Non coding RNAs and disease

(ncRNAs) = RNA transcripts that do not have a protein-coding capacity

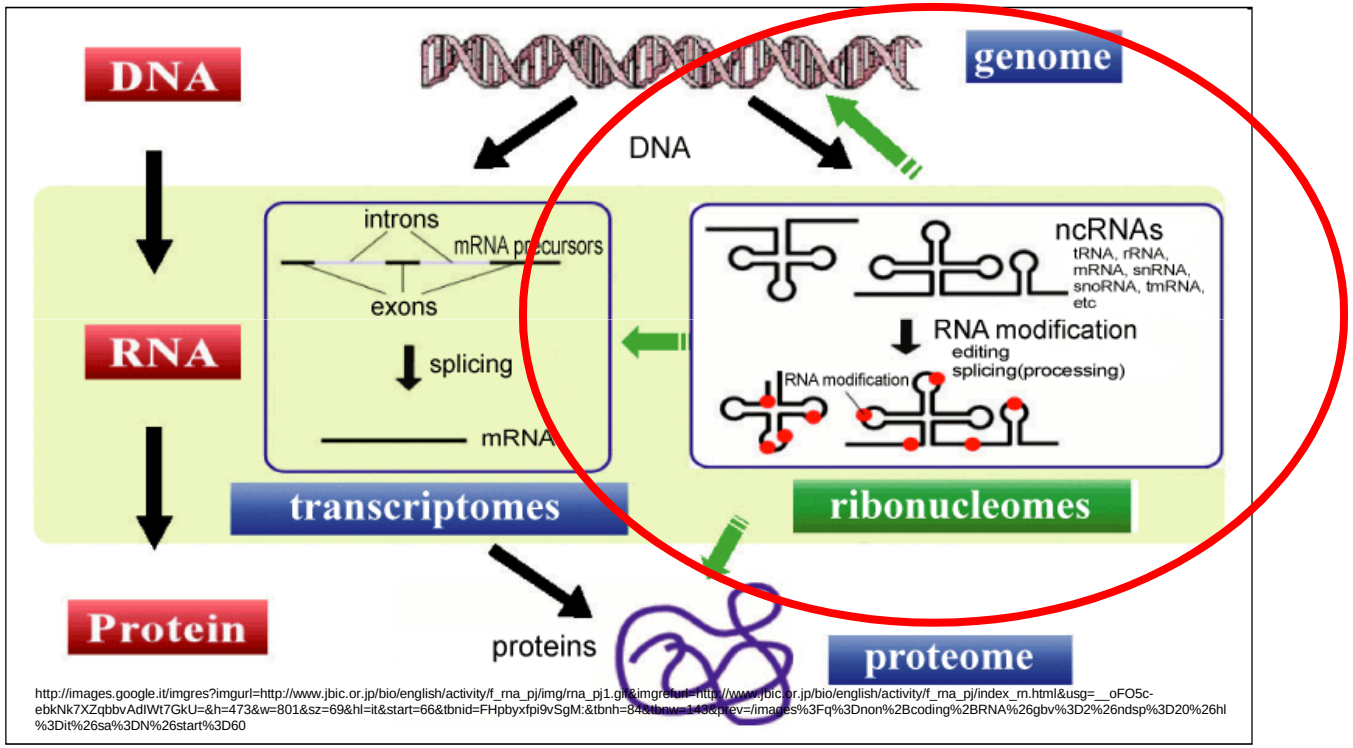
- Protein coding genes alone are not sufficient to accuont for the complexity of higher eukaryotic organisms.

Interestingly, from genomic analysis it is evident that as an organism's complexity increases, the protein-coding contribution of its genome decreases.

A portion of this paradox can be resolved through alternative pre-mRNA **splicing**, whereby diverse mRNA species, encoding different protein isoforms, can be derived from a single gene.
In addition, a range of **post-translational modifications** contributes to the increased complexity and diversity of protein species.

Genes & Development 2007 (21:11-42)

It is estimated that ~96-98% of the transcriptional output of the human genome represents RNA that does not encode protein.



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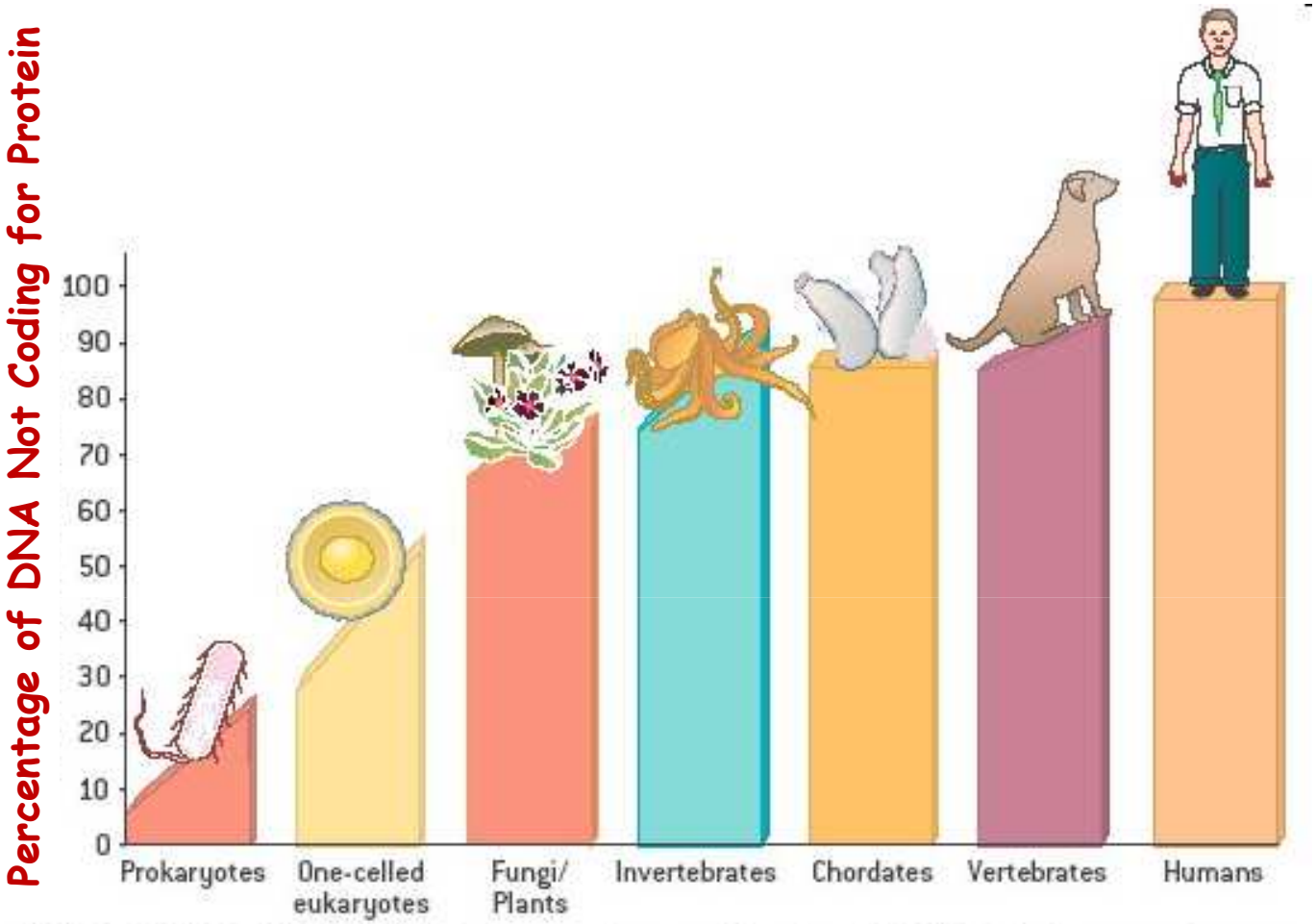
ncRNAs are fulfilling a wide range of unexpected functions in eukaryotic biology.

Recent observations strongly suggest that ncRNAs contribute to the complex networks needed to regulate cell function and could be the ultimate answer to the genome paradox.

Genes & Development 2007 (21:11-42)



NONPROTEIN-CODING SEQUENCES make up only a small fraction of the DNA of prokaryotes. Among eukaryotes, as their complexity increases, generally so, too, does the proportion of their DNA that does not code for protein. The noncoding sequences have been considered junk, but perhaps it actually helps to explain organisms' complexity.

http://www.bio.miami.edu/~cmallery/150/gene/noncoding.genes.jpg

Based on functional relevance, ncRNAs can be subdivided into two classes:

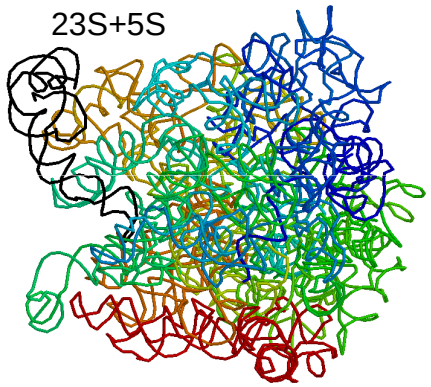
1_ housekeeping ncRNAs and

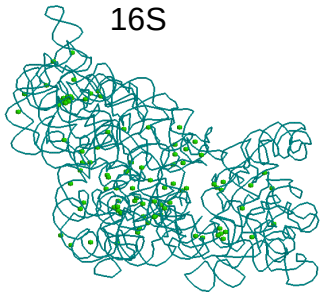
2_ regulatory ncRNAs

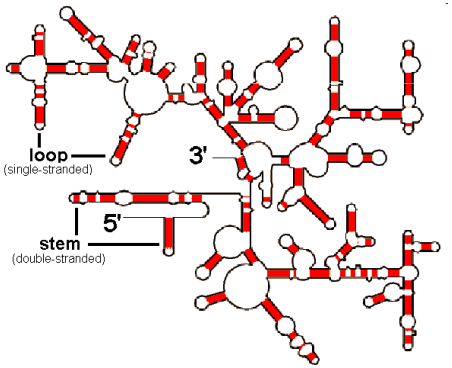
1_ housekeeping ncRNAs

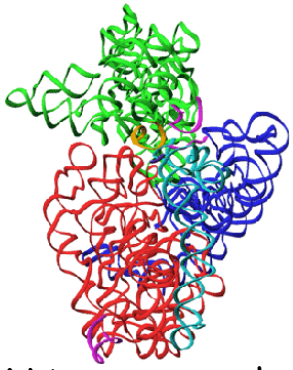
Examples are:

- tRNA, ribosomal RNAs (rRNAs)







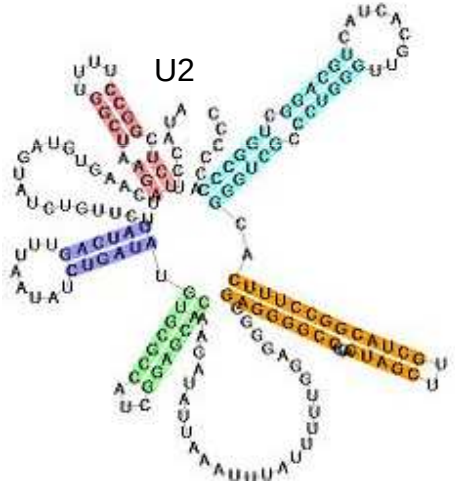


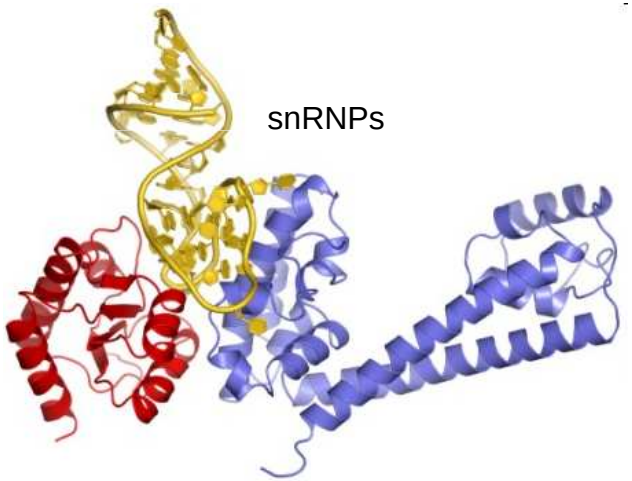
ribosomal RNA is found in the ribosomes.
Prokaryotic ribosomes have 3 rRNA molecules: 23S, 16S and 5S.
Eukaryotic ribosomes have 4 rRNA molecules: 28S, 18S, 5.8S and 5S.
rRNA was once thought to be an inert scaffold for the ribosomal proteins. We now know that the 23S (and 28S) rRNA is the catalytic agent in protein synthesis.

1_ housekeeping ncRNAs

Examples are:

- small nuclear (snRNAs)



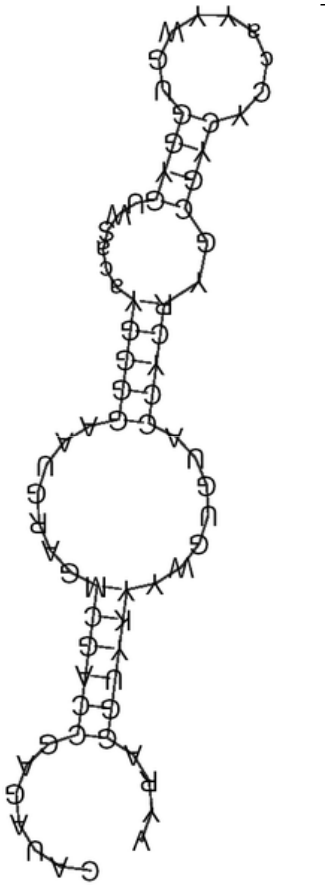


small nuclear RNA molecules are integral components of the small nuclear ribonucleoprotein particles which bring about splicing of eukaryotic mRNAs.

1_ housekeeping ncRNAs

Examples are:

- Small nucleolar (snoRNAs)

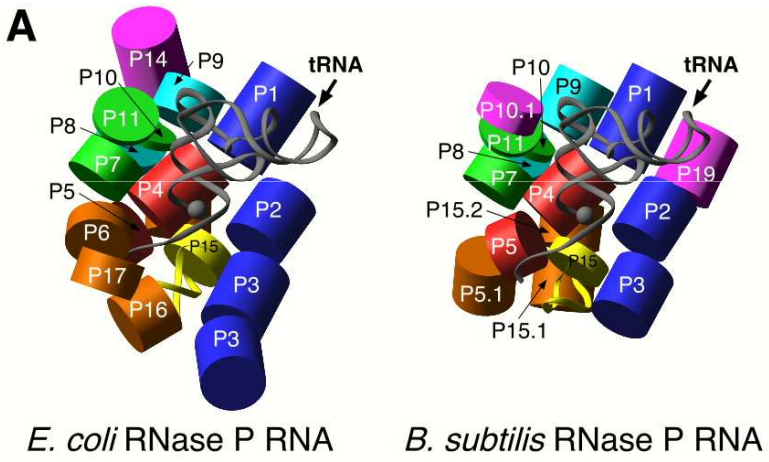


small nucleolar RNA molecules are found in the nucleolus of eukaryotic cells. They are associated with protein particles, snoRNPs, and they have been demonstrated to define sites of nucleotide modifications in rRNA. In addition, a few snoRNAs may play a role in of pre-rRNA processing in the nucleolus.

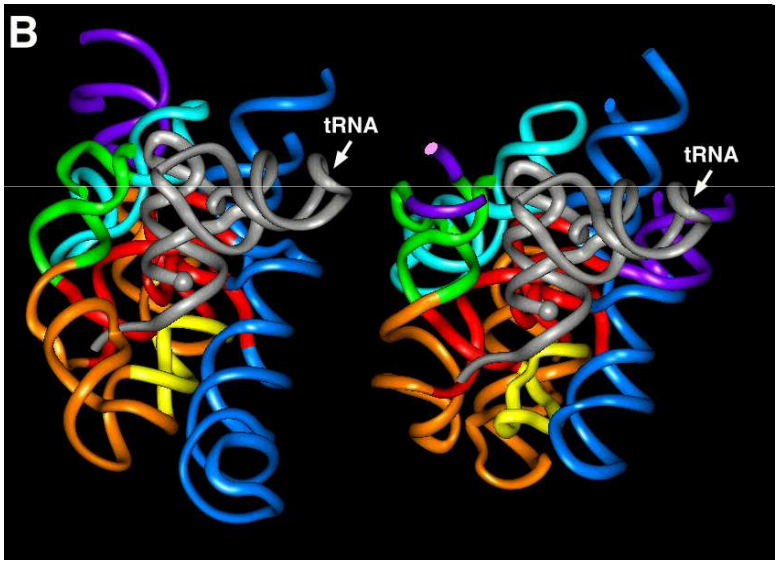
1_ housekeeping ncRNAs

Examples are:

- RNase P RNAs



E. coli RNase P RNA *B. subtilis* RNase P RNA



M1 RNA is the name given to the RNA component of **Ribonuclease P**, which functions in the processing of tRNA molecules in prokaryotes. M1 RNA is the catalytic component of the enzyme.

2_ regulatory ncRNAs or riboregulators

include those ncRNAs that are

- expressed at certain stages of development,
during cell differentiation,
- or as a responce to external stimuli

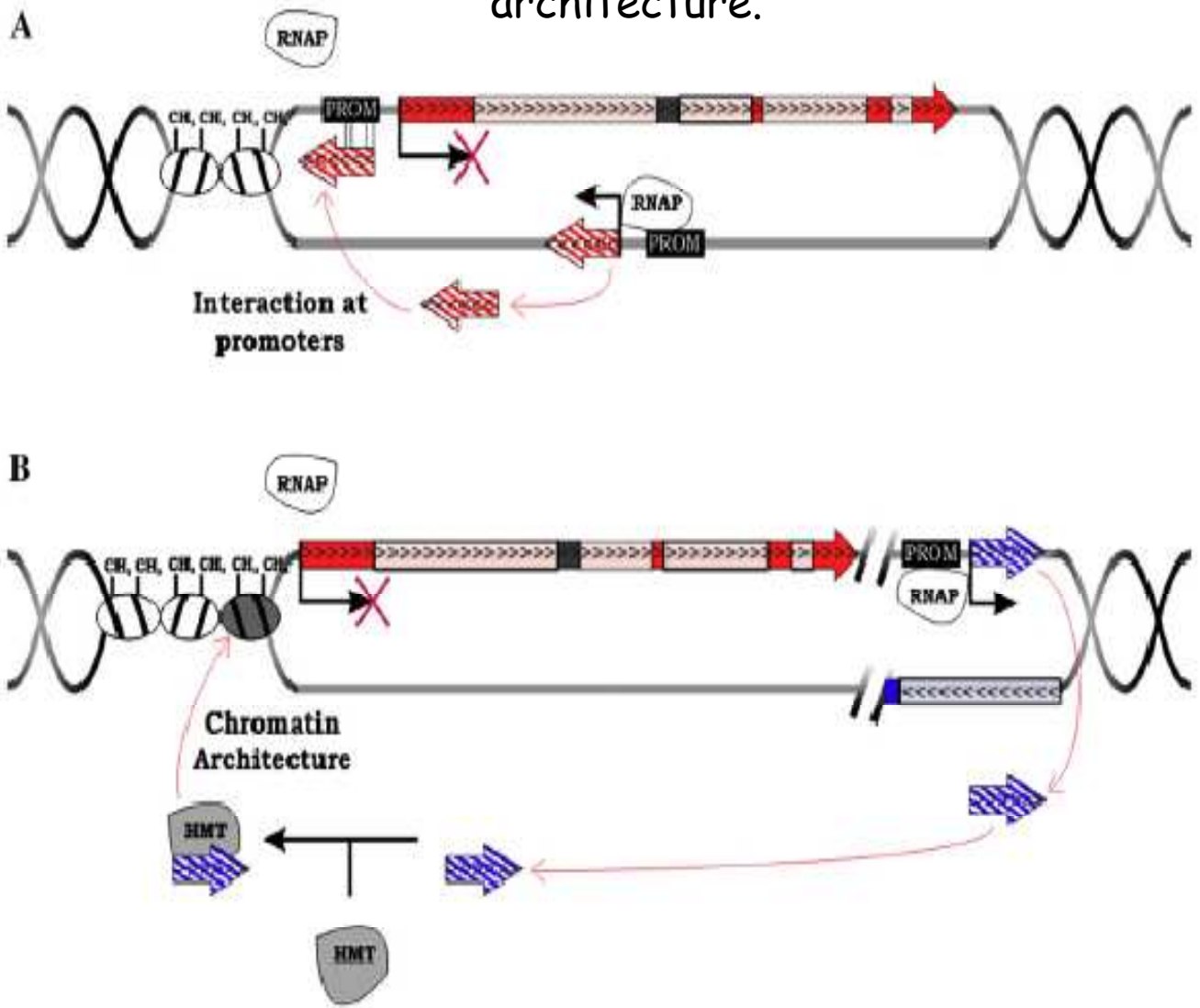
which can affect the expression of other genes
at the level of transcription or translation.

Examples are siRNAs, miRNAs and

other non coding regulatory RNAs in eukaryotic cells involved in gene organization, regulation and disease etiology.

mechanisms of **gene expression control by regulatory ncRNAs** at the **transcriptional level**.

(A) Transcriptional interference through interaction at promoters.
(B) Epigenetic control of gene-expression through modulation of chromatin architecture.



enhancer RNAs (eRNAs)
Nature 2010

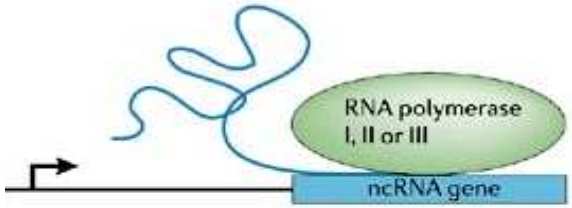
RNAPII at enhancers transcribes bi-directionally a novel class of enhancer RNAs (eRNAs)
enhancer domains defined by the presence of histone H3 monomethylated at lysine 4.

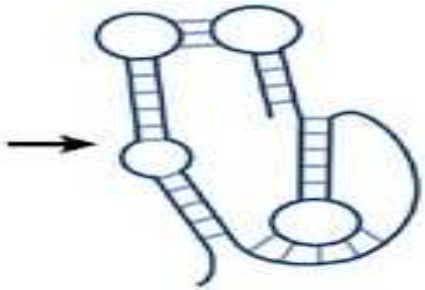
The level of eRNA expression at neuronal enhancers positively correlates with the level of mRNA synthesis at nearby genes

eRNA synthesis occurs specifically at enhancers that are actively engaged in promoting mRNA synthesis

A widespread mechanism of enhancer activation involves RNAPII binding and eRNA synthesis.

noncoding transcriptome





Non-coding RNA (ncRNA) genes can be transcribed by either RNA polymerase I, II or III, depending on the individual ncRNA

ncRNAs fold into specific structures that impart a function to the molecule

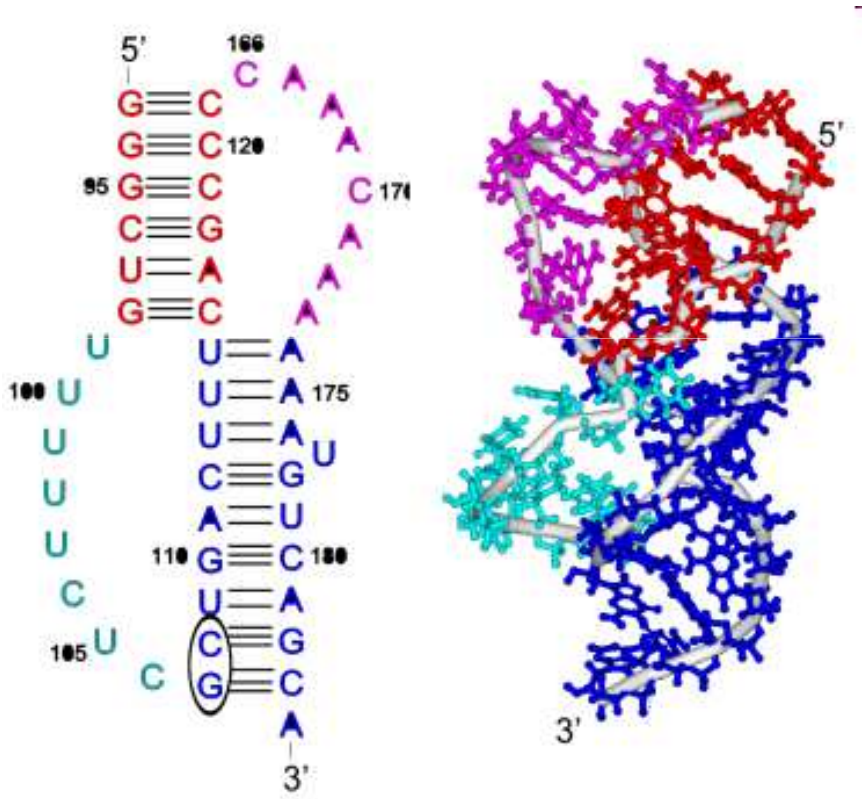
often these RNAs are incorporated into large complexes (yellow) that contain proteins and sometimes other nucleic acids.

These complexes then regulate biological reactions, and their function is strictly dependent on the presence of the ncRNA.

1_ housekeeping ncRNAs

Examples are:

- telomerase RNA



Telomerase, the enzyme that adds the telomere repeats to eukaryotic chromosomes contains an essential RNA template.

TERRA

Telomeres, the physical ends of eukaryotes chromosomes are transcribed into telomeric repeat containing RNA (TERRA), a large non-coding RNA

endogenous TERRA is bound to human telomerase

the 50-UUAGGG-30 repeats of TERRA base pair with the RNA template of the telomerase RNA moiety

TERRA contacts the telomerase reverse transcriptase (TERT) protein subunit

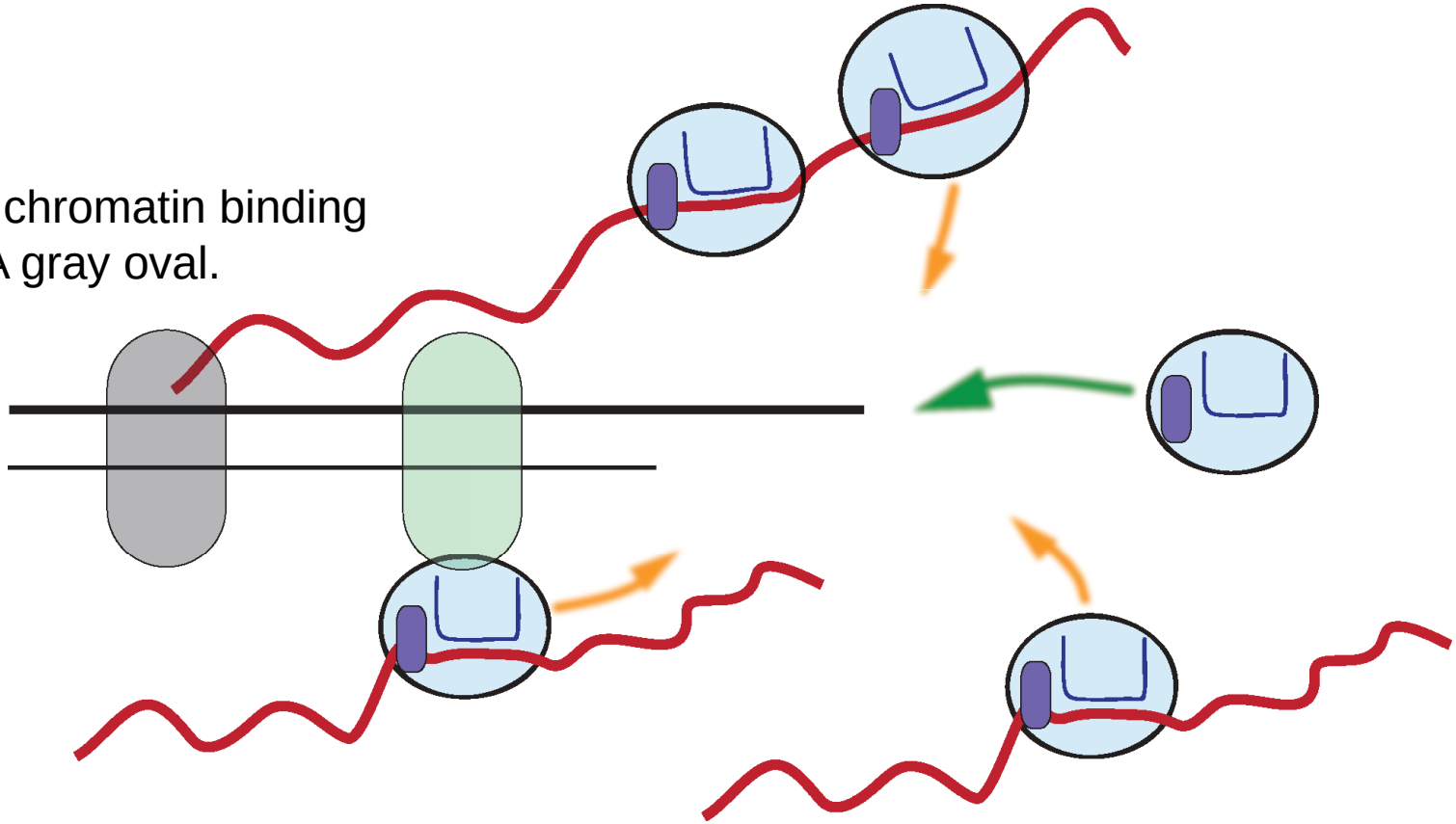
TERRA acts as a potent competitive inhibitor for telomeric DNA
TERRA is a telomerase ligand and natural direct inhibitor of human telomerase.

Telomerase regulation by the telomere substrate may be mediated via its transcription and non coding RNA

Telomerase sequestration by TERRA

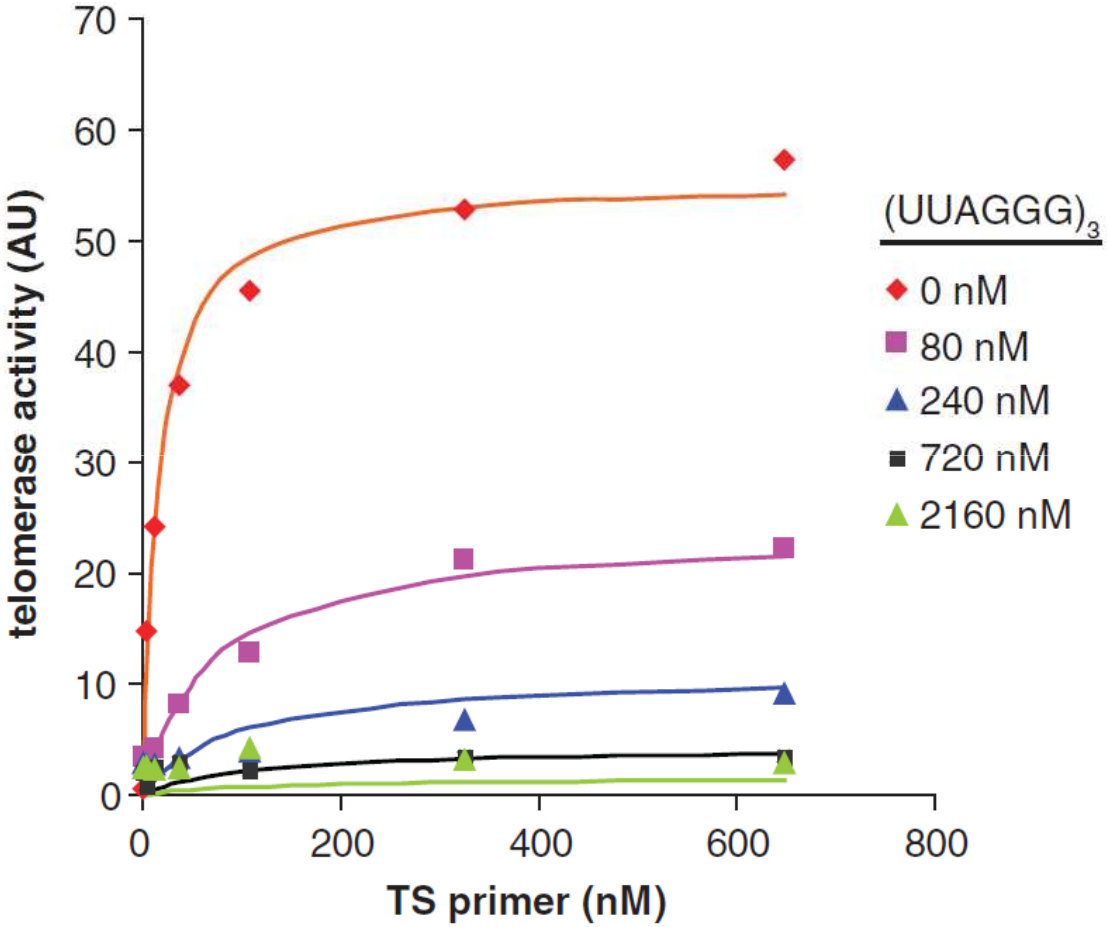
TERRA (red line)
telomerase RNA template (U-shaped blue line)
TERT polypeptide (dark-blue rounded rectangle).

telomeric chromatin binding
of TERRA gray oval.



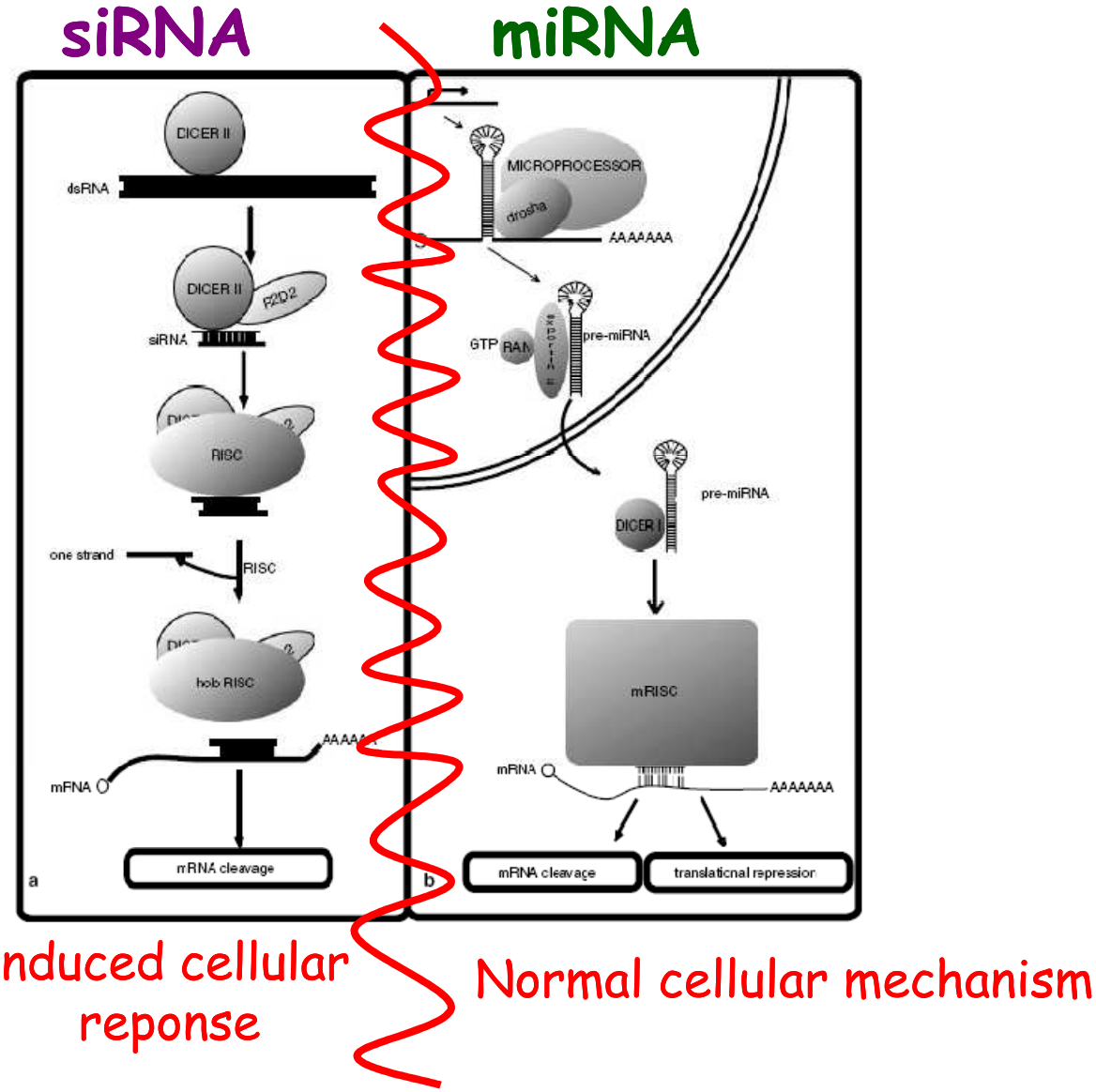
Telomeric chromatin binding of telomerase
the green oval

Quantification of telomerase activity from (A) as a function of primer in presence of increasing amounts of (UUAGGG)3



Many classes of ncRNAs are now extensively studied, and their regulatory role is broadly recognized.

The **miRNAs** represent a classical example of well-known ncRNA molecules that perform regulation at the RNA level


siRNA
miRNA
DICER II
dsRNA
DICER II
R2D2
siRNA
RISC
one strand
RISC
hob RISC
AAAAA
mRNA
mRNA cleavage
a
MICROPROCESSOR
drosha
AAAAAAA
GTP
RAN
pre-miRNA
pre-miRNA
DICER I
mRISC
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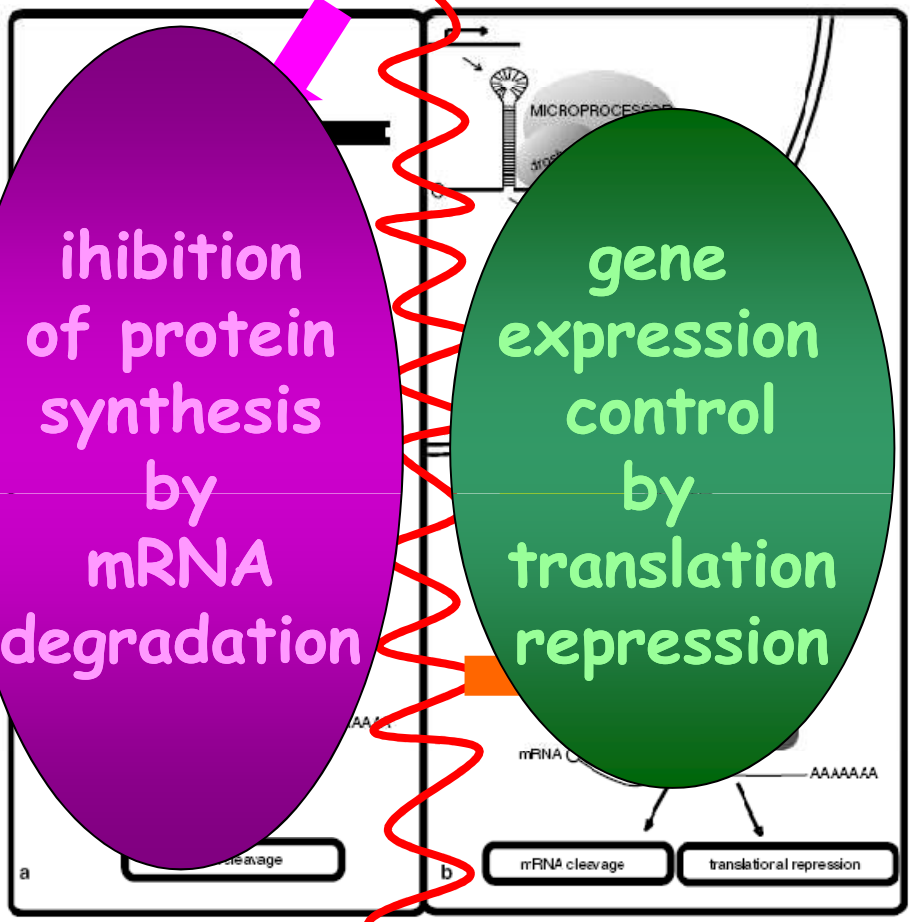
AAAAAAA
mRNA cleavage
translaticnal repressior
b
Induced cellular reponse
Normal cellular mechanism

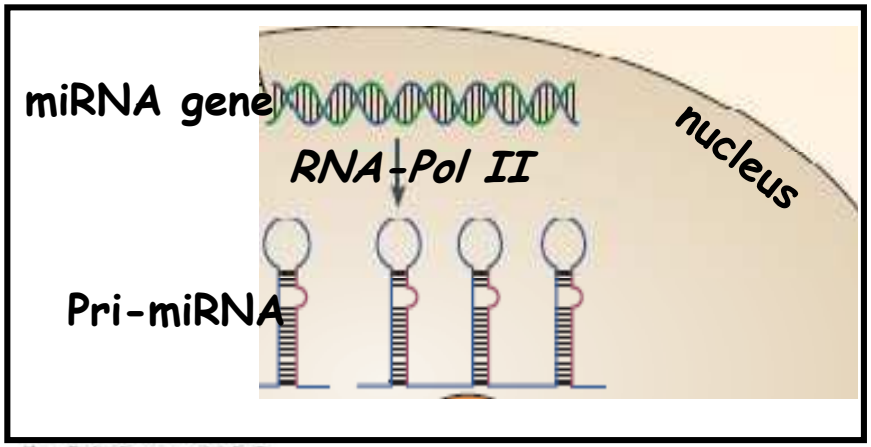
siRNA

miRNA

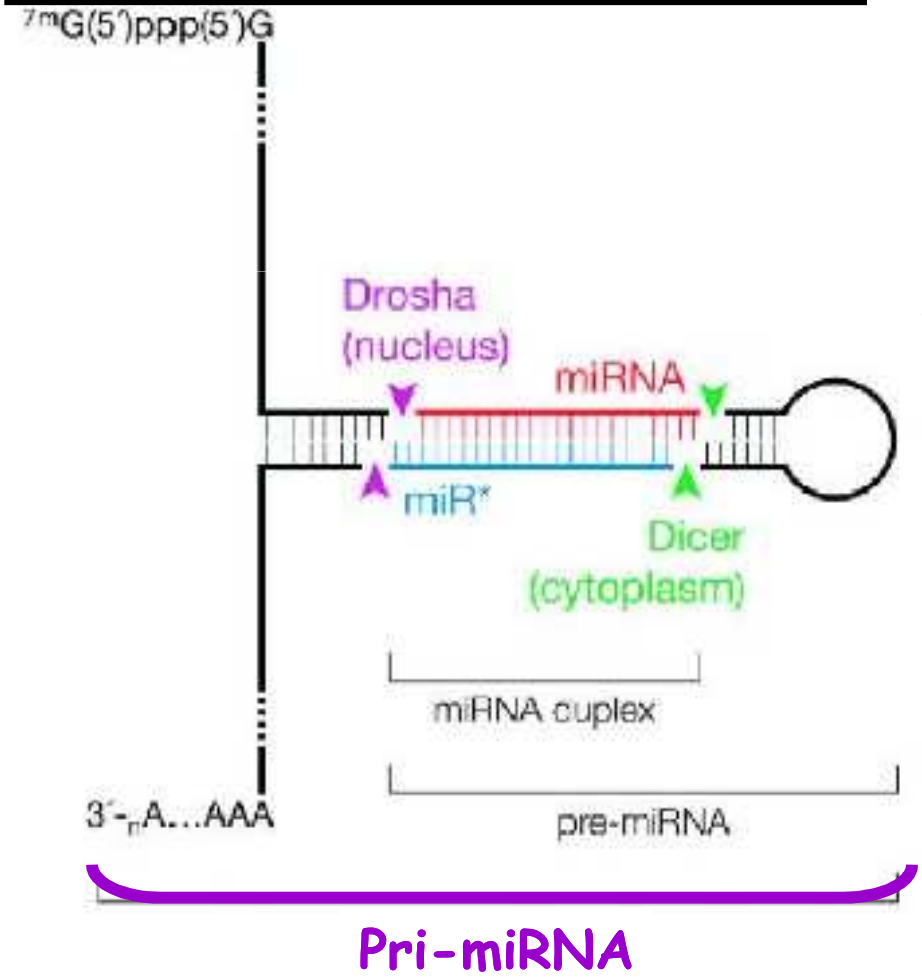


Induced cellular reponse

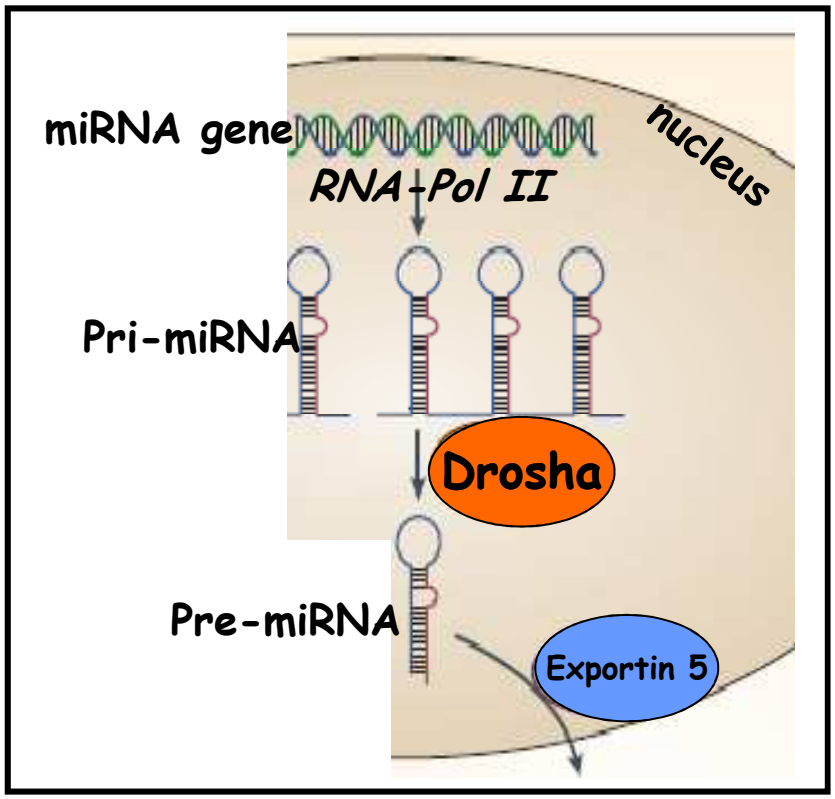
Normal cellular mechanism



Pri-miRNA (primary miRNA transcripts) dsRNA-like hairpin are generated by RNA-polymerase II transcription of several different categories of genes: **some having their own transcription units, others clustered in polycistronic transcript**



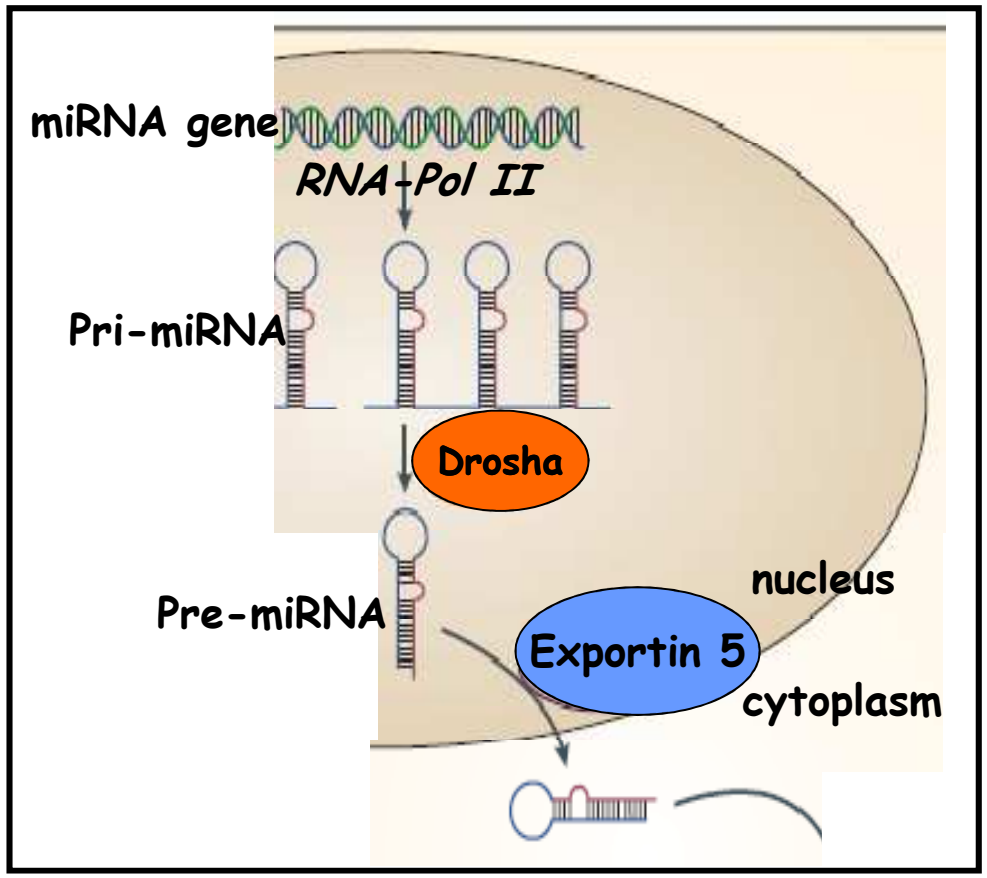
In mammals about one-half of the known miRNA are located within the transcription units of other genes and share a single primary transcript. miRNAs generally reside in the introns or in exon sequences that are not protein coding.



Pri-miRNA → Pre-miRNA

First processing step: cleavage of pri-miRNA in the nucleus by RNaseIII enzyme **Drosha**

The RNase III Drosha **is the core nuclease that executes the initiation step of microRNA (miRNA) processing in the nucleus** (Lee et al., 2003).[supplied by OMIM]

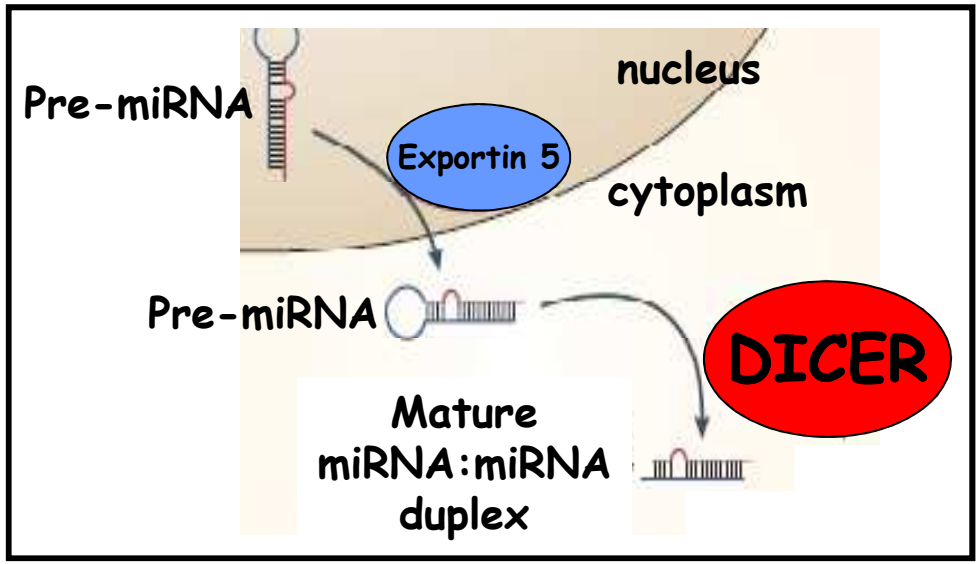


Pre-miRNAs released by Drosha are exported to the cytoplasm in an **Exportin 5-RanGTPase**-dependent manner

Exportin-5 belongs to a **large family that mediate the transport of proteins and other cargo between the nuclear and cytoplasmic compartments**.[supplied by OMIM]

Pri-miRNA —Drosha→ **Pre-miRNA** (~70 nt)

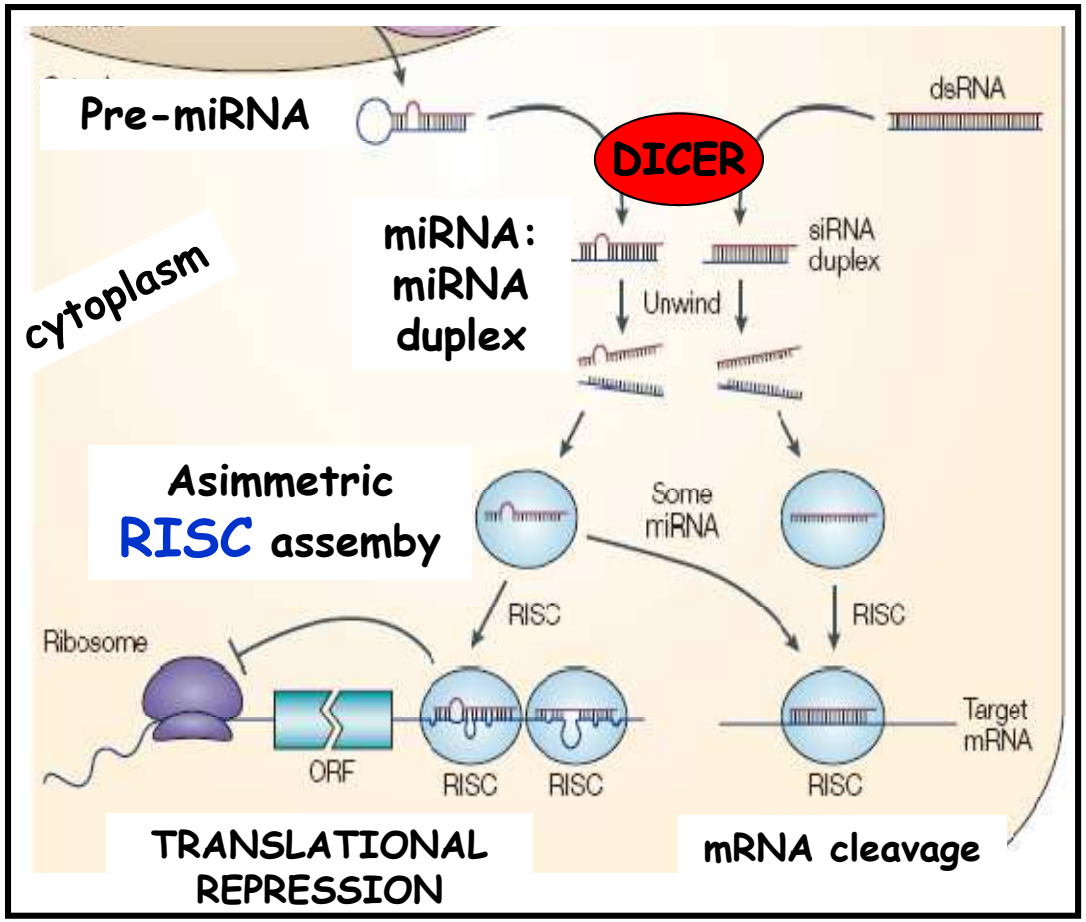
3' & 5'-end similar of end of siRNAs



In the cytoplasm a **DICER** enzyme makes a pair of cuts

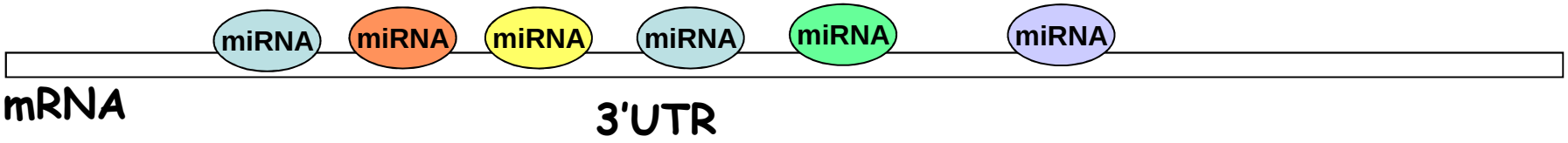
~21-25nt RNA duplex = **mature miRNA:miRNA imperfect duplex**

2-nt-long 3' overhangs at the cleavage site



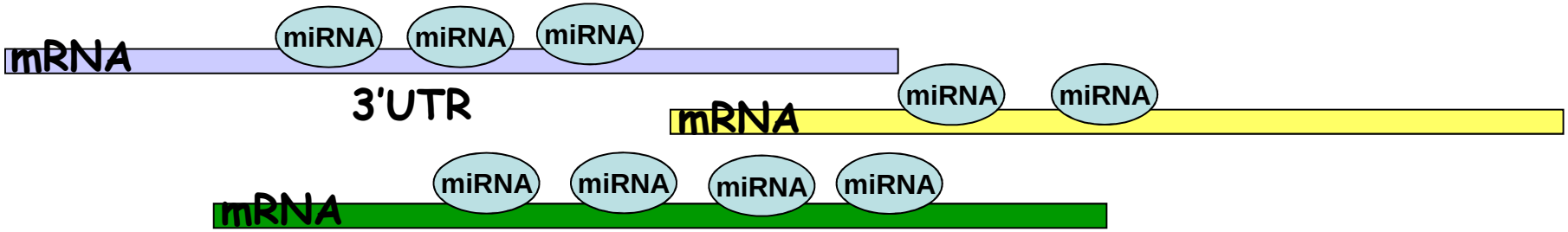
miRNAs enter the microribonucleoprotein (miRNP) complex = RISC that repress target gene expression

- Similar to siRNA duplexes, the strand whose 5′ end is less stably paired will be used as the guide/miRNA strand



Effective translational inhibition of mRNA by animal miRNAs usulally requires multiple imperfect target sites in the 3' UTR that are recognized by the same or several different miRNAs.

Hence, animal miRNAs may act combinatorially with several different miRNAs binding a single transcript.



Furthermore the same miRNA may have one to hundreds of target genes, suggesting that all the different human miRNAs may regulate as many as one-third of the protein-coding genes.

The precise molecular mechanism that underlie post-transcripional repression by miRNAs still remain largely unknown…

The repression by miRNAs has been shown to involve accumulation of the target mRNAs in processing (P)-bodies:

- large cytoplasmatic aggregates known to serve as sites of mRNA decapping, degradation and storage

- P-body environment is unfavorable for translation and this could contribute to the repression as well

The high number of putative target genes indicates that miRNAs function in a broad range of physiological and pathological processes:

- cell fate determination
- cell division and differentiation control
- apoptosis
- morphogenesis
- neurogenesis
- develpmental timing
- … … … … …

Disregulation of miRNA function might lead to human disease.

- most evidence for some **cancer** diseases (a large number of miRNAs was found to be downregulated in primary tumors)
  - hsa-mir-181a-1/2 & Acute **myeloid leukemia**
  - hsa-mir-28-3p, hsa-mir-28-5p, hsa-mir-21 & **B cell lymphoma**
  - hsa-mir-155, hsa-mir-21, hsa-let-7f-1, hsa-mir-125b-1 & **Breast cancer**
  - hsa-mir-143, hsa-mir-145, mmu-mir-143, rno-mir-145 & **Colorectal neoplasia**
  - hsa-mir-197, hsa-mir-346 & **Follicular thyroid carcinoma**
  - hsa-mir-132, hsa-mir-212 & **Hepatocellular carcinoma**
  - hsa-mir-155, hsa-mir-21, hsa-mir-34a, hsa-mir-128b, hsa-mir-191, hsa-mir-125b-1, hsa-mir-19a, hsa-let-7g & **Lung cancer**

Disregulation of miRNA function might lead to human disease.

- most evidence for some **cancer** diseases (a large number of miRNAs was found to be downregulated in primary tumors)

- miR-375, miR-124, let-7 & type 2 **diabetes**
- hsa-mir-125b-1/2 & **Alzheimer** disease
- hsa-mir-203 & **Psoriatic plaques**
- mmu-mir-195, hsa-mir-1-1/2, hsa-mir-133a-1/2, hsa-mir-133b, mmu-mir-1-1, mmu-mir-21 & **Cardiac hypertrophy**

http://www.mirbase.org/

- http://www.ma.uni-heidelberg.de/apps/zmf/argonaute/index.html

Table 1. *Examples of ncRNAs correlated with diseases/disorders*

NcRNAs	Disease/disorder	Reference
NcRNAs with altered expression levels in cancer		
Antisense intronic ncRNAs	Prostate cancer	Reis et al. 2004
BC1	Overexpressed in several cancers	Chen et al. 1997b
BC200	Overexpressed in breast, cervix, esophagus, lung, ovary, parotid, and tongue cancer	Chen et al. 1997a; Iacoangeli et al. 2004
BCMS	B-cell neoplasia	Wolf et al. 2001
C13orf25 (miR-17-92)	Elevated expression in lymphoma	Ota et al. 2004; L. He et al. 2005; O'Donnell et al. 2005
CMPD	Campomyelic displasia	Ninomiya et al. 1996
DD3	Overexpressed in prostate cancer	Bussemakers et al. 1999
H19	Overexpressed in liver and breast cancer	Lustenga et al. 1997; Lottin et al. 2002
HIS-1	Overexpressed in myeloid leukemia	Askew et al. 1994
HOST2	Expressed in ovarian cancer cells	Rangel et al. 2003
let-7 family miRNAs	Down-regulated in lung adenocarcinoma	Takamizawa et al. 2004; Johnson et al. 2005
MALAT-1	NSCLC, endometrial sarcoma, and hepatocellular carcinoma	Ji et al. 2003; Lin et al. 2006; Yamada et al. 2006
miR-143 and miR-145	Down-regulated in colorectal cancer	Michael et al. 2003
miR-146, miR-221, and miR-222	Elevated expression in papillary thyroid carcinoma	H. He et al. 2005
miR-155/BIC	Overexpressed in Burkitt and B-cell lymphomas, overexpressed in leukemia and breast cancer	Tam et al. 2002; Metzler et al. 2004; Eis et al. 2005; Iorio et al. 2005; Tam and Dahlberg 2006
miR-15a and miR-16-1	Deleted or down-regulated in B-cell lymphocytic leukaemia [B-CLL] and pituitary adenoma	Calin et al. 2002; Bottoni et al. 2005
miR-21	Elevated expression in glioblastoma cells and breast cancer	Chan et al. 2005; Iorio et al. 2005
miR-372 and miR-373	Testicular germ cell tumors	Voorhoeve et al. 2006
NC612	Prostate cancer	A.P. Silva et al. 2003
NCRMS	Elevated expression in alveolar rhabdomyosarcoma	Chan et al. 2002
OCC1	Overexpressed in colon carcinoma	Pibouin et al. 2002
PCGEM1	Overexpressed in prostate cancer	Srikantan et al. 2000
PEG8/IGF2AS	Fetal tumors	Okutsu et al. 2000
SRA	Steroid receptor activated RNA isoform expressed in breast cancer	Lanz et al. 1999
TRNG10	Various cancers	Roberts et al. 1998
U50HG	snoRNA host gene, located at the chromosomal breakpoint involved in human B-cell lymphoma	Tanaka et al. 2000

Changes in expression levels or genetic and epigenetic alterations affecting ncRNAs in cancer

role of ncRNAs in normal cellular development and differentiation (Szymanski et al. 2005).

Regulatory RNAs implicated in complex diseases

Table 1. *Examples of ncRNAs correlated with diseases/disorders*

NcRNAs	Disease/disorder	Reference
NcRNAs correlated with neurological diseases/disorders		
BC200	Alzheimer's	Lukiw et al. 1992
DISC2	Schizophrenia and bipolar affective disorder	Millar et al. 2000, 2004; Blackwood et al. 2001
IPW	Prader-Willi syndrome	Wevrick et al. 1994
Prion-associated RNAs	Prion pathologies	Deleault et al. 2003; Supattapone 2004
PSZA11q14	Reduced expression in brains of patients with schizophrenia	Polesskaya et al. 2003
RAY1/ST7	Autistic disorder	Vincent et al. 2002
SCA8 (KLHL1 antisense)	Spinocerebellar ataxia type 8	Nemes et al. 2000; Moseddi et al. 2004
UBE3A-AS	Angelman syndrome	Chamberlain and Brannan 2001
ZNF127AS	Prader-Willi syndrome	Jong et al. 1999
NcRNAs correlated with other diseases/disorders		
22h48	HIRA intronic transcript deleted in DiGeorge syndrome	Pizzuti et al. 1999
C6orf37OS	Antisense transcript from C6orf37 locus within diffuse panbronchiolitis critical region	Matsuzaka et al. 2002
COPG2IT1	Russell-Silver syndrome	Yamasaki et al. 2000
DGCR5	Disrupted in DiGeorge syndrome	Sutherland et al. 1996
H19	Beckwith-Wiedemann syndrome	Sparago et al. 2004
LIT1	Beckwith-Wiedemann syndrome	Niemitz et al. 2004
LIT1	Romano-Ward, Jervell and Lange-Nielsen syndromes	Horike et al. 2000
MESTIT 1	Russell-Silver syndrome	T. Li et al. 2002; Nakabayashi et al. 2002
PRINS	Psoriasis	Sonkoly et al. 2005

Blood. 2007 Dec 15;110(13):4144-52. Epub 2007 Aug 28.

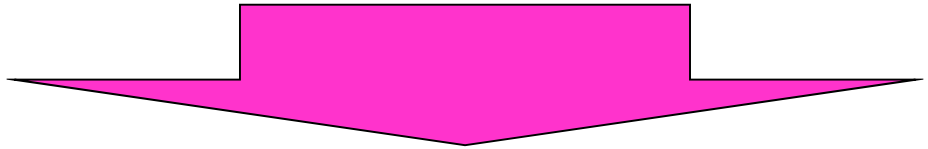
A microRNA-regulated lentiviral vector mediates stable correction of hemophilia B mice.

Brown BD, Cantore A, Annoni A, Sergi LS, Lombardo A, Della Valle P, D'Angelo A, Naldini L.
San Raffaele Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, Italy.

A long-standing goal for the treatment of hemophilia B has been the development of a strategy that can maintain sustained, endogenous production of coagulation factor IX

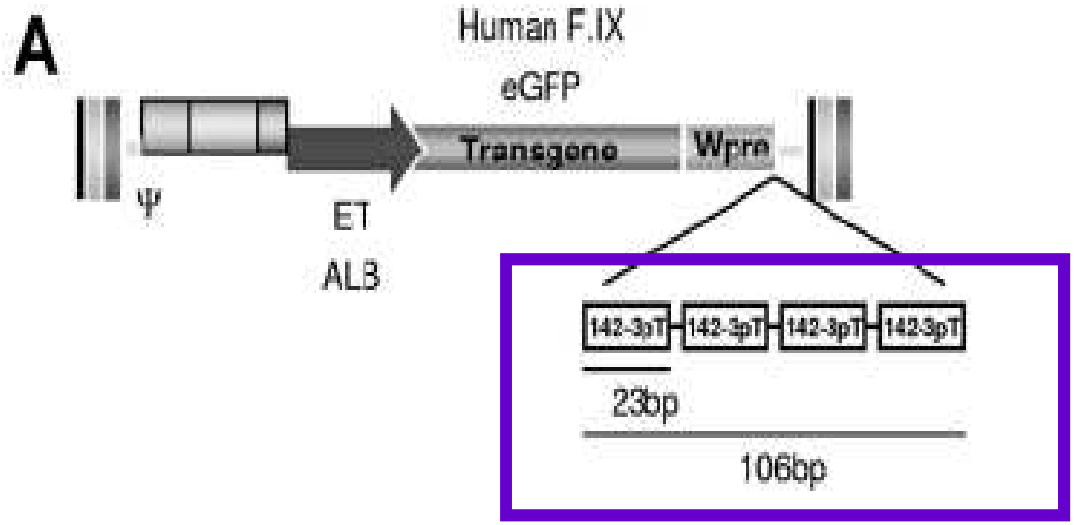
Recent clinical evidence indicates that in humans, stable hepatic delivery of AAV (adenoviral vectors) is limited by preexisting immunity to capsid antigens

Studies of intravenously delivered LVs (lentiviral vectors) encoding human factor VIII found that mice developed anti-hFVIII antibodies and we reported similar findings for LV-mediated hFIX



Instead, inclusion of a layer of post-transcriptional control mediated by endogenous miRNA regulation enabled us to achive long-term hFIX gene transfer and rescue the phenotype of adulte hemophilia B mice

Inclusion of miRNA target seq within a ubiquitously expressed vector resulted in detargeted transgene expression from cells that endogenously express the respective miRNA



Third-generation self-inactivating LV used.

The miRNA-regulated LV was generated by incorporating 4 tandem copies of a seq completely complementary to miR-142-3p (hematopoietic-specific miRNA)

Model-mice of hemophilia B (Balb/c mice) were intravenously injected with vectors with and without miR-142-3p seq and analyzed 10 days after.

In LV.ET.GFP.142-3pT-treated mice, similar to LV.ET.GFP-treated mice, high levels of GFP expression were found in a large fraction of hepatocytes within the liver.

Confocal immunofluorescence analysis of the liver

Fig.1

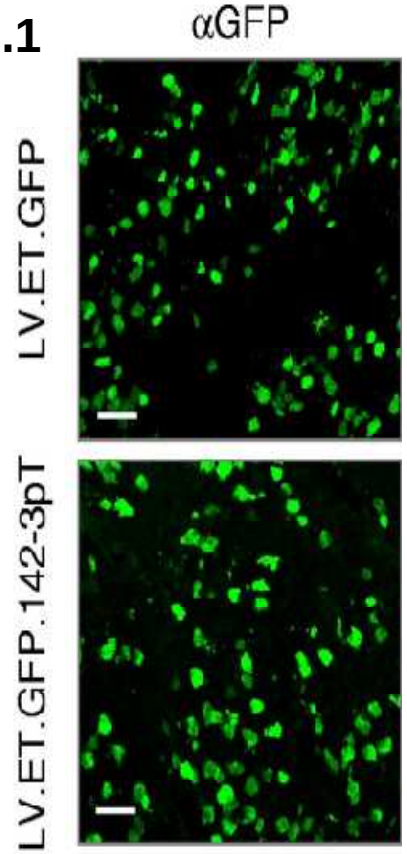


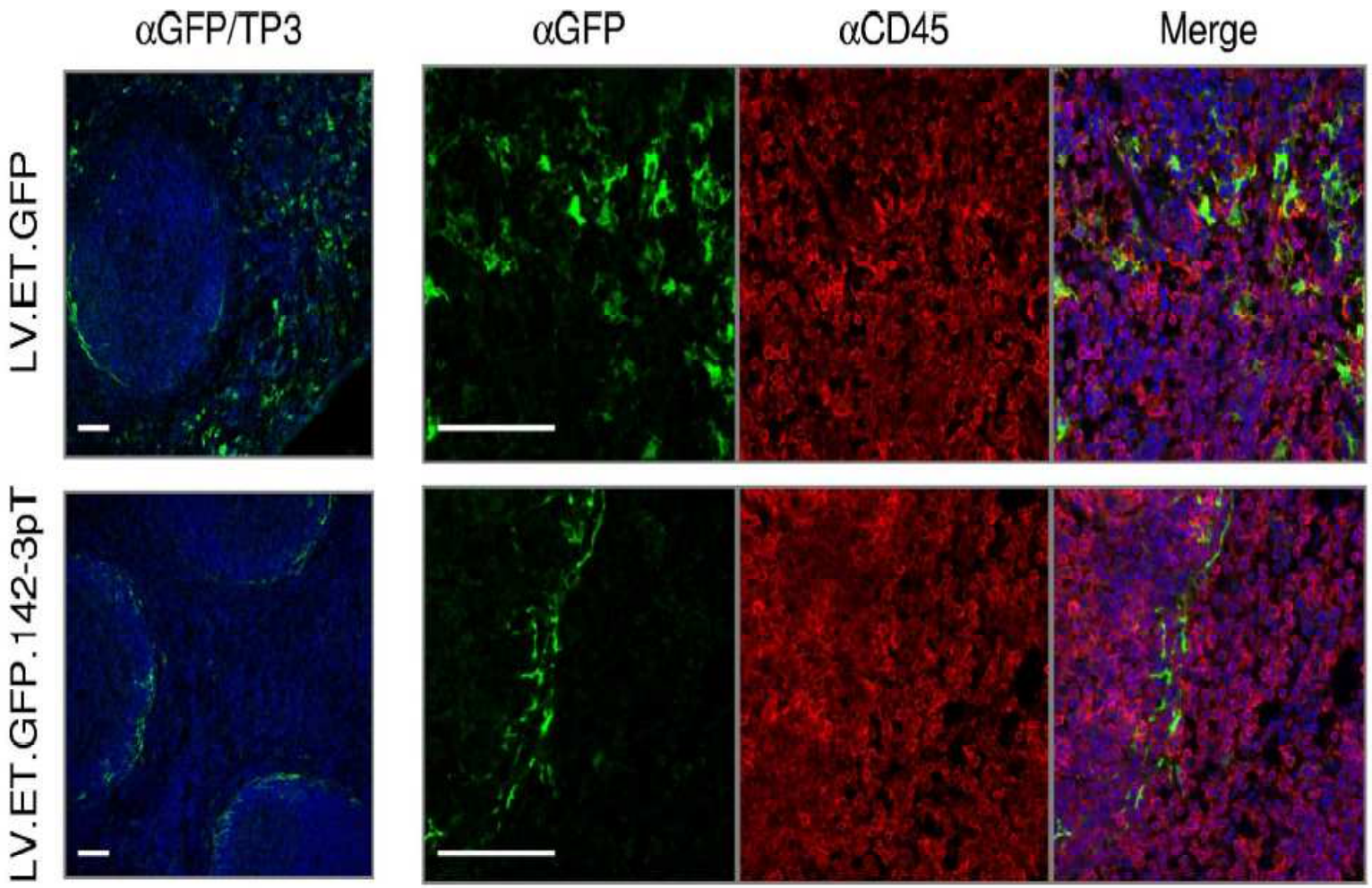
Fig.1 in verde è mostrata l'espressione del transgene (FIX) in sezioni di fegato. L'espressione è notevole sia in alto (vettore normale) sia in basso (vettore contenente le seq complementari a miR-142-3p) → l'efficienza dei due vettori non cambia

Il transgene si esprime anche nella milza

Fig.3 La figura 3 mostra in immunofluorescenze l'espressione del transgene nella milza.
c'è una grossa differenza di espressione dei due vettori in questo sito infatti:
Il vettore normale mostra un'espressione diffusa
Mentre il vettore con le sequenze complementari a miR-142-3p mostra un'espressione sicuramente minore, localizzata solo a livello periferico della milza e il segnale verde non corrisponde (colocalizza) con il segnale rosso (CD45) che è un marker delle cellule ematopoietica
→ L'espressione a livello della milza è contenuta non avviene, nemmeno in questo sito, in cellule della linea ematopoietica.

Interestingly a small fraction of cells found exclusively within the marginal zone sinus remained GFP-positive but were CD45-negative indicating that they were not from the hematopoietic lineage

Fig.3



Confocal immunofluorescence analysis of the spleen

Inclusion of the miR-142-3p target seq can **prevent "off-target" transgene expression from a tissue-specific promoter** specifically within hematopoietic cells.

Moreover, the high levels of GFP expression within the liver indicate that the miR-142-3p target seq do **not interfere with transgene expression in hepatocyte**.

LentiVirus-mediated F.IX gene transfer can achieve **successful correction** of the hemophilia B phenotype in a mouse model of the disease.

Following a single, intravenous injection of an LV encoding hF.IX, up to 15% of normal F.IX activity could be attained in hemophilia B mice for over 280 days after injection.

These levels provided clear **improvement to the phenotype** of the animal, as indicated by their ability to **survive a lethal wound**.

hF.IX expression was sustained long-term, and thus demonstrates that LVs can mediate stable transfer and expression of a transgene encoding a circulating neo-antigen without provoking an immune response.

Incorporating target sequences for the hematopoieticspecific microRNA miR-142 into an antigen-encoding transgene prevents antigen expression in antigen-presentingcells (APCs). We treated mice with a miR-142– regulated lentiviral vector encoding green fluorescent protein (GFP), and subsequently vaccinated the mice against GFP.
In contrast to control mice, no anti-GFP response was observed, indicating that robust tolerance to the transgene-encoded antigen was achieved. Detargeting antigen expression from professional APCs, coupled with expression in hepatocytes, can induce antigen-specific immunologic tolerance. (Blood. 2009;114: 5152-5161)