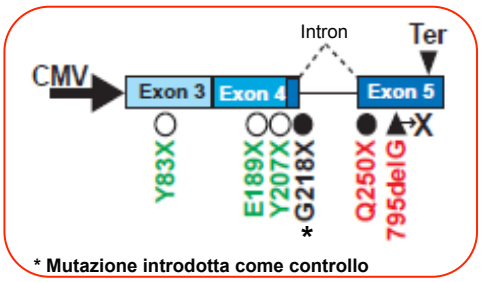
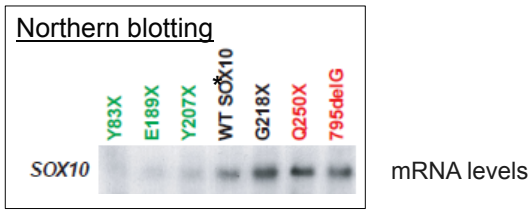
SOX10

IL FENOTIPO MENO GRAVE È DOVUTO A DOWNREGULATION DA NMD

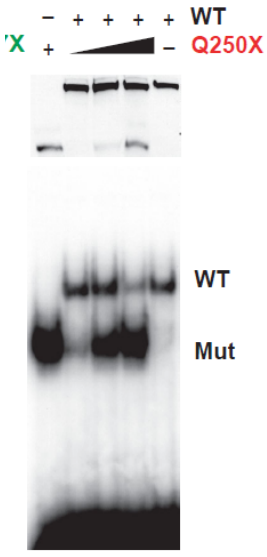
Minigene approach:



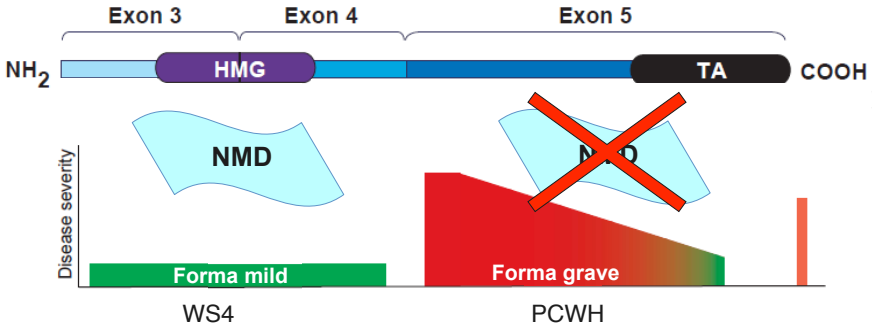


Inoue et al., Nature Genetics (2004)

EMSA - Mutant SOX10 proteins interfere with the DNA binding of wild-type SOX10



➢ **SOX10 → nonsense mutations**



DOMINANT-NEGATIVE EFFECT OF THE TRUNCATED PROTEINS
highly affinity for DNA-binding

Inoue et al., Nature Genetics (2004)

➢SIMPLE DOMINANCE

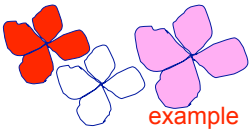
"Dominant" → any trait that is expressed in a heterozygote

Aa

aa "**Recessive**" → a character manifest only in the homozygote

➢INCOMPLETE DOMINANCE

"Haploinsufficiency" →a locus producing a normal phenotype but requiring more gene product than the amount produced by a single copy and thus describe the case where 50% reduction in the level of gene function causes an abnormal phenotype


example

Aa the mutant allele could be
•amorph (inactive)
•ipomorph (normal qualitative activity but quantitatively reduced)
•**antimorph** (antagonistic mutant genes, having an effect actually contrary to that of the gene from which they were derived)→
DOMINANT-NEGATIVE EFFECT

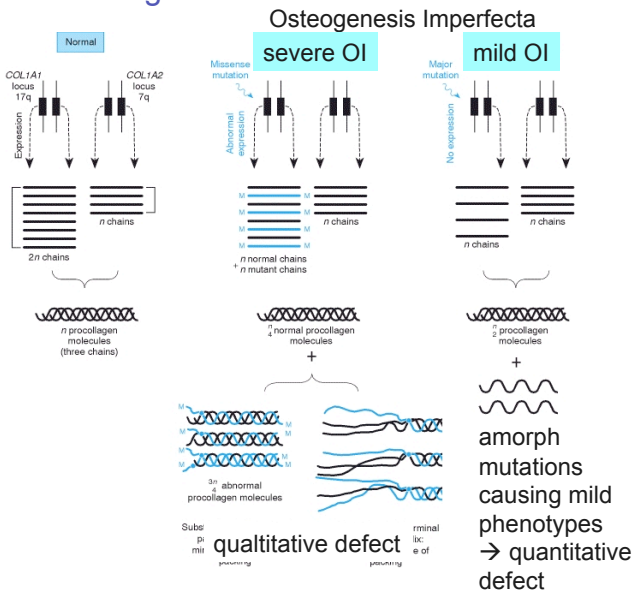
Dominant-negative mutation (antimorph):

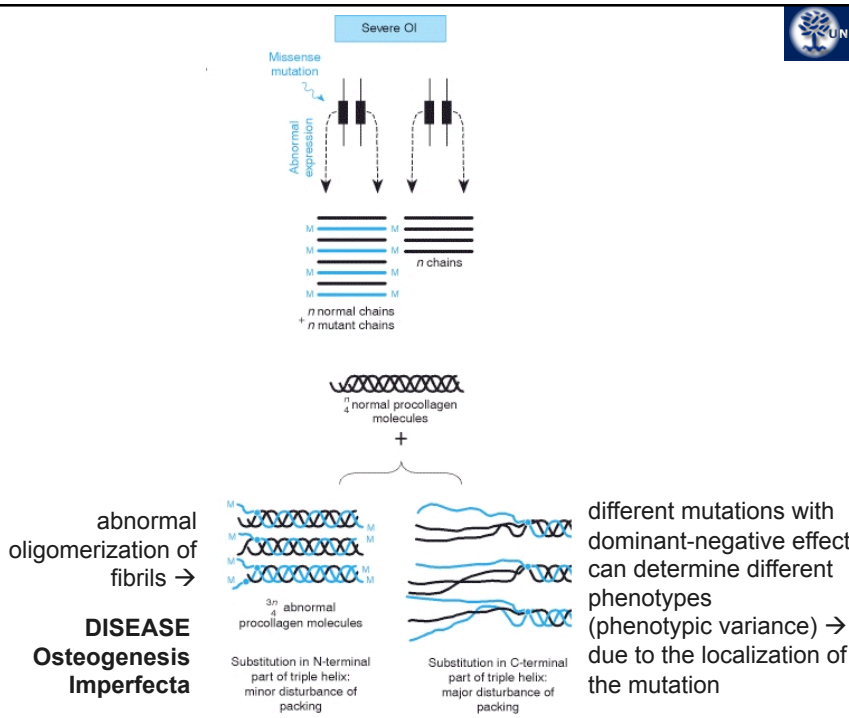
would be 'dominant' because its phenotype is manifested in the presence of wild-type gene
&
as it inactivates the wild type gene function it is referred to as a "dominant-negative" mutation

Proteins that dimerize or multimerize are vulnerable to dominant-negative effects

the classic case of the **collagen** →

fibrils are formed by triple helices of polypeptide chains



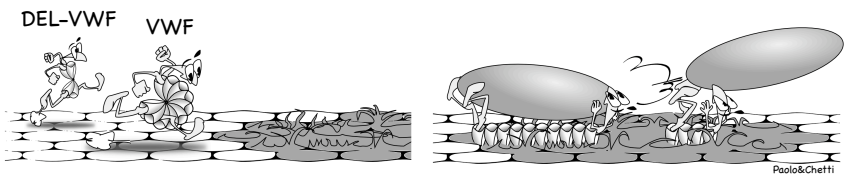


abnormal oligomerization of fibrils →

DISEASE
Osteogenesis Imperfecta

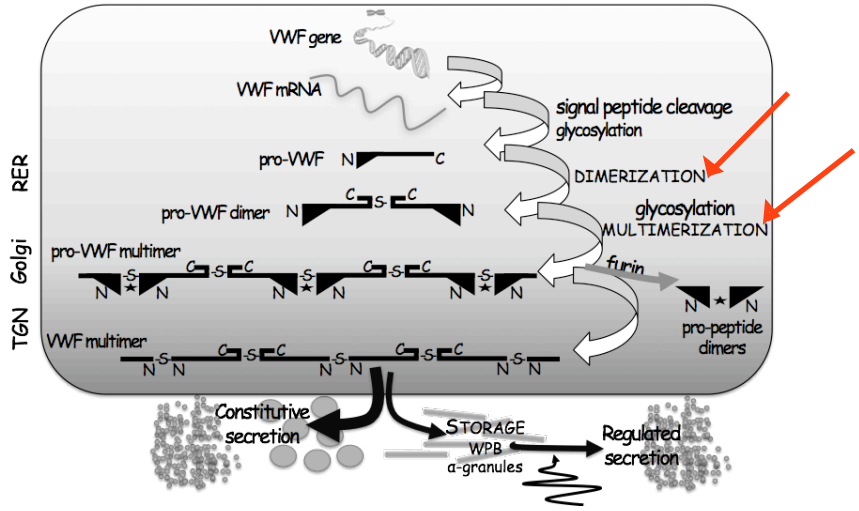
different mutations with dominant-negative effect can determine different phenotypes (phenotypic variance) → due to the localization of the mutation

A SEVERE CASE OF NEGATIVE DOMINANCE: A TYPE 2 VON WILLEBRAND DISEASE & CORRECTION APPROACHES

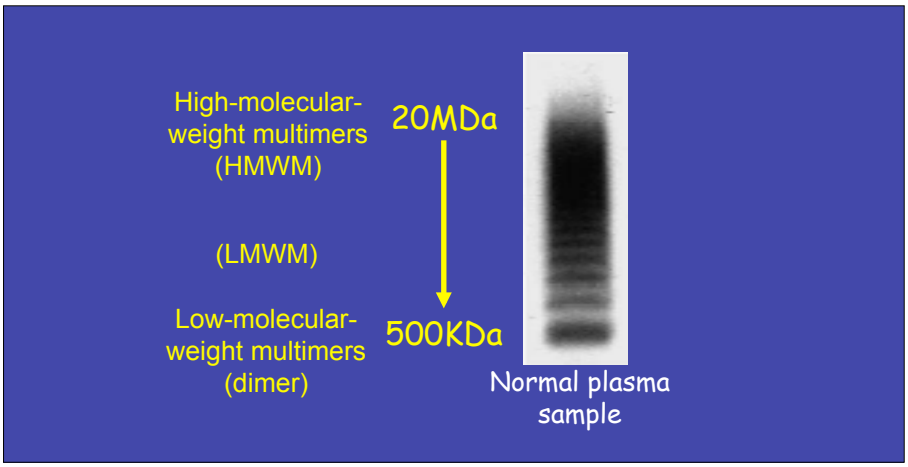


VWF biosynthesis

plasmatic, multimeric glycoprotein

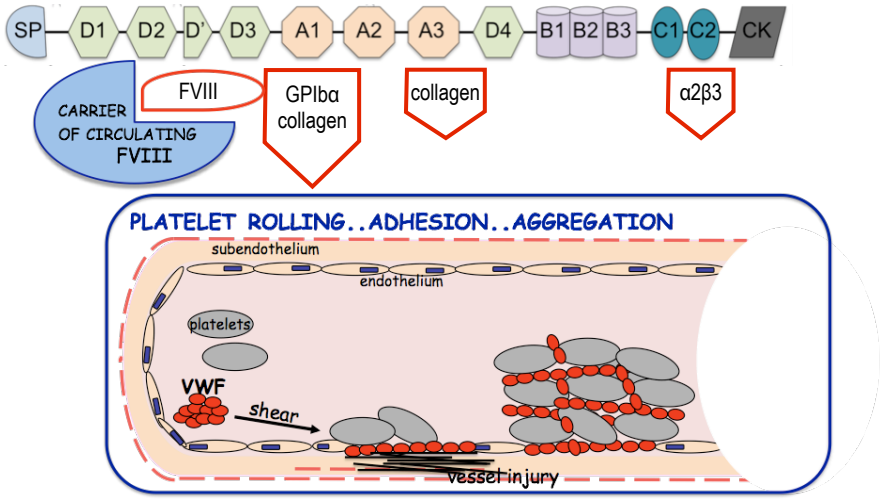


Multimer analysis:

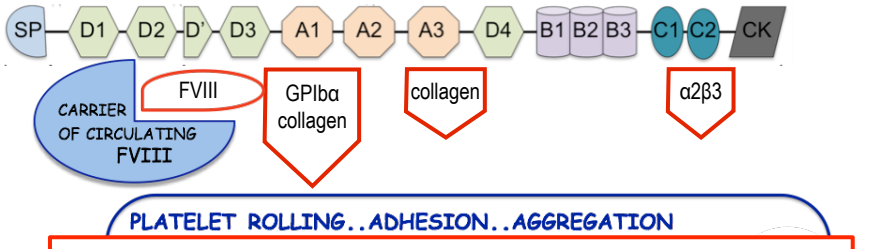


➢ **large multimers are most hemostatically active,**
because they concentrate VWF binding sites (↑mw↑function)

Von Willebrand Factor in hemostasis



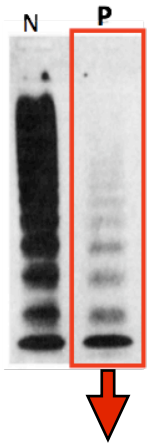
Von Willebrand Factor in hemostasis



Von Willebrand Disease (VWD)

- **more then 300 mutations**
- **the most prevalent bleeding disorder**
- **extremely heterogeneus** → It is not known if the observed heterogeneity for VWD is based on the features of VWF as multifunctional protein, or on its complex biosynthesis with extensive post-translational processing and distinct pathways of intracellular transport and secretion

Severe VWF deficiency: phenotype

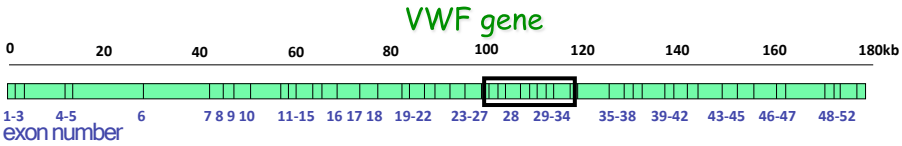


severe bleeding tendency

Bernardi F. Blood 1990.

Severe VWF deficiency: molecular defect

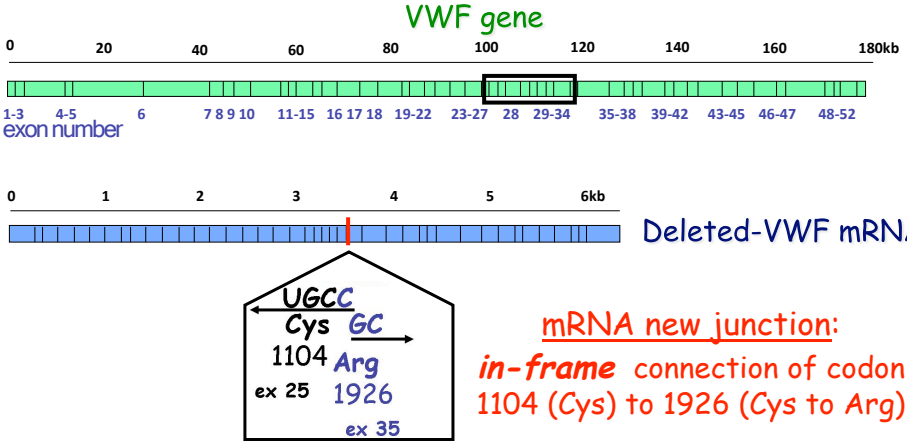
Heterozygous, *de novo*, gene deletion
(intron 25-34, 31 Kb)



Bernardi F. Blood 1990
Bernardi F. Hum Mol Genet 1993

Severe VWF deficiency: molecular defect

Heterozygous, *de novo*, gene deletion
(intron 25-34, 31 Kb)



mRNA new junction:
in-frame connection of codon 1104 (Cys) to 1926 (Cys to Arg)

Bernardi F. Blood 1990
Bernardi F. Hum Mol Genet 1993

VWF protein organization

preproVWF:

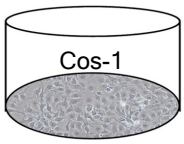
In-frame deleted-VWF: (P1105_C1926delinsR)

- **no** A domains & partial removal of D3 & D4 domains
  - Normal C-terminal domain
  - The large part of N-terminal domain

Cellular models

pWT

Wt-VWF expression vector



wild type VWF expression

Cellular models

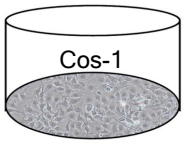
pWT

Wt-VWF expression vector

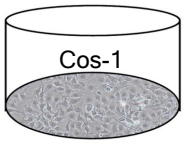
pDEL

Break-point

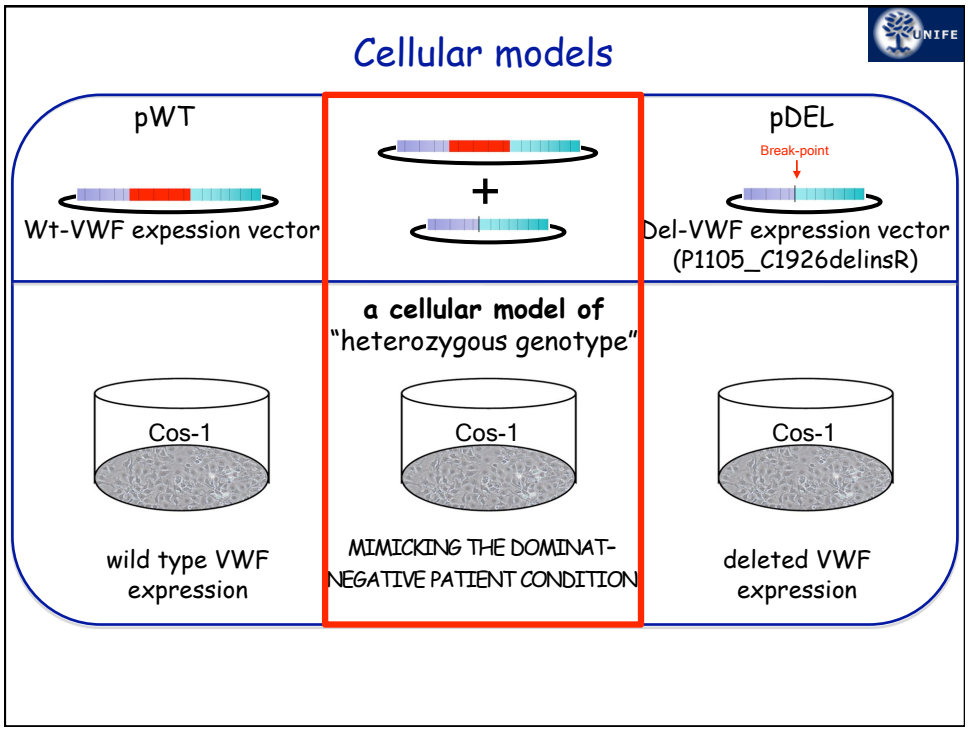
Del-VWF expression vector (P1105_C1926delinsR)

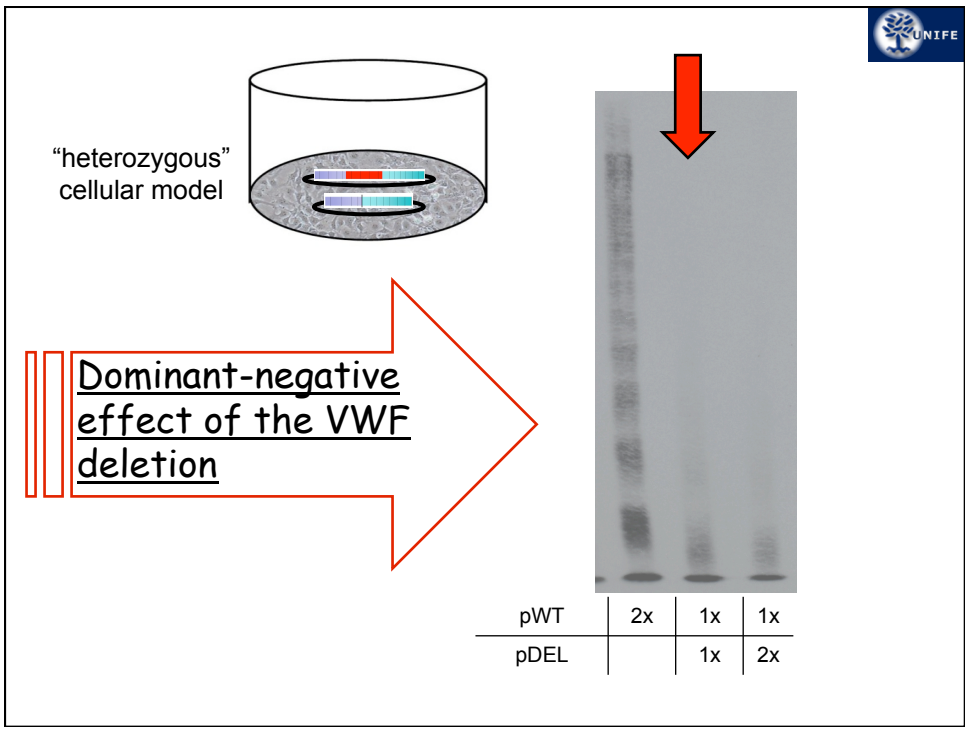


wild type VWF expression



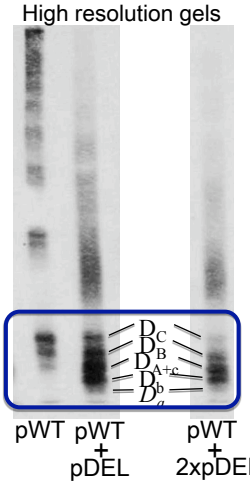
deleted VWF expression


Cellular models
pWT
Wt-VWF expression vector
+
pDEL
Break-point
Del-VWF expression vector
(P1105_C1926delinsR)
a cellular model of
"heterozygous genotype"
Cos-1
Cos-1
Cos-1
wild type VWF
expression
MIMICKING THE DOMINAT-
NEGATIVE PATIENT CONDITION
deleted VWF
expression

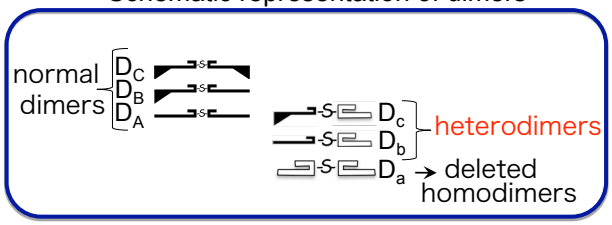

"heterozygous"
cellular model
Dominant-negative
effect of the VWF
deletion
pWT 2x 1x 1x
pDEL 1x 2x

Dominant-negative effect of the in frame deleted VWF
Formation of dimers with Wild Type VWF

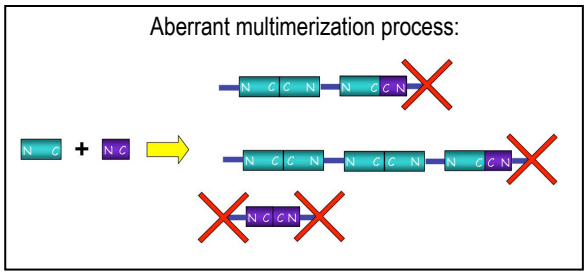
High resolution gels



Schematic representation of dimers



Dominant-negative effect of the in frame deleted VWF
Molecular mechanism: TERMINATOR HYPOTHESIS



normal and aberrant VWF proteins can form heterodimers that participate in an ineffective multimerization process

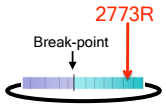
Interfering with dimerization of the in frame deleted VWF
-suppression of a key Cys residue-

Creation of the double mutant vector

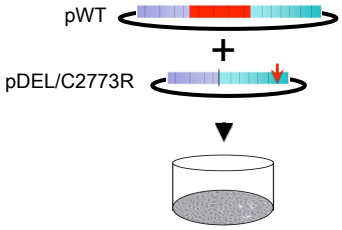
P1105_C1926delinsR

+

C2773R

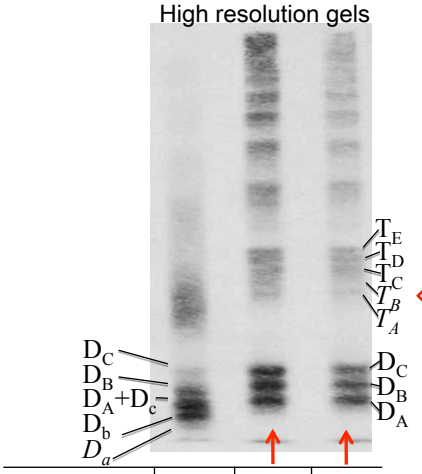


cellular model



Interfering with dimerization of the in frame deleted VWF
-suppression of a key Cys residue-

High resolution gels



pWT	+	+	+
pDEL	++		
pDEL/C2773R		+	++

correction approach at the protein level

the C2773R mutation shifts the equilibrium toward formation of normal oligomers by impairing heterodimers formation

thus favouring polymerization of normal molecules

Casari C. et al *submitted*

AIMS

Mutation based models of therapy
RNA directed therapy

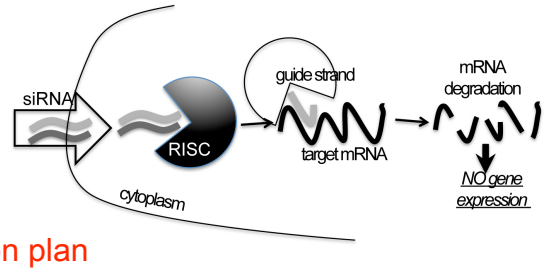
allele- & mutation-specific silencing

Rescue of gene expression

TARGET

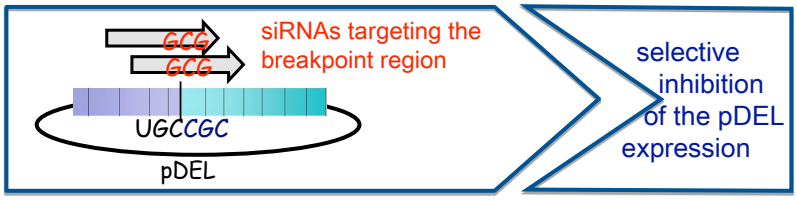
- In frame deleted VWF mRNA

Correction strategy: allele-specific RNAi



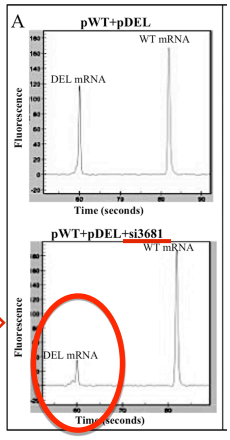
…Intervention plan

strategies preventing the expression of deleted VWF might rescue the multimerization process



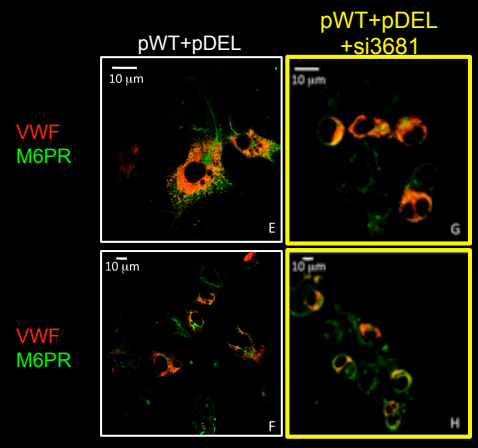
Correction strategy: allele-specific RNAi

si3681 decreased
DEL-VWF mRNA
&
did not affect
WT-VWF mRNA



Selective decrease of the deleted mRNA and thus of the deleted VWF

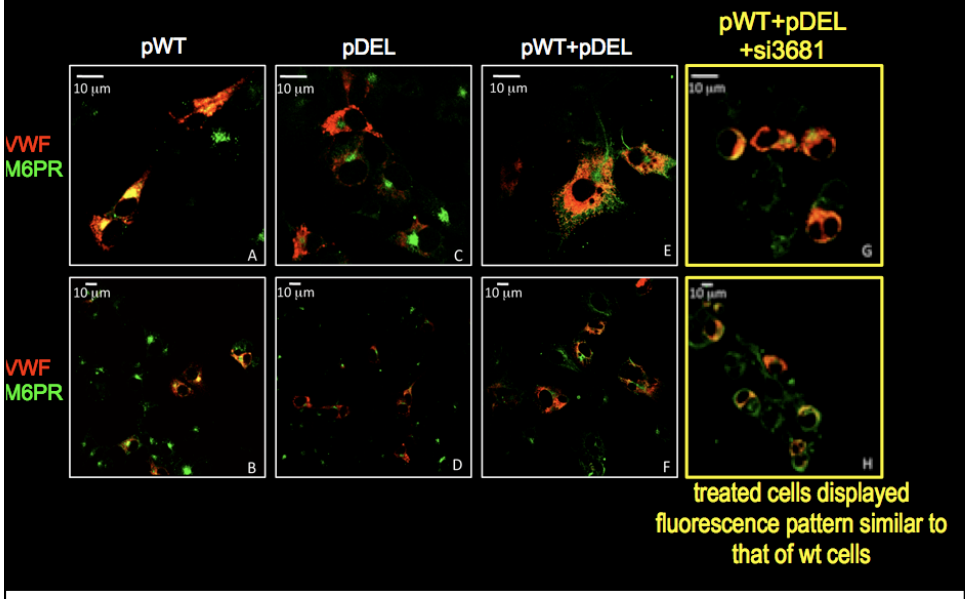
Correction strategy: allele-specific RNAi



In treated cells VWF was transported to late endosome for secretion

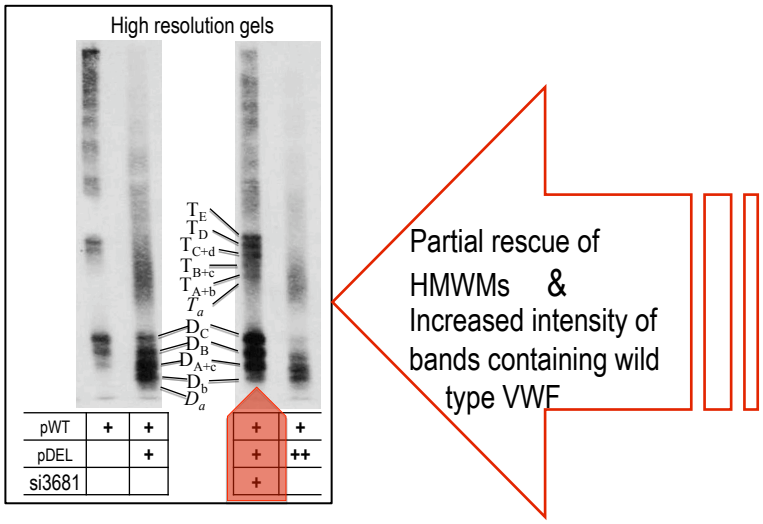
Casari C. et al *submitted*

Correction strategy: allele-specific RNAi



In treated cells VWF was transported to late endosome for secretion

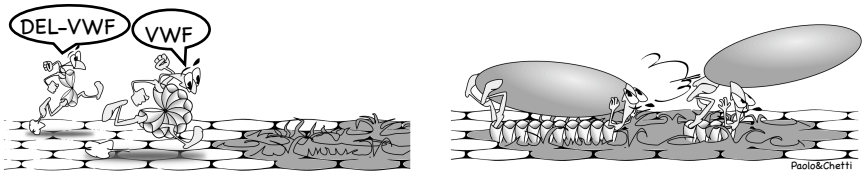
Correction strategy: allele-specific RNAi



Von Willebrand factor gene deletion with dominant-negative effect: molecular mechanism & RNAi correction

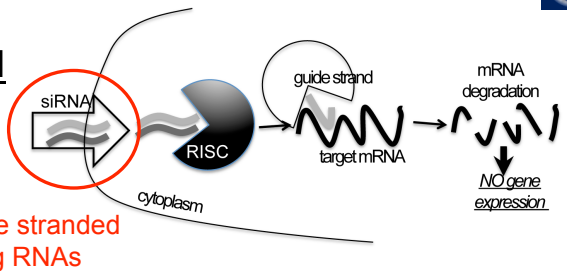
- the cellular model mirrors the severe VWF-deficiency observed in patient plasma
- the **del-VWF** is synthesized and folded in large amounts and is efficiently processed
- the del-VWF efficiently forms altered **heterodimers** with wt-VWF
- the heterodimers act as **terminator** of the multimerization process, which explains the dominant negative effects
- The allele specific **siRNAs** increases antigen & activity levels and restores HMWMs and suggests a mutation-specific therapeutic approach in this severeVWF deficiency and other human diseases caused by dominant-negative mutations

- This cellular model gets new insight on the complex biosynthesis of VWF variants and on their dominant character



methods

siRNA-mediated silencing

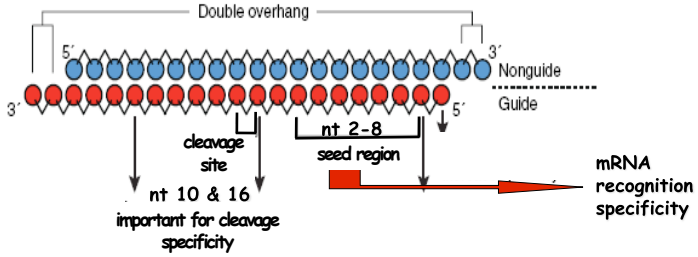


synthetic double stranded small interfering RNAs

methods

siRNA-mediated silencing

siRNAs ≈20nt



Prediction of the sequence and chemical modification to yield an ideal siRNA duplex remains a work in progress:
at present we can use some on-line free programs that predict library of siRNAs that we must test to identify the best

❖in addition to specific siRNAs
scrambled negative control must be tested

… ^{3658}gtggtgacctggaggacggccacattgtgc**c↓g**cgtgtgcacaggcagctccactcggcacatcgtgacctttg^{6194}…

si3681sense: 5′CCAUUGUGC**CG**CGUGUGCACUU3′
antisense: 3′UUGUAACACG**GC**GCACACGUG5′

si3675sense: 5′CGGCCACAUUGUGC**CG**CGUUU3′
antisense: 3′UUGCCGGUGUAACACG**GC**GCA 5′

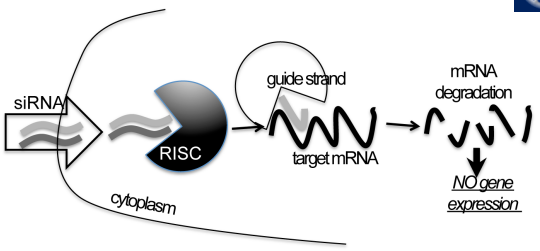
siRNAs directed to the brakpoint

siNC (sense, 5'CCUCAGUCCUAUAGCGCUUUU3'; antisense 5'AAGCGCUAUAGGACUGAGGUU3') — scrambled, negative control

siRNA 852 (sense, 5'CCUCGGACCCUUAUGACUUUU3'; antisense 5'AAGUCAUAAGGGUCCGAGGUU3'), — positive control

methods

siRNA-mediated silencing



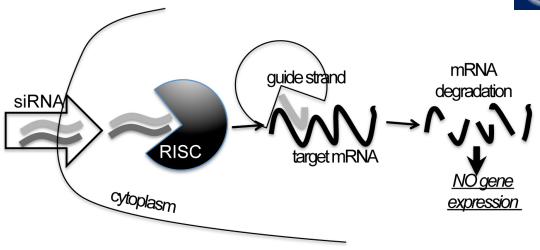
delivery
of the siRNAs inside cells

numerous strategies are now available for delivery
in mammalian cells
- liposomes
- lipid complexes
- conjugates with small molecules, polymers, protein and antibodies
- viral or non -viral vectors

BUT some of them have cytotoxic effect or can interfere with normal cellular processes.

methods

siRNA-mediated silencing

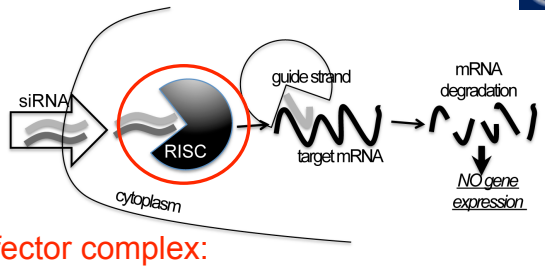


delivery
of the siRNAs inside cells

delivery remains the greatest obstacle for therapeutic *in vivo* silencing treatment

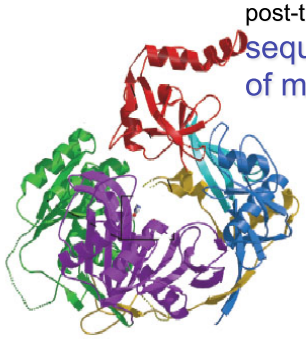
methods

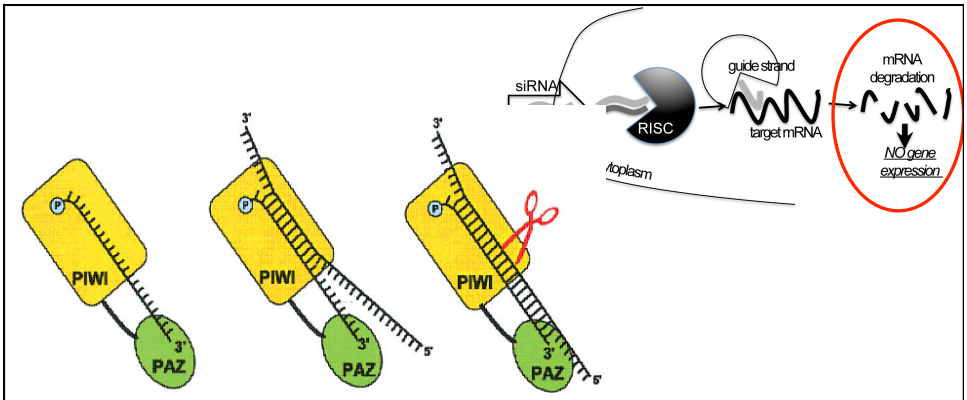
siRNA-mediated silencing



The effector complex:
RNA-induced silencing complex (RISC)

post-transcriptional silencing through
sequence-specific degradation of mRNA target

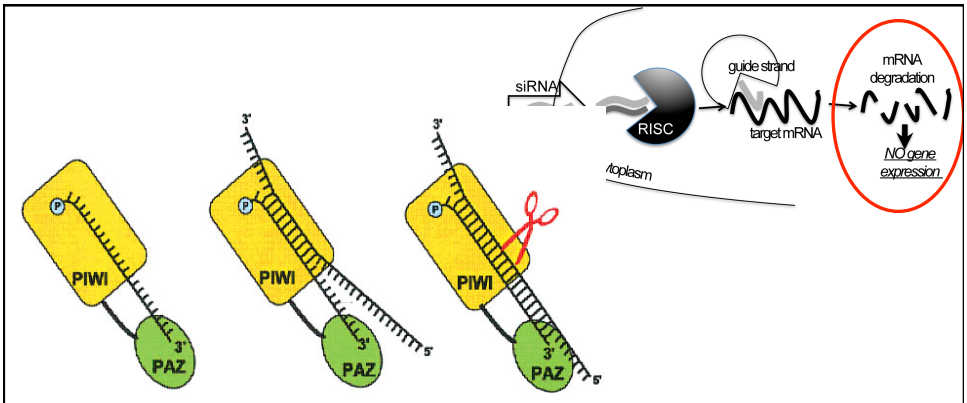




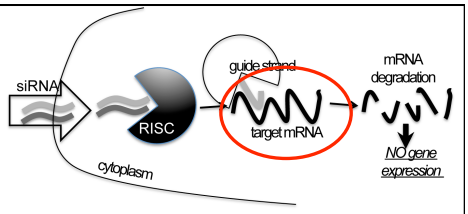
PAZ allows the identification of siRNA (3'-overhangs of 2 nt)

Pairing between the siRNA and the target mRNA is initiated in the seed region and extends to the 3' end

The position of the target mRNA cleavage is mesured from the 5' end of the siRNA and occurs at a position between nucleotides 10 and 11 on the complementary antisense strand



The activated RISC complex can then move on, destroy additional target mRNAs which further propagate gene silencing.



The accessibility of the target site correlates directly with the efficiency of cleavage,

indicating that the RISC cannot unfold structured RNA

➢Mfold program

small part of the entire RNA structure of the deleted molecule surrounding the breakpoint (bold line).