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Doutor Ricardo Jorge

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HUMAN GENETICS
University of Oldenburg

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Short **t**erm **s**cientific **m**ission

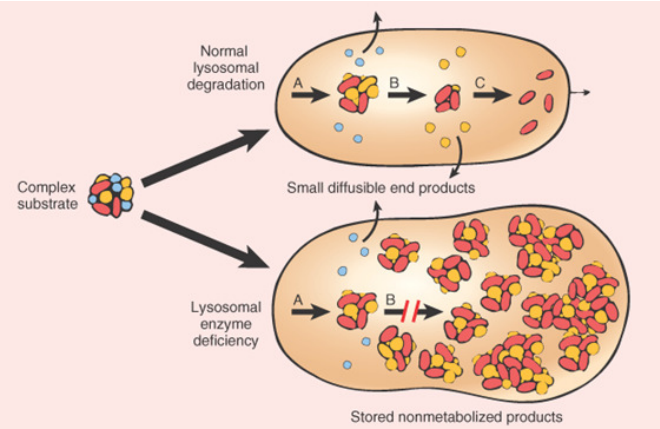
Development of antisense U1 snRNA-mediated therapeutic strategies to modulate splicing in lysosomal storage disorders

Liliana Matos

COST Action BM1207
Porto Meeting
22nd March 2017

Lysosomal Storage Disorders (LSDs)

- Large group of inherited metabolic disorders (>50)
- Autosomal recessive (majority)
- Individually rare
- Prevalence as a group is estimated in 1:4000 – 1:8000 live births
- The majority are caused by deficiencies in specific lysosomal enzymes



Progressive lysosomal accumulation of undegraded metabolites results:

- generalised cell and tissue dysfunction
- multi-systemic pathology

- Clinical presentation:

can vary

early and severe phenotype

late-onset mild disease

Reviewed in Filocamo and Morrone, 2011

Work in progress :

Project SPLICETHER from FCT (PTDC /BBB-BMD/6301/2014)

- Study of 2 different splice donor site mutations

found → 2 patients diagnosed with different LSDs

Patient	Allele 1	Allele 2	Location
Patient 1 (MPS I)	**c.1650+5G>A**	c.1205G>A (p.W402X)	**Intron 11**/Exon 9
Patient 2 (ML III α/β)	**c.3335+6T>G**	c.2864C>T (p.A955V)	**Intron 17**/Exon 14

⇘ Splicing mutations are frequent in these pathologies (10 to ~16% of the mutations described)

5' donor site mutations { ~64% MPS I, ~47% ML III α/β } → Good targets for mutation specific U1 snRNA therapeutic approaches

Main aim:

☞ To correct the pathogenic effect of both splice donor site mutations through U1 snRNA-mediated therapeutic strategies

Mucopolysaccharidosis I
(Hurler; Hurler-Scheie; Scheie syndromes)

- Autossomal recessive disorder
- Caused by mutations in *IDUA* gene (14 exons)

α-L-Iduronidase

Responsible for the degradation of dermatan and heparan sulfate

Therapy:

- Enzyme replacement therapy – laronidase (Aldurazyme®)
- Hematopoietic stem cell transplantation

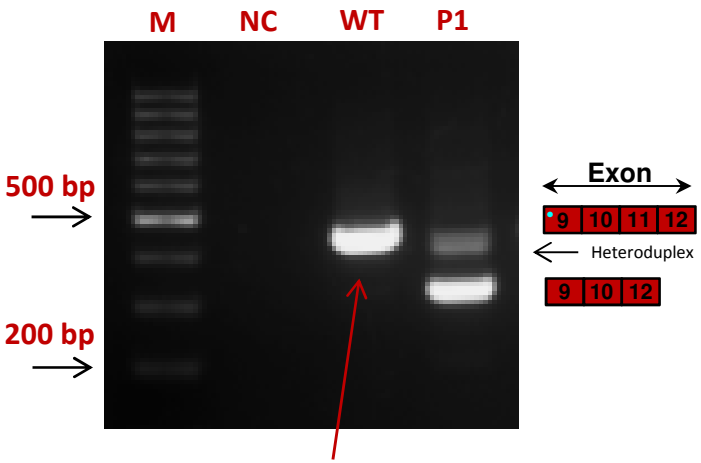
Still with some limitations → Development of alternative or adjunct therapies would be important

Mucopolysaccharidosis I

☞ ***IDUA* gene mutation analysis (RT-PCR)**

c.1650+5G>A/p.W402X

Primers for exon 9 and 12:



- Normal length transcript

Patient 1: two transcripts

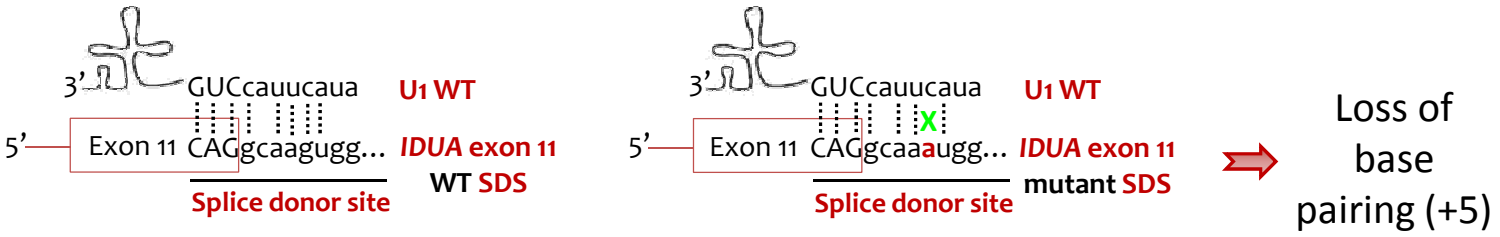
- One with normal length arising from the nonsense allele

- One with exon 11 skipping due to the donor site mutation

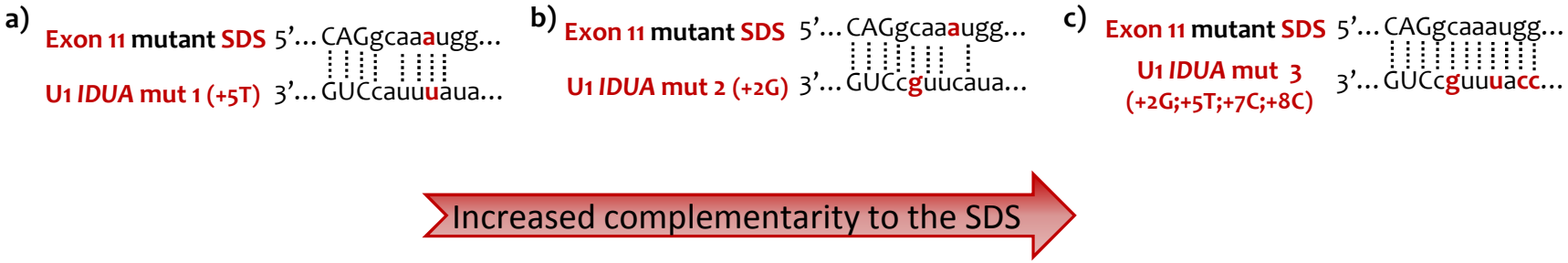
Mucopolysaccharidosis I

☞ Splicing therapeutic approach for the +5 *IDUA* gene mutation with modified U1 vectors

1. Analysis of *IDUA* exon 11 splice donor sites (SDS)



2. Design and construction of U1 modified vectors (pG3U1)

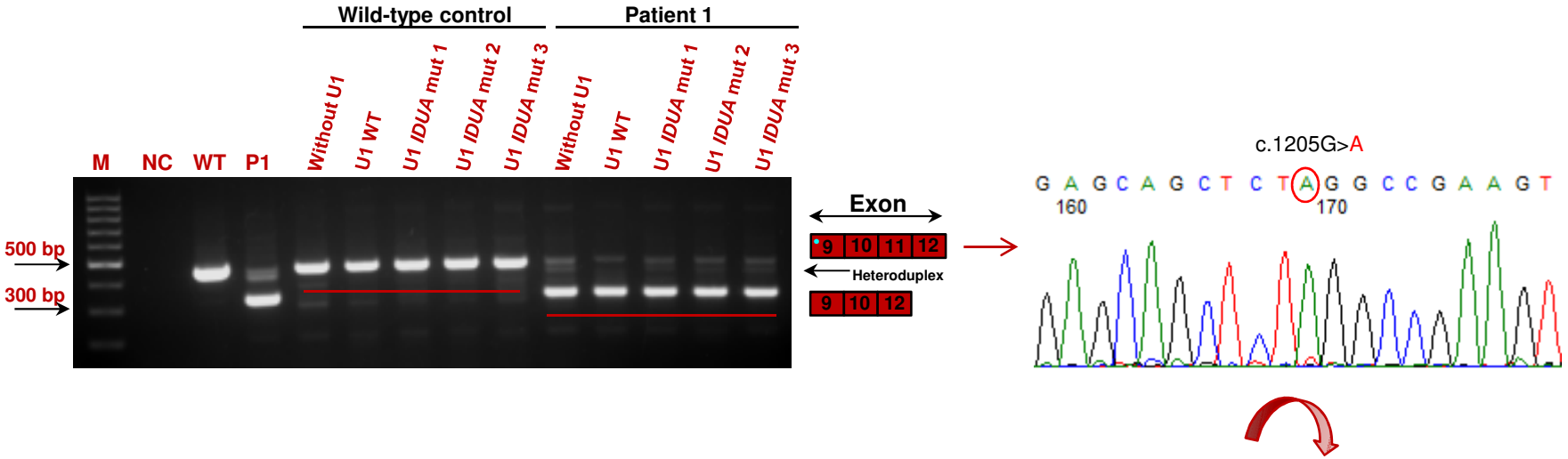


Mucopolysaccharidosis I

☞ **Transfection of wild-type and patient 1 fibroblasts with 2.5 µg of modified U1 vectors for 24 h**

- **RT-PCR analysis**

Primers for exon 9 and 12:



→ No correction with 3.5 or 4.5 µg of each U1 vector or even in a 48 h treatment

No heterozygous peak

Not even a partial correction observed

Mucolipidosis III alpha/beta (pseudoHurler polydystrophy)

- Autossomal recessive disorder
- Caused by mutations in *GNPTAB* gene (21 exons)

↓

α/β-subunit of the N-acetylglucosamine (GlcNAc)-1-phosphotransferase

↓

Formation of the mannose-6-phosphate residues on lysosomal enzymes

Therapy:

- No causal therapies available for treatment
- Disease management only symptomatic

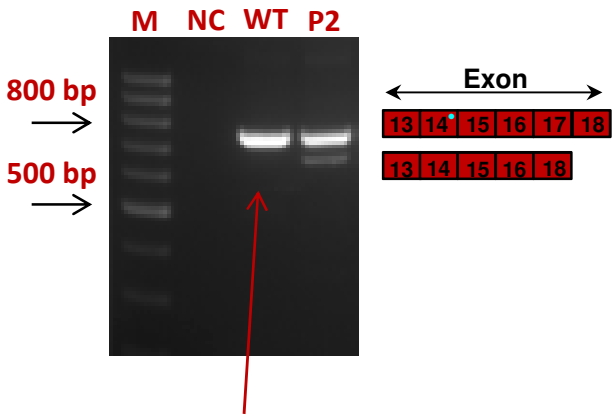
↓ Development of therapeutic strategies is essential

Mucolipidosis III alpha/beta

☞ ***GNPTAB*** **gene mutation analysis (RT-PCR)**

c.3335+6T>G/p.A955V

Primers for exon 13 and 18:



- Normal length transcript

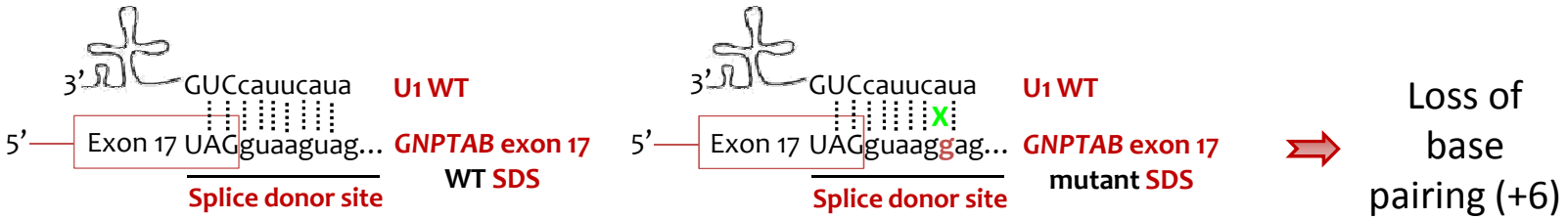
Patient 2: two transcripts

- One with normal length arising from the missense allele
- One with exon 17 skipping due to the donor site mutation

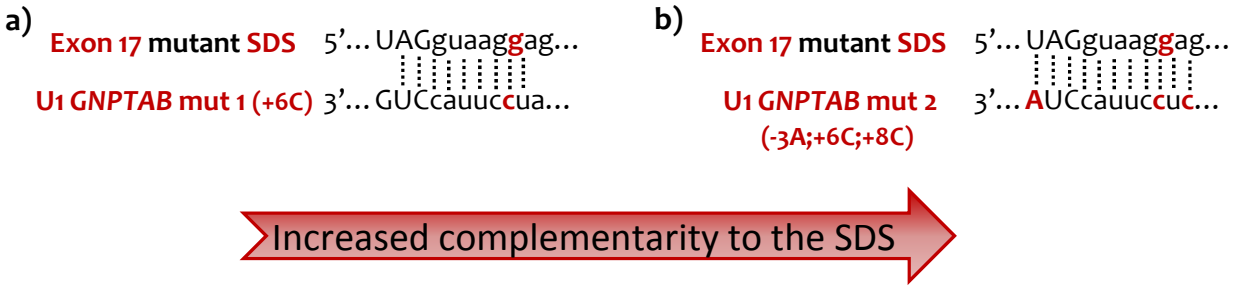
Mucolipidosis III alpha/beta

☞ **Splicing therapeutic approach for the +6 *GNPTAB* gene mutation with modified U1 vectors**

1. Analysis of *GNPTAB* exon 17 splice donor sites (SDS)



2. Design and construction of U1 modified vectors (pG3U1)

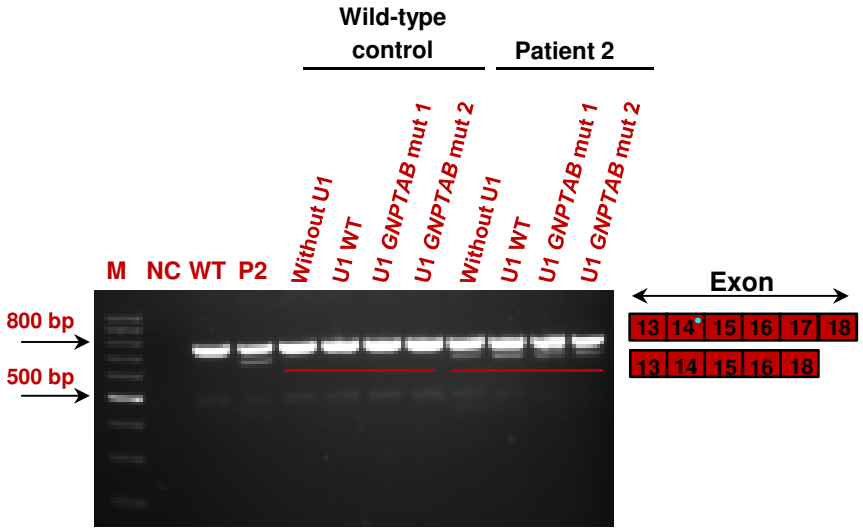


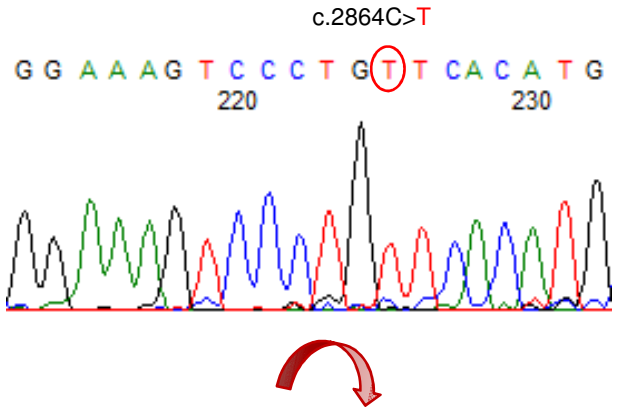
Mucolipidosis III alpha/beta

☞ **Transfection of wild-type and patient 2 fibroblasts with 2.5 µg of modified U1 vectors for 24 h**

- **RT-PCR analysis**

Primers for exon 13 and 18:





No heterozygous peak

→ No correction with 3.5 or 4.5 µg of each U1 vector or even in a 48 h treatment

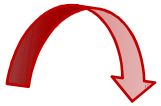
Not even a partial correction observed

Transfection results conclusions

↳ Absence of correction for both 5' splice site mutations

- High level of cell death after transfection

  - Presence of different modified U1's (?)
  - Lipo-mediated transfection toxicity (?)



Low transfection eficiency?

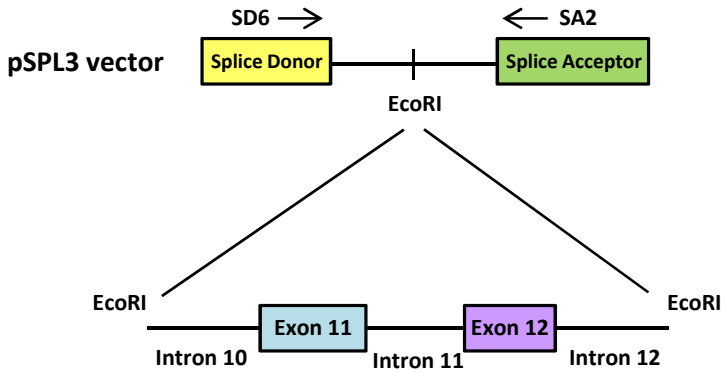
<u>Next steps:</u>

☞ Co-transfection of minigene constructs with the U1's (Hep 3B cell line)

☞ Lentiviral transduction of U1's in patients fibroblasts

Minigenes expression (Hep 3B)

- **MPS I (*IDUA* minigenes)**

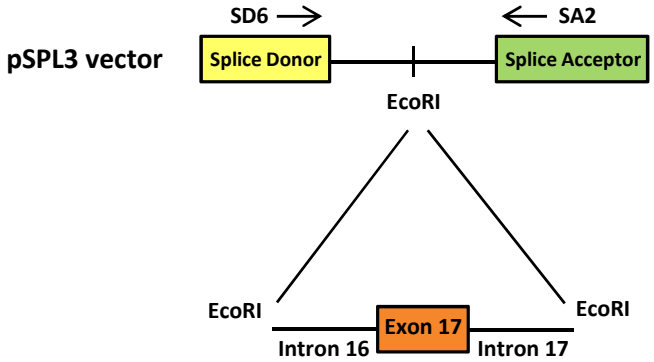


Exon 11/Intron 11

Wild-type: …CCGGGCAGgcaagtgg…
Mutant: …CCGGGCAGgcaaatgg…

c.1650+5G>A

- **ML III α/β (*GNPTAB* minigenes)**

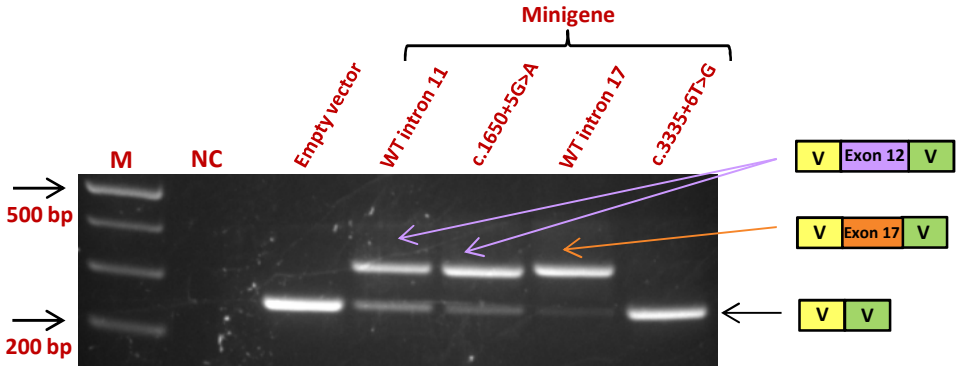


Exon 17/Intron 17

Wild-type: … AAATATAGgtaagtag…
Mutant: … AAATATAGgtaaggag…

c.3335+6T>G

- **RT-PCR analysis**

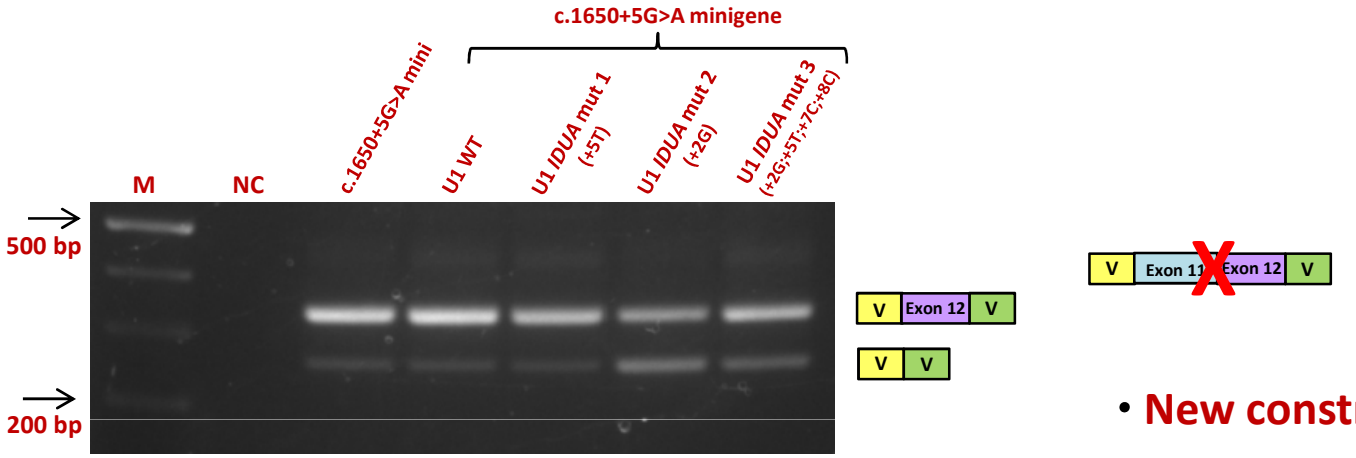


Minigenes:

- WT intron 11 ✗
- c.1650+5G>A ✓
- WT intron 17 ✓
- c.3335+6T>G ✓

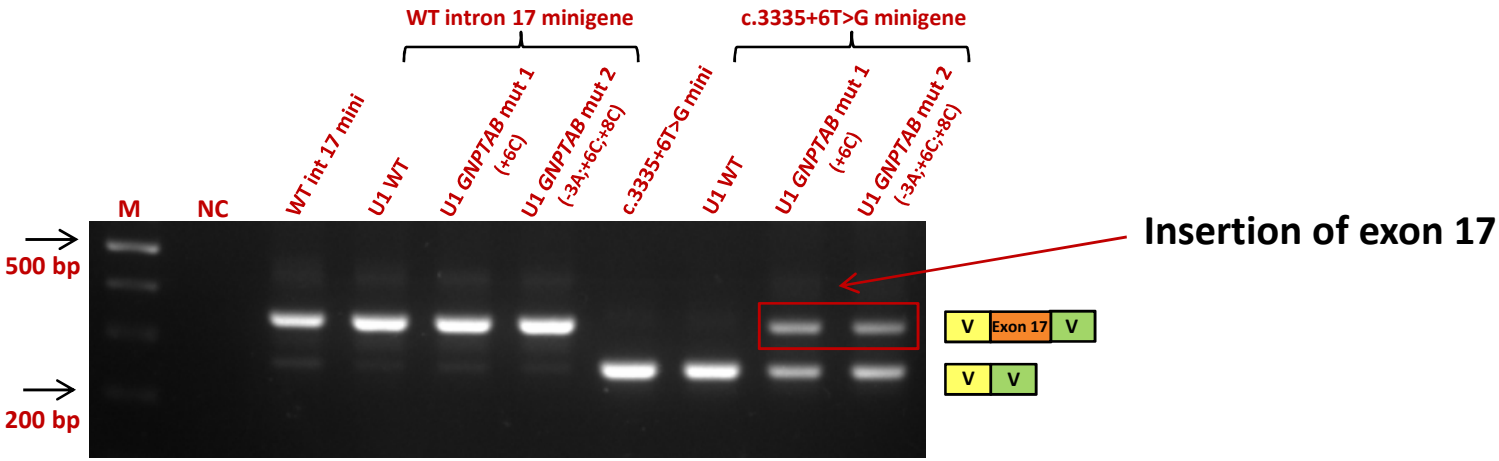
Minigenes co-transfection with different U1's (Hep 3B)

- **MPS I – *IDUA* gene** (RT-PCR analysis)



- **New constructs!**

- **ML III α/β – *GNPTAB* gene** (RT-PCR analysis)



Short **t**erm **s**cientific **m**ission

HUMAN GENETICS
University of Oldenburg

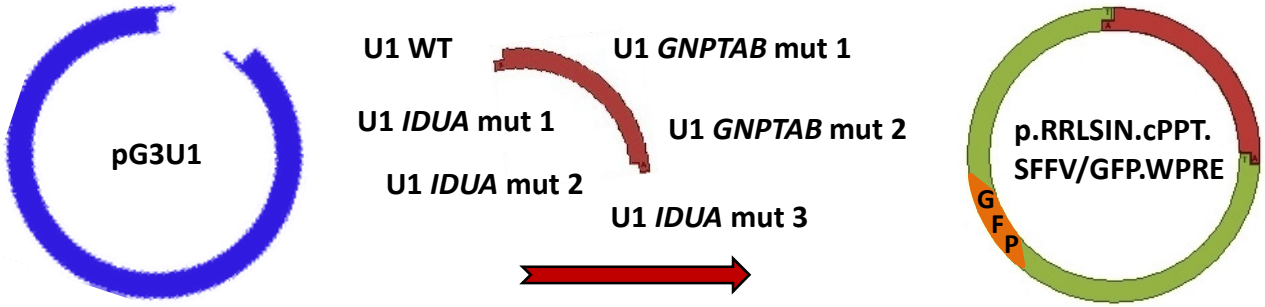
Main aim:

Learn and perform the lentiviral transduction technique of the different modified U1's into patients' fibroblasts to try the therapeutic rescue of the 5' splice site mutations, c.1650+5G>A (*IDUA* gene) and c.3335+6T>G (*GNPTAB* gene)

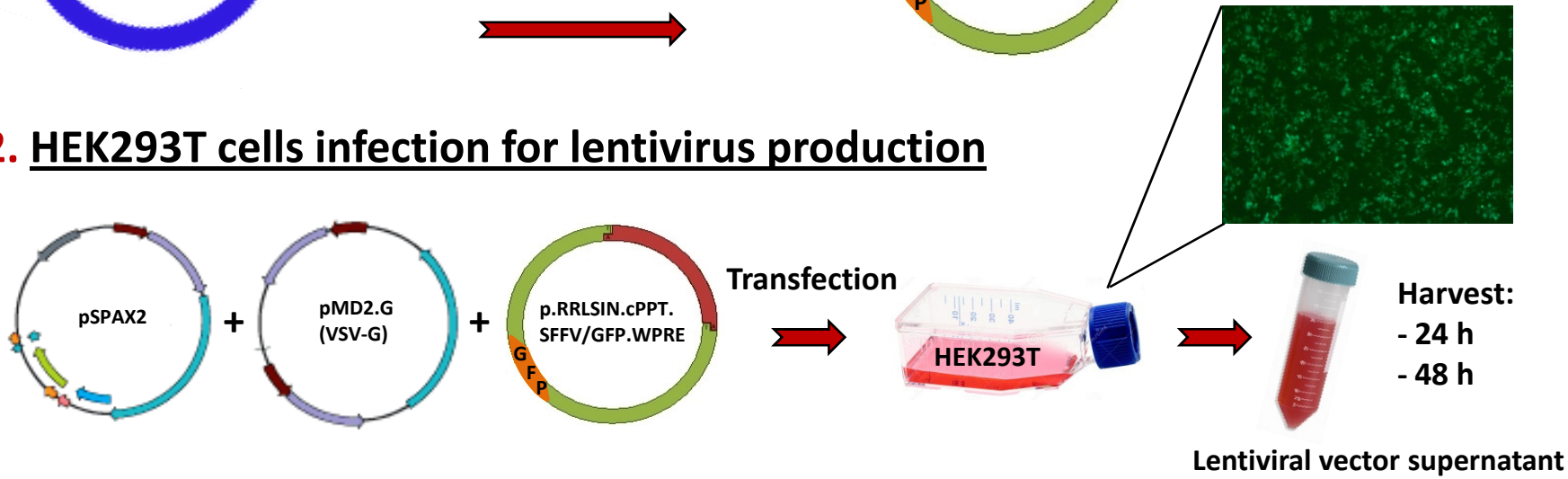
- Lentiviral transduction of U1 constructs in fibroblasts
  - ✓ More efficient technique to the acquisition of external vectors
  - ✓ Less toxic for cells than lipo-mediated transfection (↓ mortality)
  - ✓ Some different splice donor site mutations have been partially or totally rescued through viral transduction of U1 snRNAs (Glaus *et al.*, 2011; Schmid *et al.*, 2011; Dal Mas *et al.*, 2015)

Workflow

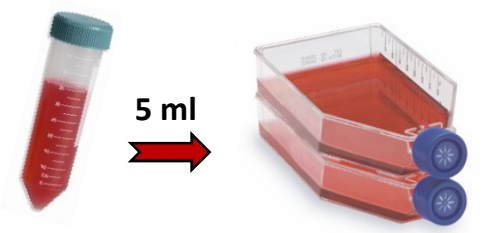
1. Cloning of the different U1 snRNAs in the lentiviral vector



2. HEK293T cells infection for lentivirus production



3. Lentivirus transduction in fibroblast cells



- Wild-type control
- MPS I patient (c.1650+5G>A)
- ML III α/β patient (c.3335+6T>G)
- Positive control (*BBS-1* gene)

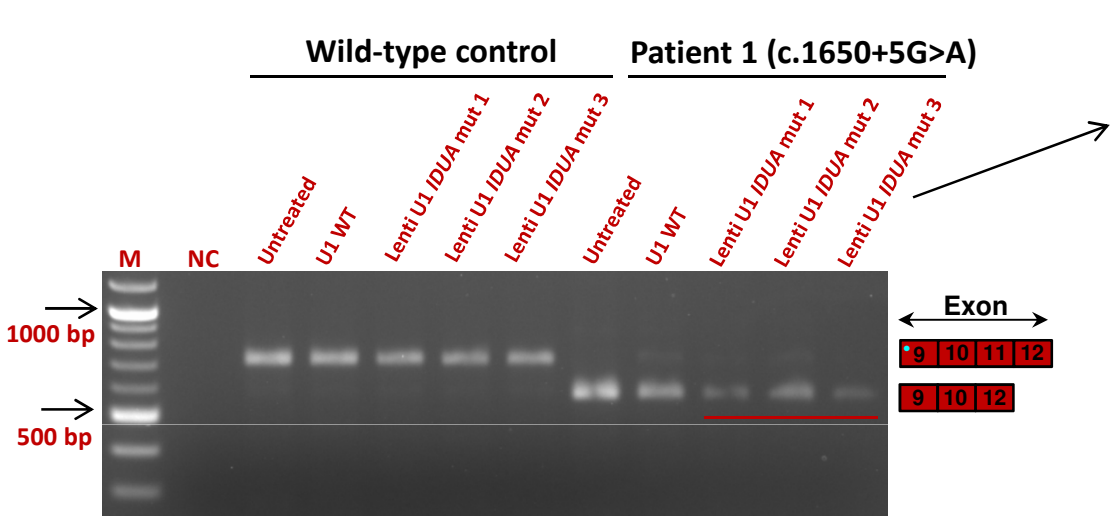
Schmid *et al.*, 2011

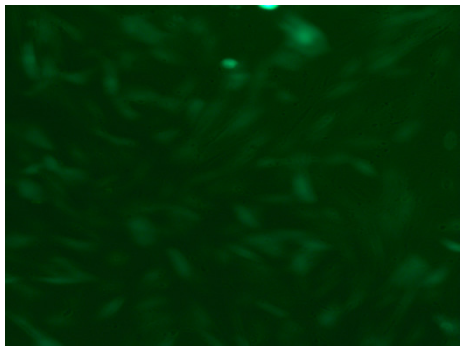
Cells harvest after 4 days

- **RT-PCR analysis**
  - Splicing pattern
- **qPCR analysis**
  - Nr of lentivirus copies

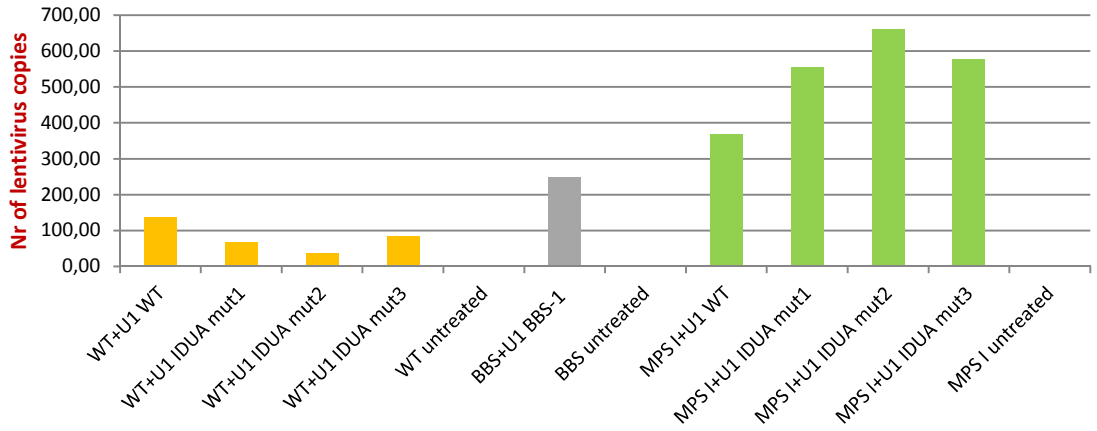
Lentiviral transduction results: MPS I

- **RT-PCR analysis**





- **qPCR analysis**



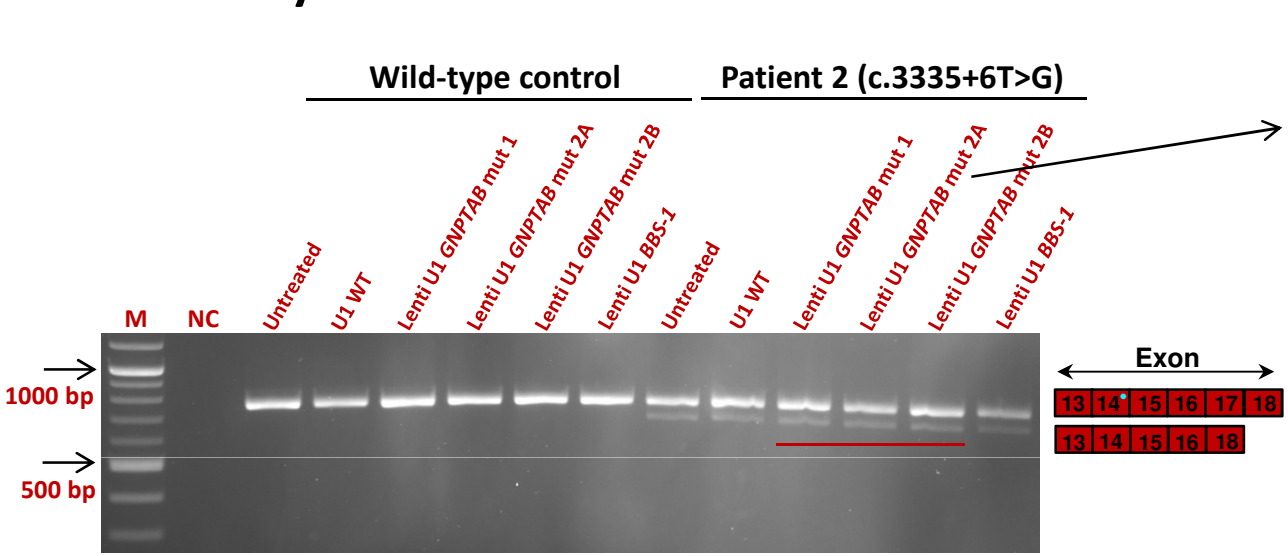
Nr of lentivirus copies = $2^{(1+\Delta Cq)}$

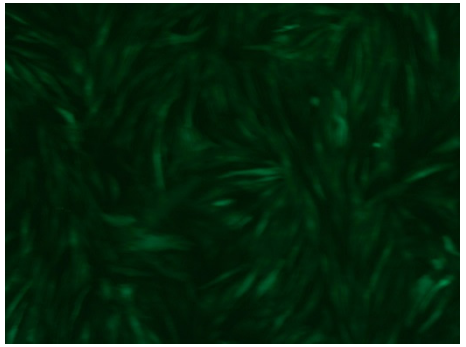
Reference gene vs Target gene:

Albumin (n=2) vs GFP (n=?)

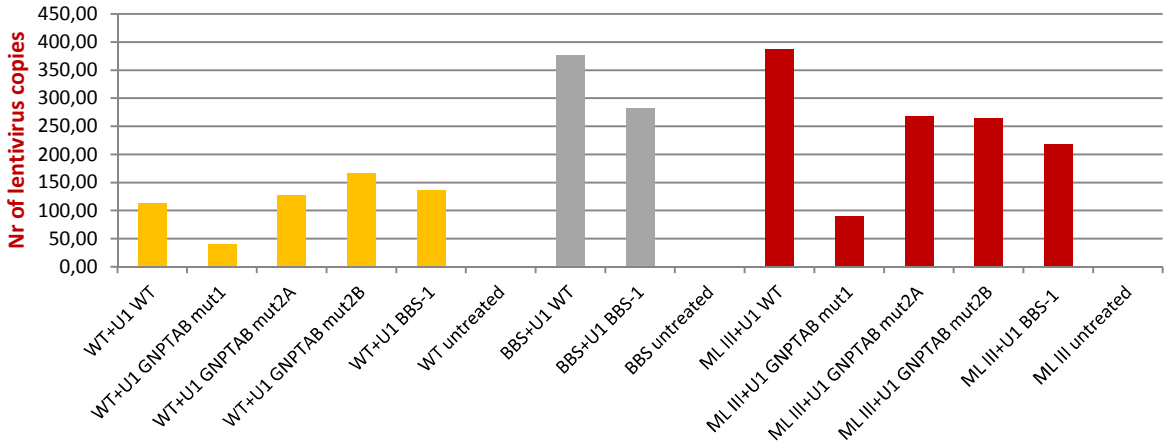
Lentiviral transduction results: ML III α/β

- **RT-PCR analysis**





- **qPCR analysis**



Nr of lentivirus copies = $2^{(1+\Delta Cq)}$

Reference gene vs Target gene:

Albumin (n=2) vs GFP (n=?)

Lentiviral transduction results: BBS (+ control)

RESEARCH ARTICLE

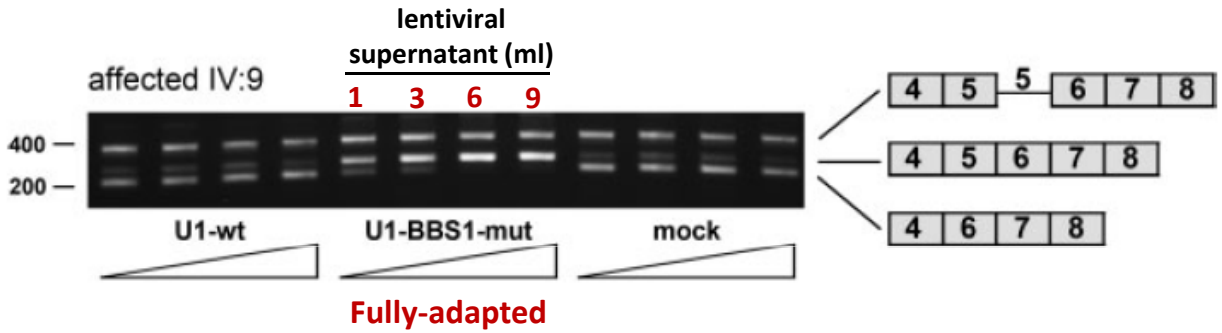
c.479G>A Human Mutation

Last nt exon 5

OFFICIAL JOURNAL HGVS HUMAN GENOME VARIATION SOCIETY www.hgvs.org

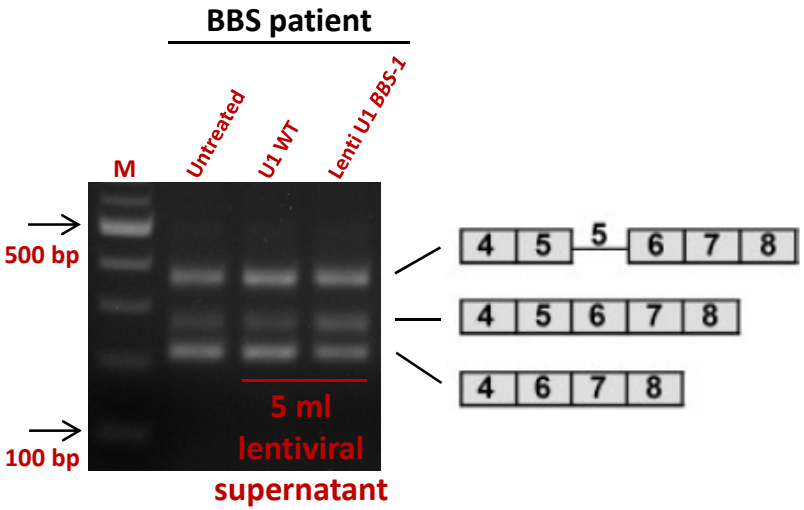
U1 snRNA-Mediated Gene Therapeutic Correction of Splice Defects Caused by an Exceptionally Mild BBS Mutation

Fabian Schmid,[1] Esther Glaus,[1] Daniel Barthelmes,[2] Manfred Fliegauf,[3] Harald Gaspar,[4] Gudrun Nürnberg,[5] Peter Nürnberg,[5] Heymut Omran,[6] Wolfgang Berger,[1] and John Neidhardt[1]*



Fully-adapted

- **ML III α/β parallel experiment**



Our cases:

- MPS I (c.1650+5G>A)
- ML III α/β (c.3335+6T>G)

  - ↑ lentivirus volume
  - Optimization !!!

Main STSM achievements

✓ **Cloning of different modified U1's in a lentiviral vector** (p.RRLSIN.cPPT.SFFV/GFP.WPRE)

✓ **Learning and performing the lentiviral transduction technique in human fibroblasts**

✓ **Obtaining the preliminary results of the lentiviral transduction of different modified U1's into MPS I and ML III α/β patients' fibroblasts to try the therapeutic rescue of the 5' ss mutations c.1650+5G>A and c.3335+6T>G**

✓ **Acquisition of the lentiviral transduction technique allowing, in a near future, its implementation in our laboratory to be applied not only to the 5' ss mutations in study as to other donor site mutations reported in LSDs patient's**

Acknowledgments

Lysosomal storage disorders research group

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Dr. Christoph Jüschke
Human Genetics group

Dr. Mirella Filocamo

Prof. Mª João Prata

Prof. Belén Pérez
Prof. Lourdes Ruiz-Desviat

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(PTDC /BBB-BMD/6301/2014)

BM1207

Thank you for your attention