BASI MOLECOLARI DI MALATTIE -2010

introduzione

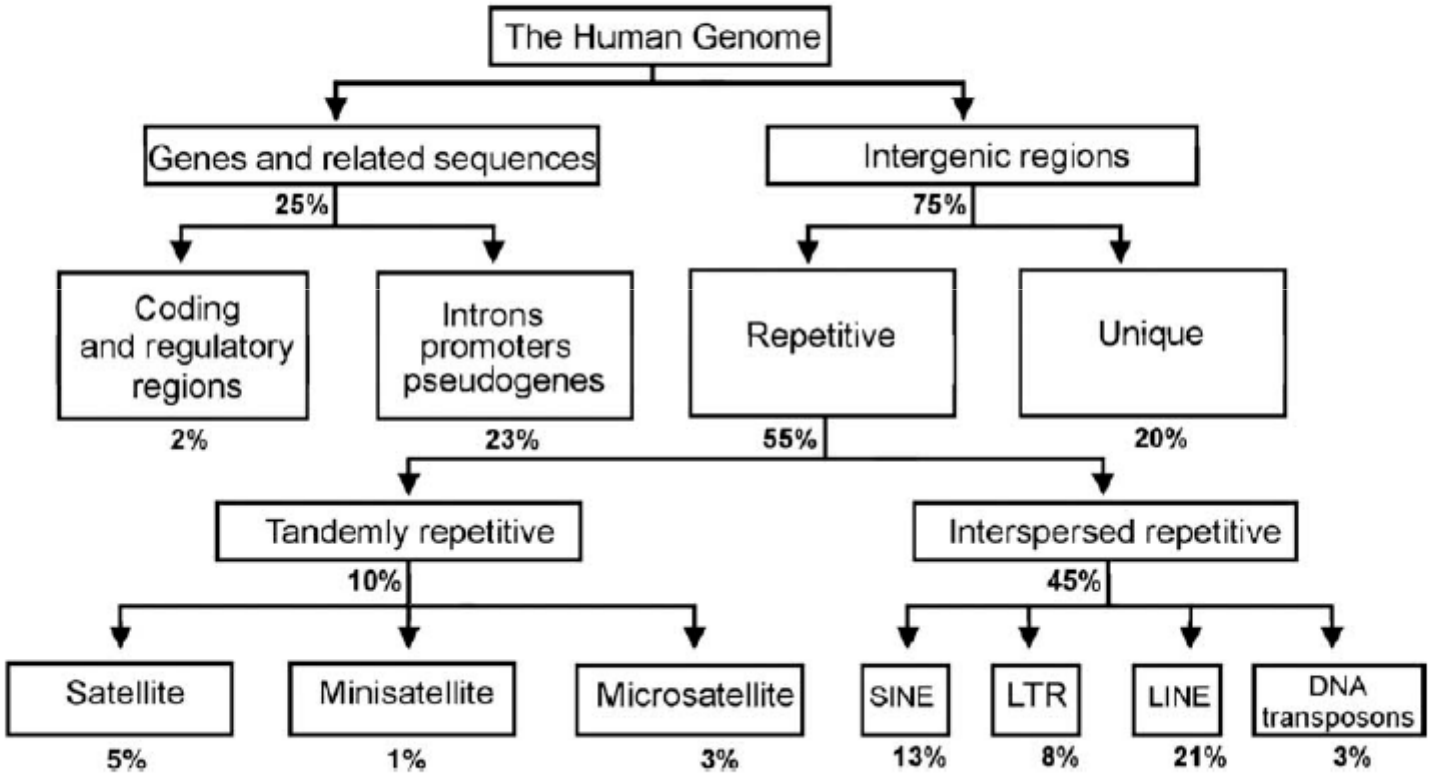

Fig. 1. Composition of the human genome. The percentage shares of various functional and non-functional sequences are shown.

TRIPLET EXPANSION AND DISEASE

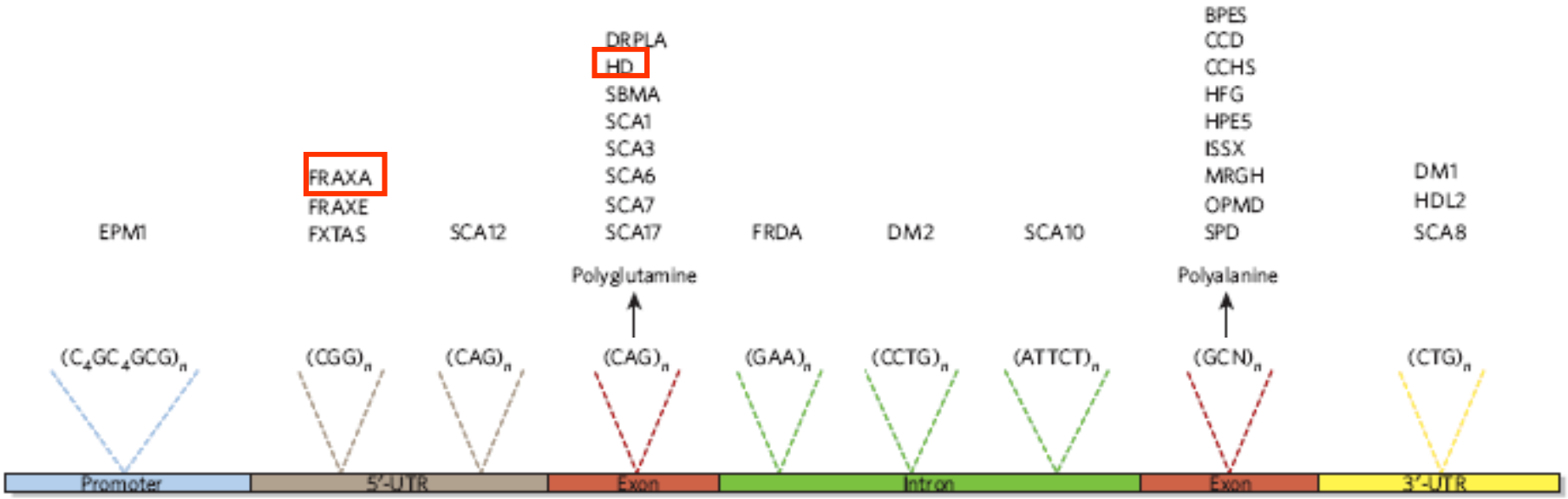

Figure 1 | Location of expandable repeats responsible for human diseases. The sequence and location within a generic gene of expandable repeats that cause human diseases are shown, and the associated diseases are listed. BPES, blepharophimosis, ptosis and epicanthus inversus; CCD, cleidocranial dysplasia; CCHS, congenital central hypoventilation syndrome; DM, myotonic dystrophy; DRPLA, dentatorubral–pallidoluysian atrophy; EPM1, progressive myoclonic epilepsy 1; FRAXA, fragile X syndrome; FRAXE, fragile X mental retardation associated with *FRAXE* site; FRDA, Friedreich's ataxia; FXTAS, fragile X tremor and ataxia syndrome; HD, Huntington's disease; HDL2, Huntington's-disease-like 2; HFG, hand–foot–genital syndrome; HPE5, holoprosencephaly 5; ISSX, X-linked infantile spasm syndrome; MRGH, mental retardation with isolated growth hormone deficiency; OPMD, oculopharyngeal muscular dystrophy; SBMA, spinal and bulbar muscular atrophy; SCA, spinocerebellar ataxia; SPD, synpolydactyly.

TRIPLET EXPANSION AND DISEASE: Anticipation

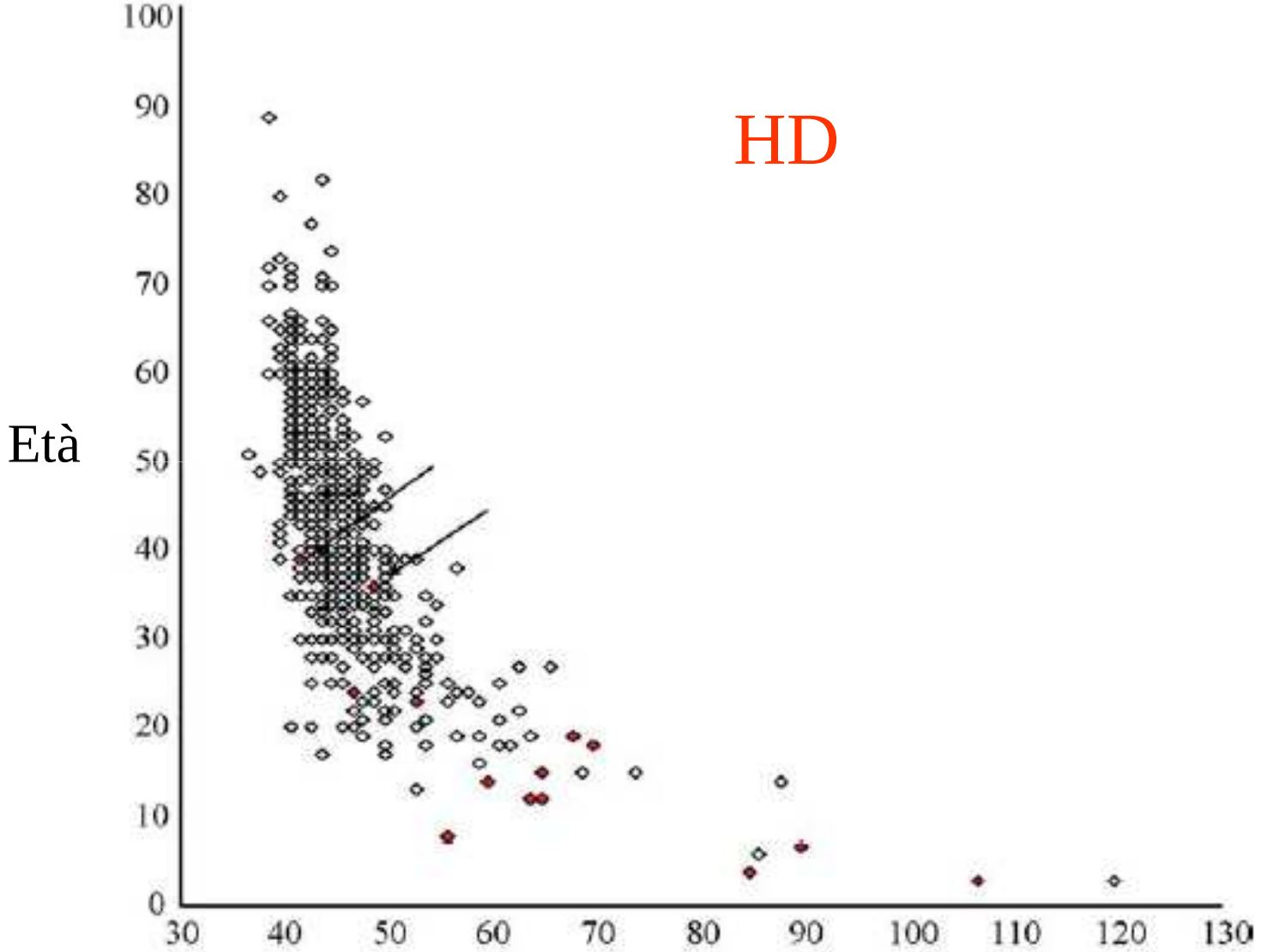

Table 1 | **Triplet repeat expansion disorders**

Disease	Symptoms	Gene	Locus	Protein
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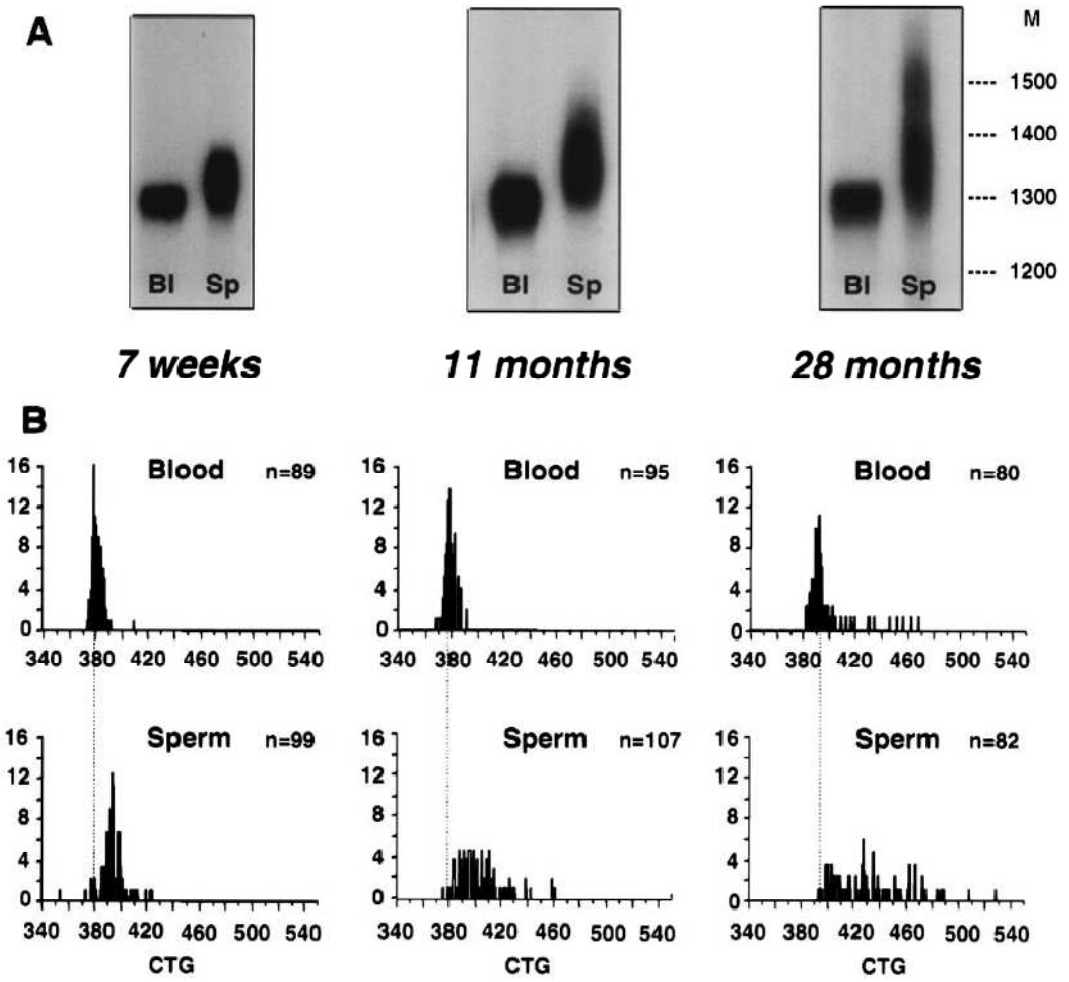
Evolution of CTG repeat-length mosaicism in blood and sperm from transgenic mice (DM1) throughout life



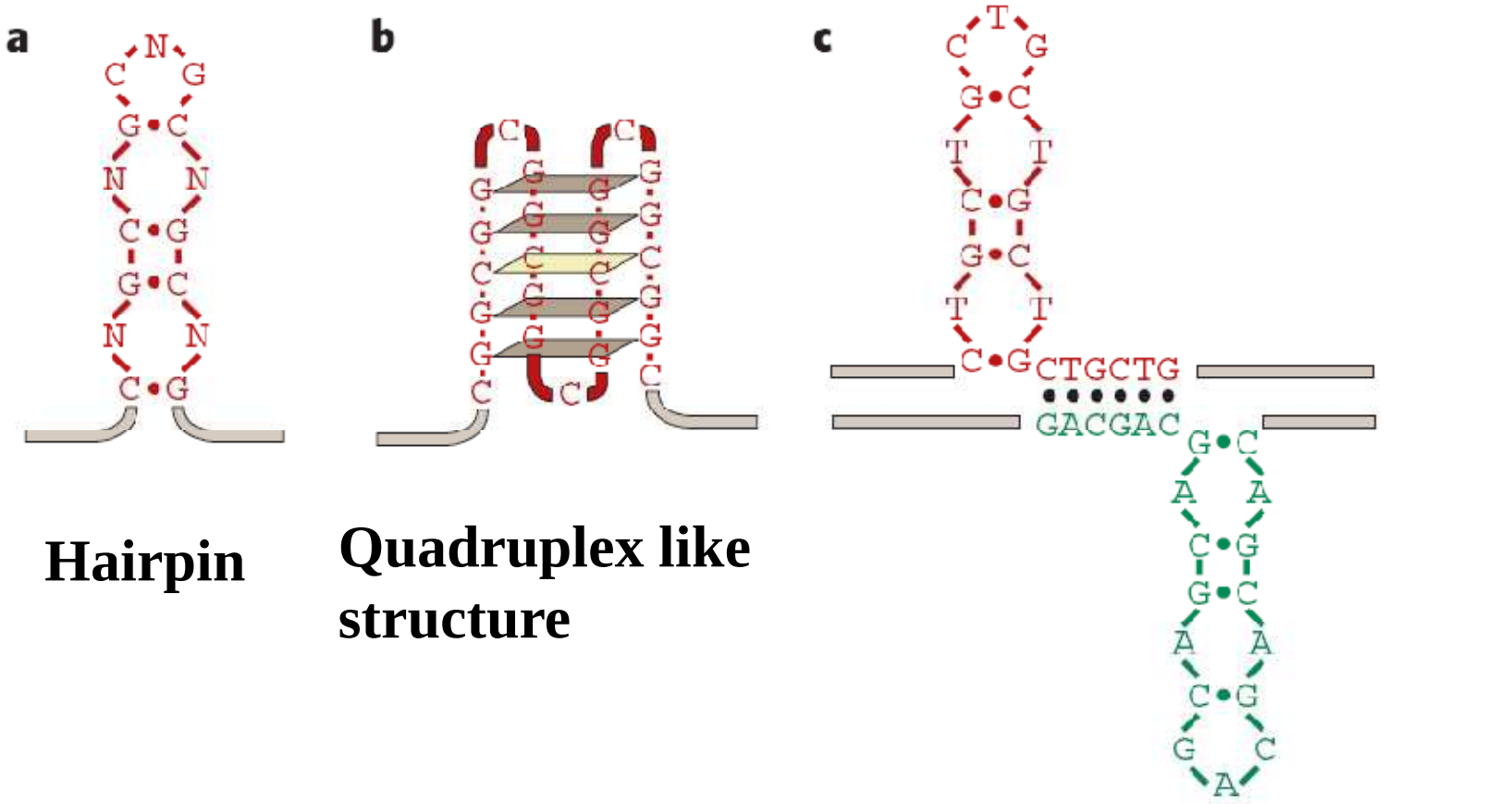
TRIPLET EXPANSION AND DISEASE: molecular mechanisms

Expandable repeats are predisposed to instability, as a result of ‘confusion’ between

- the DNA replication,
- repair and
- recombination machineries

Expandable repeats have unusual structural characteristics

Unusual DNA structures formed by expandable repeats

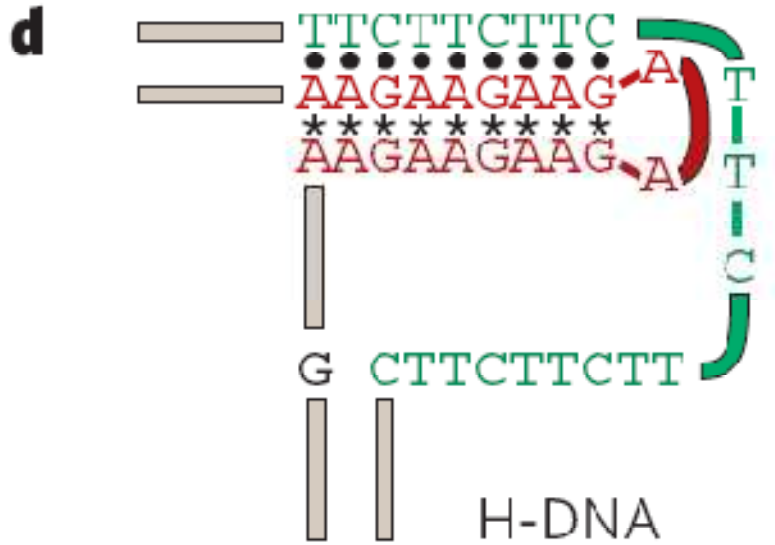


Hairpin

Quadruplex like structure

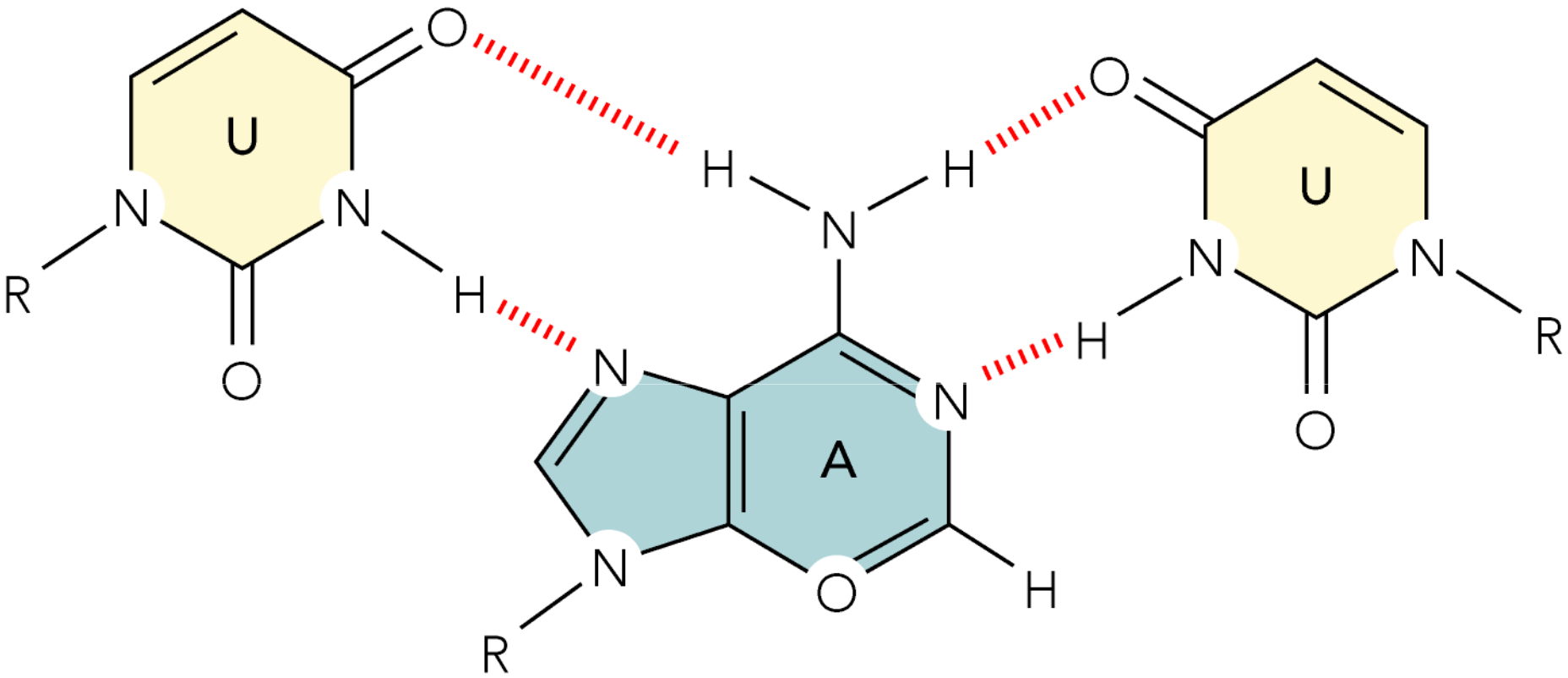
Slipped stranded structure

Unusual DNA structures formed by expandable repeats



A(GAA)*n*•(TTC)*n* (homopurine–homopyrimidine) repeat can convert into an intramolecular triplex called H-DNA under the influence of negative supercoiling

Reverse Hoogsteen pairing is indicated by asterisks



tripletta di basi U:A:U

Unusual DNA structures formed by expandable repeats

- **A DNA-unwinding element formed by the (ATTCT)*n*•(AGAAT)*n* repeat**
- This repeat belongs to the class of DNA elements that unwinds progressively with increasing negative superhelical stress

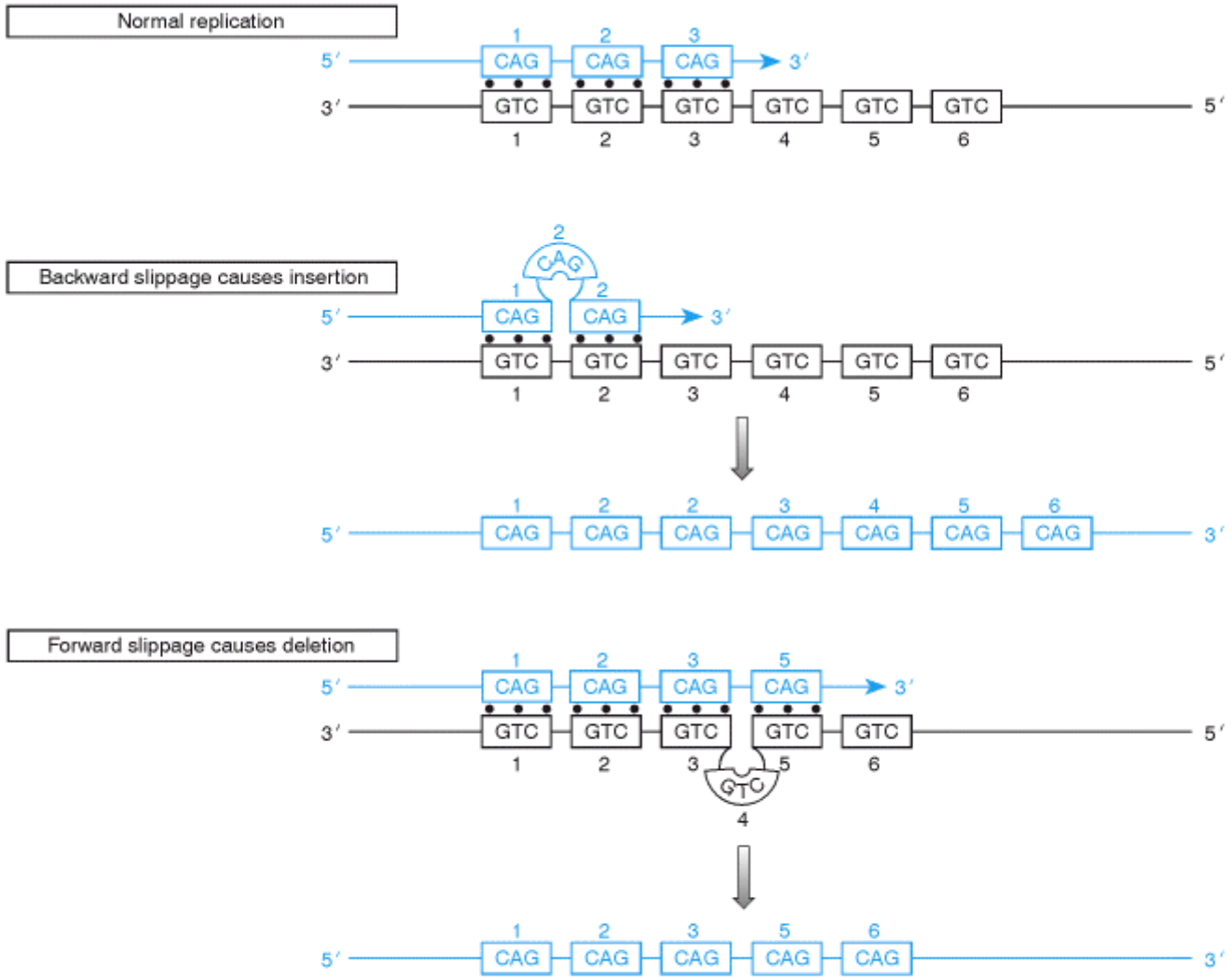
- slipped-stranded DNA is intrinsically asymmetrical.
- When the (CTG)*n*•(CAG)*n* repeat converts into the slipped-stranded form, CAG slip-outs are mainly in the random-coil state, whereas CTG slipouts are in the hairpin state.

TRIPLET EXPANSION AND DISEASE: molecular mechanisms

Expandable repeats are predisposed to instability, as a result of ‘confusion’ between

- the DNA replication,
- repair

- Unusual DNA structures that are formed by expandable repeats during DNA synthesis *in vitro* stall various DNA polymerases.
- Occasionally, this stalling results in the misalignment of repetitive DNA strands, causing repeat expansions or contractions



ıispairing during DNA replication can cause insertions or deletions.

- The frequencies of repeat expansions and contractions are affected markedly by mutations in several genes that encode proteins involved in DNA replication:

flap endonuclease (Fen1; also known as Rad27),

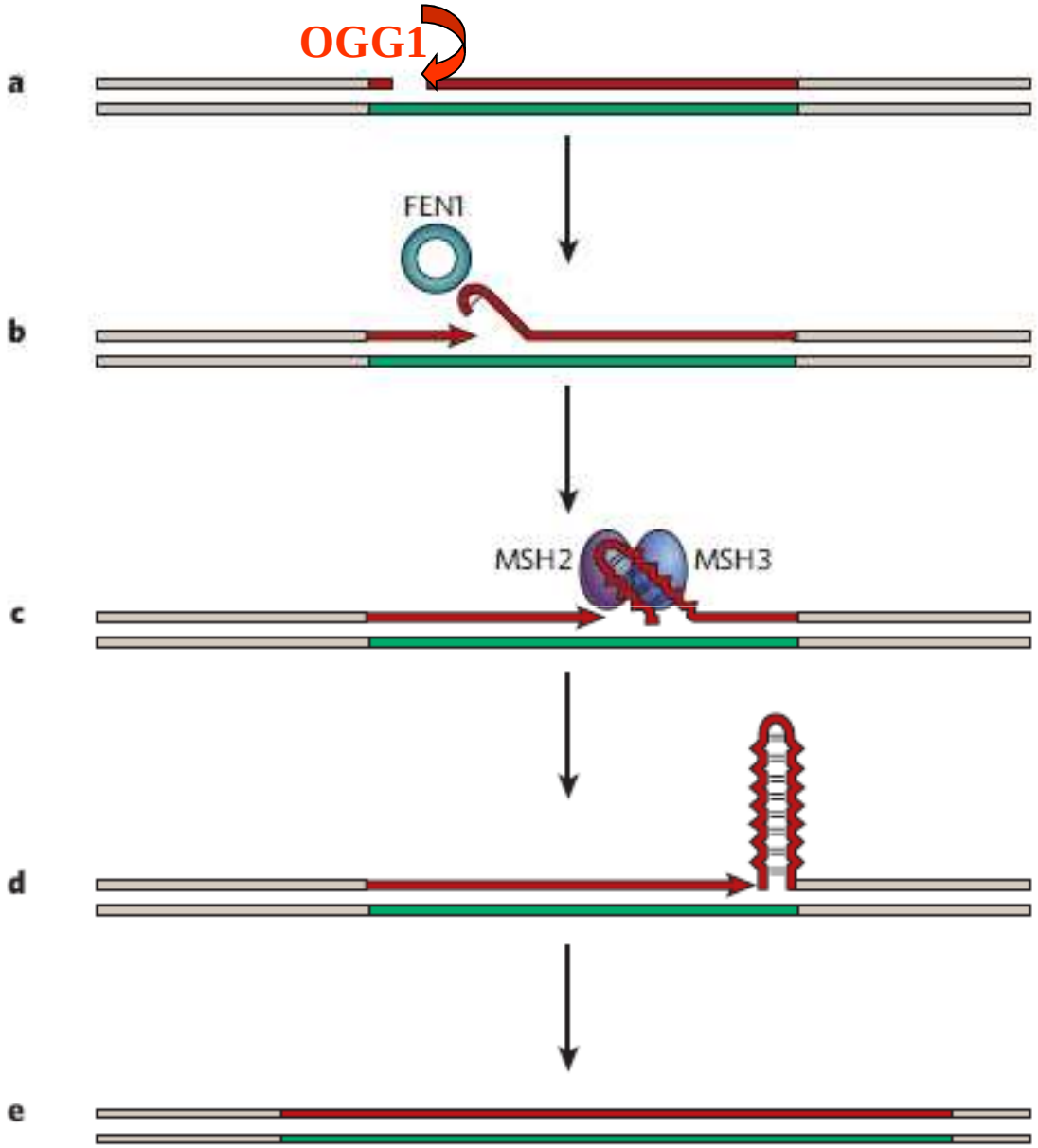
DNA polymerase-δ

MSH2

proliferating cell nuclear antigen PCNA, the large subunit of the clamp-loading complex,

the helicase Srs2 (also known as Hpr5)

•Gap repair model for repeat expansions in non-dividing cells.



a, Oxidizing radicals generate a small gap in the structure-prone strand of a repetitive run.

b, The loading of FEN1 onto a repetitive flap generated during the DNA-repair synthesis is impaired by hairpin formation.

c, The binding of MSH2–MSH3 stabilizes the repetitive hairpin, preventing flap removal.

d, A stable slipped-stranded DNA intermediate is formed on the completion of the repair synthesis.

e, The slipped-stranded intermediateis converted into an expansion by an error-prone repair pathway.

red -structure-prone strand of the repetitive run green complementary strand beige flanking DNA

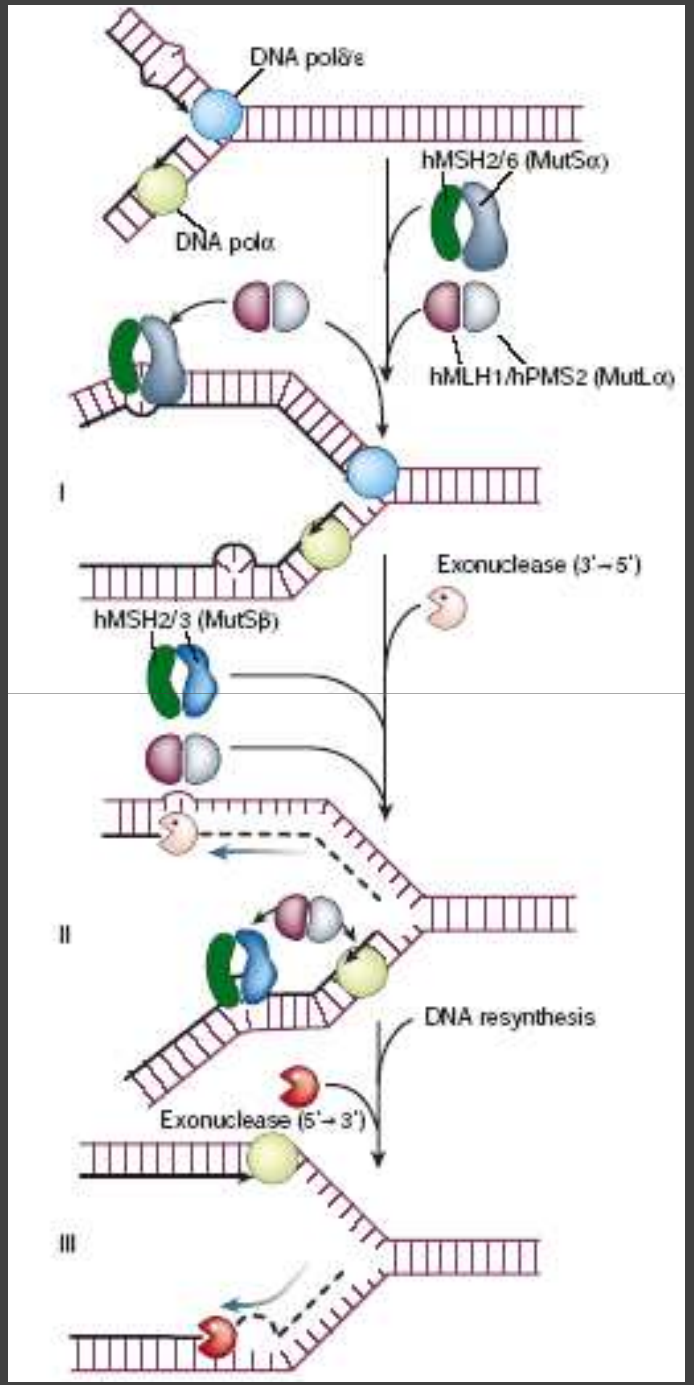
- recent studies using a transgenic mouse model of Huntington’sdisease have i mplicated base-excision repair as central to this process 53.
- In these mice, age-dependent repeat expansions in somatic cells depended on a single base-excision repair enzyme, 8-oxoguanine DNA glycosylase (OGG1)
- Removal of an oxidized guanine by OGG1 generates a nick in the repetitive run (Fig. 4a), and DNA-repair synthesis is then needed to heal this lesion. During this repair synthesis, the non-template DNA strand is displaced, forming a flap (Fig. 4b).
- Normally, a flap is removed by FEN1; this endonuclease is loaded onto the 5′ end of the flap, migrates to the junction with the duplex, and cleaves the flap. If a flap contains the structure-prone strand of a repetitive run, however, it can fold into a hairpin-like conformation, complicating FEN1 loading54,55.
- MSH2–MSH3 can further stabilize this hairpin preventing flap removal (Fig. 4c).
- Completion of the repair process will yield a stable slipped-stranded DNA intermediate with a repeat extension in the nicked strand (Fig. 4d).

MSH2 forma un eterodimero con **MSH6** (misappaiamento) o **MSH3** (loop di inserzione-delezione) e si lega al DNA segnalando l'elica templato

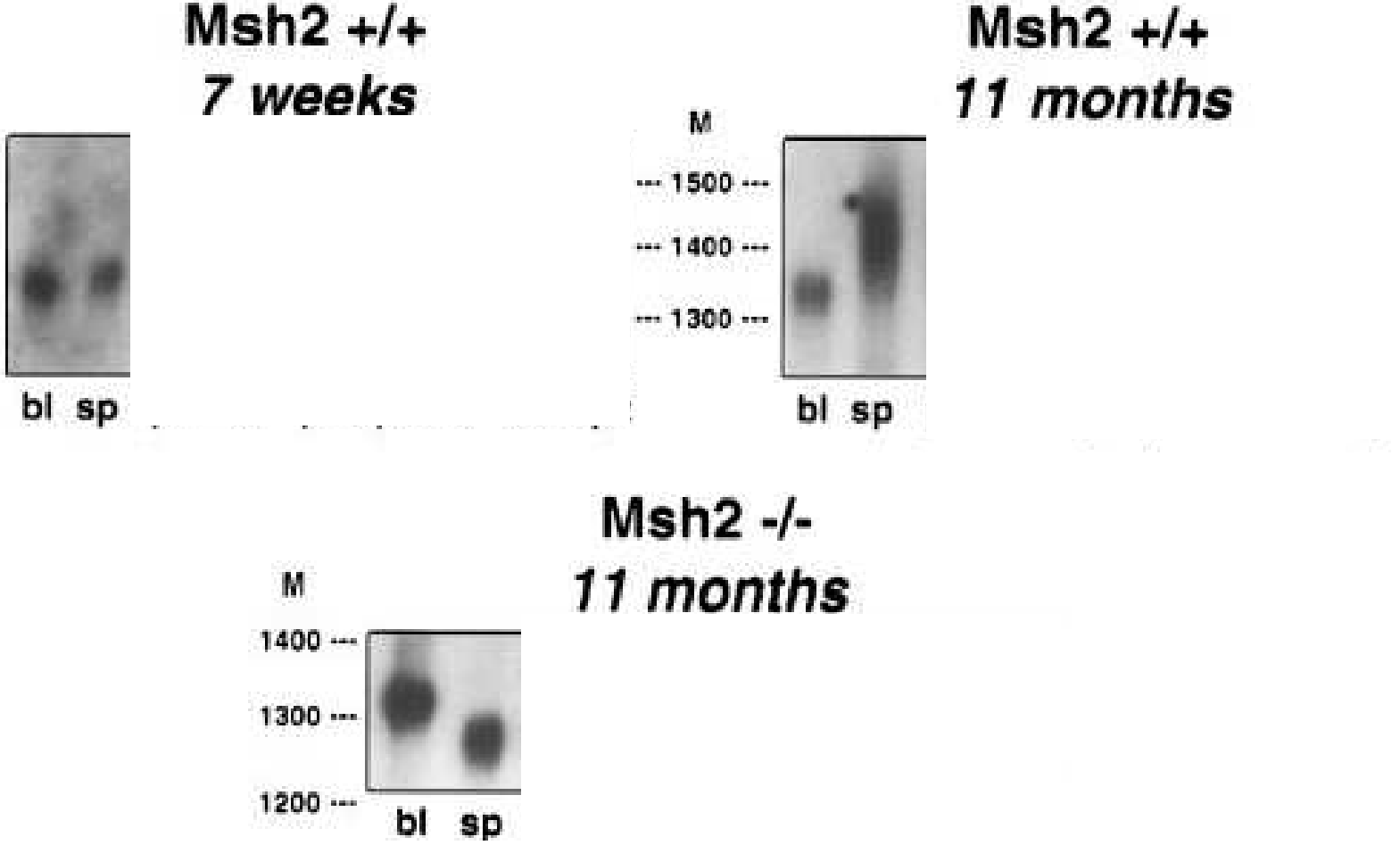
L'eterodimero **MLH1-PMS2**, talvolta legato anche a **PMS1**, coordina il legame con l'esonucleasi **EXO1** 3'- 5' ed una o più elicasi

EXO1 rimuove le basi errate e il gap è riempito da DNA polimerasi e ligasi

MSH2, MSH3 e MSH6 sono omologhi a **mutS** di E.coli; **MLH1**, **PMS1** e **PMS2** sono omologhi a **mutL** di E.coli



CTG repeat-length mosaicism in blood, in spermatozoa in Msh2/++ transgenic males at 7 weeks and 11 months of age and in an 11-month-old Msh2/-/- transgenic male.

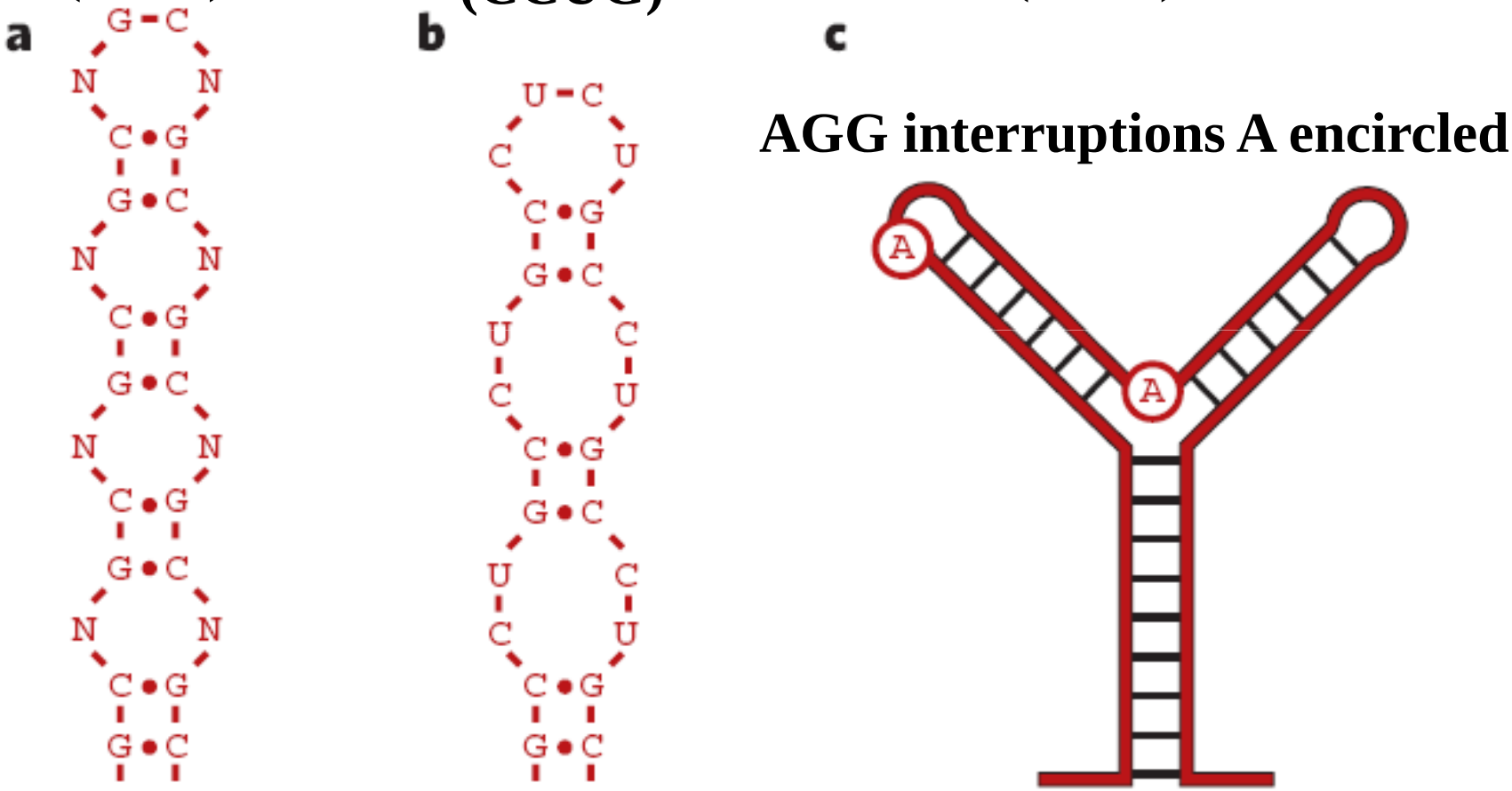


Meccanismi di “malattia”

- RNA gain-of-function

RNA gain-of-function effects could be grounded in the unusualstructural features of repeat-containing RNAs

(CNG) **(CCUG)** **(CGG)**



- The stability of RNA hairpins (CNG)*n* depends on
- the mismatched base pairs, decreasing in the order of CGG > CUG > CCG > CAG.

- The study was extended to all 20 possible triplet repeats in RNA.
- It seems that six repetitive motifs — (CGU)*n*, (CGA)*n*, (CAG)*n*, (CUG)*n*, (CCG)*n* and (CGG)*n* — can form stable RNA hairpins.

RNA gain-of-function

- The earliest support for this idea came from the observation that (CUG)*n* repeats in the natural sequence context of the *DMPK* transcript formed imperfect,mismatched hairpins, the stability of which increased with the length of the repeat.

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MD1 (1)

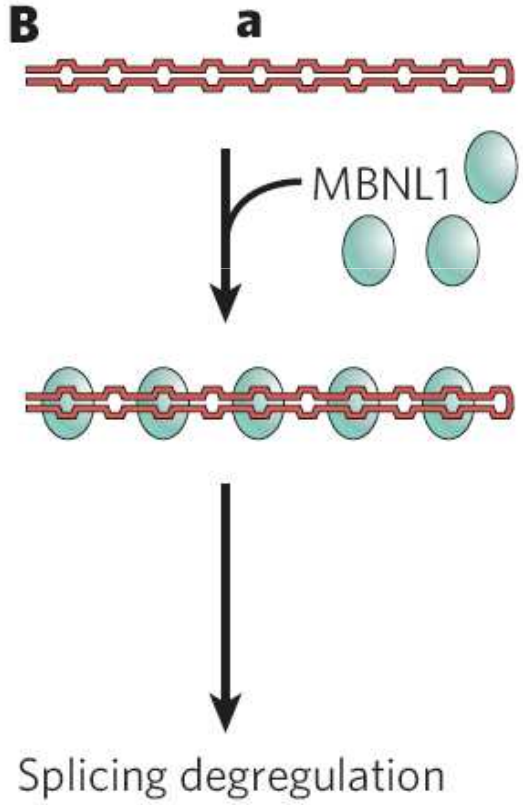
Myotonic dystrophy (DM) type 1 is associated with an expansion of (>50) CTG repeats within the 3' untranslated region (UTR) of the dystrophin myotonin protein kinase gene (dmpk).
Regulation by oligomerization is a key feature of the dystrophia myotonica protein kinase (DMPK) related family of Ser/Thr kinases.
DMPK is functionally related to reorganization events in the cytoskeleton during cellular processes such as cytokinesis, and smooth-muscle contraction.

MD1 (2)

In the mRNA transcript, the CUG repeats form an extended stem-loop structure.

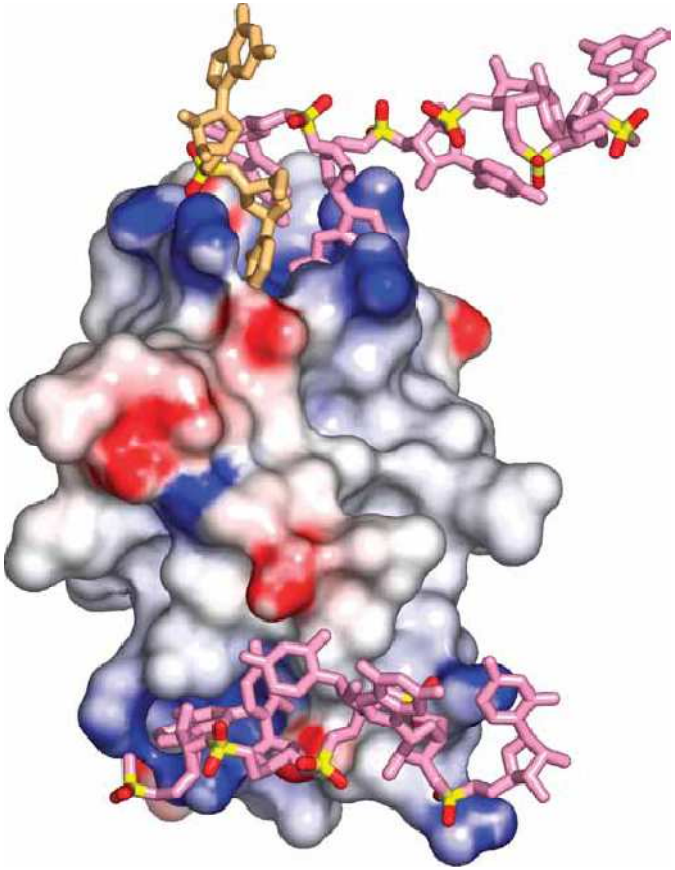
The double-stranded RNA of the stem sequesters RNA binding proteins away from their normal cellular targets resulting in aberrant transcription, alternative splicing patterns, or both, thereby leading to DM.

Molecular models of RNA gain of function in MD1

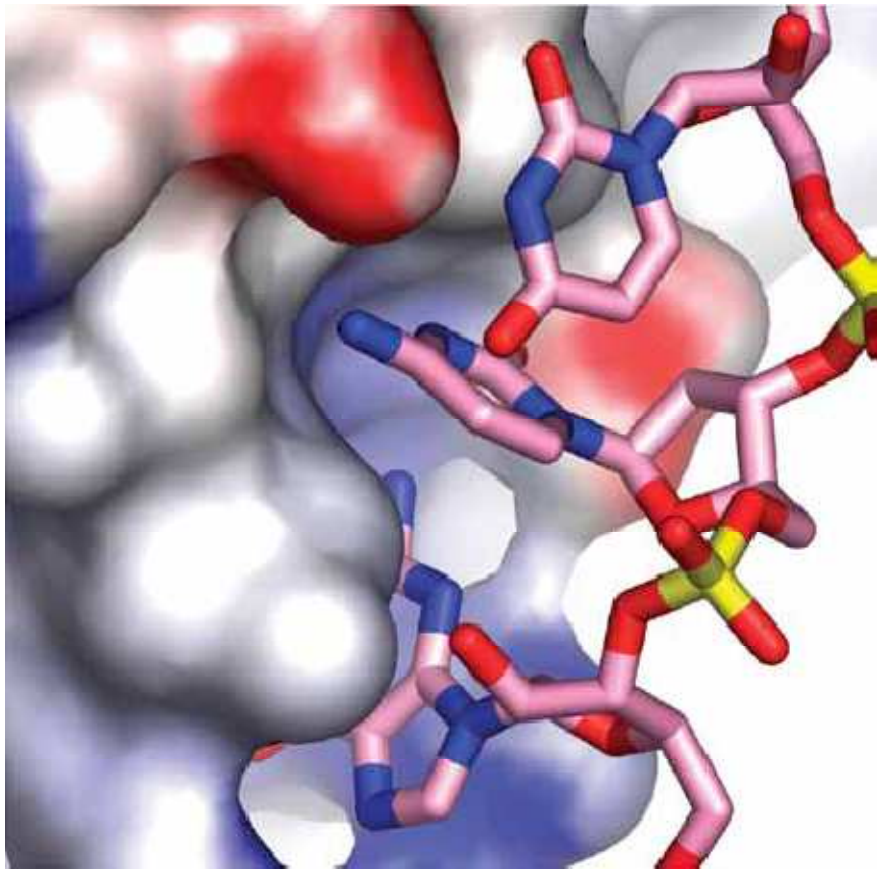


A repetitive RNA hairpin sequesters the protein MBNL1

MBNL1 Protein-RNA interactions



MBNL1 Protein- CUG interaction



DM1 2009

- Te expanded CUG repeat (CUG(exp)) cause muscle dysfunction by interfering with biogenesis of other mRNAs.
- The toxic effects of mutant RNA are mediated partly through sequestration of splicing regulator Muscleblind-like 1 (Mbnl1), a protein that binds to CUG(exp) RNA.
- the majority of changes induced by CUG(exp) RNA in skeletal muscle can be explained by reduced activity of Mbnl1
- The pathway most affected comprises genes involved in calcium signaling and homeostasis.
- Some effects of CUG(exp) RNA on gene expression are caused by abnormal alternative splicing or downregulation of Mbnl1-interacting mRNAs.

MD1

gain of function at the RNA level

- dominantly inherited disease(s)
- (CUG)*n* repeats in the *DMPK* transcript formed imperfect,mismatched hairpins, the stability of which increased with the length of the repeat
- transcripts of *DMPK* containing expanded CUG repeats were shown to be retained in the nuclei of fibroblasts and myoblasts, forming distinct foci.
- The ability of normal myoblasts to undergo myogenic differentiation in cell culture seemed to be suppressed by overexpression of RNA containing a (CUG)200 repeat
- a transgenic mouse expressing (CUG)250 repeats within the 3′-UTR of a heterologous gene (skeletal actin) showed major symptoms of myotonic dystrophy

myotonic dystrophy 1 and 2.

- Intranuclear foci that are characteristic of these diseases contain at least seven RNA-binding proteins associated with (CUG)*n*- and (CCUG)*n*-containing transcripts : three MBNL)-family proteins and two different CUG RNA-binding proteins (CUG-BPs).

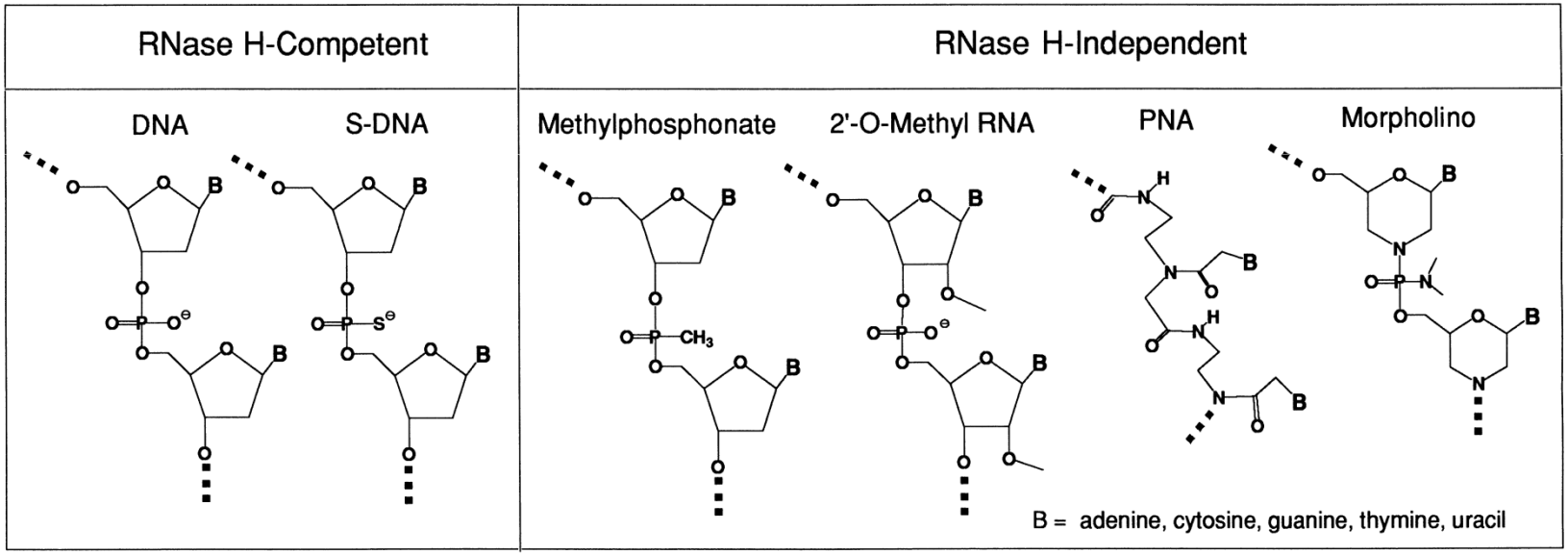
- a key molecular event leading to myotonic dystrophy is the deregulation of alternative RNA splicing during development.
  At least 13 splicing events are disturbed in muscle, heart and brain tissues from patients with myotonic dystrophy, and an embryonic 'blueprint' for splicing is almost always retained
- Both MBNL1 and CUG-BP1 are implicated in these splicing events, and the splicing pattern characteristic for myotonic dystrophy is consistent with the loss of MBNL1 and gain of CUG-BP1 activities.
- most of the data suggest that MBNL1 sequestration by long mismatched hairpins is a key event leading to splicing deregulation and, eventually, disease.
- *Mbnl1*-knockout mice show splicing deregulation and phenotypic manifestations characteristic of myotonic dystrophy, such as skeletal muscle myotonia and cataracts.
- The myotonic-dystrophy-like phenotype of mice expressing (CUG)*n* from a heterologous gene could be reversed by overproduction of MBNL1

Reversal of RNA Dominanceby Displacement of Protein Sequestered on Triplet Repeat RNA
Thurman M. Wheeler Science 2009

- Genomic expansions of simple tandem repeats can give rise to toxic RNAs that contain expanded repeats.
- In myotonic dystrophy, the expression of expanded CUG repeats (CUGexp) causes abnormal regulation of alternative splicing and neuromuscular dysfunction.
- We used a transgenic mouse model to show that derangements of myotonic dystrophy are reversed by a morpholino antisense oligonucleotide, CAG25, that binds to CUGexp RNA and blocks its interaction with muscleblind-like 1 (MBNL1), a CUGexp-binding protein. CAG25 disperses nuclear foci of CUGexp RNA and reduces the overall burden of this toxic RNA.
- As MBNL1 is released from sequestration, the defect of alternative splicing regulation is corrected, thereby restoring ion channel function.
- These findings suggest an alternative use of antisense methods, to inhibit deleterious interactions of proteins with pathogenic RNAs.

RNase H-independent morpholino antisense oligos provide complete resistance to nucleases, generally good targeting

predictability, generally high in-cell efficacy, excellent sequence specificity



phosphorothioate-linked DNA analogs (S-DNAs)

morpholine rings+

phosphorodiamidate linkages

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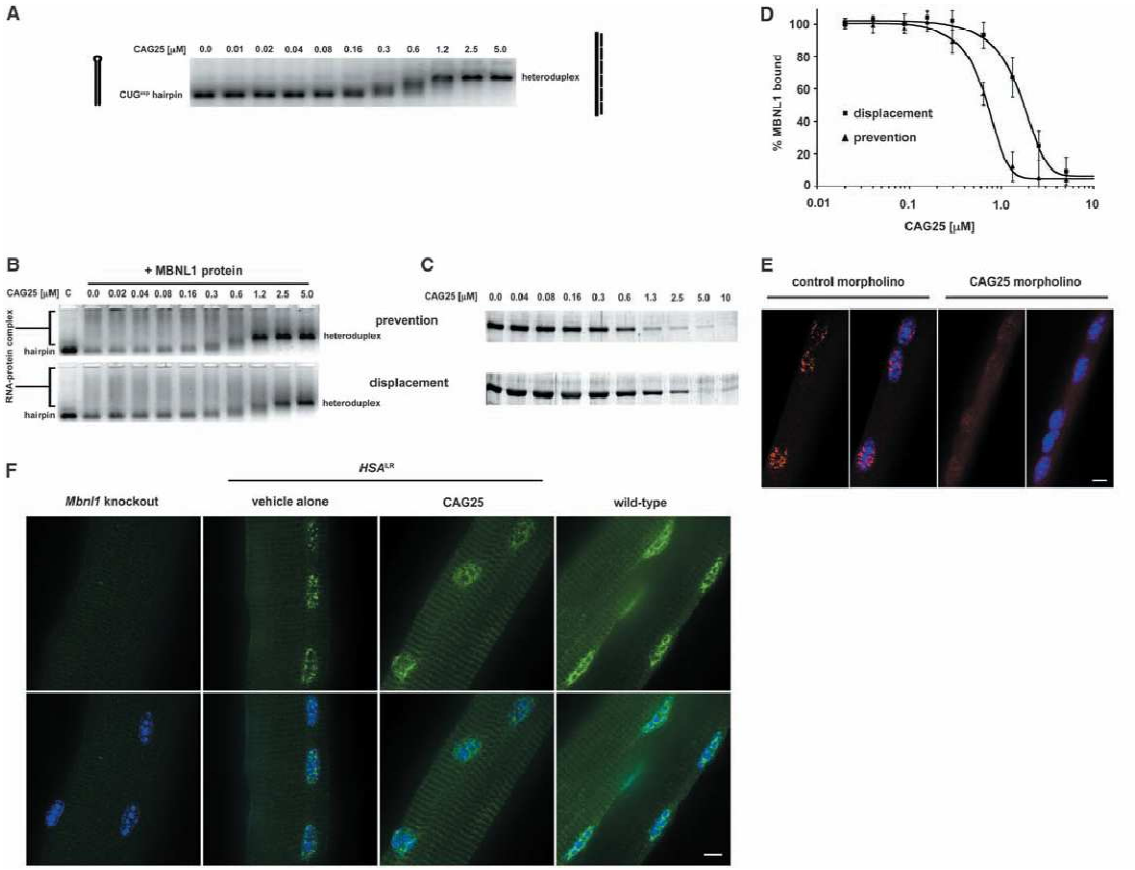

HSALR transgenic mice express human skeletal actin transcripts that have (CUG)250 inserted in the 3′UTR.

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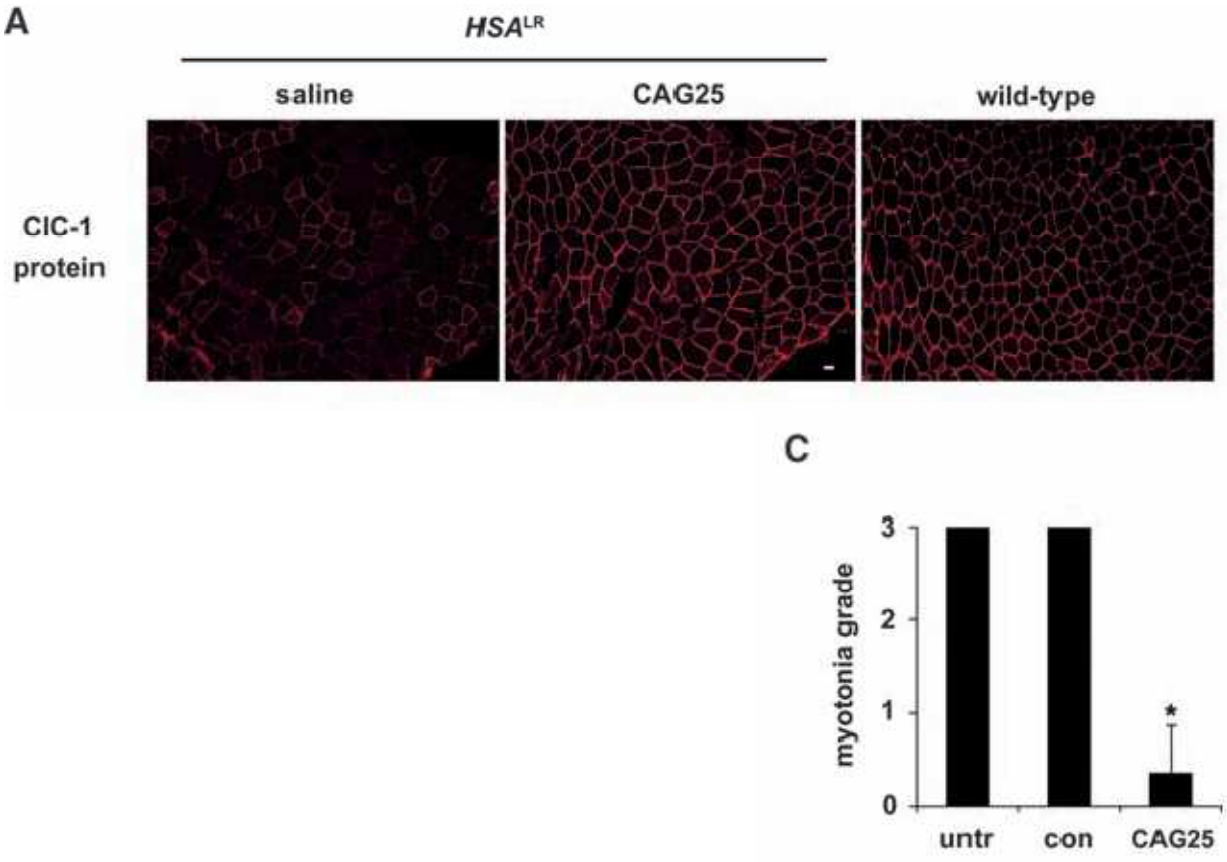
chloride channel 1
(ClC-1)
the muscle-specific
chloride channel



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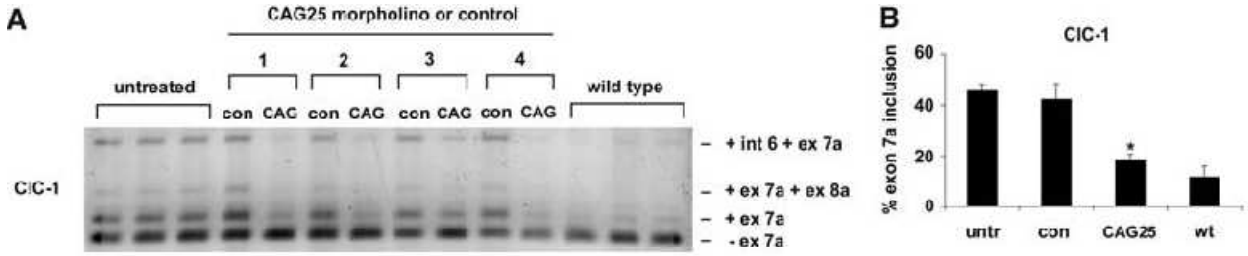

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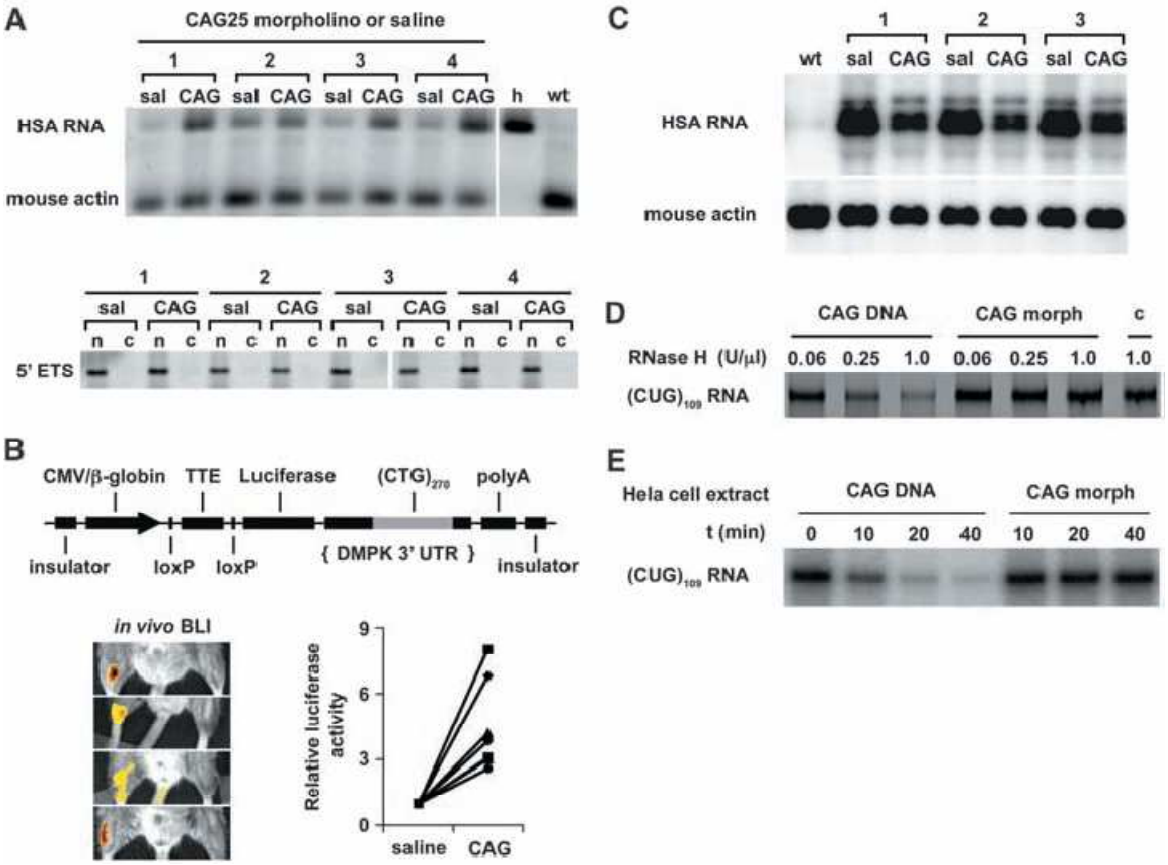
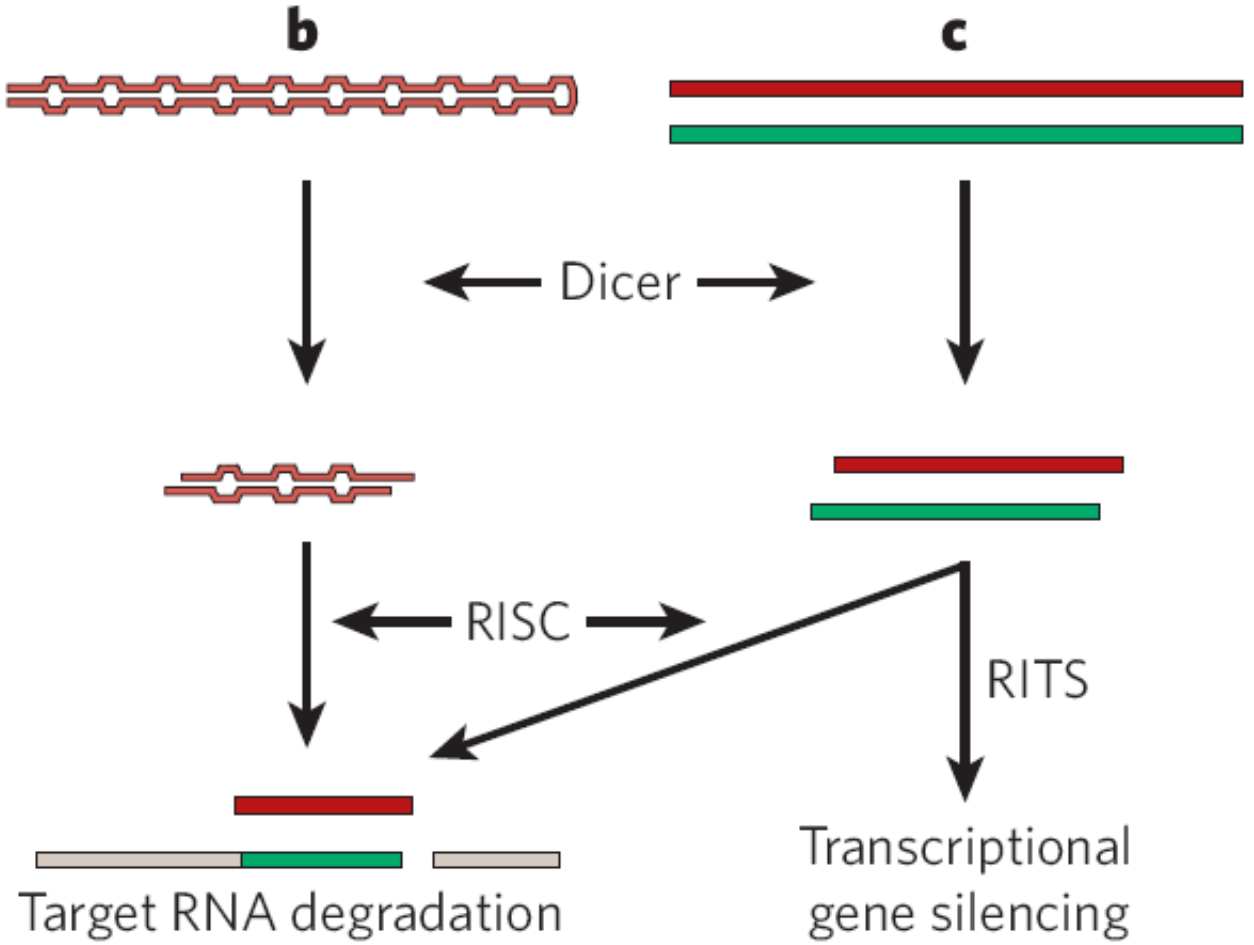


Molecular models of RNA gain of function



RNA-induced
Silencing complex

RNA-induced
transcriptional silencing

RNA-induced transcriptional silencing
RITS

- The maintenance of centromeric heterochromatin in yeast relies on the RNAi-dependent complexes, RITS to recruit histone H3 K9 methyltransferase

- RITS complex:
- centromeric siRNAs
- Ago1, which binds siRNAs
- Chp1, which binds K9 methylated histone H3,
- the adaptor protein, Tas3, which links Ago1 to Chp1.

- Chp1 associates with Tas3 for recruitment of histone H3 K9 methyltransferase activity to centromeres

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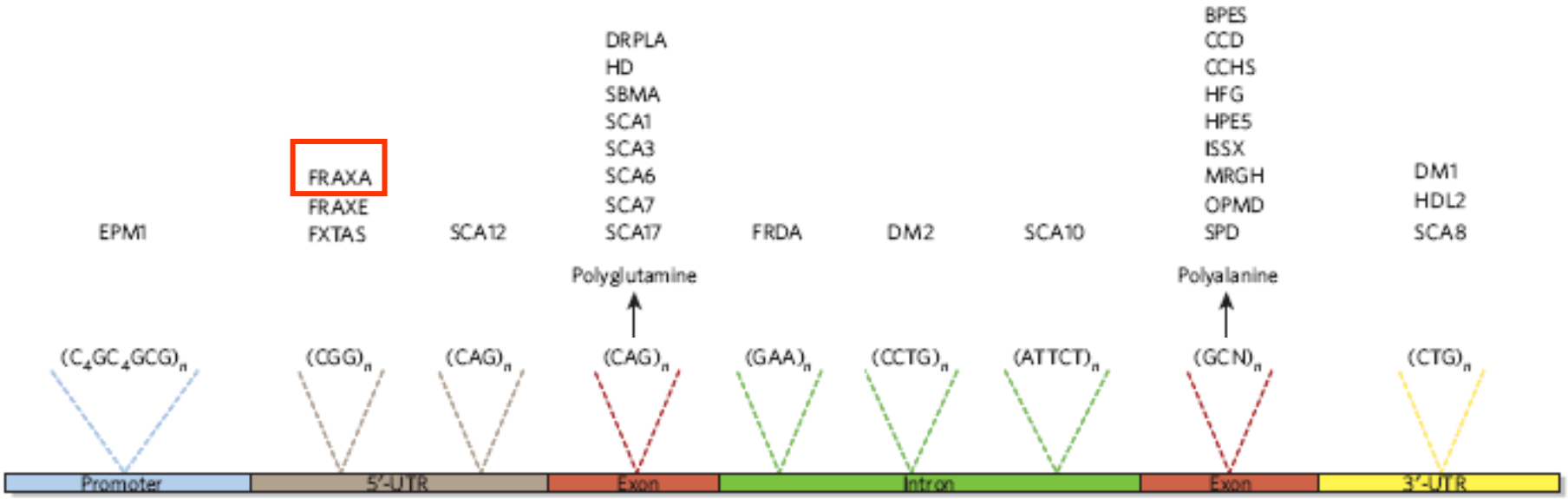

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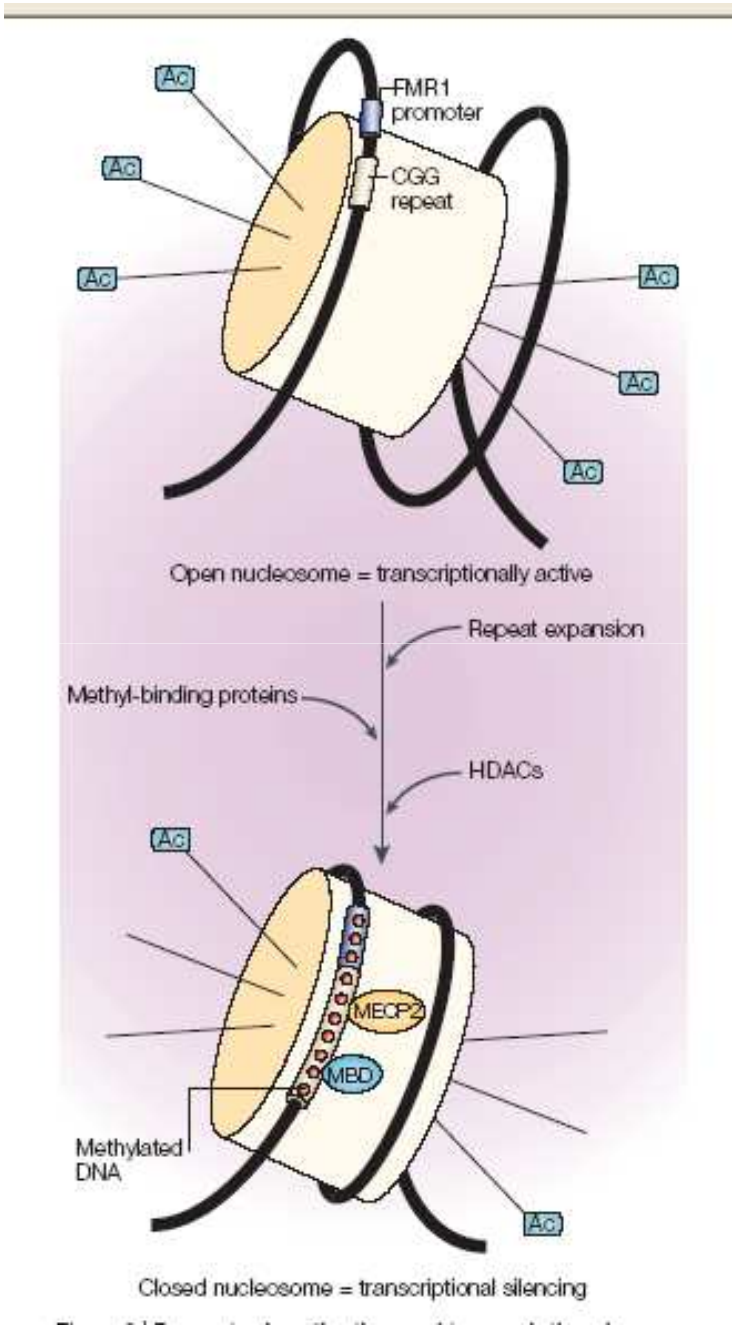


Promoter inactivation and transcriptional repression in fragile X syndrome. The expansion of a CGGrepeat in the 52 UTR of the fragile X mental retardation 1 (*FMR1*) gene results in hypermethylation of the repeat region which recruits methyl-DNA-binding proteins, such as methyl-CpG-binding protein 2 (MECP2)and methyl-CpG-binding domain protein (MBD) that repress transcription.

These proteins also recruit histone-modifying enzymes, including histone deacetylases (HDACs), whichfurther repress transcription (lower panel). Ac, acetylation.

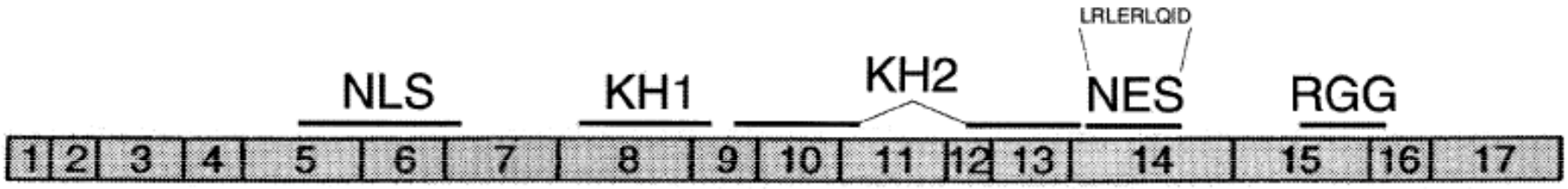


FIG. 1. Structural organisation of the full-length Fragile X mental retardation gene 1 gene. All 17 exons are present in the ISO1 isoform (632 amino acids). All the functional domains present in the protein (NLS, NES, KH1, KH2, RGG box) are indicated. The KH2 domain has been defined according to Musco et al. [53a] and Adinolfi et al. [2].

an RNA-binding protein that regulates
the transport/translation of specific mRNAs at synapses

Fig. 1. NMR structure of the KH1 domain from FMRP (PDB code 2FMR). The KH1 domain has a typical KH β/α fold. The helices pack against a three-stranded antiparallel β-sheet forming the hydrophobic core of the protein. The conserved isoleucine residue is shown in orange.

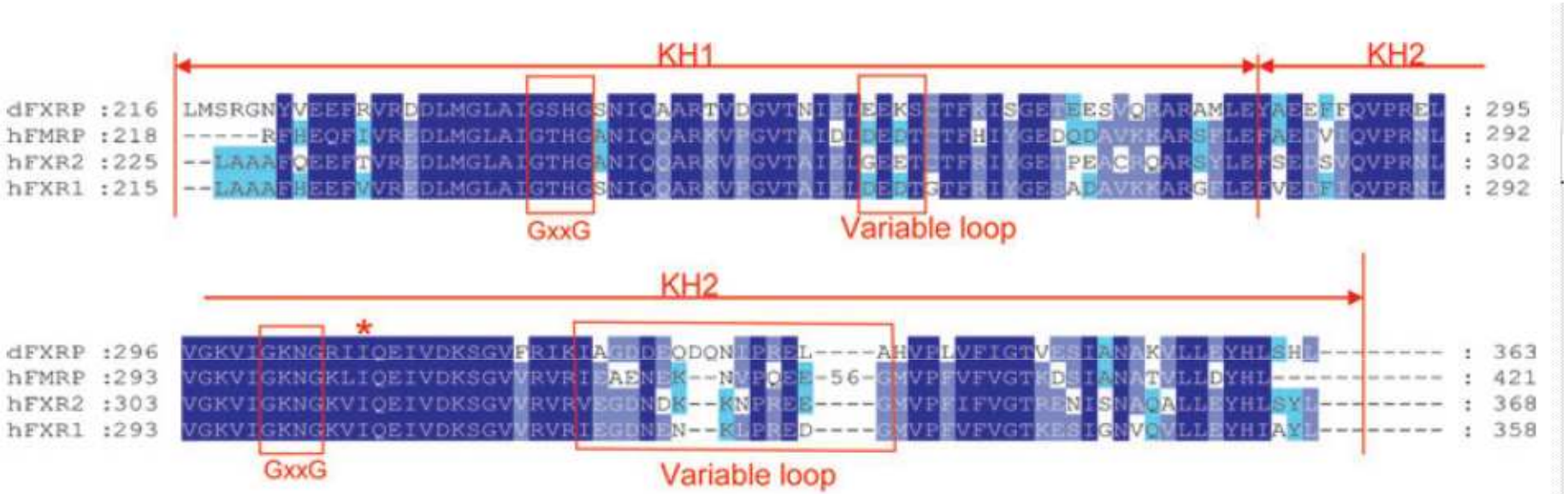


Fig. 3. Multiple-sequence alignment of KH domains from dFXRP and the human Fragile X proteins (in this figure named hFMRP, hFXR1, hFXR2). Conserved residues are colored in dark blue and semiconserved residues are colored in lighter shades of blue. The domain boundaries, the conserved signature motif GxxG and a variable loop between β-strands β2 and β3 which was proposed to have functional significance are indicated. The conserved isoleucine residue is marked with an asterisk. Residue numbering corresponds to the full-length proteins.

Fragile X mental retardation protein (FMRP) binds to Amyloid Precursor Protein (APP) mRNA

- Fragile X mental retardation protein (FMRP) is a cytoplasmic mRNA binding protein whose expression is lost in fragile X syndrome.
- FMRP binds to the coding region of Amyloid precursor protein (APP) mRNA
- APP facilitates synapse formation in the developing brain, while beta-amyloid (Ab) accumulation, which is associated with Alzheimer disease, results in synaptic loss and impaired neurotransmission.

Sequenza di eventi che portano alla patologia di Alzheimer

Mutazioni nei geni APP, PS1, PS2

Alterata proteolisi di APP

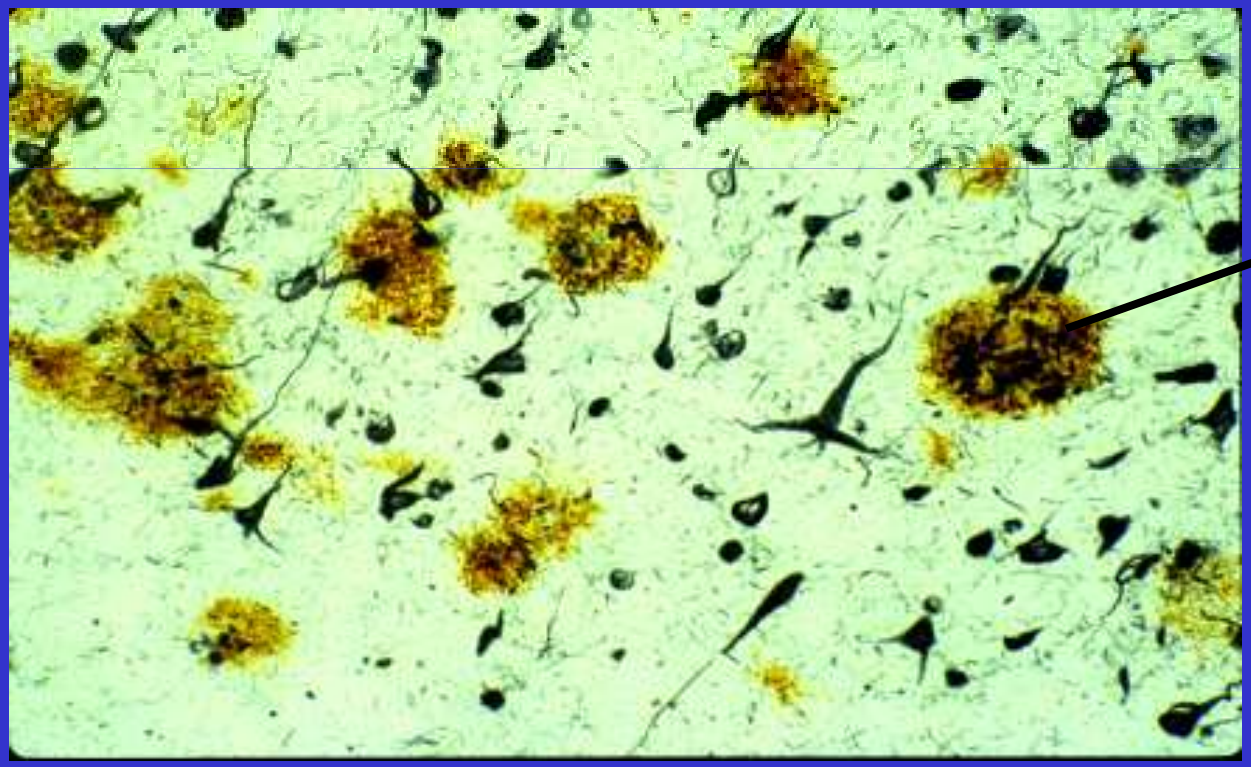
Aumentata produzione di Aβ42

Progressivo accumulo e aggregazione di Aβ42

Placche di Aβ42

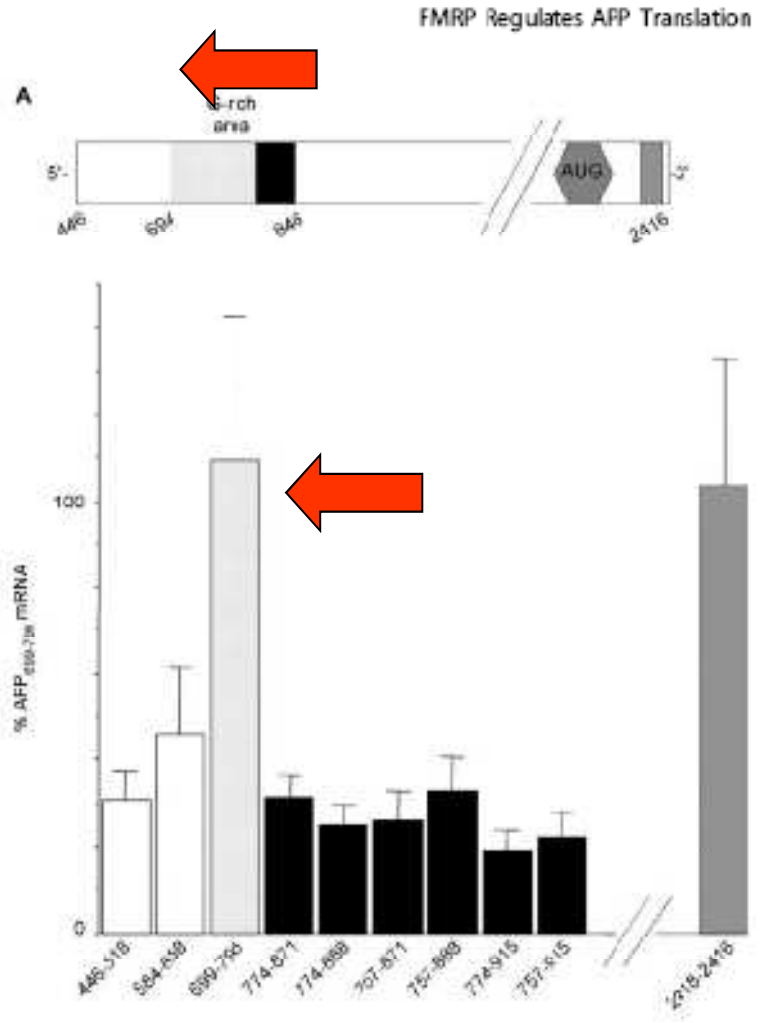
APP e Patologia di Alzheimer

La principale modificazione patologica associata all'Alzheimer è l'accumulo di PLACCHE AMILOIDI nel cervello



Aggregati di un peptide di 42 aa (Aβ42)

Fragile X mental retardation protein (FMRP) binds to a G-Rich Sequence in the Amyloid Precursor Protein (APP) mRNA

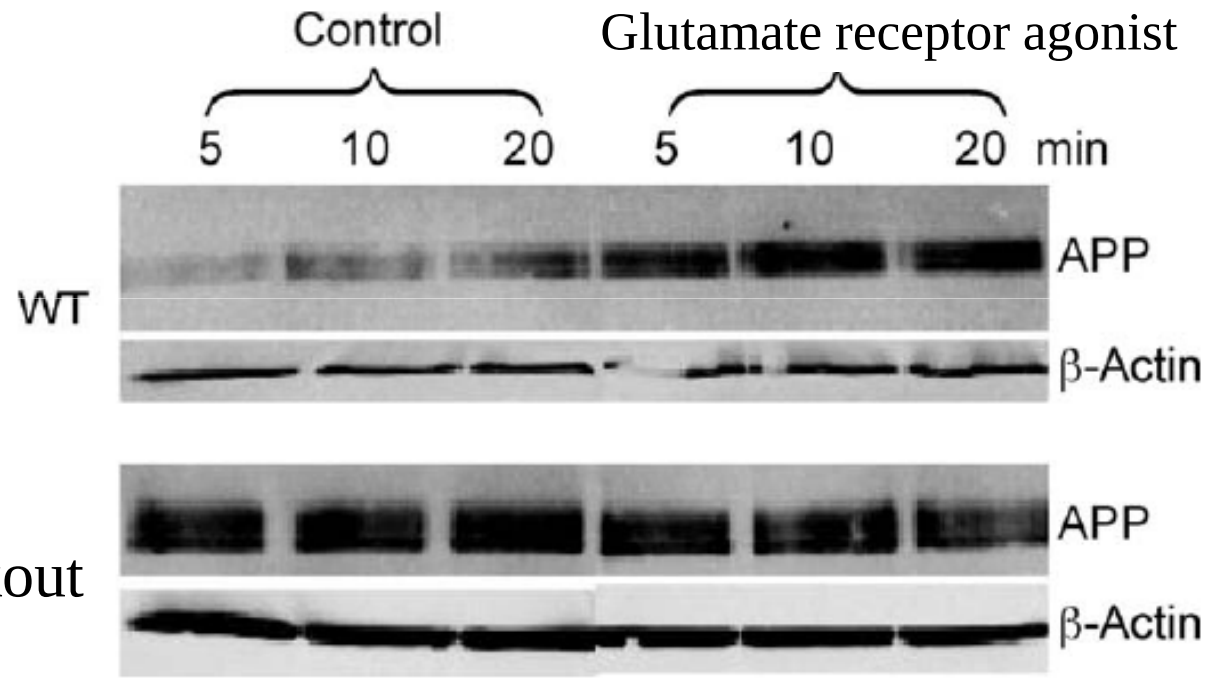


position of the G-rich, predicted G-quartet element in APP mRNA

FMRP IPs digested with RNAse T1 and analyzed by RTqPCR

Nucleotides 699–796 in the coding region of APP mRNA possess a G-rich sequence that is protected from nuclease digestion

Amyloid Precursor Protein regulation is lost in FMR-1 knockout mice



fmr-1 knockout

Figure 4. Differential Regulation of APP Levels in WT and KO SNs

Western blots of WT (top panel) and KO (bottom panel) SN treated with or without DHPG (5, 10, and 20 min) and hybridized with anti-APP and anti–β-actin antibodies. The data are representative of three experiments, and quantitation with ImageQuant software demonstrates a 1.6–1.8-fold increase in APP between untreated and DHPG-stimulated WT SNs at all of the times tested.