylation prediction sites; Protein stability
- Literature overview

Full length product detected by PCR

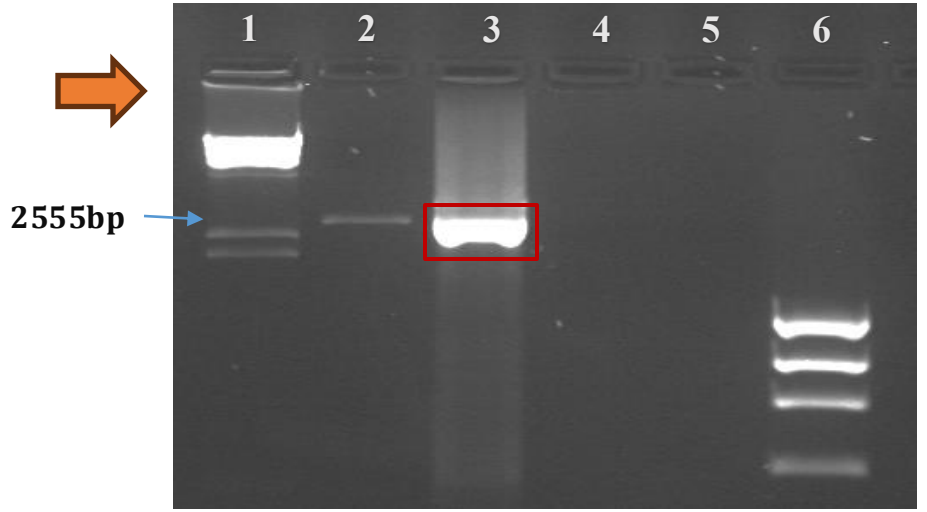

Fig 12. 1.3% (w/v) Agarose electrophoresis gel of the following PCR products: 1) MII; 2); Plasmid (+ control) 3) ED-SRGN-transient; 4) HEK293T (- control); 5) NTC; 6) MIX

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What's next?

- **Improving WB efficiency** → **New Ab Anti-TREX2**
- **New construct design**

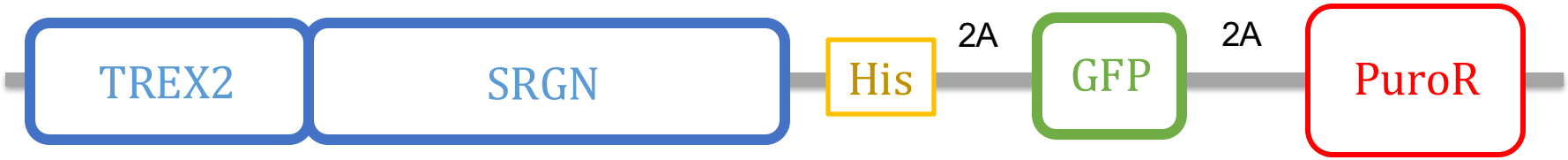

Fig 13. Schematic representation of the optimised pKLV2 construct

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Thanks to:
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In the meantime…

thanks for your attention!