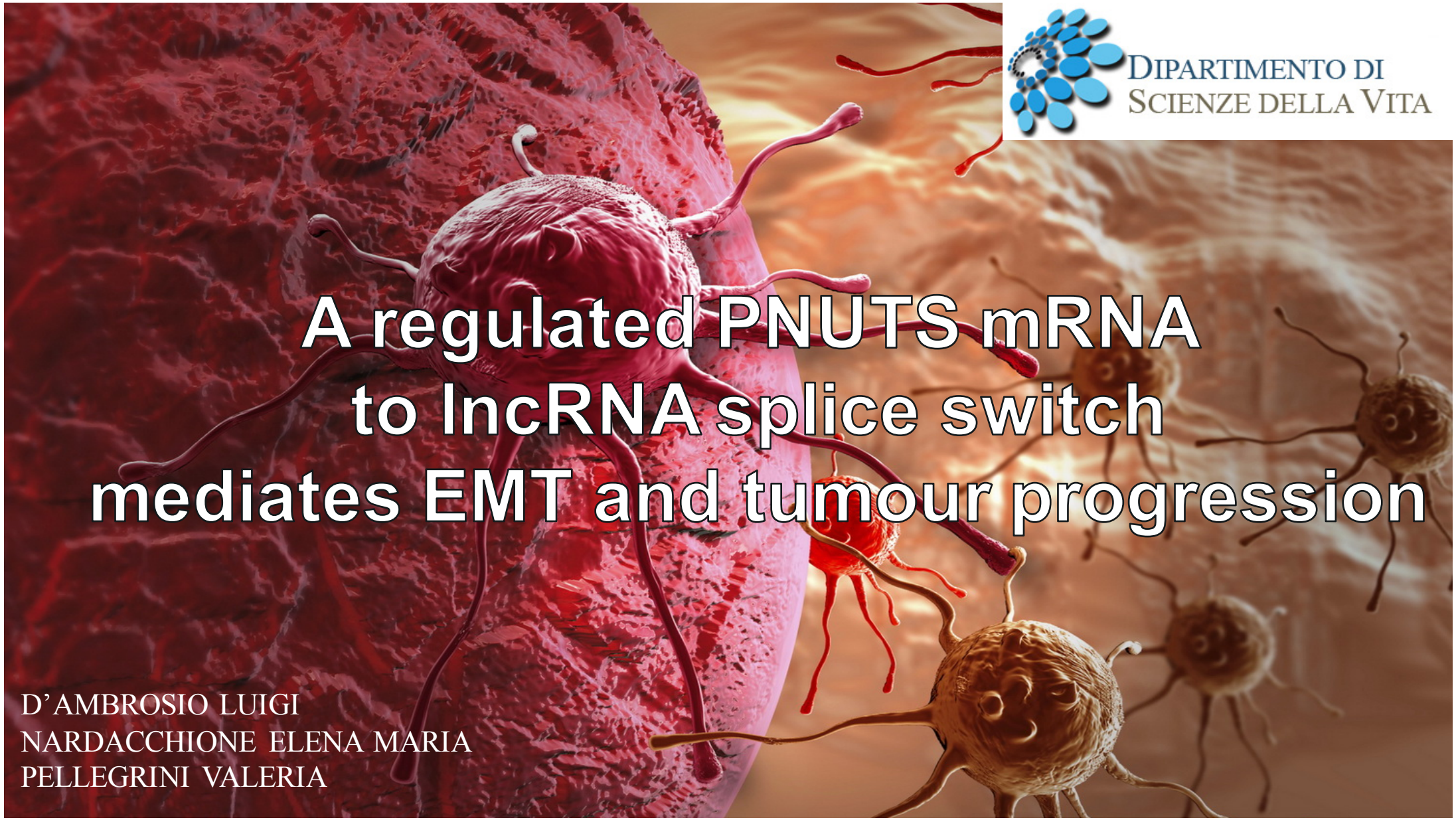


A regulated PNUTS mRNA to lncRNA splice switch mediates EMT and tumour progression

D'AMBROSIO LUIGI
NARDACCHIONE ELENA MARIA
PELLEGRINI VALERIA

DIPARTIMENTO DI SCIENZE DELLA VITA

OUTLINE:

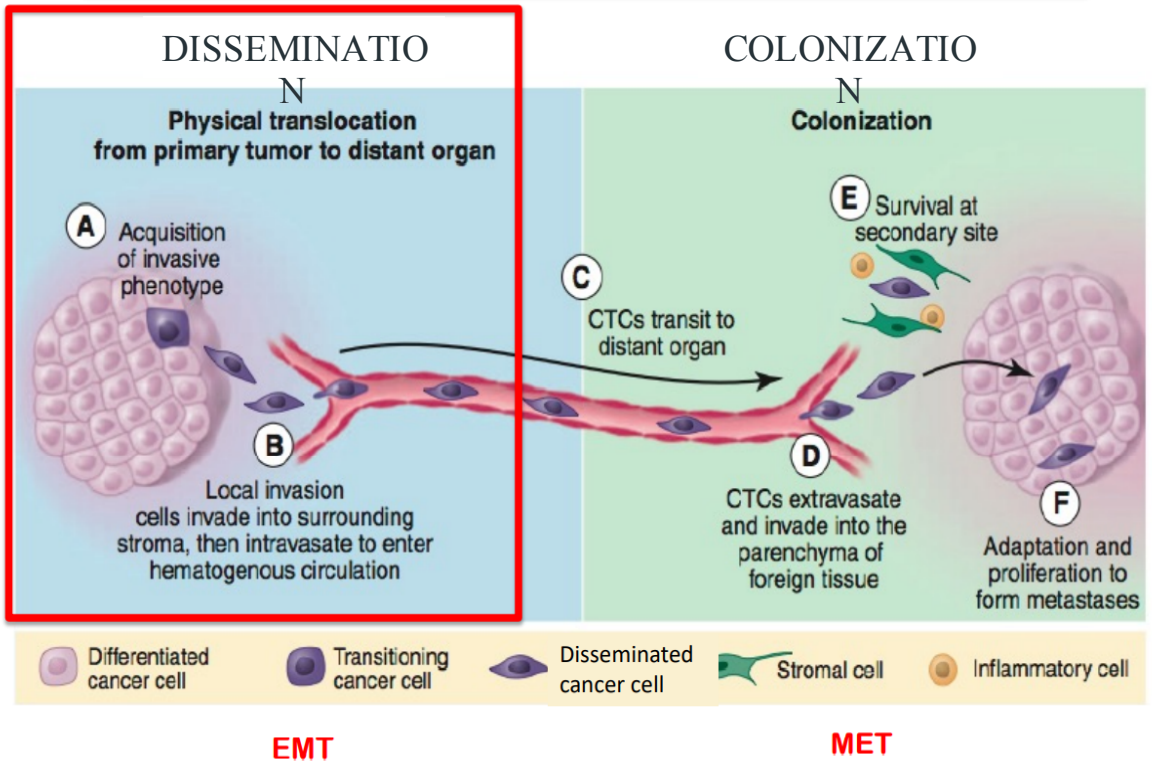
1. EMT and its characteristics;
2. Transcriptional factors involved;
3. EMT mediated by TGF-β;
4. lncRNAs in cancer;
5. hnRNPs;
6. hnRNP E1;
7. Paper discussion;
8. Conclusions and future perspectives.

Epithelial-mesenchymal transition in cancer

Epithelial-mesenchymal transition (EMT) is a complex developmental program that enables carcinoma cells to suppress their epithelial features changing to mesenchymal ones.

This change allows cells to acquire mobility and the capacity to migrate from the primary site.

EMT provides a new insight for understanding the several steps of the metastatic process, from dedifferentiation to a more aggressive phenotype.

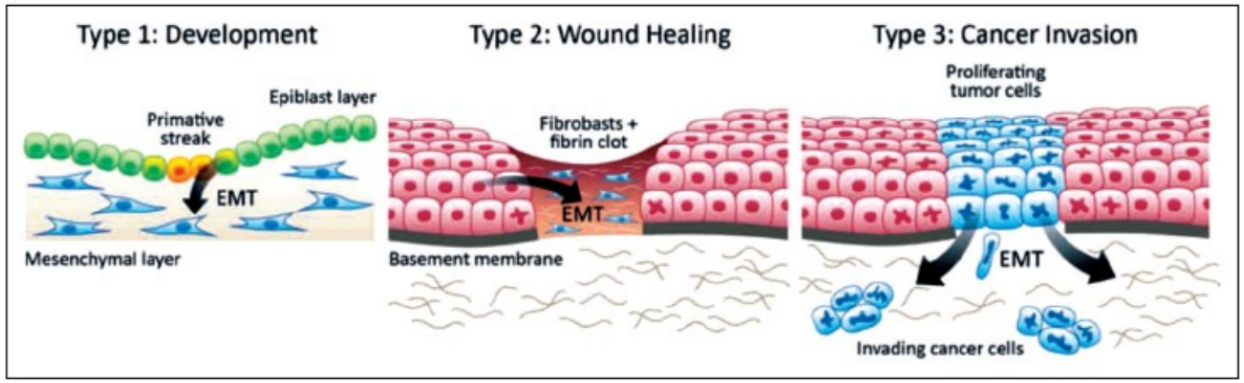


Epithelial-mesenchymal transition in cancer

The term transition started to be used due to the reversibility of the process (EMT-MET).

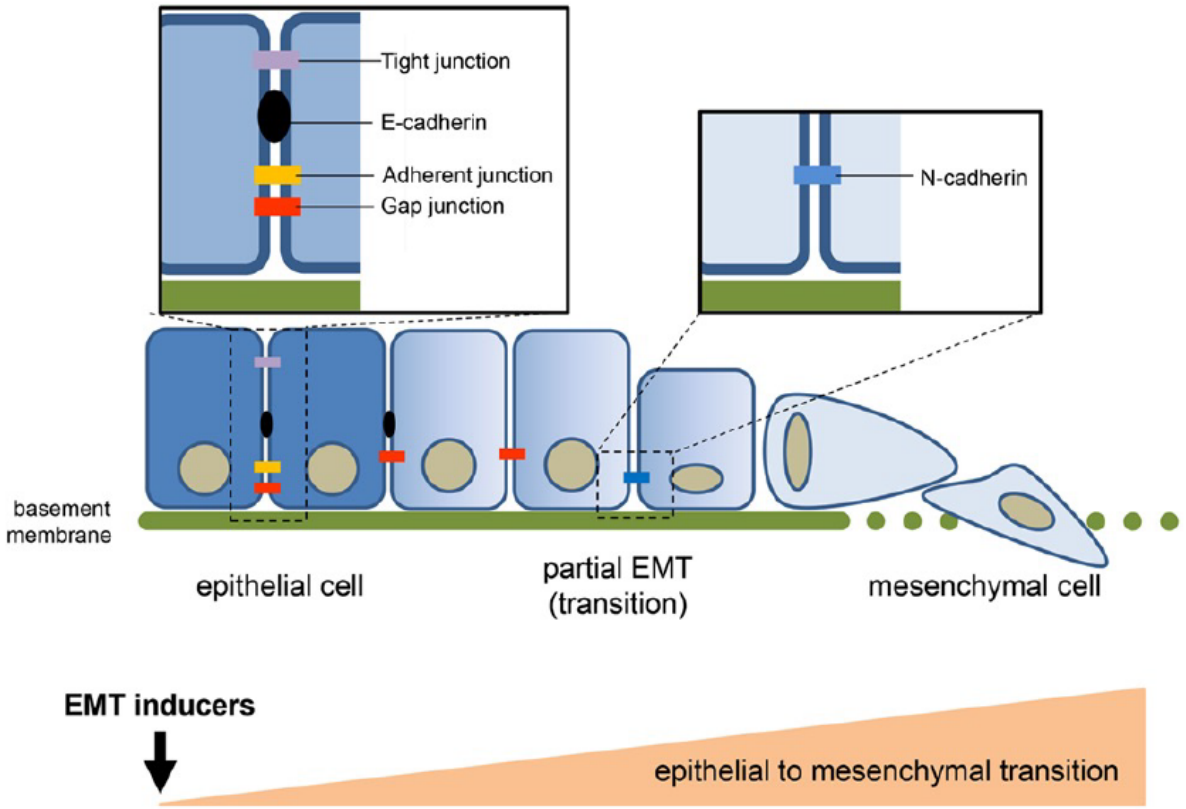
EMT can be classified in three types according to the process involved:

- type 1 for gastrulation and embryogenesis,
- type 2 for regeneration and wound healing,
- type 3 for carcinogenesis (metastasis, malignancy and invasion).



Deconstructing cell junctions and polarity

- Dissolution of **tight junctions** → decreased claudin and occludin expression.
- Destabilization of **adherens junctions** → switch from E-cadherin to N-cadherin.
- Disrupts **desmosomes**.
- Decreased connexin levels → **gap junction** integrity compromised

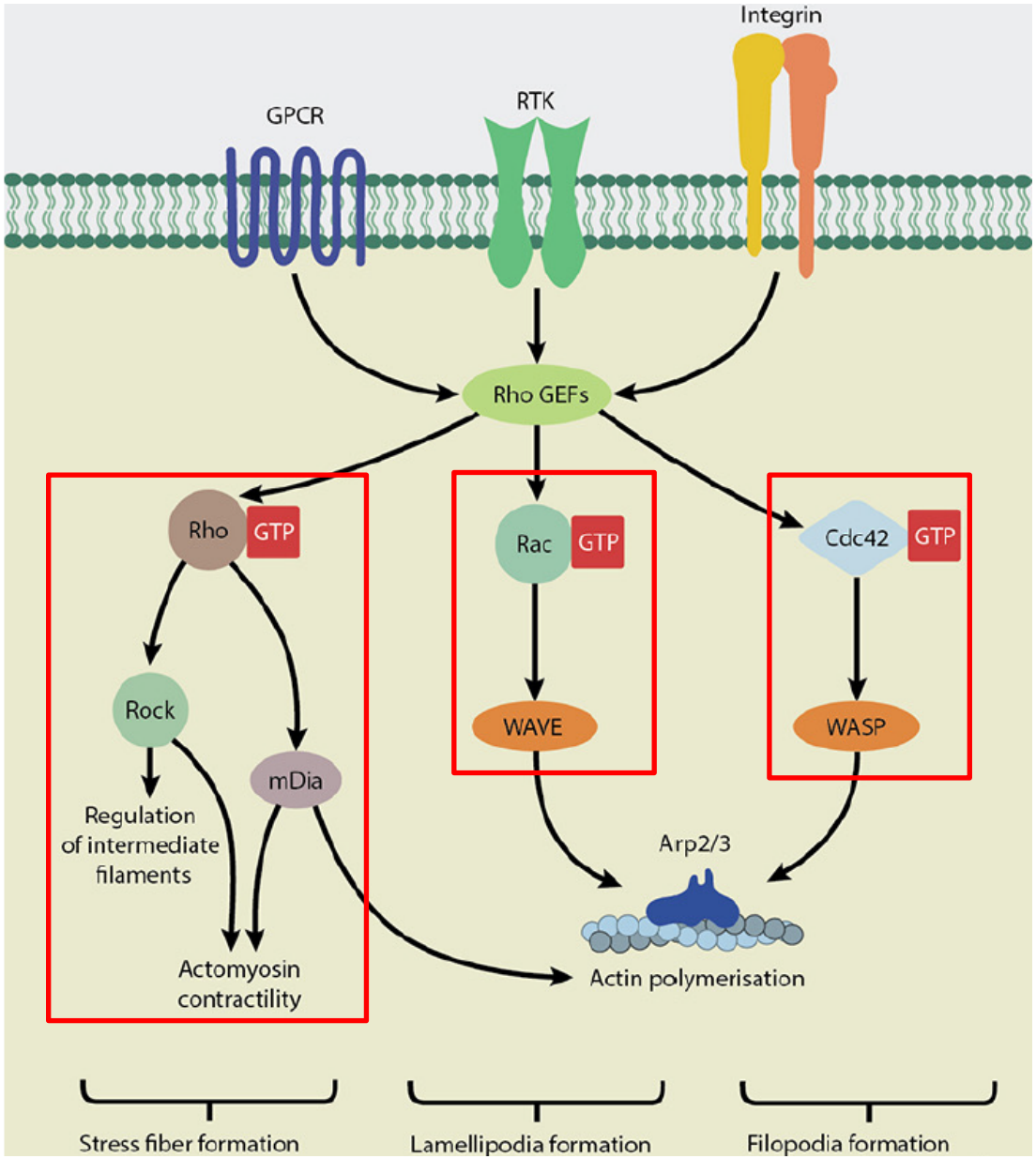


Cytoskeletal changes and motility

The activation of RHO GTPases is **tightly regulated** by:

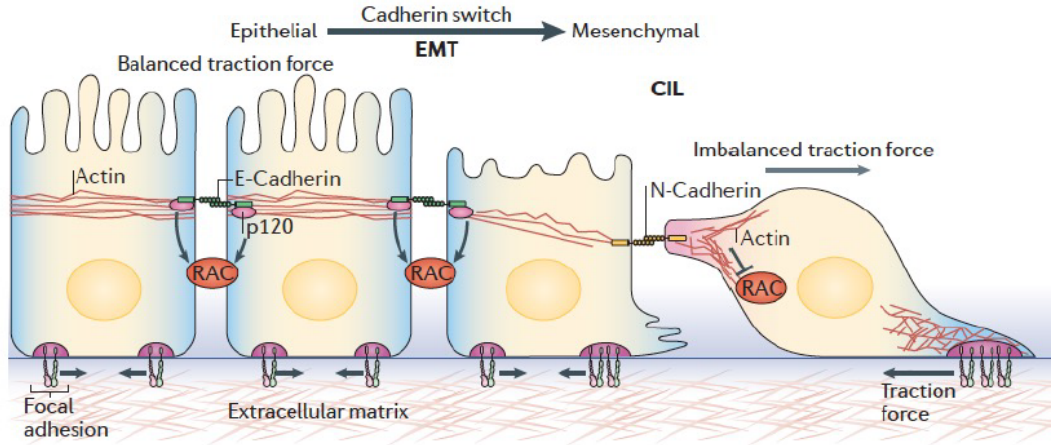
- GEFs,
- GAPs,
- GDIs.

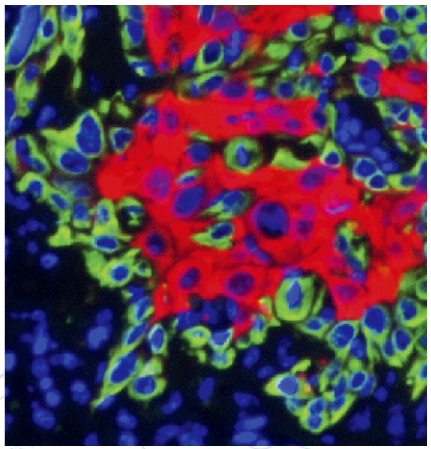
This results in the formation of **protrusions** and the formation of **actin stress fiber** that facilitate cell mobility and invasion.



Overview of changes in gene expression

Cadherin switch in adherens junctions.





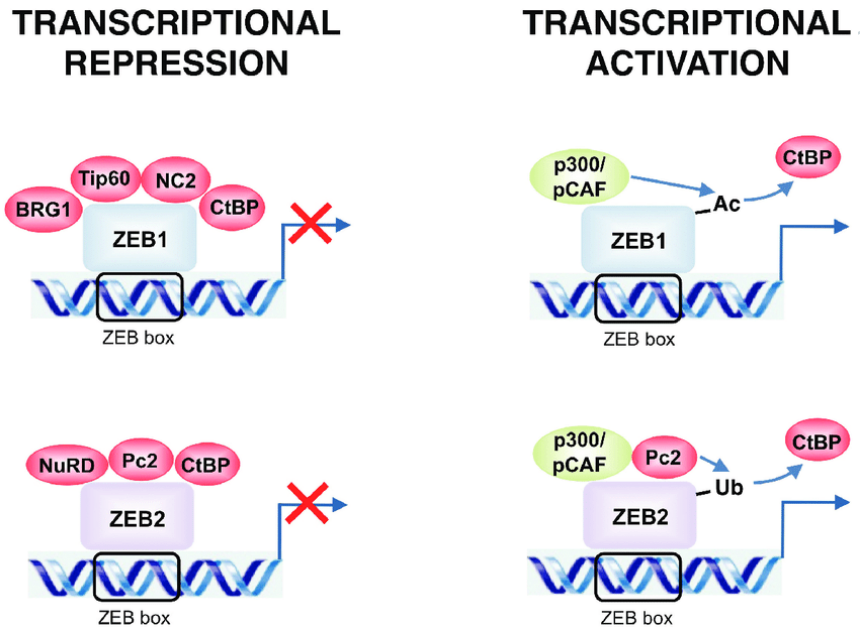
Cytokeratin

Vimentin

Repression of cytokeratin and activation of **vimentin expression** in intermediate filaments.

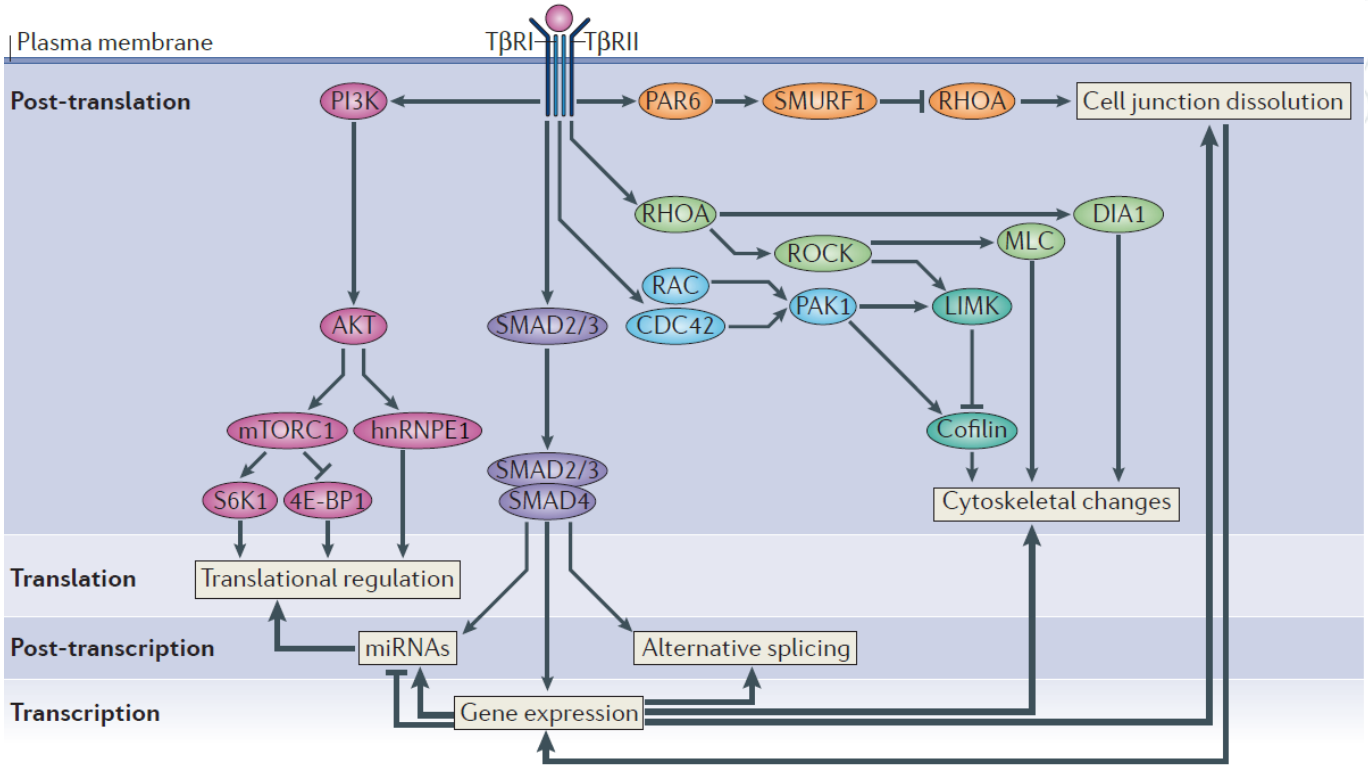
Transcription factors driving EMT

- SNAIL;
- TWIST;
- SLUG;
- ***ZEB***: ZEBs bind E-boxes and function as transcriptional repressors and activators, thereby repressing some epithelial junction and polarity genes and activating mesenchymal genes that define the EMT phenotype.



Molecular mechanisms of TGFβ-induced EMT

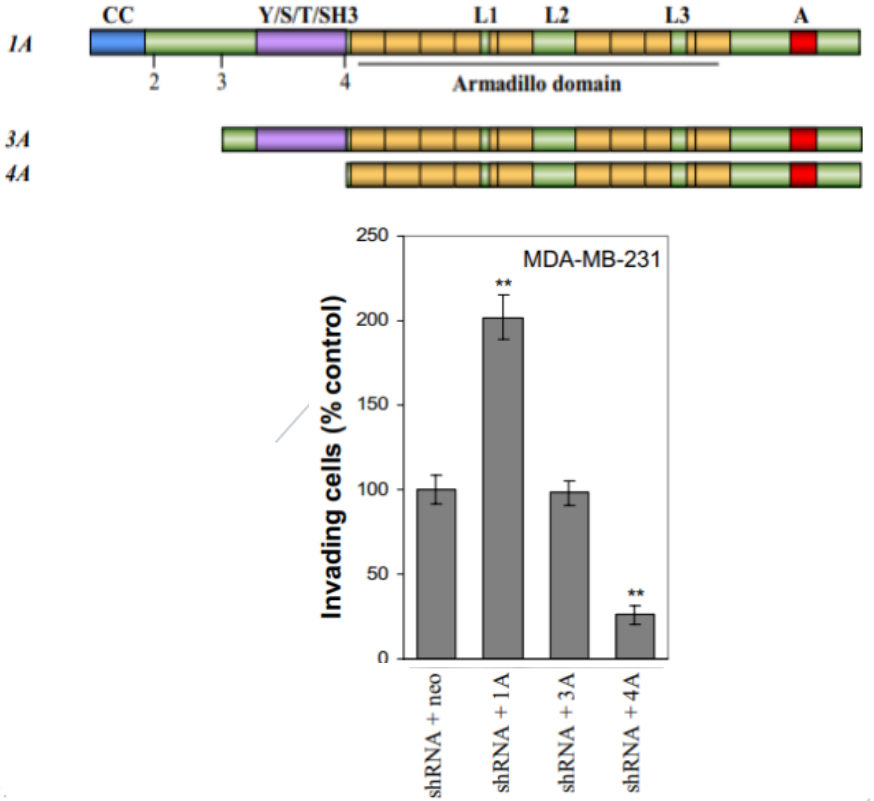
The initiation of, and progression through, epithelial–mesenchymal transition (EMT) are regulated at the transcriptional, post-transcriptional, translational and post-translational levels. Transforming growth factor-β induces EMT by acting at several of these levels and through SMAD-mediated and non-SMAD signalling.



Alternative splicing in EMT

The functional consequences of differential splicing in EMT are well illustrated by p120 catenin, the adhesion protein cluster of differentiation 44 (CD44), the RTK FGFR2 and extensive isoform changes, as a result of alternative splicing, in various additional proteins that regulate EMT.

These changes in splicing impose another layer of complexity on the gene expression changes that occur during EMT.



A p120 Catenin Isoform Switch Affects Rho Activity, Induces Tumor Cell Invasion, and Predicts Metastatic Disease[*][S]

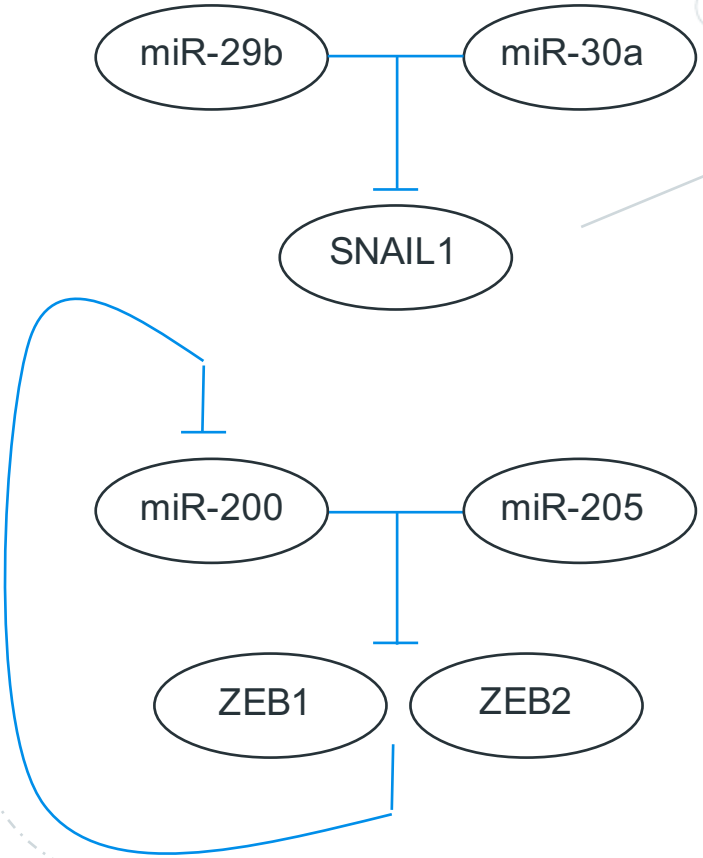
Received for publication, February 13, 2008, and in revised form, April 9, 2008 Published, JBC Papers in Press, April 11, 2008, DOI 10.1074/jbc.M801192200

Masahiro Yanagisawa[‡], Deborah Huveldt[‡], Pamela Kreinest[‡], Christine M. Lohse[§], John C. Cheville[¶], Alexander S. Parker[‖], John A. Copland[‡], and Panos Z. Anastasiadis[‡1]

miRNA-mediated control of EMT

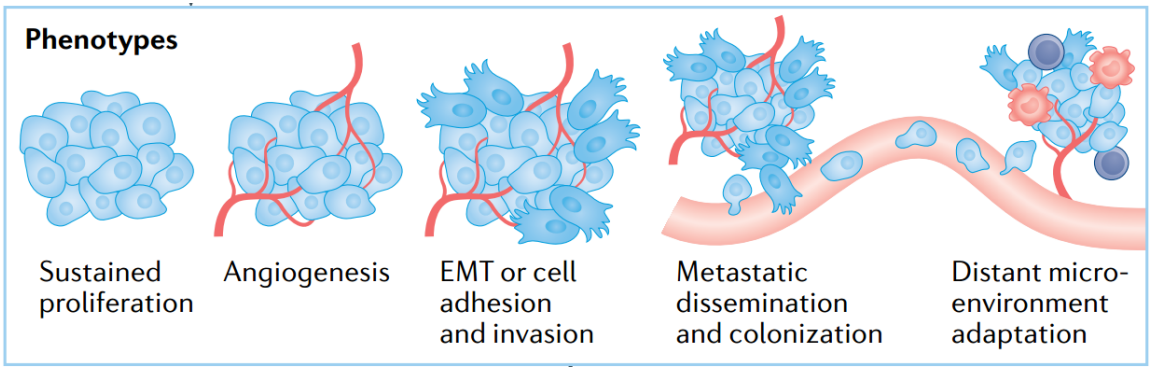
Non-coding miRNAs that selectively bind mRNAs, thus inhibiting their translation or promoting their degradation, also regulate the epithelial phenotype and EMT.

- Some of these control the expression of EMT master transcription factors.
- miRNAs also target genes that help to define the epithelial or mesenchymal phenotype, such as those encoding adhesion junction and polarity complex proteins and signalling mediators.



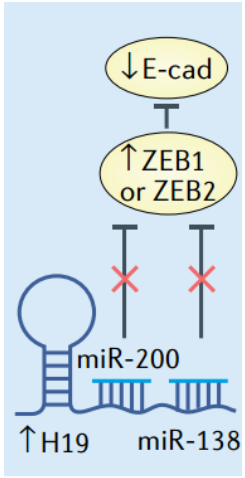
lncRNAs in cancer

described in most (if not all) of the classic hallmarks of cancer:



- lncRNAs are **amplified, delated or mutated** in malignancies (ex: PVT1)
- A broad set of lncRNAs is located in recurrent copy number-altered regions in genomes of tumors
- **Epigenetic aterations** are also possible
- Their **regulatory roles** are diverse and still emerging (ex: PVT1 acts both as a DNA regulatory element and as an RNA transcript)

H19 in the **EMT**: one of the earliest lncRNAs described, is overexpressed in several cancers and involved in tumor cell invasion.



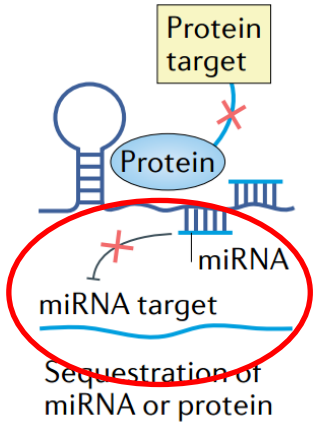
Competition for miRNA or protein

+ a different miRNA is encoded in the first exon of H19 gene

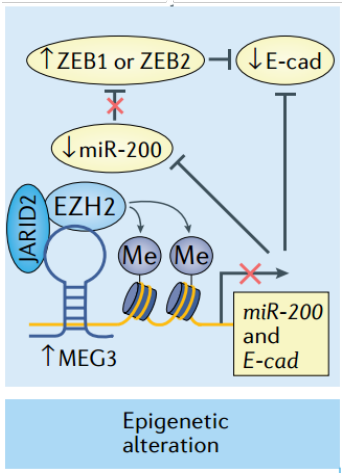
Additional lncRNAs implicated in the EMT

- PTAR (pro-transition associated RNA)
- lncRNA-ATB (lncRNA-activated by TGFβ)
- **lncRNA-*PNUTS***

ceRNAs →



- MEG3 → mediates epigenetic alterations →



- MUF → mediates transcriptional regulation

+ other possible mechanisms: RNA/protein modification

They tend to function as **'fine tuners'** rather than master regulators **of metastasis.**

hnRNPs: heterogenous nuclear RNA binding Proteins

- They associate with hnRNAs in **large complexes** (highly dynamic) → major/minor proteins.

hnRNP proteins are among the most abundant nuclear proteins and, when complexed to hnRNAs, form major components of the nucleus.

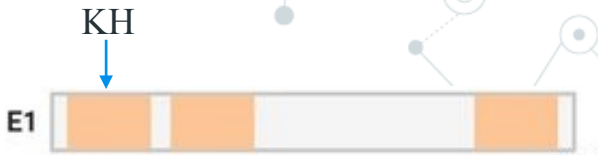
- They are **multifunctional**:
  - participate in pre-mRNA processing
  - important determinants of mRNA export, localization, translation, and stability
  - (may also participate in RNA pol II transcription)

  → hnRNP proteins are principally involved in **RNA metabolism.**

Nuclear-cytoplasmic shuttling

- They have a **modular structure**: RNA binding motif + at least one auxiliary domain.
- They undergo many **PTMs.**
- Different predicted **mechanisms** for hnRNP functions (transcript-specific/non specific).

hnRNP E1



- **Ubiquitously expressed.**
- **Structure:** three KH domains (RNA binding domains).
- **Localization:** mostly nuclear, but also cytoplasmatic.
- **Functions:**
  - transcriptional activator;
  - regulator attenuating alternative splicing;
  - mRNA stability;
  - translational regulation.

This functional diversity *could* be achieved trhough PTMS.

- **Binding to BAT:** novel 33-nt structural element of transcripts

identified in Dab2 and ILEI

↓

BAT inhibits translation

↓

relieved by treatment with TGFβ → BAT: TGFβ activated translational element

activates Akt2, which phosphorylates E1, detaches from BAT

hnRNPE1 binds to BAT elements

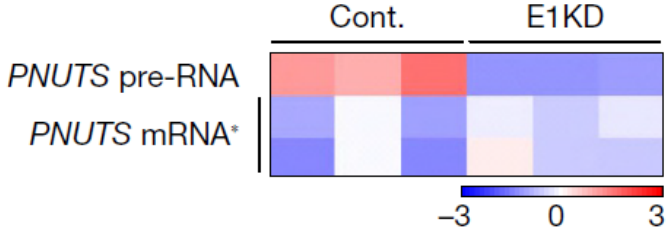
Investigating the link between lncRNA-*PNUTS* and EMT

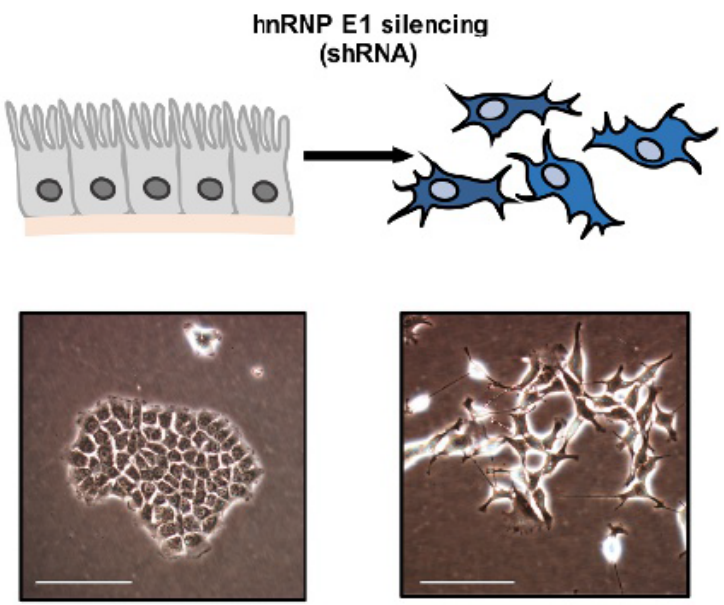
A regulated *PNUTS* mRNA to lncRNA splice switch mediates EMT and tumour progression

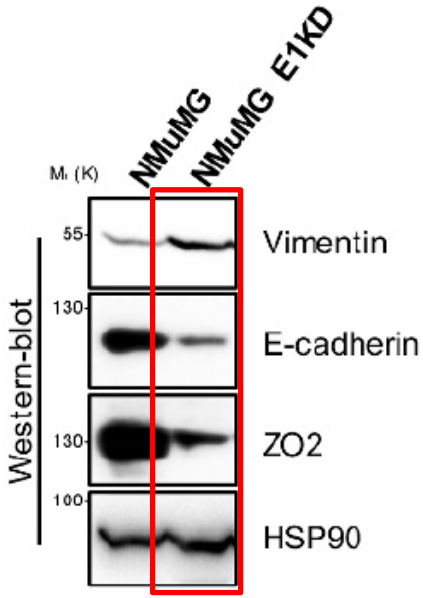
Simon Grelet[1], Laura A. Link[1], Breege Howley[1], Clémence Obellianne[1], Viswanathan Palanisamy[2,3], Vamsi K. Gangaraju[1,3], J. Alan Diehl[1,3] and Philip H. Howe[1,3,4]

The influence of *hnRNP E1* silencing

Affymetrix array analysis in NMuMG cells.





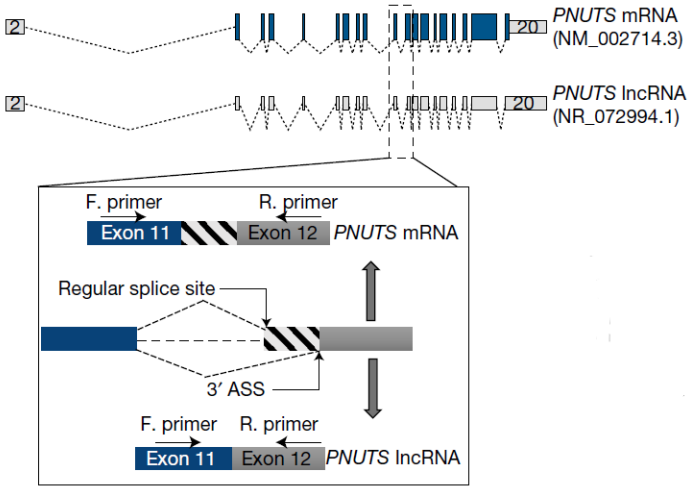


Immunoblotting analysis using antibodies to the mesenchymal marker vimentin and the epithelial markers ZO2 and E-cadherin.

CONCLUSION:

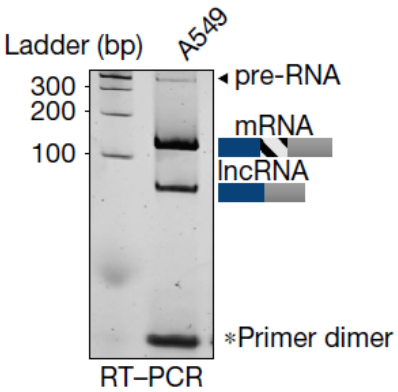
- **The silencing of *hnRNP E1* results in a differential processing of *PNUTS* pre-RNA.**
- **The silencing of *hnRNP E1* results in a morphological change in cellular phenotype.**

The difference between *PNUTS* mRNA and lncRNA-*PNUTS*



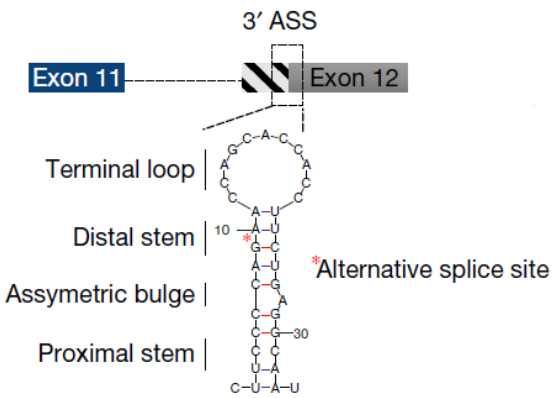
The **NCBI database** predicted the generation of lncRNA-*PNUTS* as a result of the removal of 61 bases in the 5' region of exon 12 → break in the ORF of the transcript.

Confirmed by **RT-PCR** using flanking primers.

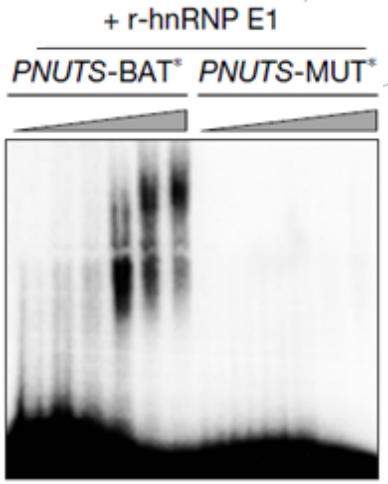


CONCLUSION:
lncRNA-*PNUTS* is upregulated following *hnRNP E1* knockdown.

The inhibitory mechanism of alternative splicing site utilization



RNA electromobility shift assay using a combination of *PNUTS*-BAT or mutated *PNUTS*-MUT α-^{32}P-labelled probes with increasing concentration of recombinant *hnRNP E1* protein.

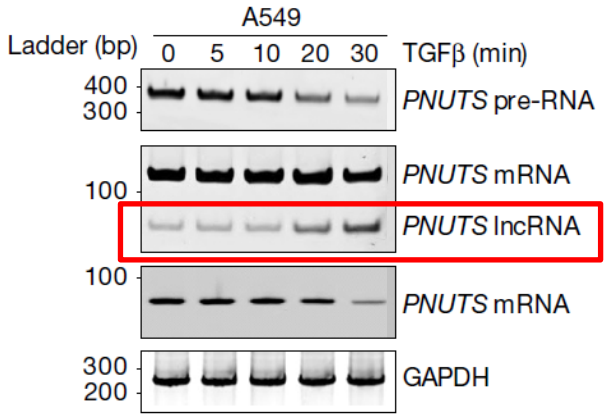


CONCLUSION:
***hnRNP E1* prevents the splicing of the lncRNA-*PNUTS* isoform by binding to a BAT structural element located at the alternative splice site.**

Can *hnRNP 1 removal from the BAT element mediate alternative splicing?*

1° METHOD:

TGFβ-induced Akt2 phosphorylation of *hnRNP E1* leads to its loss of binding and release from the BAT element.



Treatment with **TGFβ**

RT-PCR analysis of *PNUTS* gene processing.

CONCLUSION:
if *hnRNP E1* does not bind BAT element, alternative splicing occur.

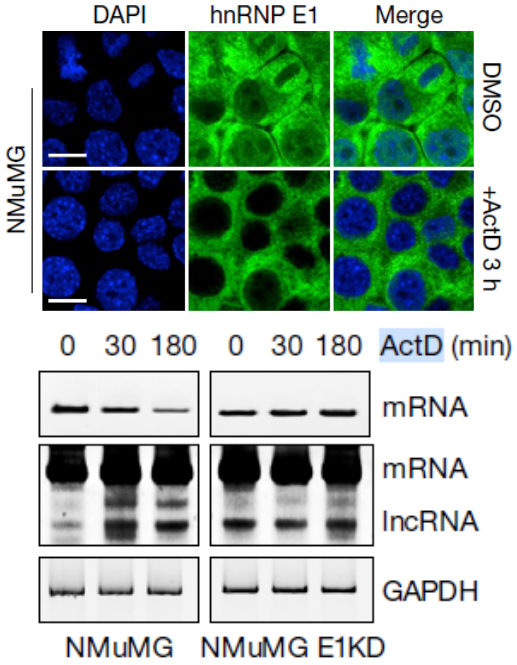
Can *hnRNP E1 removal from the BAT element mediate alternative splicing?*

2° METHOD:

Treatment with actinomycin D (**ActD**)

Immunofluorescence analysis

Cell franctionation



ActD induced the release of *hnRNP E1* from pre-RNAs

↓

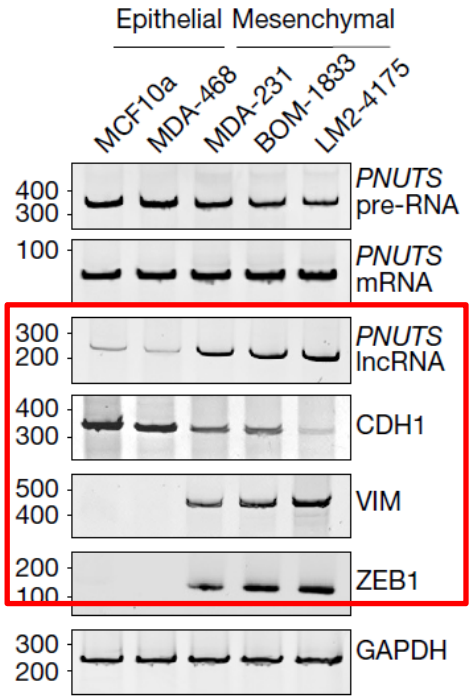
Nuclear/cytoplasmic shuttling

↓

Cytoplasmic accumulation.

CONCLUSION:
Cytoplasmic accumulation of *hnRNP E1* results in a strong induction of *PNUTS* alternative splicing.

The biological significance of *PNUTS* pre-RNA differential processing

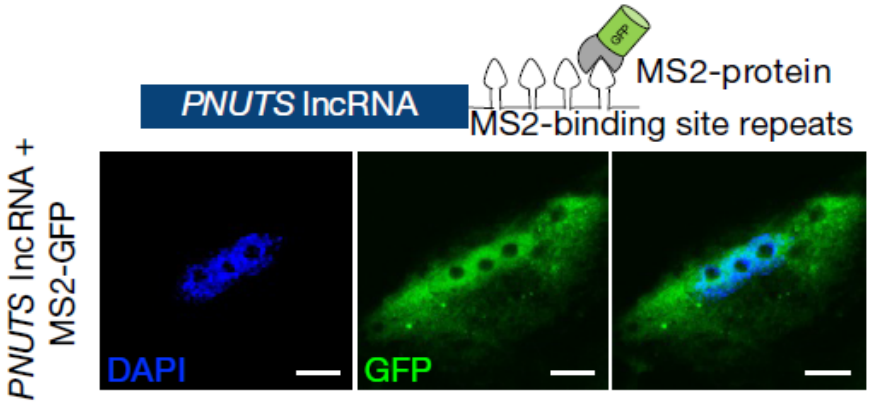


PNUTS isoform expression screening by **RT-PCR analysis** in different cell lines using *E-cadherin* as an epithelial marker while *vimentin* and *ZEB1* as mesenchymal-cells-specific markers.

CONCLUSION:
There is a correlation between lncRNA-*PNUTS* expression and the epithelial/mesenchymal status of cells.

Where is lncRNA *PNUTS* located?

Confocal microscopy imaging of subcellular localization of lncRNA-*PNUTS* using **co-transfection** of an MS2-tagged-RNA construct of lncRNA-*PNUTS* and a fused MS2-GFP protein construct.

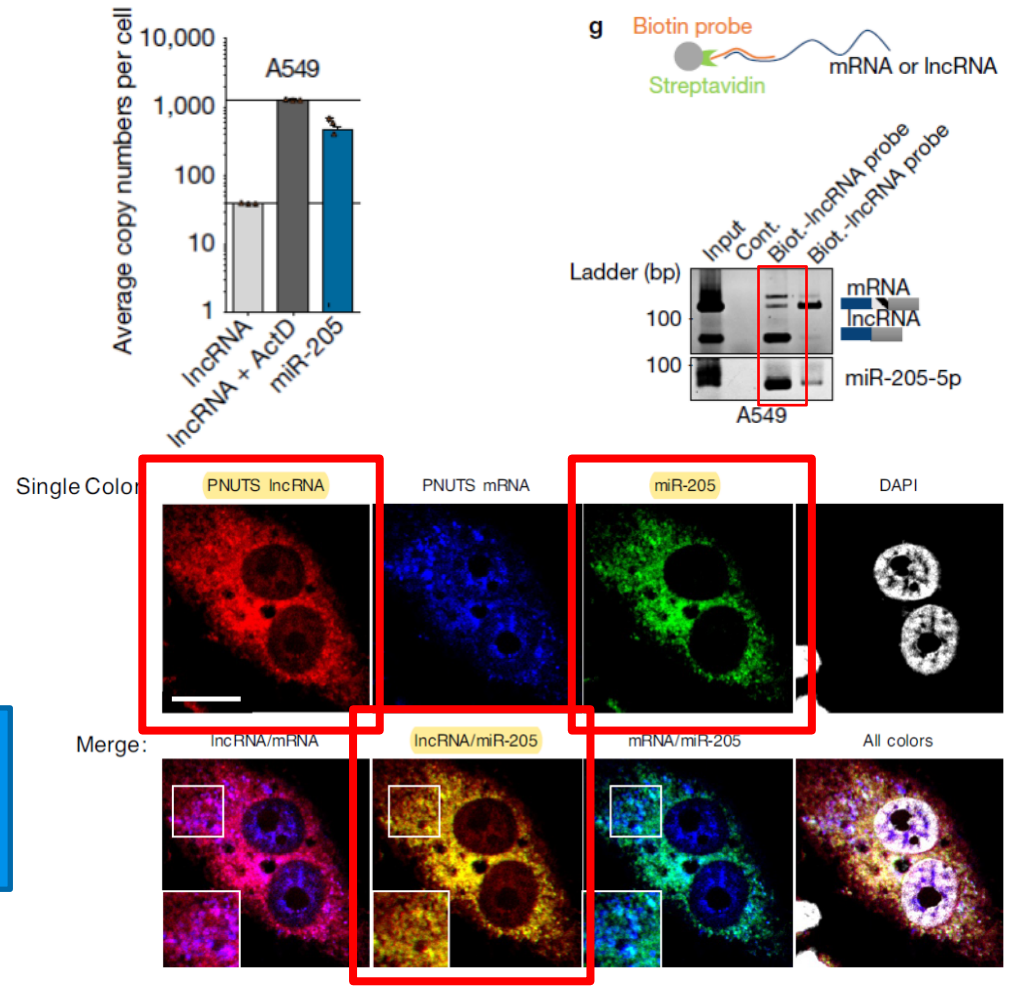


CONCLUSION:
lncRNA-*PNUTS* is located in both the cytoplasmic and nuclear compartements.

lncRNA-PNUTS interacts with miR-205

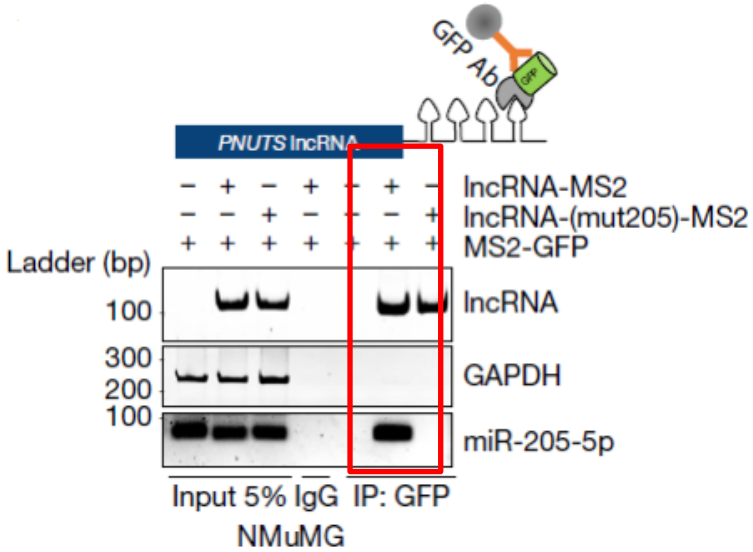
Given the subcellular localization of lncRNA-PNUTS, we next explored its biological function as a presumed competing-endogenous RNA (ceRNA).

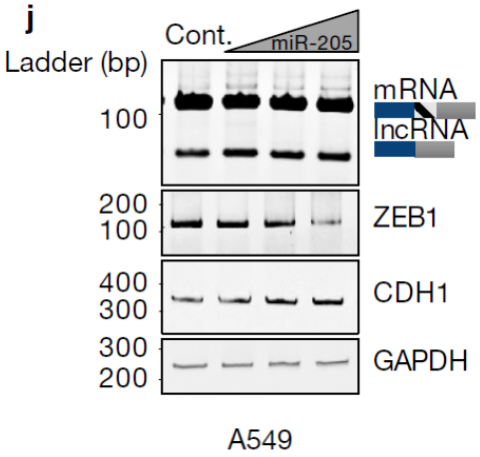
CONCLUSION:
miR-205 interacts with lncRNA-PNUTS and not interacts with PNUTS mRNA.



lncRNA-PNUTS interacts with miR-205

LncRNA-PNUTS has seven miR-205 sites, including one located in the 3'-UTR of PNUTS mRNA. To ensure that the part including the first six miR-205-binding sites is functionally active, we cloned this portion, either wild type or mutated for the miRNA-205-binding sites, into the MS2-TRAP vector and validated the specific binding by an MS2-tagged RNA affinity purification strategy and by avidin-affinity pulldown of cellular lysates.





CONCLUSION:
miR-205 interaction with lncRNA-PNUTS can convert the mesenchymal phenotype into the epithelial.

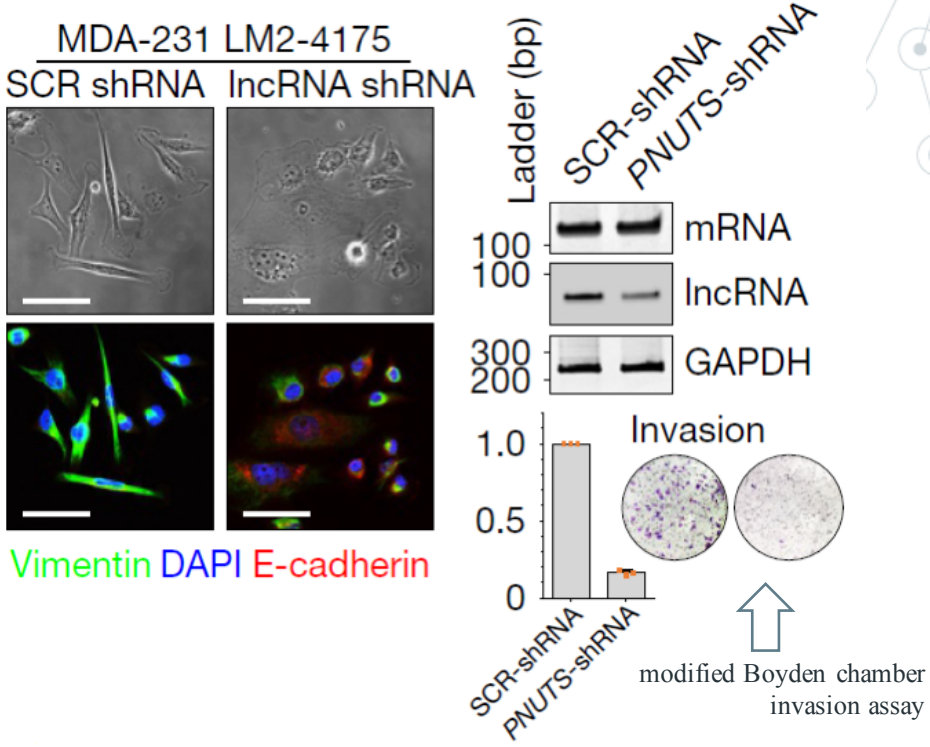
lncRNA-PNUTS regulates EMT migration and invasion in vitro through its miR-205 interaction

LncRNA-PNUTS regulates EMT and cell migration/invasion in vitro.

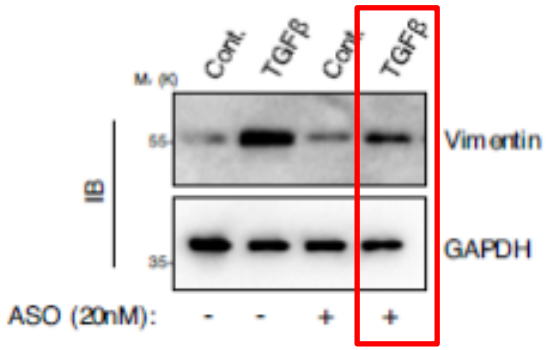
MDA-231-LM2-4175 cells stably silenced for lncRNA-PNUTS were analysed by immunofluorescence using antibodies against vimentin and E-cadherin.

CONCLUSION:

lncRNA-PNUTS silencing led to a significant decrease in cell invasion correlating with reduced vimentin expression and re-expression of epithelial marker E-cadherin concomitant to morphological changes.

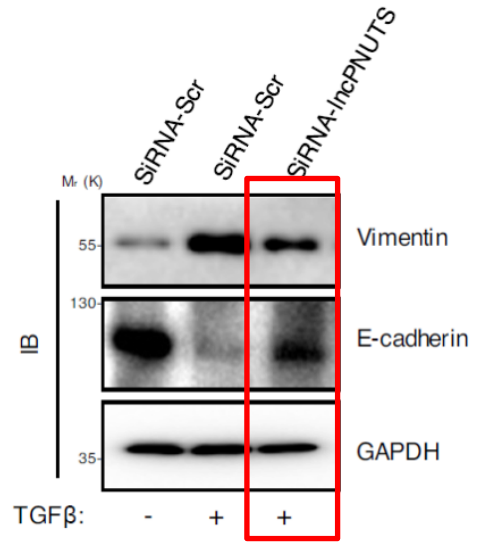


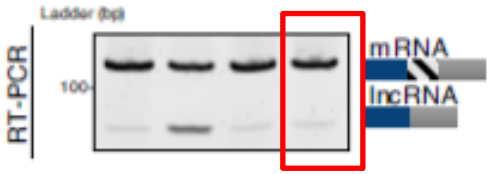
lncRNA-PNUTS regulates EMT migration and invasion in vitro through its miR-205 interaction



A549 cells with ASO against the alternative splice site.

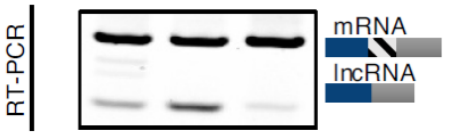
Confirmed using short interfering RNA (siRNA) specifically targeting lncRNA-PNUTS to prevent either TGFβ-mediated.





CONCLUSION:

lncRNA-PNUTS generation is a prerequisite for TGFβ-mediated EMT.

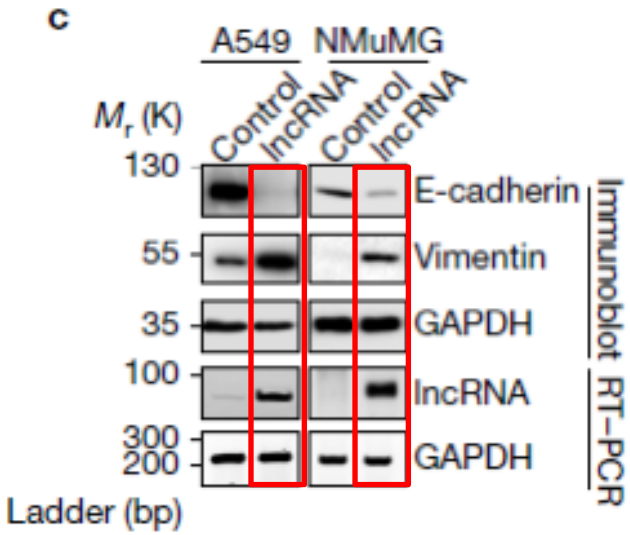


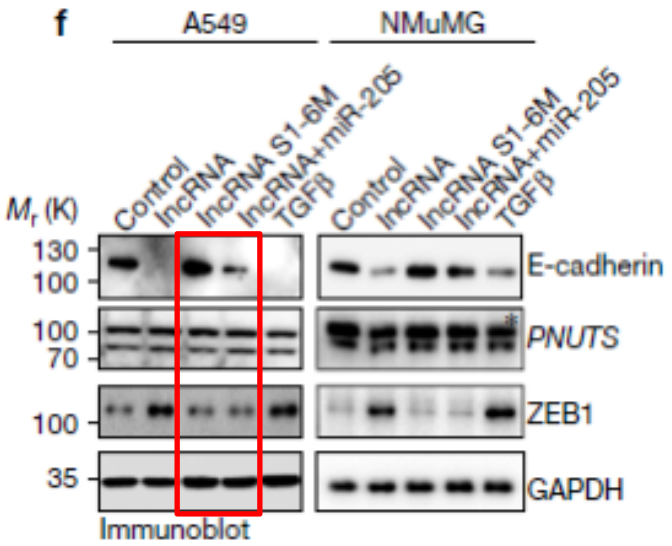
lncRNA-PNUTS regulates EMT migration and invasion in vitro through its miR-205 interaction

While the wild-type lncRNA-PNUTS induced an EMT associated with a downregulation of E-cadherin and upregulation of ZEB1, co-transfection with miRNA-205 as well as the overexpression of the miR-205-mutant form of the lncRNA-PNUTS abolished this effect.

CONCLUSION:

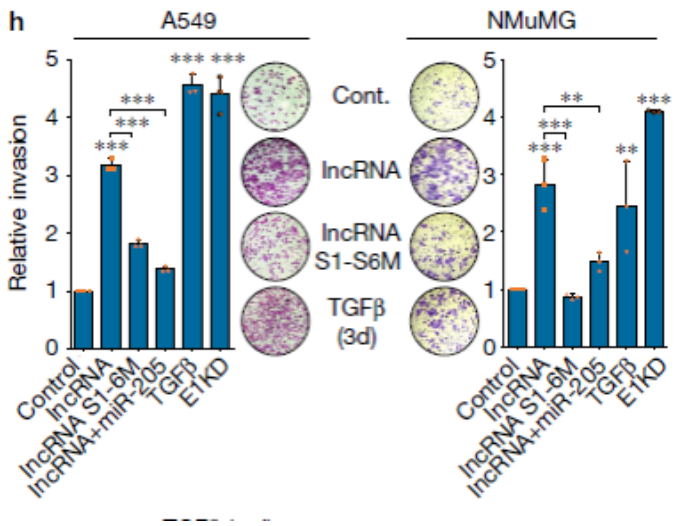
lncRNA-PNUTS induced an EMT and presence of miR-205 cancel this event.

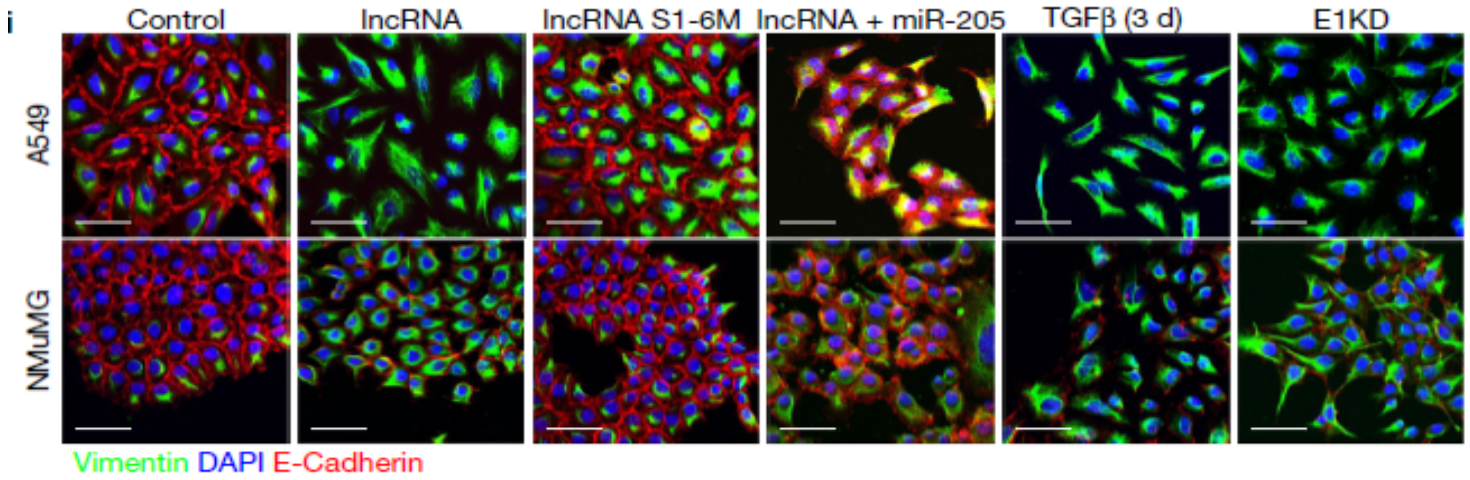




lncRNA-PNUTS regulates EMT migration and invasion in vitro through its miR-205 interaction

The lncRNA-PNUTS controls both migration and invasion of A549 and NMuMG cells in a manner dependent on its miR-205-binding sites, and miR-205 overexpression is able to abolish this effect.



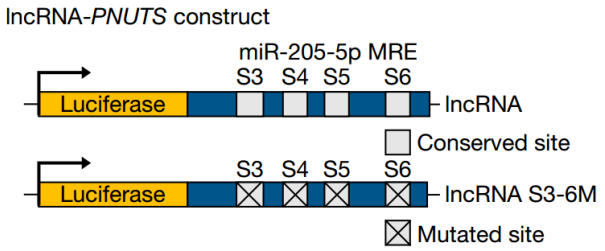


Investigating the lncRNA-*PNUTS* control of the miR-205/ZEB/E-cadherin axis

-using dual-luciferase reporter assays-

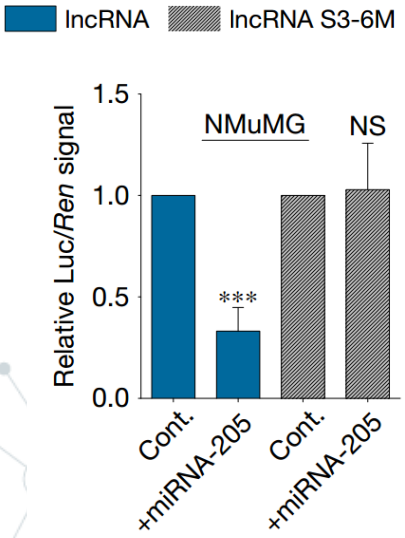
Does miR-205 actually bind to lncRNA-*PNUTS*?

3'UTR-reporter assay:



Mutated for the MRE

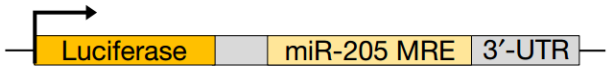
Cotrasfection with synthetic miR-205 mimic, in NMuMG cells



CONCLUSION:

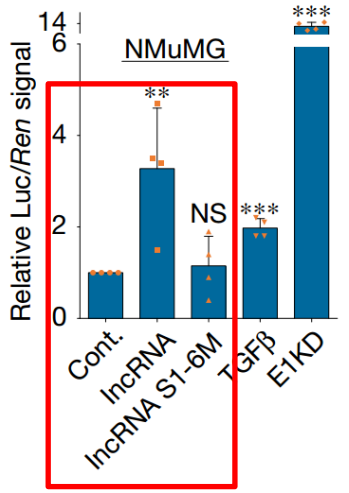
miRNA-205 binds to the lncRNA-PNUTS, reducing its bioavailability.

miR-205-5p reporter construct

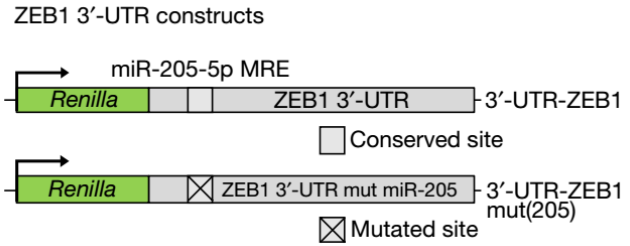


construct whose stability is dependent on miR-205 binding

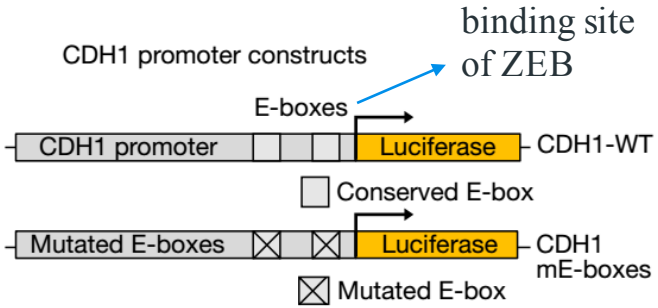
cotrasfection with wt and mutated lncRNA-*PNUTS*, in NMuMG cells



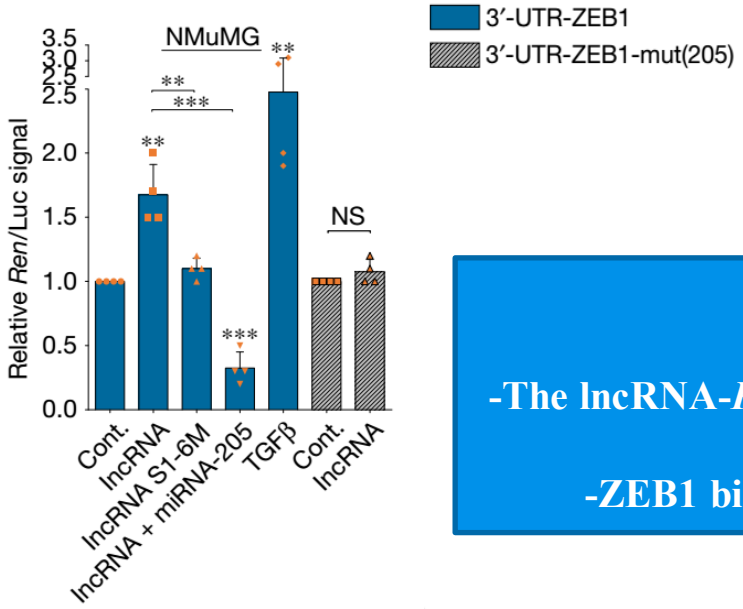
Does this binding effect ZEB1 and CDH1 expression?



Transfected in NMuMG cells

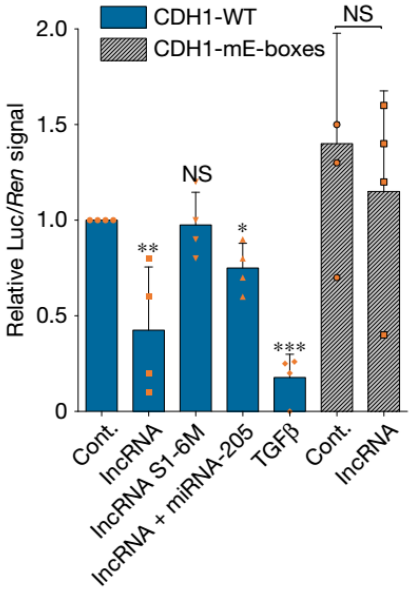


Transfected in A549 cells

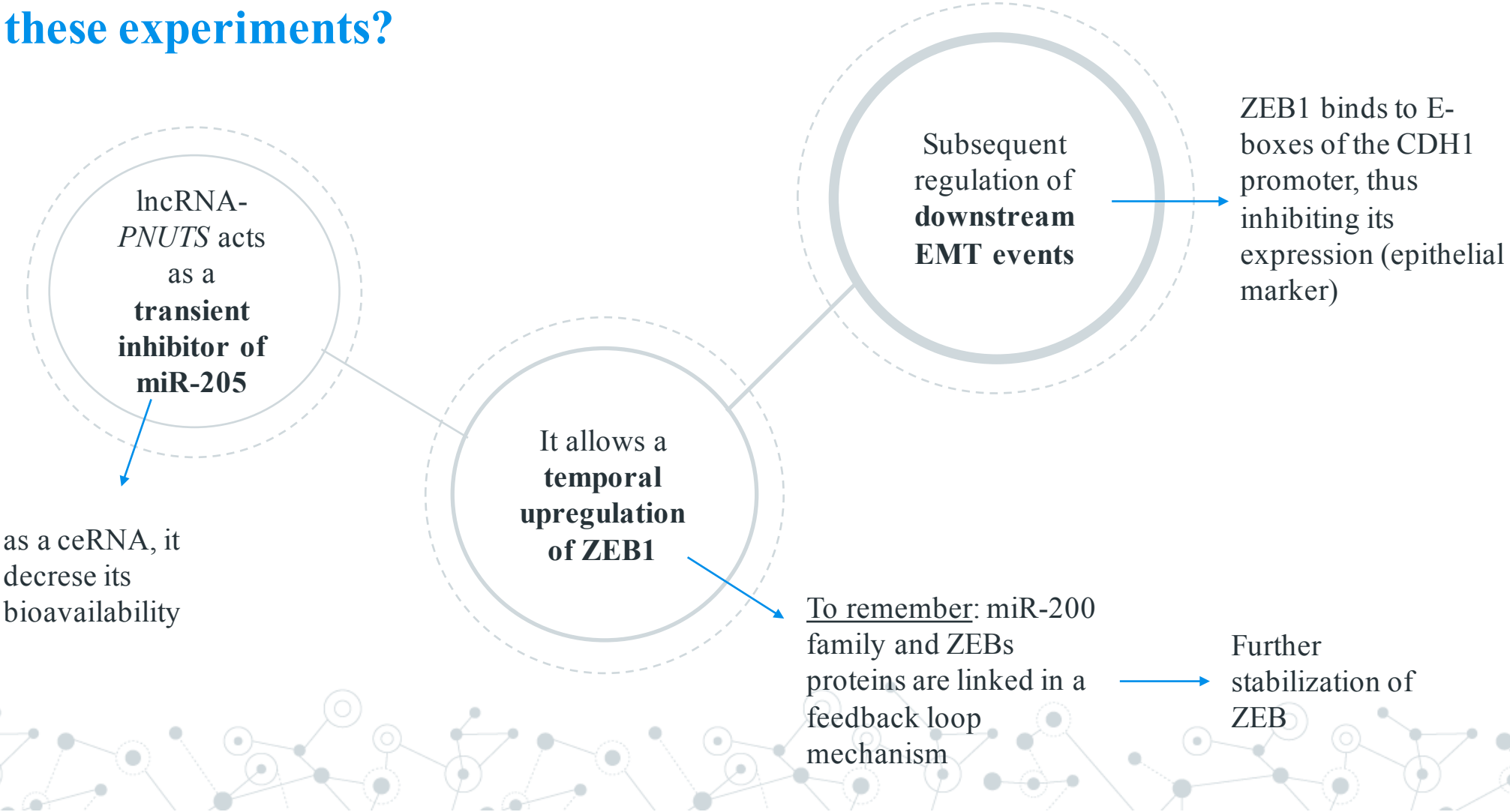


CONCLUSION:

-The lncRNA-*PNUTS* regulates ZEB1 3'-UTR through its control of miR-205;
-ZEB1 binds to CDH1 promoter → repression.



So, what have been demonstrated with these experiments?



Investigating the lncRNA-*PNUTS* contribution to tumor initiation, growth and metastasis

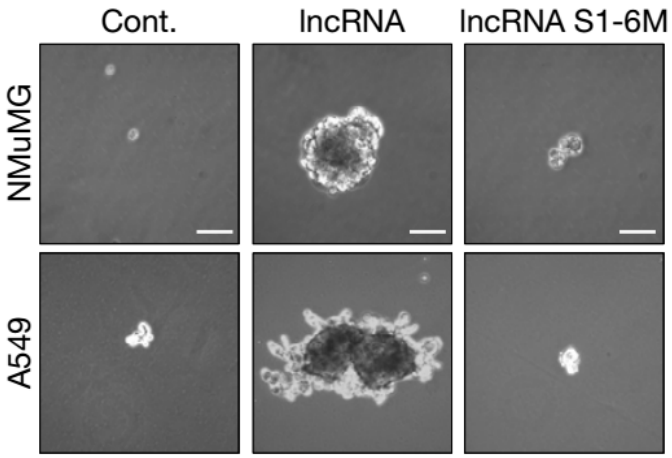
Given the role of miR-205 in regulating mammary stem cell fate and tumorigenesis through EMT
→ does the lncRNA-*PNUTS* contributes to these phenotypes?

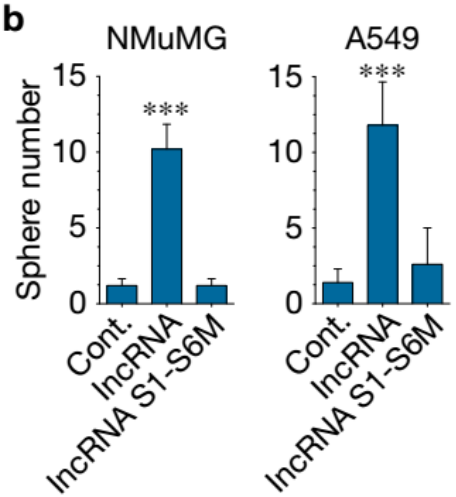
What is the effect of lncRNA-PNUTS overexpression in epithelial cells?

Mammosphere/oncosphere-formation assay

grown in non-adherent serum-free gel matrix

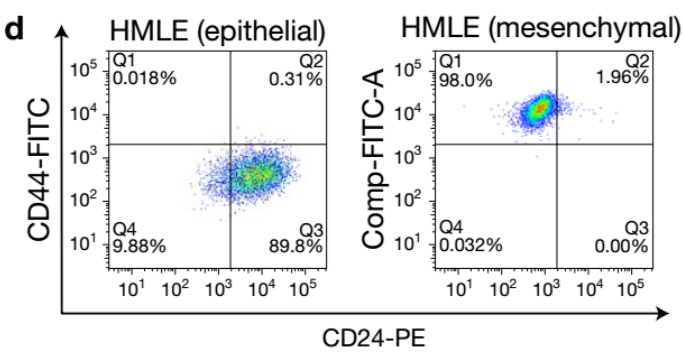
- Mouse mammary gland epithelial cells →
- Adenocarcinomic human alveolar basal epithelial cells →



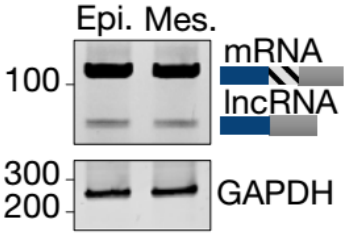


CONCLUSION:
lncRNA-PNUTS overexpression leads to an increase in sphere formation (increased number of stem cells), dependent on its miR-205 binding site.

Is lncRNA-*PNUTS* upregulated in stem cells?



FACS-sorted HMLE cells (immortalized human mammary epithelial cells) → **RT–PCR analysis** of lncRNA-*PNUTS* expression level

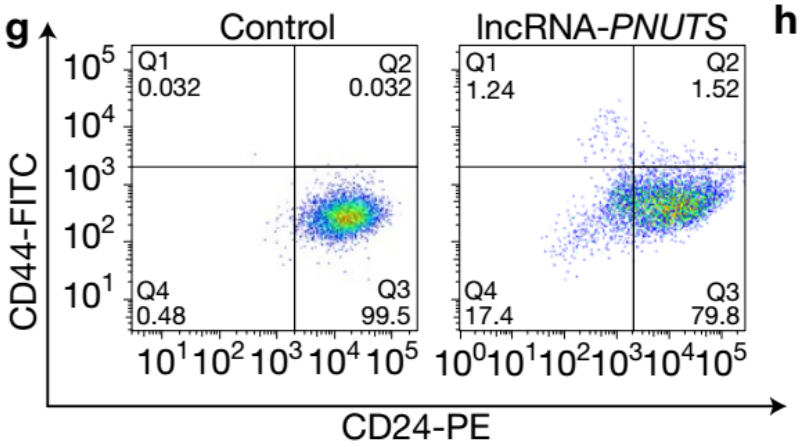


CD24+/CD44− → epithelial marker
CD24−/CD44+ → mesenchymal marker

CONCLUSION:
There is no upregulation of lncRNA-PNUTS in CD24-/CD44+ sorted cells.

Overexpression of the lncRNA-*PNUTS*

In HMLE cells already sorted for epithelial markers.



CONCLUSION:
-Significant decrease in the number of CD24+/CD44− cells;
-Minor subpopulation of cells harbouring the CD24−/CD44+ phenotype.

Effects of lncRNA-*PNUTS* on tumor progression and metastasis *in vivo*

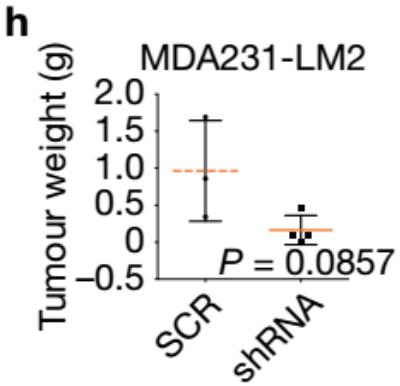
MDA-231-LM2 cells
(human breast adecarcinoma cell line, with the ability to metastasize to lung tissue in vivo)
silenced for lncRNA-*PNUTS*

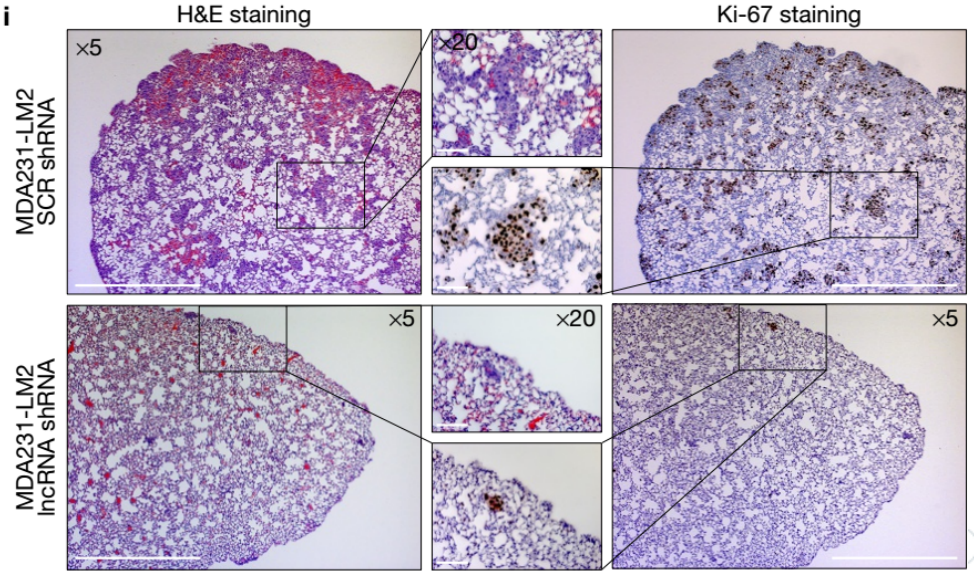
↓

mammary fat pad injection
in SCID mice

↓

-better recapitulates the location of the disease
-proper stromal compartment



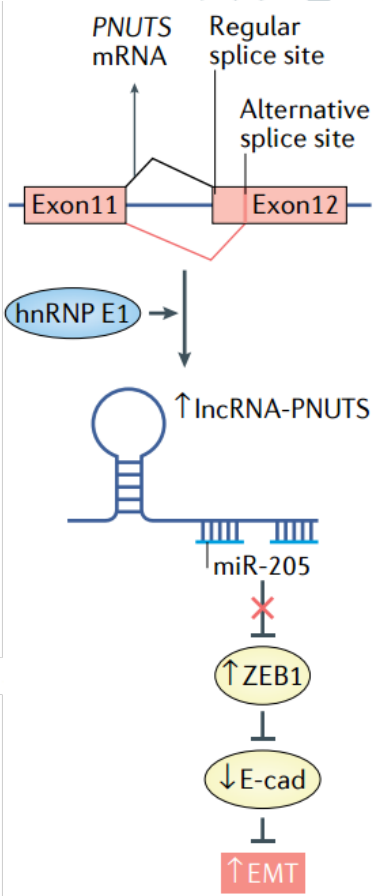


CONCLUSION:
lncRNA-*PNUTS* contributes to tumor formation and lung colonization *in vivo*.

TAKE HOME MESSAGE:

- *PNUTS* gene harbours a **bifunctional RNA**: a functional mRNA and a lncRNA
- lncRNA-*PNUTS* is localized in both the cytoplasm and the nucleus
- **lncRNA-*PNUTS*** (cytoplasmic) is involved in EMT, how?

1. **TGF-β** phosphorylates **hnRNP E1**
2. hnRNP E1 loses its binding to **BAT element**
3. **Alternative splicing** is allowed
4. Formation of lncRNA-*PNUTS*
5. lncRNA-*PNUTS* sponges away **miR-205 (ceRNA)**
6. **ZEB1** is translated
7. **EMT**



Future perspectives

- Given the role of EMT in drug resistance + contribution of miR-205 in chemotherapy sensitivity → evaluate the contribution of lncRNA-*PNUTS* in EMT-mediated drug resistace mechanisms

- hnRNP E1 and lncRNA-*PNUTS* as potential predictive markers of metastasis and/or chemoresistance

- hnRNP E1 and lncRNA-*PNUTS* as potential targets for anti-metastatic therapies

QUESTIONS?