UNIVERSITÀ DEGLI STUDI DI TRIESTE

Dipartimento di Scienze della Vita

Micropeptides CIP2A-BP encoded by LINC00665 inhibits triple-negative breast cancer progression

Binbin Guo, Siqi Wu, Xun Zhu, Liyuan Zhang, Jieqiong Deng, Fang Li, Yirong Wang, Shenghua Zhang, Rui Wu, Jiachun Lu & Yifeng Zhou

Presented by Chiara Mion and Lorenzo Murru

Outline

1. Theoretical framework: Unveiling the Hidden Microproteome;
2. The candidate: LINC00665 and its canonical oncogenic role;
3. Discovery of CIP2A-BP: From translational silencing to molecular decoy;
4. Translational impact: *In Vivo* validation and peptide therapy
5. Discussion and Conclusion

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Breaking the Central Dogma: The "Non-Coding" paradox

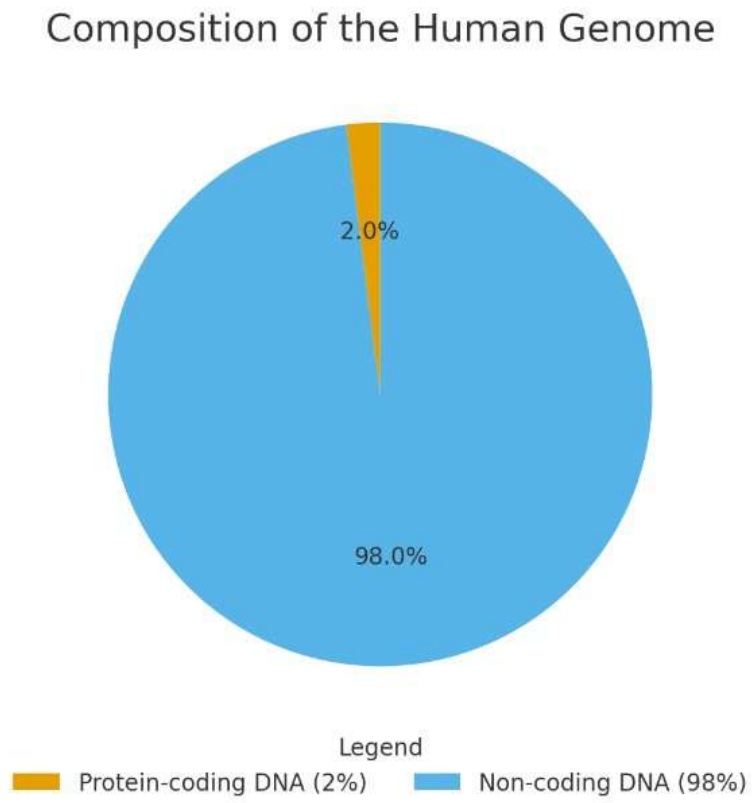

Figure 1: Proportion of protein-coding vs. non-coding DNA in the human genome.

The historical Paradigm:

- Central Dogma: DNA → RNA → Protein;
- *The "100-Codon rule"*: ORFs < 100 aminoacids were discarded as noise.

The reality:

- ~ *98%* of the genome is transcribed as ncRNA;
- Are lncRNAs truly non-coding?

How do we see the invisible?

- **The limit of RNA-seq:** Detects abundance, not translation.
- **The solution: Ribosome profiling (Ribo-seq):**
  - Sequences «Ribosome Protected Fragments« (RPFs);
  - Maps precise position of active ribosomes.
- **Discovery:** Thousands of lncRNAs are actively translated.

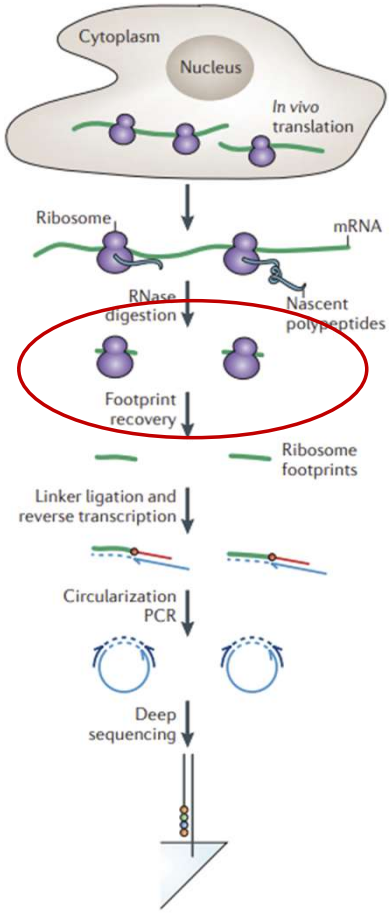

Figure 2: Schematic of the Ribo-seq Method [Ingolia, N. (2014)]

Where are micropeptides encoded?

- **Types of Non-canonical ORFs (ncORFs):**
  - **uORFs/dORFs:** Located in mRNA untranslated region (5'UTR and 3'UTR);
  - **lncORFs:** Within long non-coding RNAs (intergenic/pseudogenes);
  - **circORFs:** Encoded by circular RNAs (back-splicing junction).
  - **pri-miORF:** Within primary miRNA transcripts.

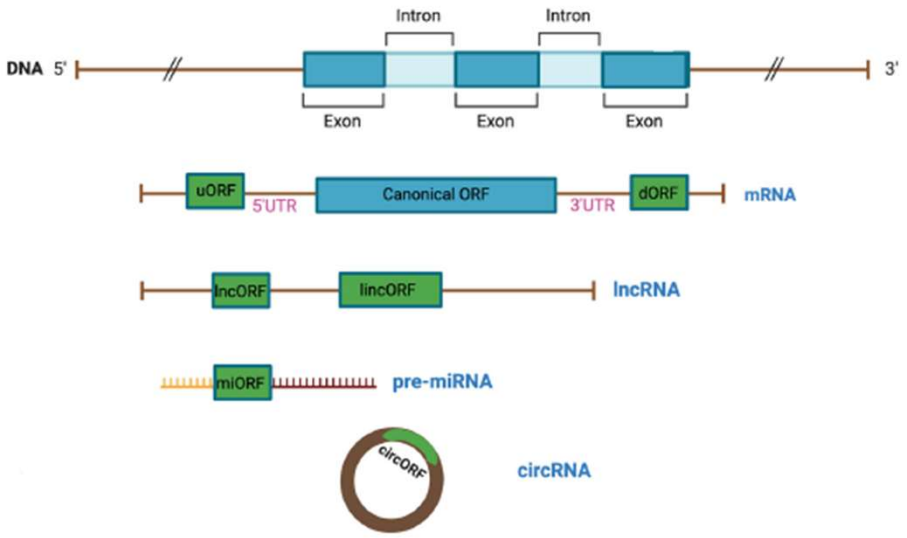

Figure 3: Genomic Classification of Non-Canonical ORFs [Ge, A.(2024)]

Decoding the "Non-Coding": Translation Mechanisms

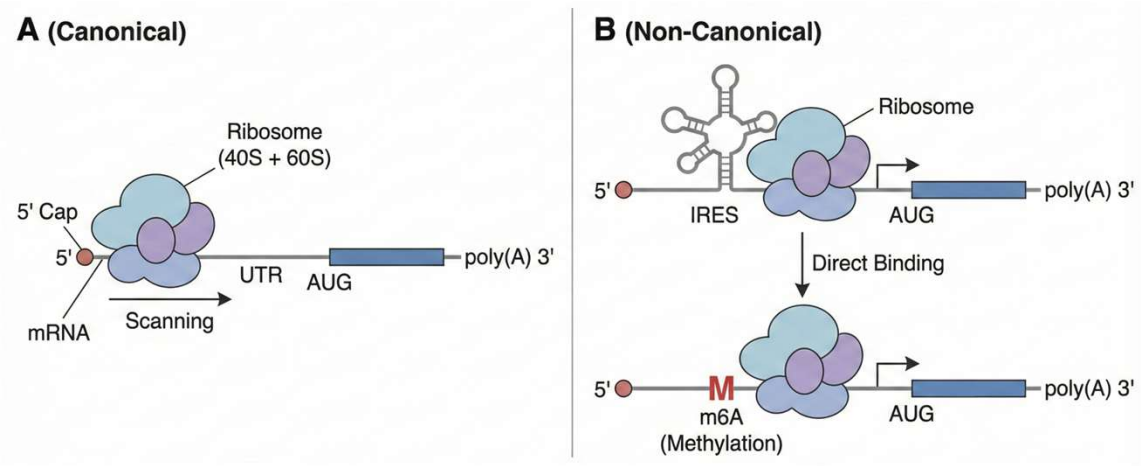

Figure 4: Canonical (A) vs. Non-Canonical (B) Translation Initiation

- **Challenge:** Many lncRNAs/circRNAs lack a 5'cap.

- **Alternative entry mechanisms:**
  - **Internal Ribosome Entry Site (IRES):** Direct ribosome recruitment (crucial for circRNAs);
  - **m^{6}A modification:** N^6-methyladenosine recruits factor (e.g., eIF4G2).

Micropeptides: Definition & Features

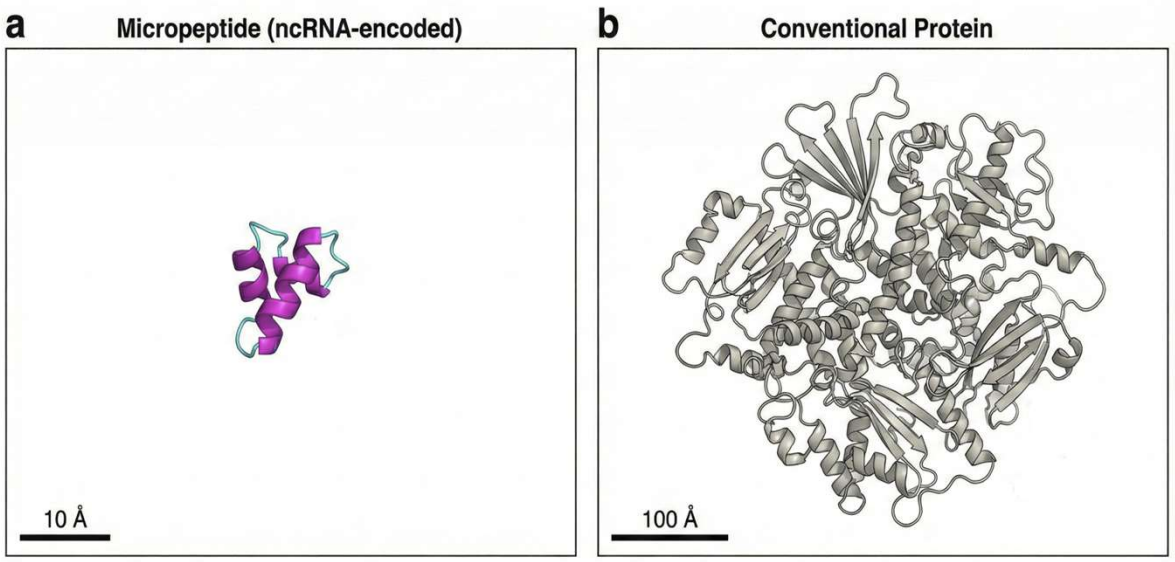

Figure 5: Structural contrast between a micropeptide (A) and a conventional protein (B)

- **Definition:** < 100 aminoacids (smORFs).
- **Key features:**
  - **Simple structure:** Single a-helix or transmembrane domain;
  - **No enzymatic activity:** Too small for catalytic pocket;
  - **Evolutionary conservation:** High conservation implies biological function.
- **Role:** Modulators/Fine-tuners of larger complexes.

Mechanism I: Scaffolding & Stabilization

- **Concept:** Micropeptides stabilize protein complexes.

- **Example: ASAP (encoded by LINC00467)**
  - **Target:** Mitochondrial ATP synthase;
  - **Action:** Binds subunits ATP5A + ATP5C;
  - **Effect:** Boosts ATP production →Promotes Colon Cancer growth.

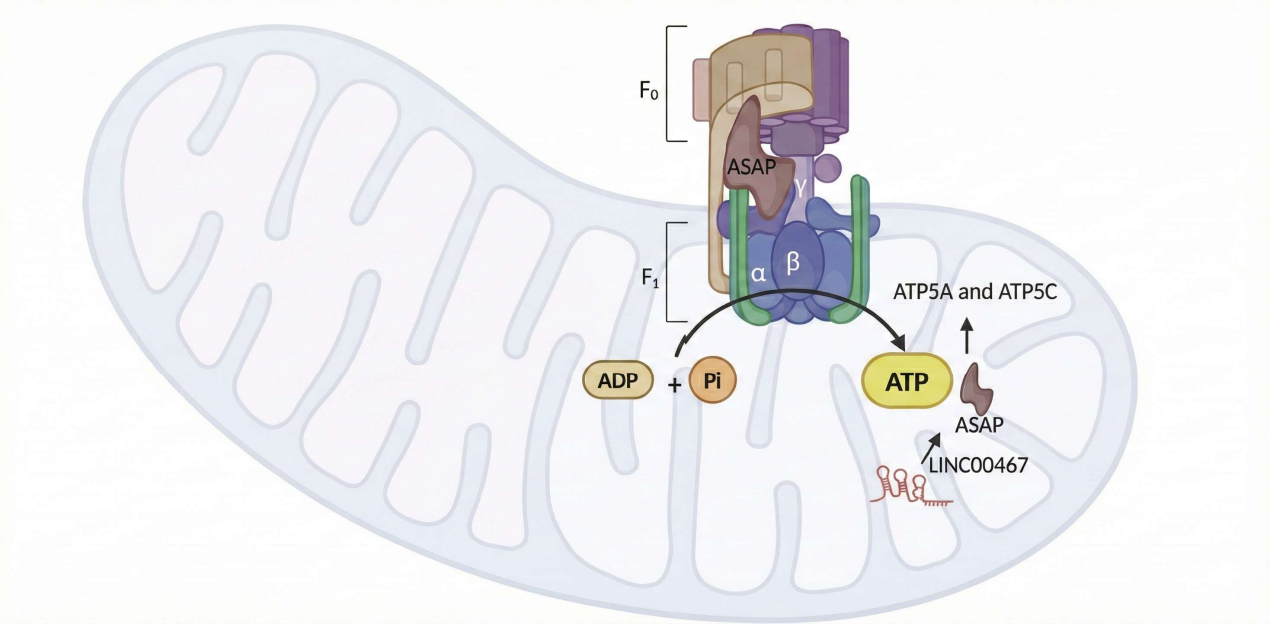

Figure 6: ASAP micropeptide regulates ATP synthase activity [Xiao, Y. (2024)]

Mechanism II: Competitive inhibition (Decoy)

- **Concept:** Mimicking binding sites to displace partners.

- **Example: HOXB-AS3 Peptide**
  - **Target:** RBP hnRNP A1;
  - **Action:** Blocks hnRNP A1 binding to PKM mRNA
  - **Effect:** Suppresses metabolic reprogramming (Tumor suppressor)

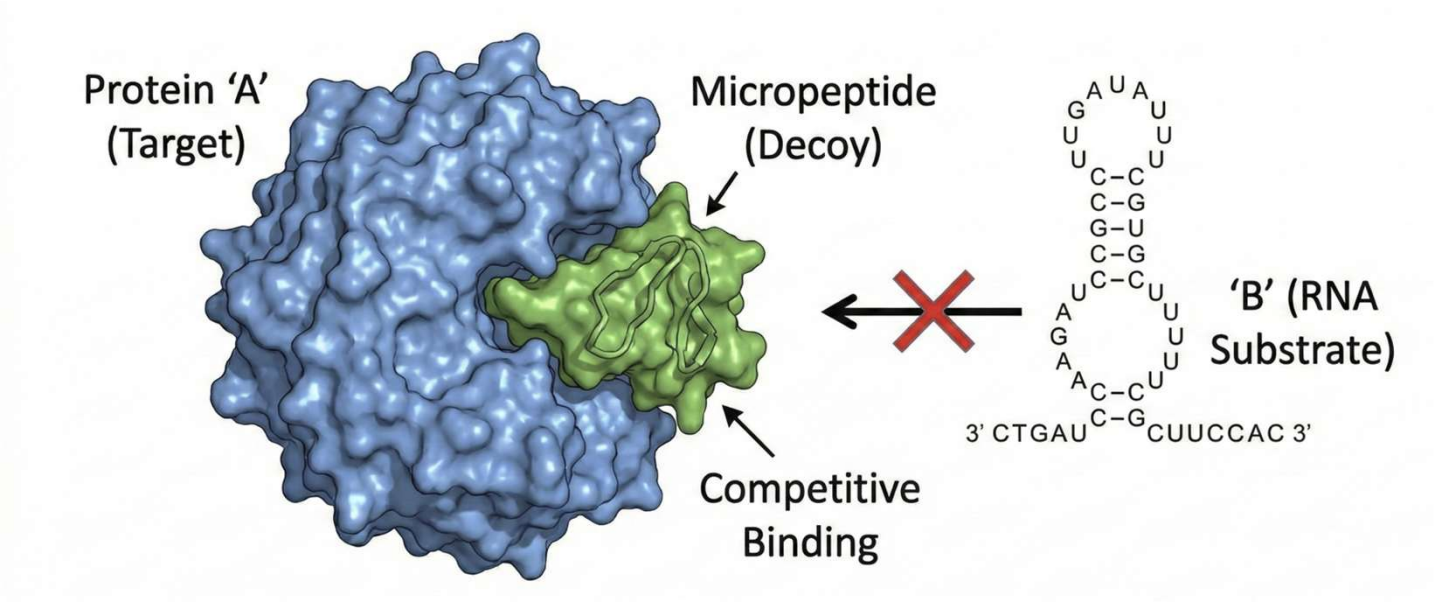

Figure 7: Mechanism of the Decoy effect

Micropeptides in Physiology & Disease

- **Versatile Biological Roles:**
  - **Muscle:** Regulation of calcium pumps (e.g., Myoregulin/MLN);
  - **Metabolism:** Oxidative phosphorylation and glycolysis;
  - **Immunity:** Antigen presentation and inflammation;
  - **DNA repair:** Recruitment of repair factors (e.g., DDUP).

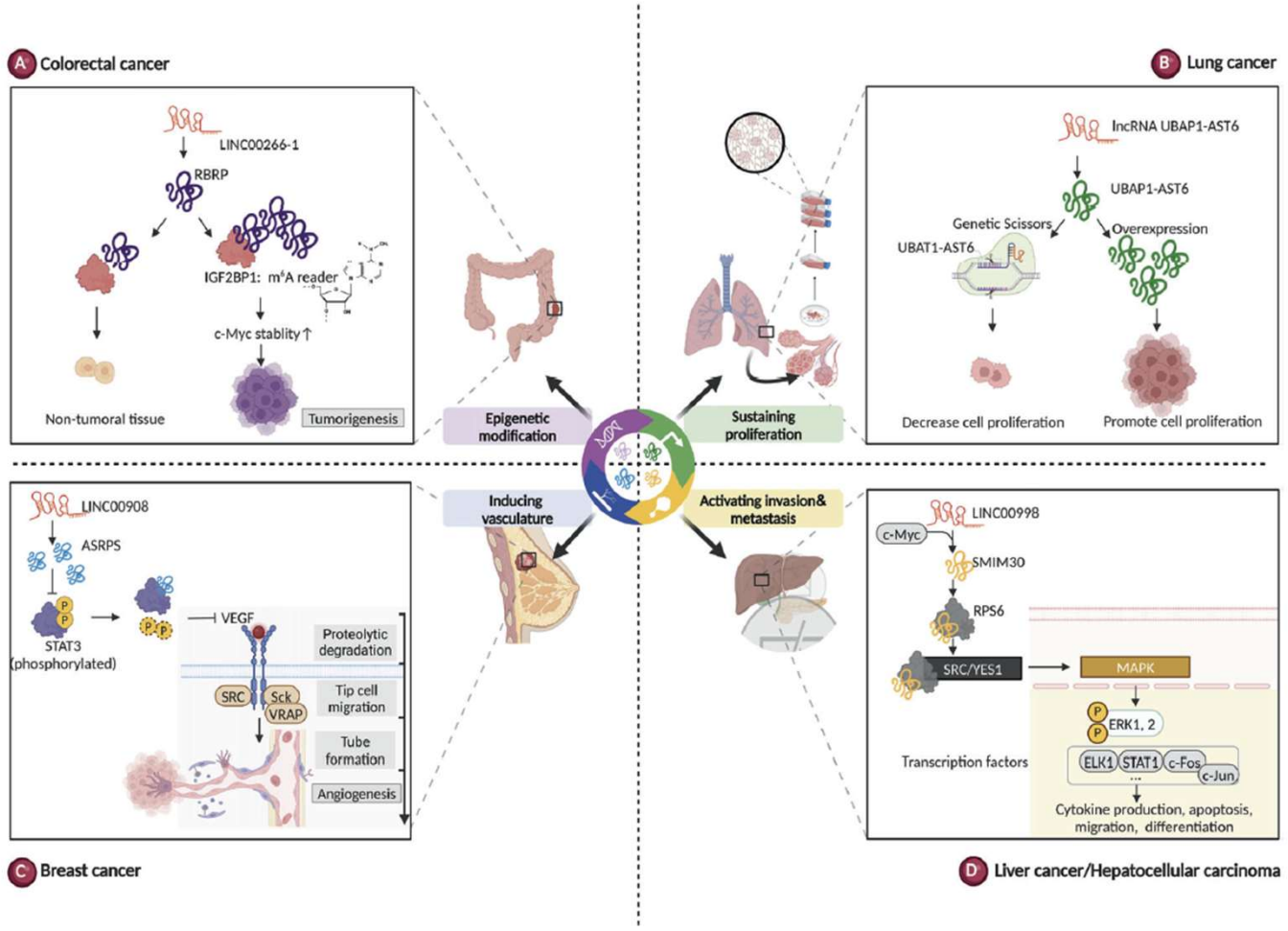

Figure 8: Diverse mechanisms of lncRNA-encoded micropeptides [Xiao, Y. (2024)]

From general principles to specific target

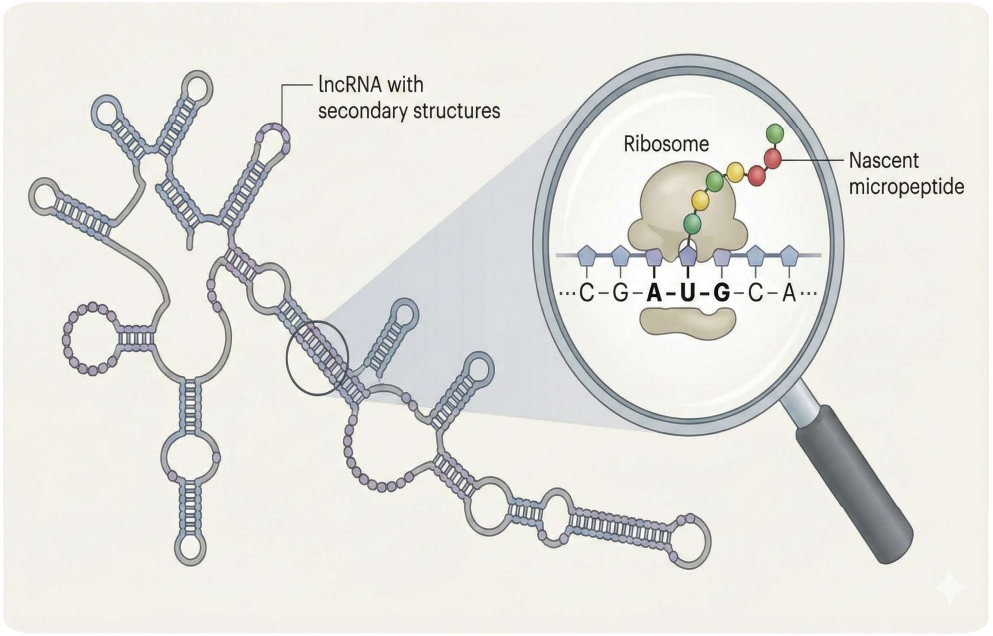

Figure 9: Hidden coding potential of lncRNAs

The Theoretical Framework:

lncRNAs can harbor hidden, functional micropeptides.

The research Gap:

Re-evaluating known cancer-associated transcripts for coding potential.

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lncRNAs: long non-coding RNAs

«Transcripts > 200 nucleotides with limited or no protein-coding capacity»

Recap from previous slides...

LINC00665: Long Intergenic Non-Coding RNA 00665

- Located on **human chromosome 19q13.12**
- First reported in 2018 by Dong-Yue Wen in hepatocellular carcinoma (HCC)
- **Pol II-transcribed lncRNA with a 5' cap**
- **Contains an ORF 1** encoding CIP2A-BP
- Length: ~400-9.000 nt, with **tissue-specific alternative splicing**

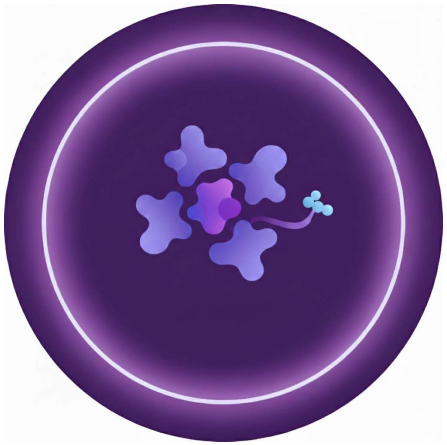
LINC00665: Long Intergenic Non-Coding RNA 00665

- **Context-dependent role**
- Aberrantly expressed in **>10 cancer types**
- Associated with **poor prognosis** and **aggressive clinical features**

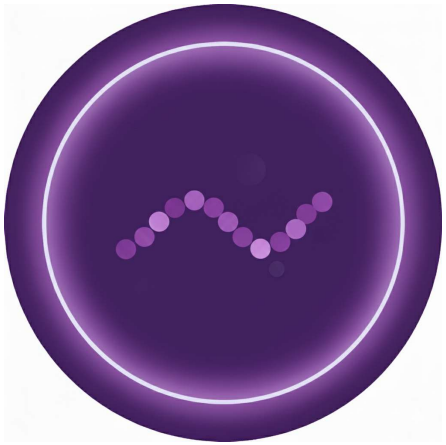
LINC00665 is a promising diagnostic biomarker for breast cancer, gastric cancer and hepatocellular carcinoma

Direct and indirect molecular interactions

COMPETING ENDOGENOUS RNA (ceRNA): miRNA SPONGE



DIRECT TARGET THE INTERACTION OF 10 PROTEIN CODING GENES



ENCODES A MICRO-PEPTIDE CIP2A-BP

ceRNA: Competing Endogenous RNA

"miRNAs sponging to regulate target mRNAs"

Complex interaction network

25miRNAs ⟶ 26mRNAs

Promotes tumor progression in 16 cancers

LINC00665 acts as an oncogene through competitive miRNA binding

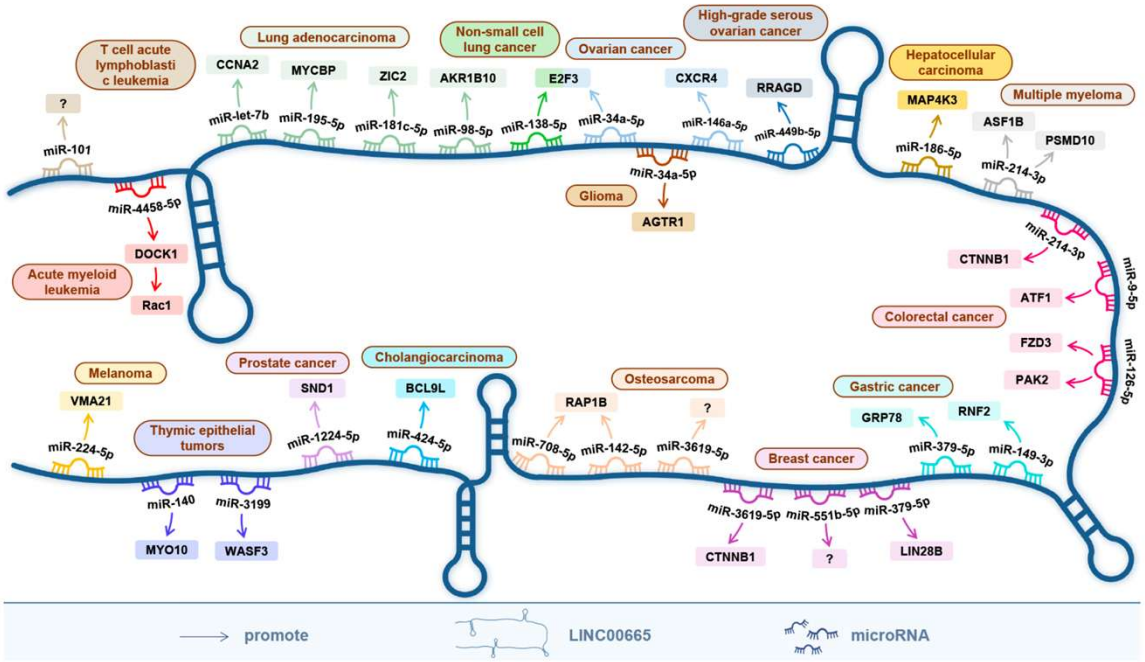


Fig. 10. ceRNA network centered on LINC00665

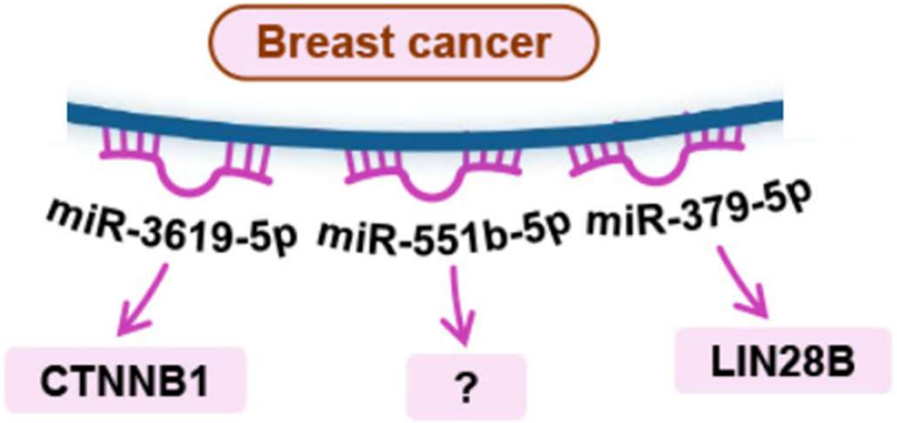


Fig.11. ceRNA in breast cancer

Examples in breast cancer:

- **miR-3619-5p** → CTNNB1 → Wnt/β-catenin pathway
- **miR-551b-5p** → proliferation and invasion
- **miR-379-5p** → LIN28B → Epithelial-Mesenchymal Transition (EMT)

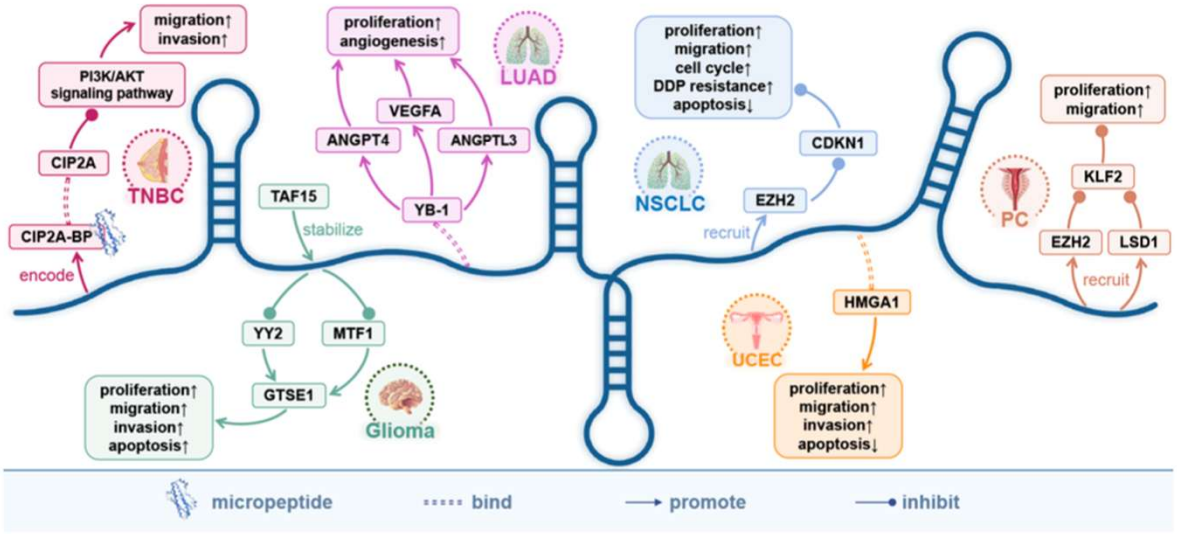


Fig.12. Direct targeting of LINC00665

RNA-Protein Interaction

LINC00665 directly regulates ~10 protein-coding genes

- Chromatin regulatory complex: binds PRC2 → transcriptional activation of targets
- RNA-binding proteins (RBPs): alters mRNA stability, transport, translation

Changes protein levels independently of miRNAs

Micropeptide Function: CIP2A-BP

- LINC00665 contains a smORF encoding CIP2A-BP (~52 aa)
- Effect ⟶ suppress invasion and metastasis in Triple Negative Breast Cancer (TNBC)
- **In TNBC the LINC00665 expression is lower than in normal cells**

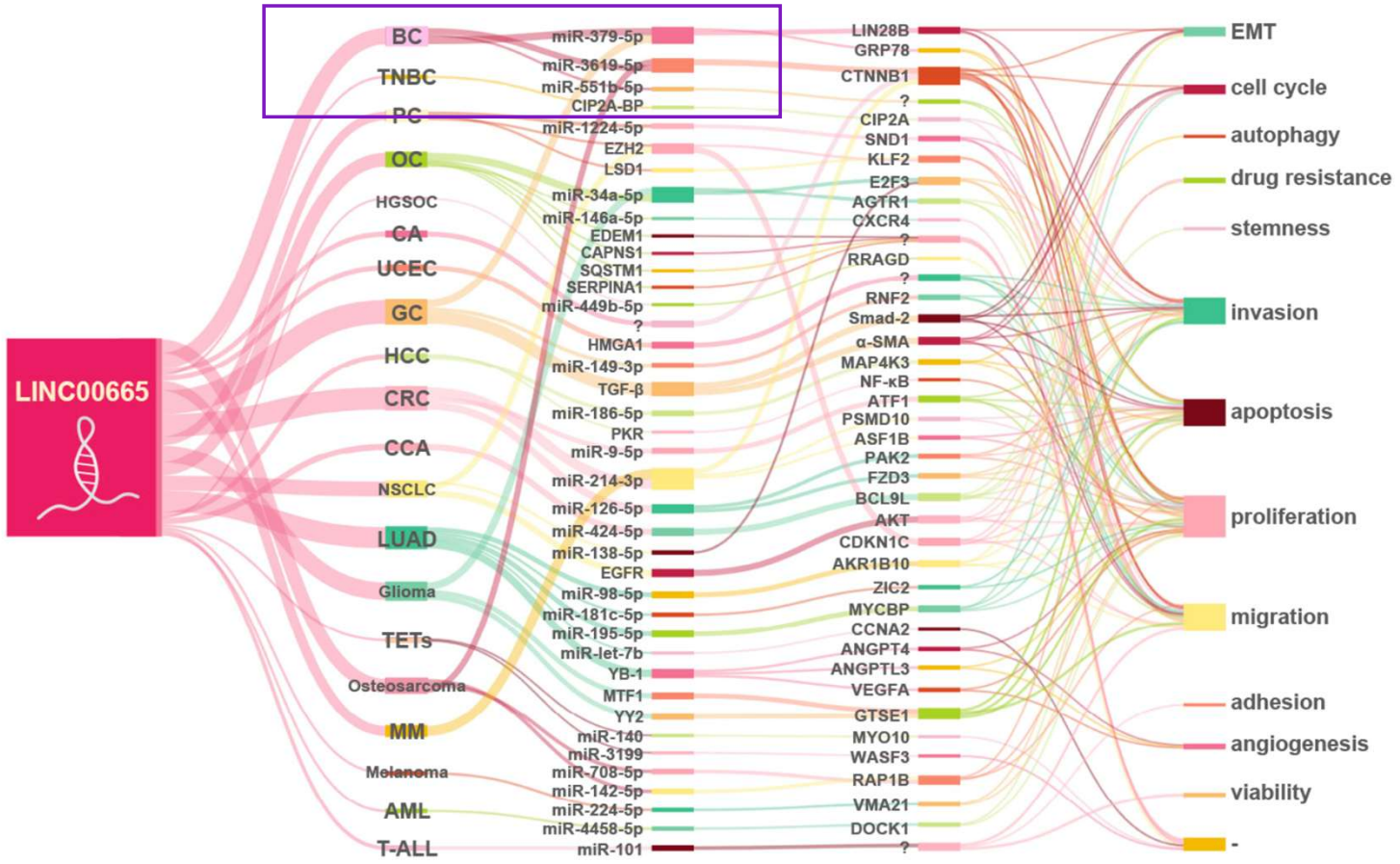


Fig. 13. The complex molecular regulatory mechanism of LINC00665 in tumors.

Clinical relevance

1. **Promising diagnostic and prognostic biomarker** in breast, gastric and hepatocellular carcinoma
2. **Predictive marker** of response to cisplatin–paclitaxel in breast cancer therapy
3. **Involved in the modulation of different pathways** like Wnt/β-catenin, NF-κB, PI3K/AKT and TGF-β/Smad
4. **Aberrant expression in ≥18 types of cancers**, including reproductive, digestive, respiratory, neurological, endocrine

LINC00665 as a promising prognostic biomarker

System	Tumor Type	Sample Size	Expression	Prognostic/Diagnostic Value	Ref.
Digestive system	Gastric cancer	49 patients	Upregulation	Positively associated with poor differentiation and TNM stage	[23]
		116 patients	Upregulation	Positively associated with tumor depth, lymph node metastasis and TNM stage; prognostic factor of OS ($p < 0.05$, HR = 2.703); AUC = 0.828	[24]
	Hepatocellular carcinoma	122 patients	Upregulation	Positively associated with lymph node metastasis and TNM stage; prognostic factor of OS ($p < 0.05$)	[26]
		39 patients	Upregulation	Positively associated with poor differentiation, TNM stage and vascular invasion; prognostic factor of OS (MST: 46 versus 70 months, $p = 0.027$, HR = 1.477); AUC = 0.614, sensitivity = 0.55, specificity = 0.53	[3]
		76 patients	Upregulation	Positively associated with tumor size and poor differentiation; prognostic factor of OS ($p < 0.05$)	[27]
	Colorectal cancer	46 patients	Upregulation	Positively associated with lymph node metastasis and poor differentiation	[28]
	Cholangiocarcinoma	100 patients	Upregulation	Positively associated with lymph node metastasis and TNM stage; prognostic factor of OS ($p = 0.0375$, HR = 1.835) and RFS ($p < 0.001$, HR = 2.554)	[8]
Respiratory system	Non-small cell lung cancer	60 patients	Upregulation	Positively associated with tumor size, lymph node metastasis and TNM stage; prognostic factor of OS ($p = 0.005$) and PFS ($p = 0.002$)	[7]
	Lung adenocarcinoma	60 patients	Upregulation	Positively associated with tumor size, lymph node metastasis and TNM stage	[32]
		80 patients	Upregulation	Positively associated with tumor size, lymph node metastasis and TNM stage; prognostic factor of OS ($p = 0.0115$) and RFS ($p < 0.001$)	[1]
		84 patients	Upregulation	Prognostic factor of OS ($p = 0.035$, HR = 1.44)	[33]
Nervous system	Glioma	-	Upregulation	Prognostic factor of OS ($p < 0.05$)	[35]
		-	Downregulation	Negatively associated with pathological grade	[43]
		48 patients	Upregulation	Prognostic factor of OS ($p = 0.0241$)	[36]
Endocrine system	Thymic epithelial tumors	-	Upregulation	Prognostic factor of OS ($p = 0.047$)	[37]
Endocrine system	Osteosarcoma	33 patients	Upregulation	Prognostic factor of OS ($p < 0.05$)	[38]
		42 patients	Upregulation	Prognostic factor of OS ($p = 0.011$)	[2]

TNM, Tumor-node-metastasis; OS, Overall survival; DFS, Disease-free survival; RFS, Recurrence-free survival; PFS, Progression-free survival; AUC, Area under the curve; MST, Median survival time; HR, Hazard ratio; FIGO, Federation internationale of gynecologie and obstetrotrique.

Fig. 14. aberrant expression of LINC00665 in different types of cancers

Signaling pathways involved in LINC00665

1° TGF-β/SMAD signaling pathway

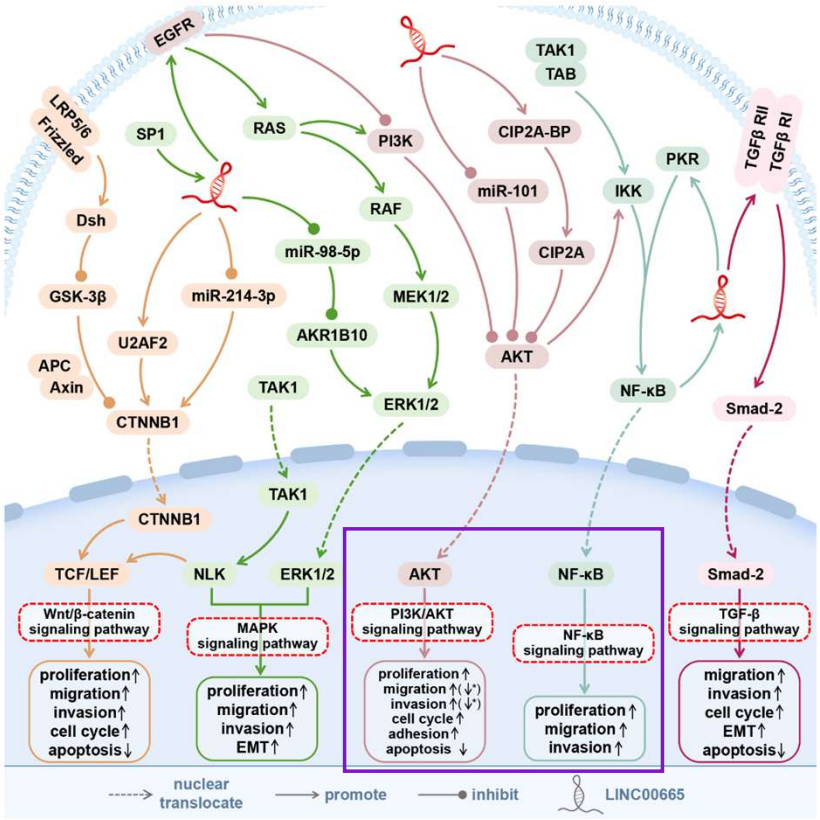


Fig. 15. Signaling pathways involved in LINC00665

2° PI3K/AKT/NF-kB signaling pathway

LINC00665 and the efficacy of Anticancer Drugs

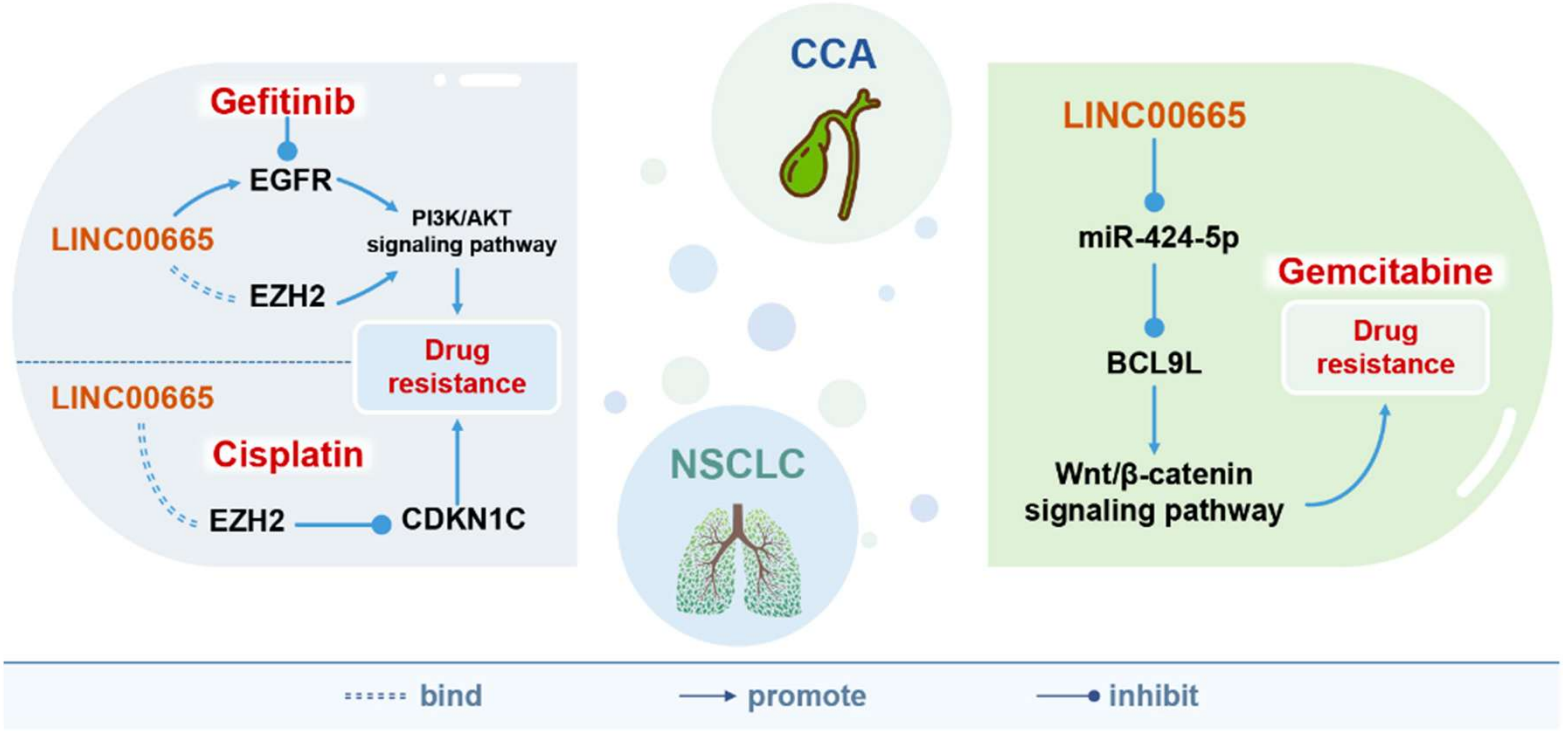


Fig. 16. Role of LINC00665 in resistance to anticancer drugs

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Article

TRANSPARENT PROCESS

THE EMBO JOURNAL

Micropeptide CIP2A-BP encoded by LINC00665 inhibits triple-negative breast cancer progression

Binbin Guo[1,†], Siqi Wu[1,†], Xun Zhu[2,†], Liyuan Zhang[3,†], Jieqiong Deng[1], Fang Li[1], Yirong Wang[1], Shenghua Zhang[1], Rui Wu[1], Jiachun Lu[4] & Yifeng Zhou[1,*]

The clinical Challenge: TNBC

- **Triple-Negative Breast Cancer (TNBC):**
  - ~15% of breast cancers;
  - Lacks ER, PR, and HER2 amplification;
  - **Clinical Features:** Aggressive behavior, early relapse, high metastatic risk;
- **The Problem:** Lack of targeted therapies (e.g., Tamoxifen/Herceptin do not work).

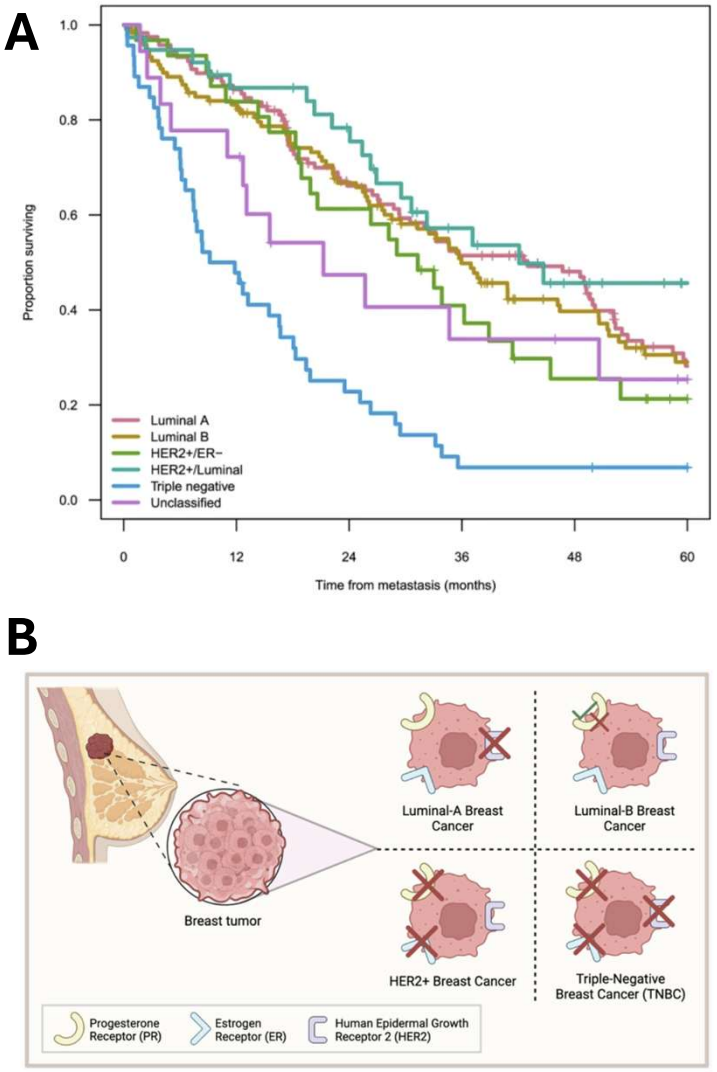


Figure 17: TNBC definition and poor prognosis. (A) Kaplan-Meier curve showing reduced survival probability for TNBC compared to other subtypes [Sears, A.J. (2025)]. (B) Breast cancer subtypes classified by ER, PR, and HER2 receptor status [Kirbly, M. (2023)]

TGF-β and lncRNAs

- **TGF-β Signaling:** Known driver of EMT and metastasis in late-stage cancer;
- **lncRNAs:** >200nt transcripts, historically considered non-coding;
- ***The Hypothesis:*** *Does TGF-β regulate cancer progression by modulating the* ***translation*** *of specific lncRNAs?*

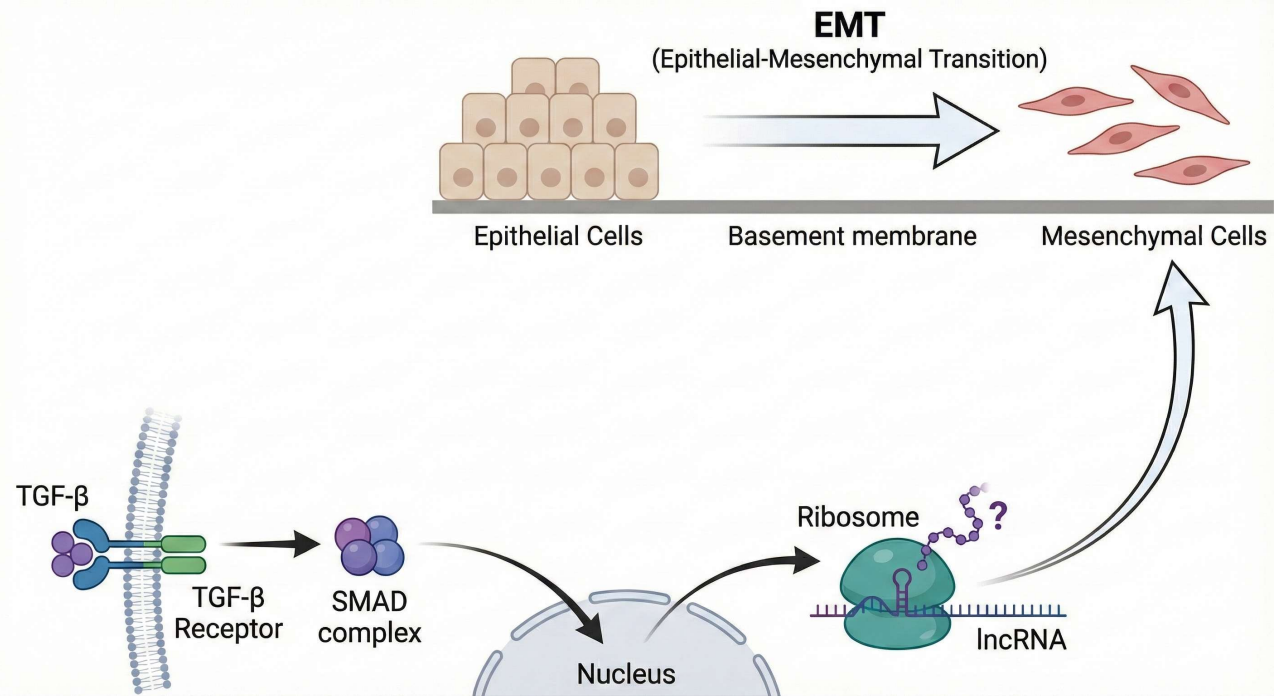


Figure 18: TGF-β signaling may promote EMT by modulating the translation of specific lncRNAs

Principal aims of the study

- **Aim 1:** Screen for lncRNAs regulated by TGF-β at the **translational level**;
- **Aim 2:** Validate the coding potential and endogenous expression of the candidate;
- **Aim 3:** Determine clinical significance in TNBC patients;
- **Aim 4:** Elucidate the biological function (Tumor Suppressor vs. Oncogene).

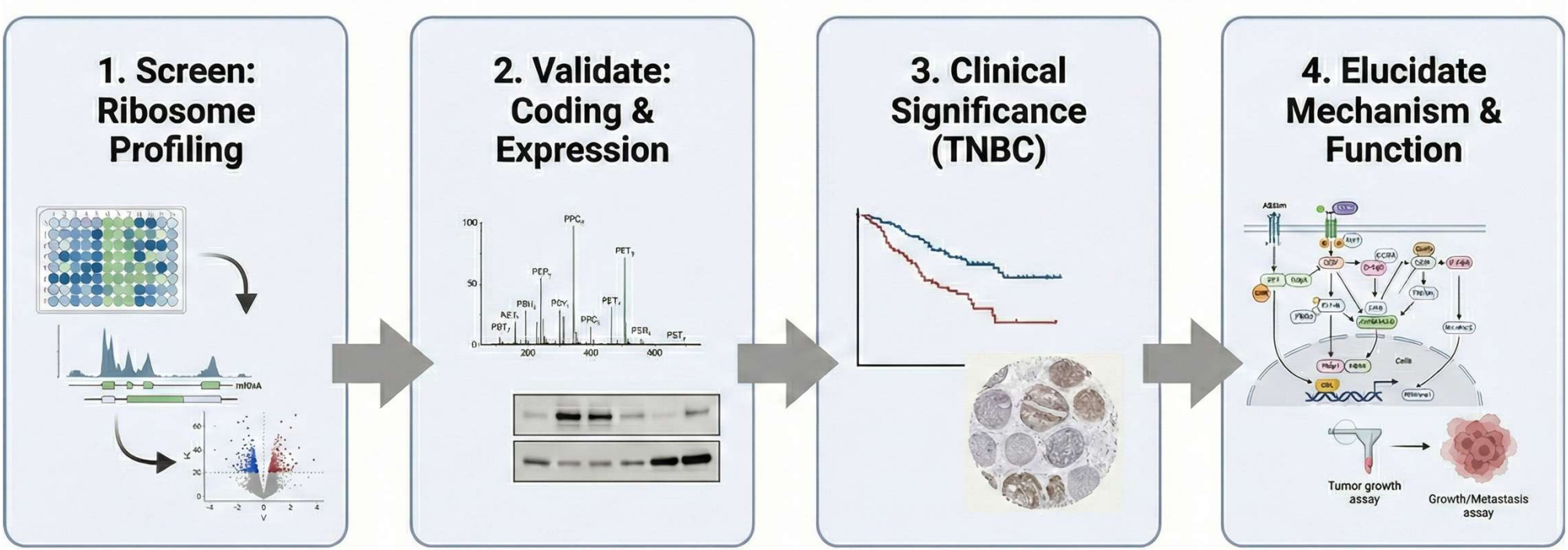
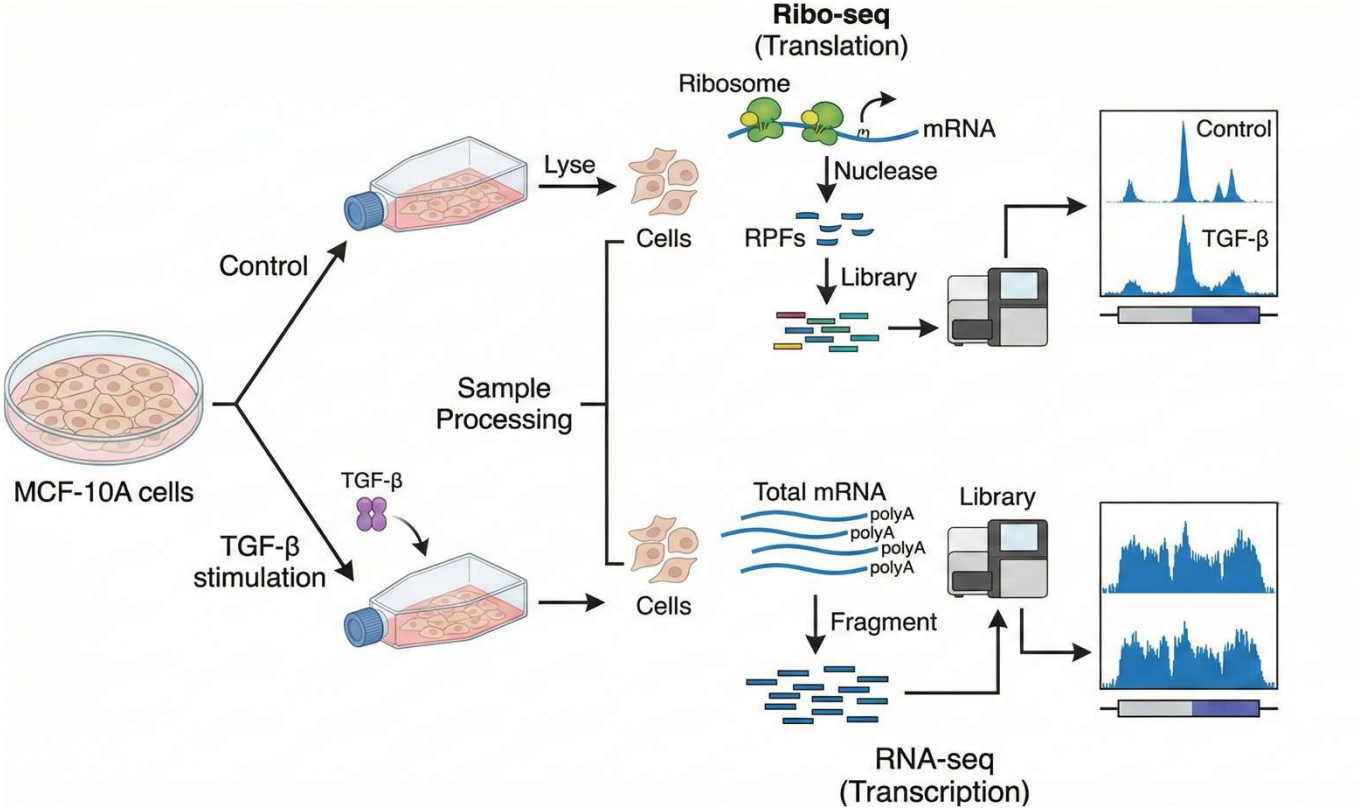


Figure 19: Experimental workflow for the identification, validation, and functional characterization of novel micropeptides in TNBC

The screening strategy



- **Model:** MCF-10A.
- **Treatment:** TGF-β stimulation.
- **Method:** Comparative Ribo-seq (Translation) vs. RNA-seq (Transcription).
- **Goal:** Find genes with changed translation but stable transcription.

Figure 20: Experimental schematic for identifying TGF-β-induced translational regulation via comparative Ribo-seq and RNA-seq

Identifying the Candidate: LINC00665

- **Candidate:** *LINC00665*.
- **Observation:**
  - ➢ TGF-β significantly reduces ribosome binding (Polysome fraction);
  - ➢ TGF-β has **NO effect** on total RNA levels.
- **Conclusion:** TGF-β suppresses the translation of *LINC00665* without degrading the RNA.

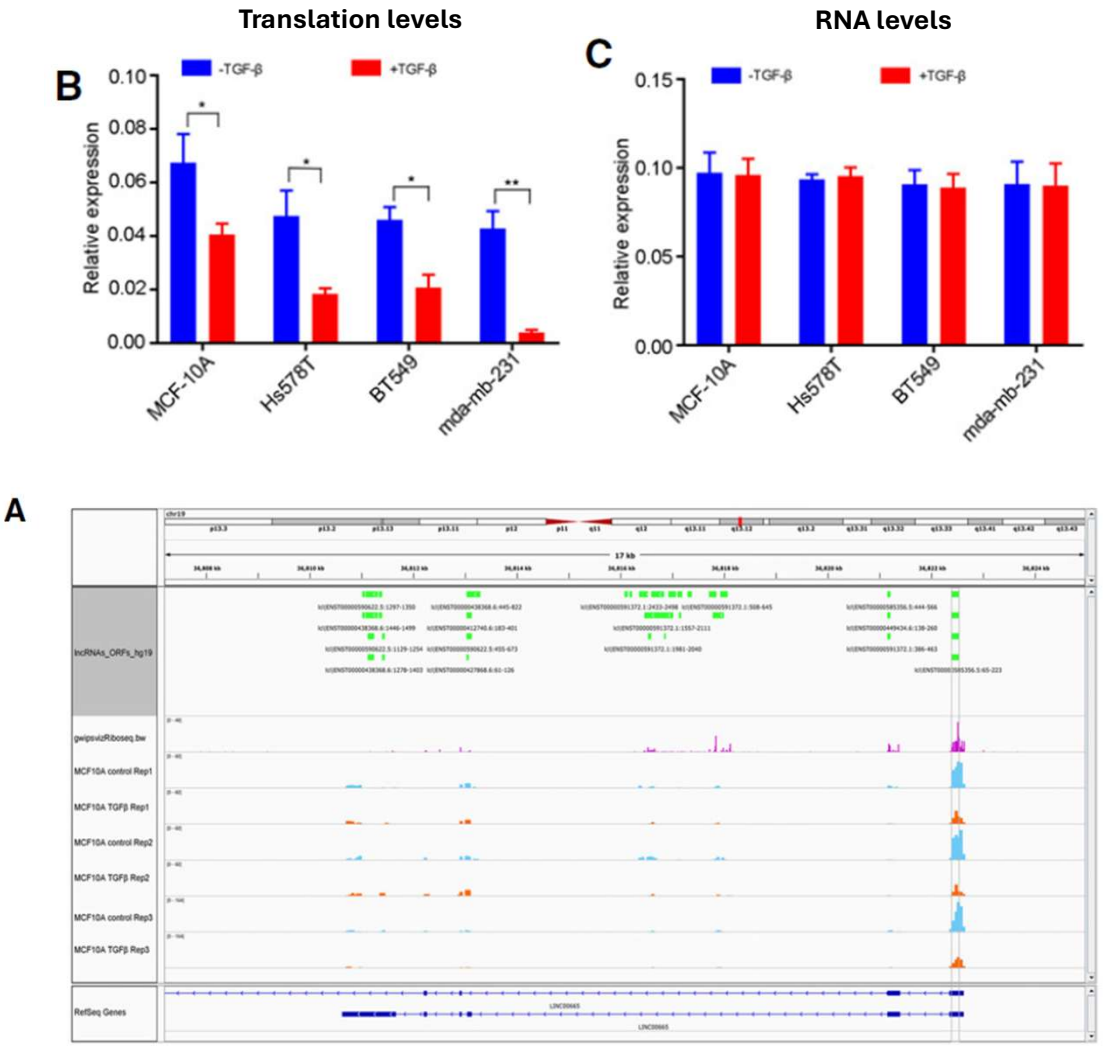


Figure 21: TGF-β represses LINC00665 translation but not transcription.
(A) Ribosome occupancy map of the LINC00665 locus;
(B) TGF- β treatment significantly decreases translation (polysome levels), while (C) total mRNA levels remain unchanged across cell lines [Guo, B. (2020)]

Coding potential analysis

- **In Silico Prediction:** 4 potential Open Reading Frames (ORFs).
- **Validation:** Vectors containing each ORF fused with a **His-tag**.
- **Result:** Only **ORF1** produced a detectable protein (~5.5 kDa).
- **Location:** Exon 1 (Chr 19).
- **Peptide:** 52 amino acids. Named **CIP2A-BP**.

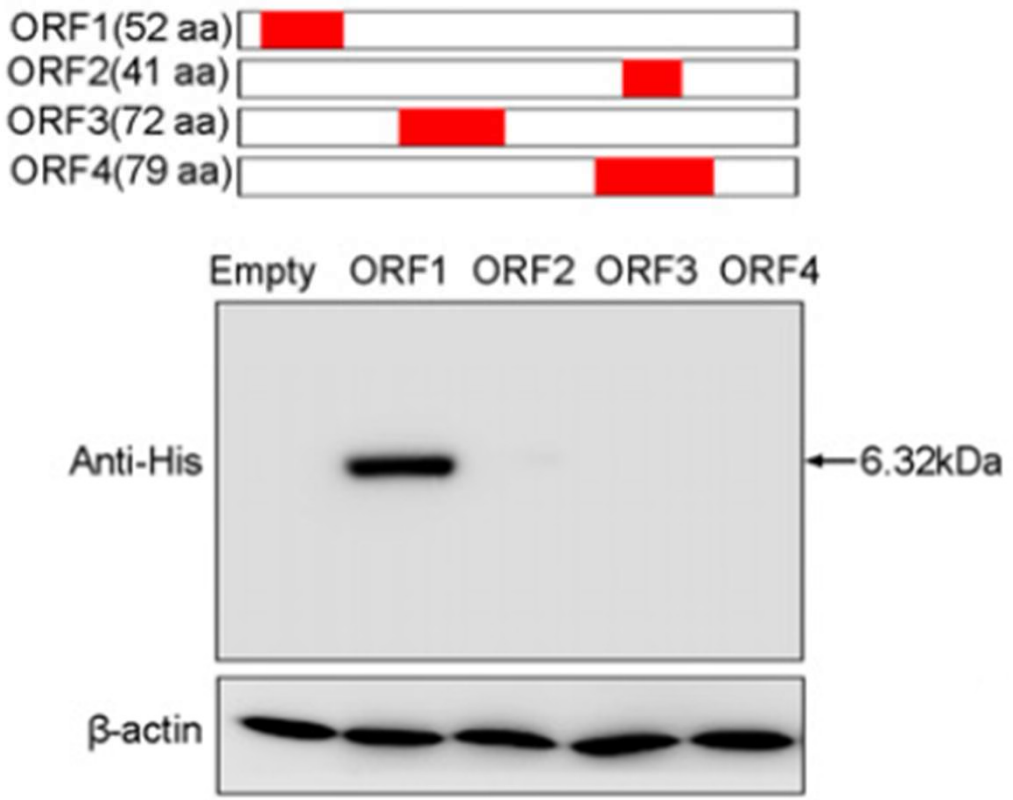


Figure 22: Western blot analysis of four putative open reading frames confirms that only ORF1 encodes a detectable micropeptide [Guo, B. (2020)]

Proving Translation: The GFP-Fusion Experiment

Strategy:

- Fusion of the ORF with GFP.

Constructs:

- *Wild-Type (WT):* Normal ATG start codon.
- *Mutant (Mut):* Start codon mutated to ATT.

Result :

- WT → Green Fluorescence.
- Mutant → No Fluorescence.

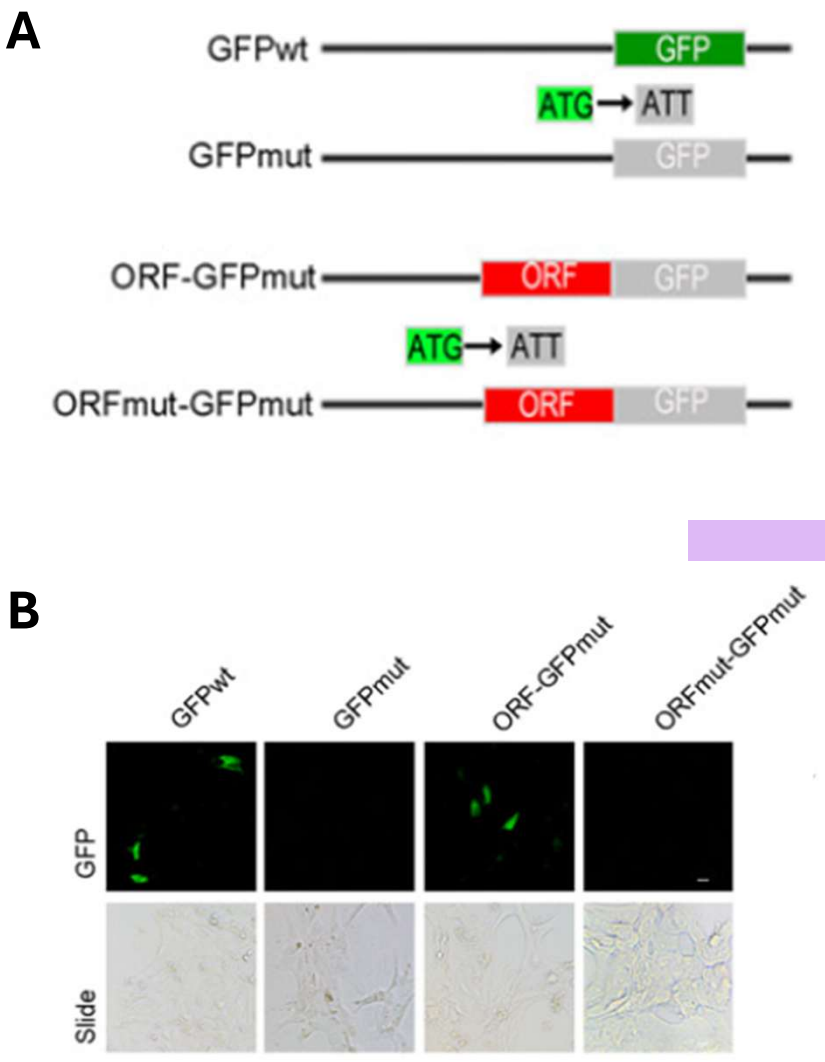


Figure 23: (A) Strategy: Mutated GFP requires the upstream ORF to initiate translation; (B) Fluorescence confirms the LINC00665 start codon is functional [Guo, B. (2020)]

Endogenous peptide detection

- **Tool:** Custom polyclonal antibody generated against CIP2A-BP;
- **Localization:** Mainly **Cytoplasmic**;
- **Regulation:**
  - Expressed in normal cells (MCF-10A);
  - **Lower** in TNBC cells (Hs578T, BT549, MDA-MB-231);
  - **Reduced** by TGF-β treatment in all cell lines.

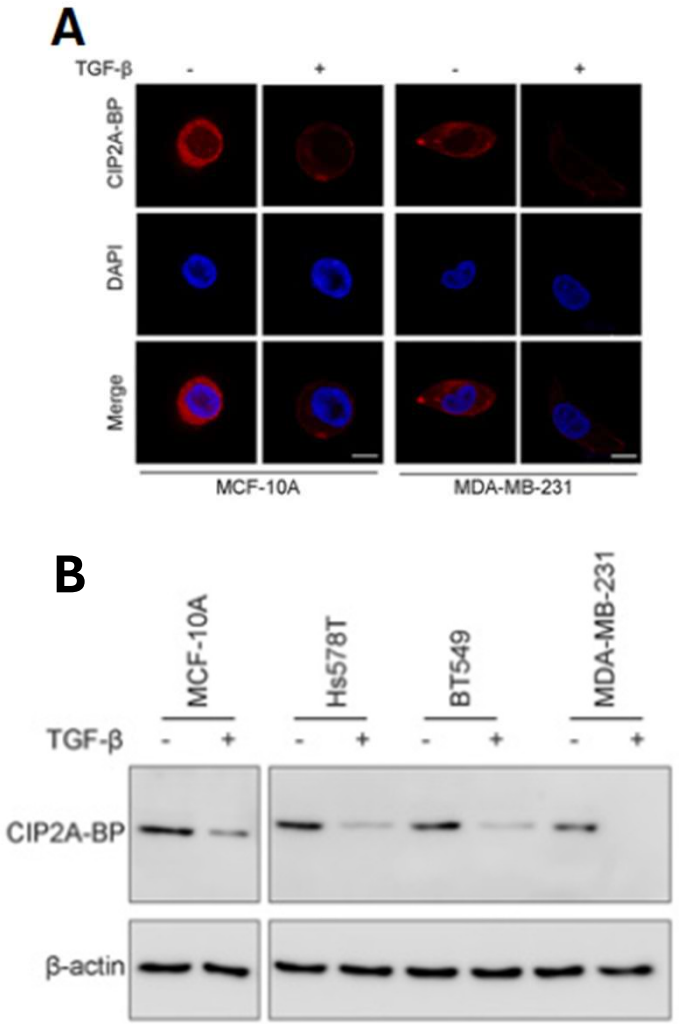


Figure 26: Detection of endogenous CIP2A-BP protein;
(A) Immunofluorescence staining of MCF-10A and MDA-MB-231 cells, and
(B) Western blot analysis of peptide expression in normal and TNBC cell lines treated with TGF-β [Guo, B. (2020)]

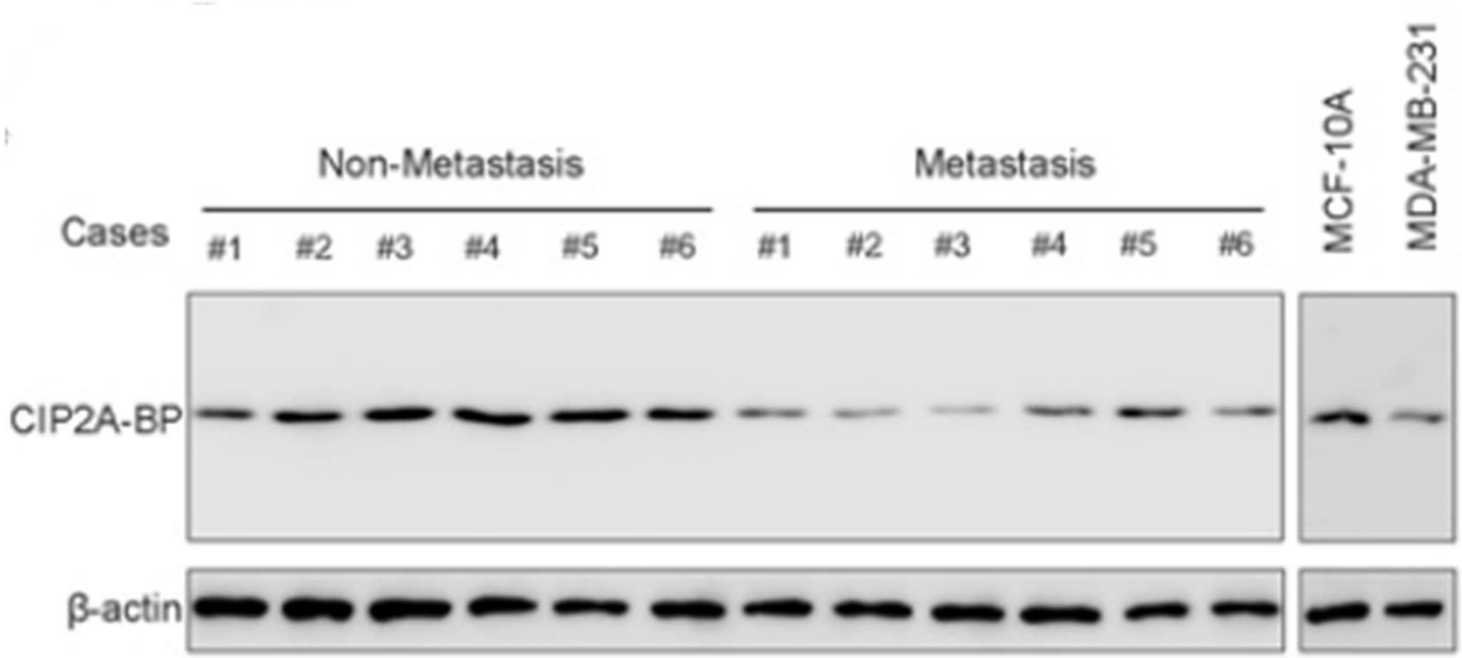


Figure 27: Western blot analysis comparing CIP2A-BP protein levels in non-metastatic versus metastatic TNBC patient tissues [Guo, B. (2020)]

Clinical correlation: Metastasis

- **Sample:** Tissue from TNBC patients.
- **Comparison:** Non-Metastatic vs. Metastatic tumors.
- **Finding:** CIP2A-BP protein levels are markedly **lower** in metastatic tissues.

Overall survival

- **Cohorts:** Two independent cohorts (Suzhou & Guangzhou).
- **Analysis:** Immunohistochemistry (IHC) on clinical samples
- **Result:** Low CIP2A-BP expression is significantly associated with **Poor Overall Survival**

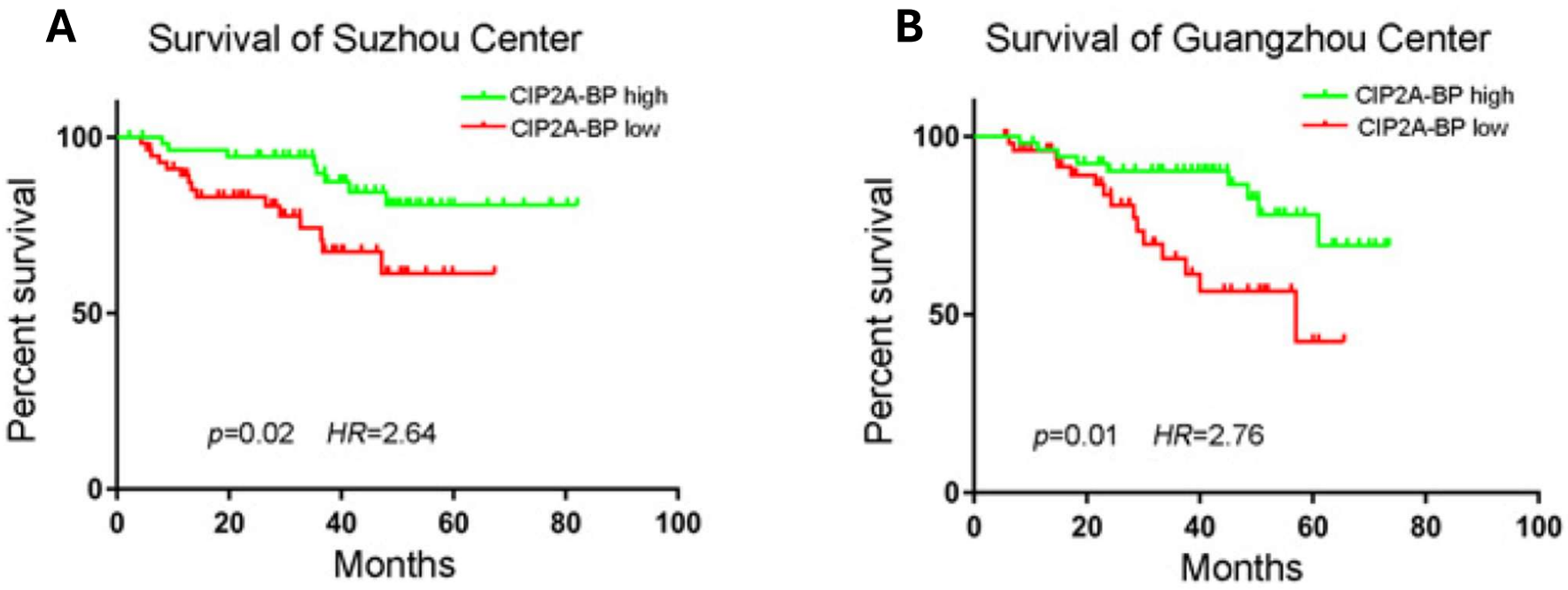


Figure 28: Kaplan-Meier overall survival curves for TNBC patients stratified by high vs. low micropeptide expression in the (A) Suzhou and (B) Guangzhou cohorts [Guo, B. (2020)]

RNA vs. Peptide

Observation: The *LINC00665* **RNA transcript levels** do NOT correlate with survival;

Conclusion: The prognostic marker is **CIP2A-BP**, not the lncRNA;

Significance: Highlights the importance of studying the "hidden proteome".

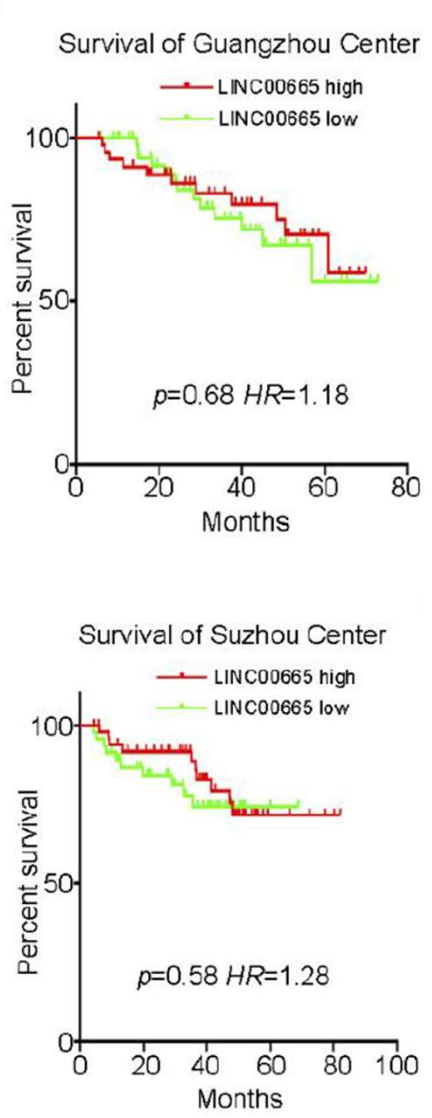


Figure 29: Kaplan-Meier overall survival curves for TNBC patients stratified by high vs. low LINC00665 RNA expressionin the (A) Guangzhou and (B) Suzhou cohorts show no significant difference [Guo, B. (2020)]

Investigating Function: The Setup

- **Gain of Function:** Overexpression of CIP2A-BP (ORF).
- **Loss of Function:** CRISPR/Cas9 Knockout (KO) of the peptide.
- **Rescue:** Re-expression of the peptide in KO cells.
- **Models:** Hs578T and MDA-MB-231 cell lines.

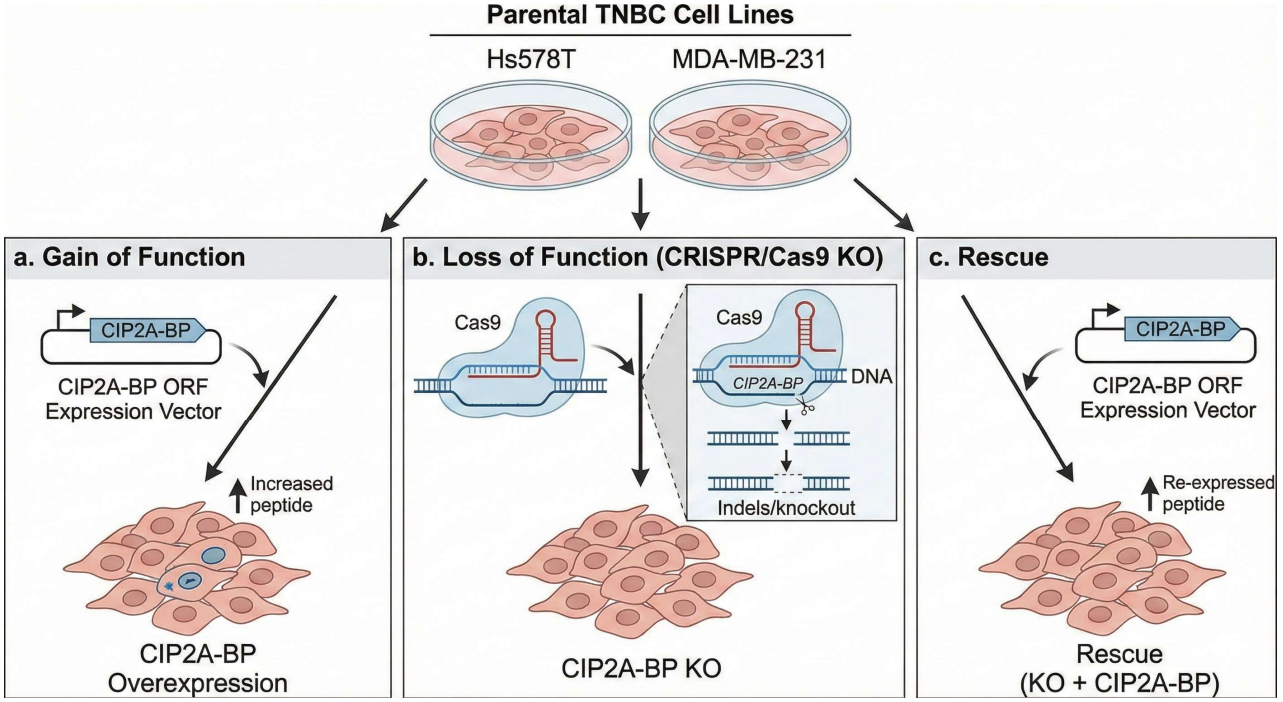


Figure 30: Experimental setup for genetic manipulation of CIP2A-BP in TNBC cell lines

In Vitro Results: Migration and Invasion

- **Overexpression:** Significantly **reduces** migration and invasion;
- **Knockout:** Significantly **increases** migration and invasion;
- **Rescue:** Restores the phenotype to baseline.

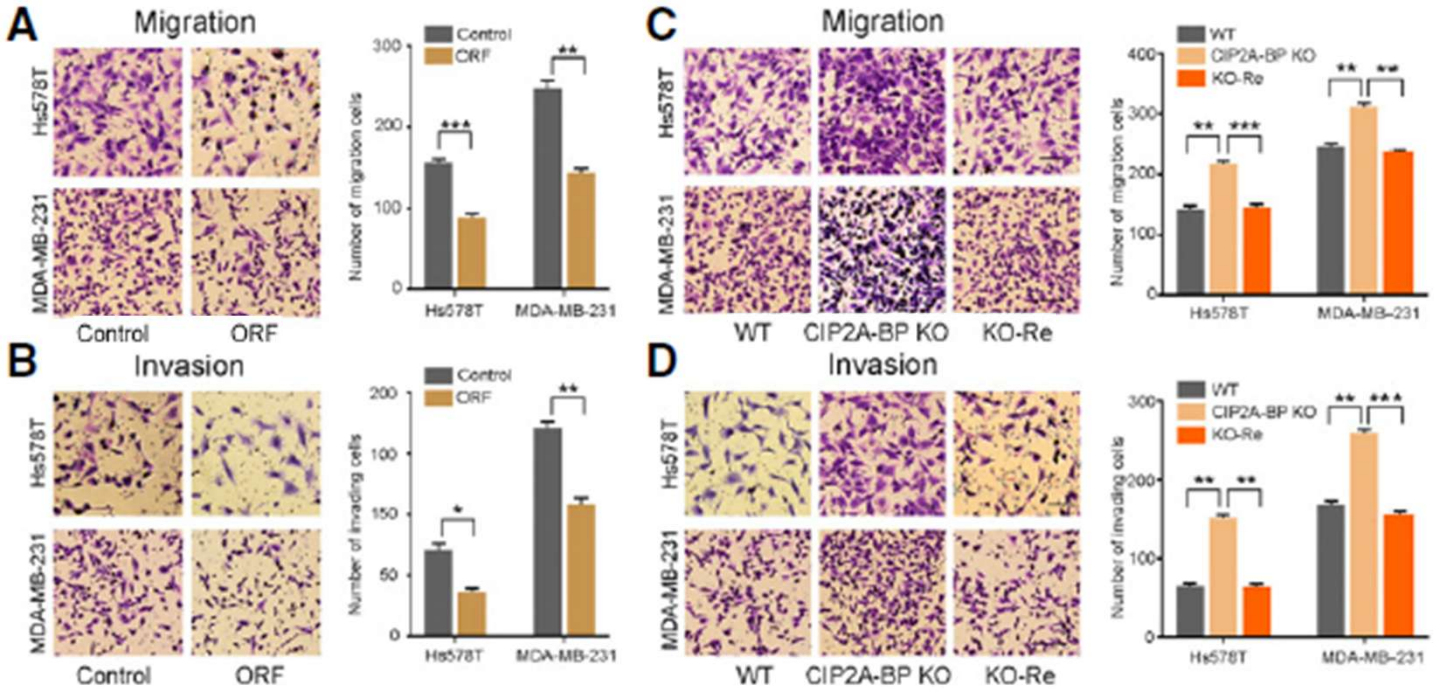


Figure 31: CIP2A-BP inhibits TNBC cell motility. Overexpression reduces (A, B), whereas knockout increases migration and invasion, a phenotype reversed by rescue experiments (C, D) [Guo, B. (2020)]

In Vivo validation: Lung metastasis

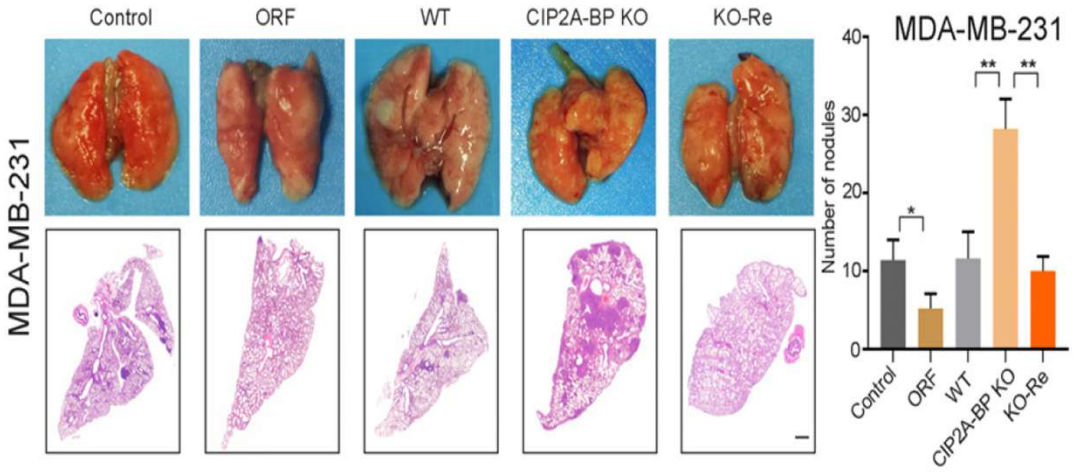


Figure 32: CIP2A-BP inhibits lung metastasis *in vivo*. (A, B) Overexpression reduces, while knockout increases metastatic nodule formation, a phenotype reversed by rescue ***[Guo, B. (2020)]***

- **Model:** Tail vein injection of TNBC cells into nude mice;
- **Groups:** Control, ORF (Overexpression), WT, KO (Knockout), Rescue;
- **Result:**
  - ORF → Fewer lung nodules;
  - KO → **More** lung nodules;
- **Conclusion:** CIP2A-BP suppresses metastasis *in vivo*.

Short Summary

- **Summary:**
  - *LINC00665* encodes a 52-aa peptide: **CIP2A-BP**;
  - TGF-β specifically blocks its translation;
  - CIP2A-BP acts as a **Tumor Suppressor**;
  - Loss of CIP2A-BP drives metastasis in TNBC;
- **Next:** *How* does TGF-β block it? And *what* is the molecular target?

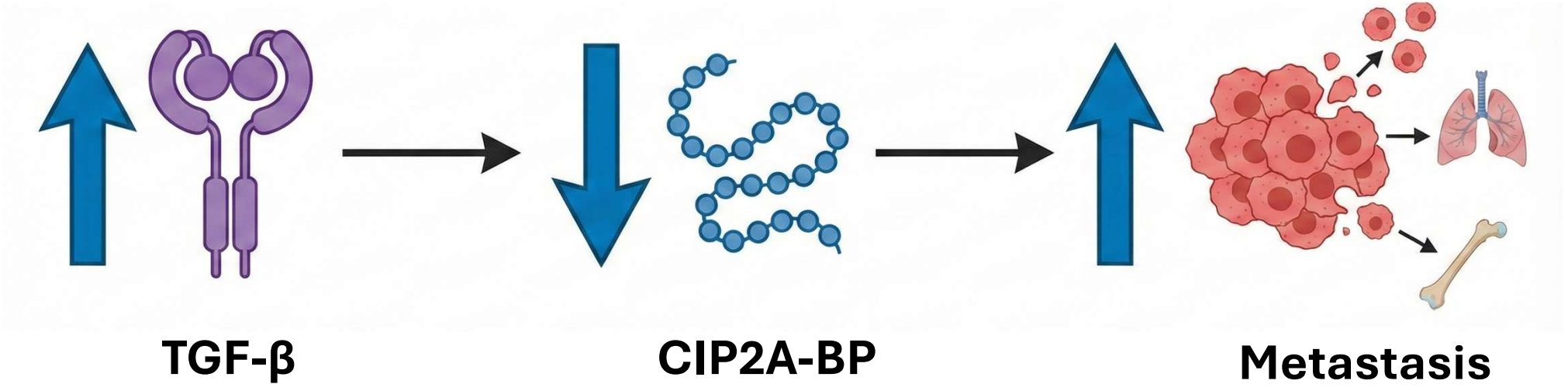


Figure 33: TGF-β blocks translation of CIP2A-BP, thereby driving TNBC metastasis

Outline

1. Theoretical framework: Unveiling the Hidden Microproteome;
2. The candidate: LINC00665 and its canonical oncogenic role;
3. Discovery of CIP2A-BP: From translational silencing to molecular decoy;
4. Translational impact: *In Vivo* validation and peptide therapy
5. Discussion and Conclusion

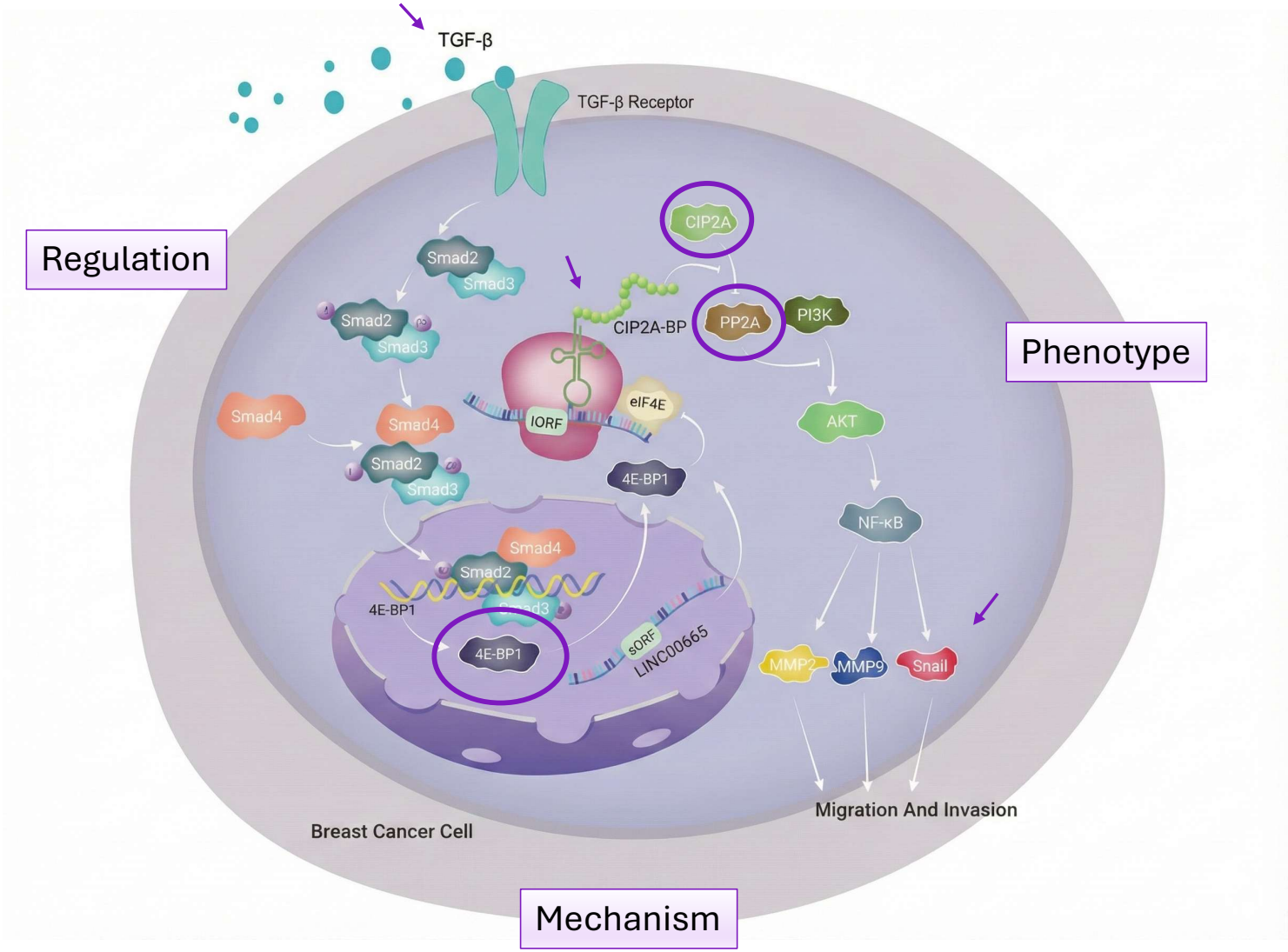


Figure 34. molecular Landscape of the LINC00665/CIP2A-BP Axis in Triple-Negative Breast Cancer (TNBC)

Does TGF-β treatment reduce CIP2A-BP translation through 4E-BP1?

TGF-β treatment reduced the levels of CIP2A-BP and activated epithelial mesenchymal transition (EMT) and Smad signaling pathway in TNBC cell lines.

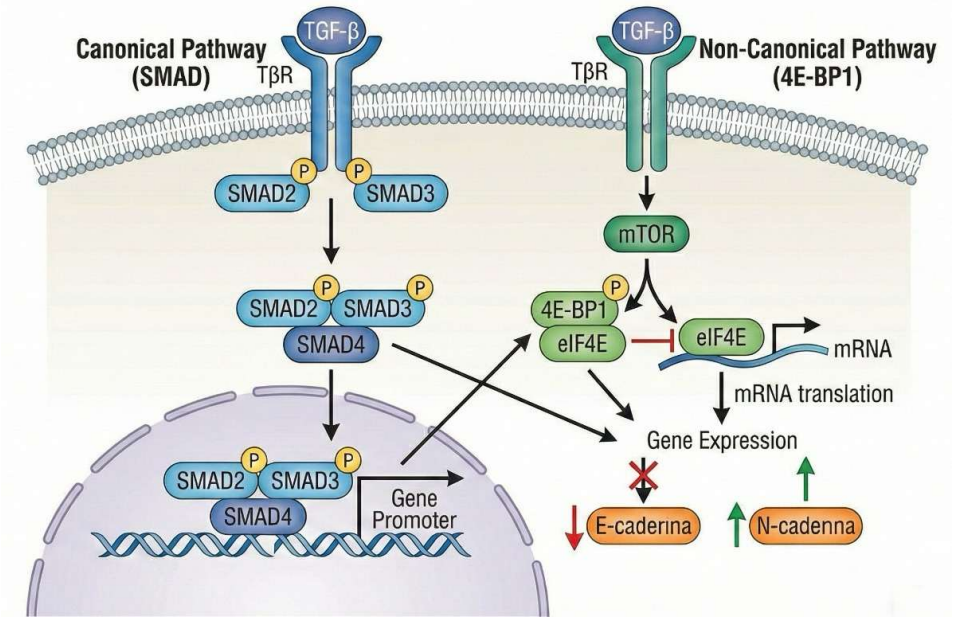


Figure 35. Schematic representation of TGF-β signaling mechanism in TNBC cells

Does TGF-β treatment reduce CIP2A-BP translation through 4E-BP1?

Immunoblotting analysis

- Downregulation of E-cadherin
- Upregulation of N-cadherin
- Increased level of p-Smad2 and p-Smad3

- **Upregulation of 4E-BP1**

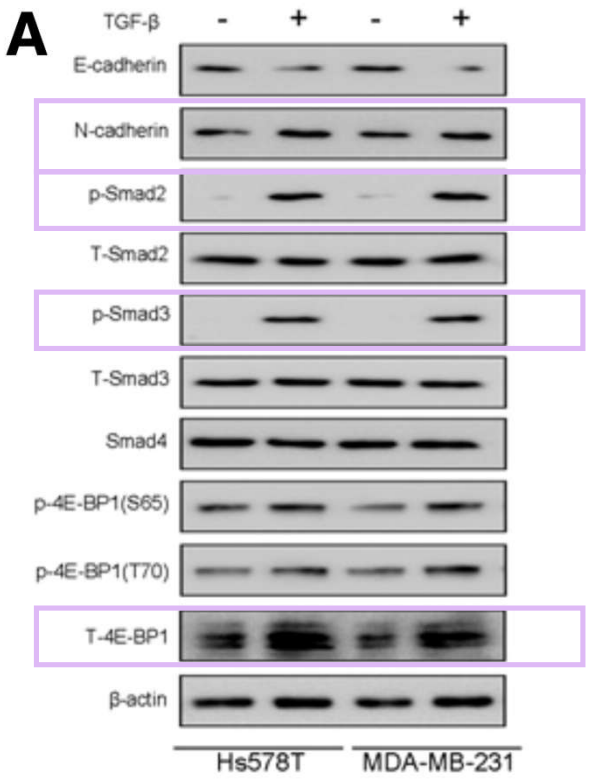
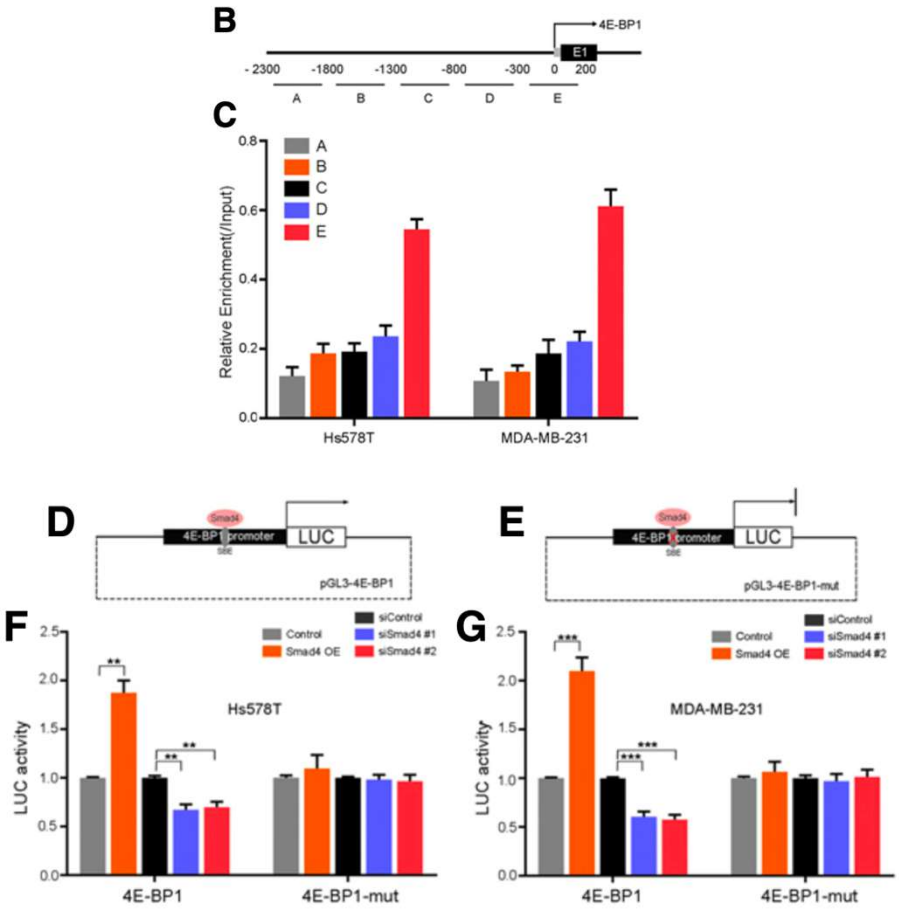


Figure 36. Effects of TGF-β on the micropeptides CIP2A-BP through 4E-BP1
(A) Immunoblotting analysis of expression of the indicated proteins in TNBC cells cultured with or without TGF-β



- ChIP
- Luciferase reporter assay

✓Smad4 bind to the 4E-BP1 promoter adjacent to exon 1 which contained a potential Smad4 binding element

✓Significantly upregulated luciferase activity in Smad4 overexpressed cells rather then down-regulated cells by Smad4 KD

Smad4 is the transcription factor for 4E-BP1

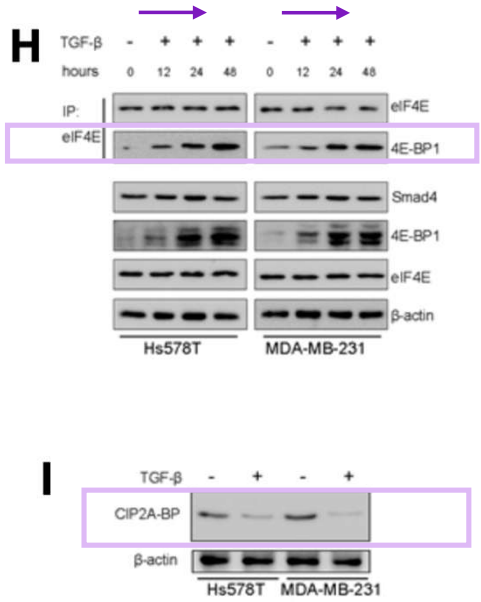
(B) & (C) Chromatin Immunoprecipitation assay
(D) Luciferase reported assay wt 4E-BP1 promoter
(E) Luciferase reported assay mutant 4E-BP1 promoter
(F) & (G) Luciferase reported assay using mutant and wt 4E-BP1 promoter construct in TNBC cells with Smad4 OE or KD

Does TGF-β treatment in TNBC cell lines increase the binding of 4E-BP1 to eIF4E?

Co-IP

- ✓ The amount of eIF4E-associated 4E-BP1 increased in a time-dependent manner after TGF-β treatment of TNBC cell lines, correlated with decreased level of CIP2A-BP.
- ✓ TGF-β/Smad4 signaling pathway significantly reduce the expression of CIP2A-BP

Activation of TGF-β/Smad pathway leads to increased expression of 4E-BP1, which reduced expression of CIP2A-BP through directly binding to eIF4E



(H) Regulation of 4E-BP1 expression and binding to eIF4E by TGF-β
(I) Immunoblotting analysis of expression of micropeptide CIP2A-BP

Does 4E-BP1 phosphorylation level influence LINC00665 translation?

mTOR/4E-BP1 signaling pathway regulates protein translation through hypophosphorylated 4E-BP1 but:

- Under high level of TGF-β, LINC00665 translation is insensitive to regulation by mTOR signaling pathway

Downregulation of CIP2A-BP in TNBC was a direct consequence of activation of TGF-β/Smad signaling pathway.

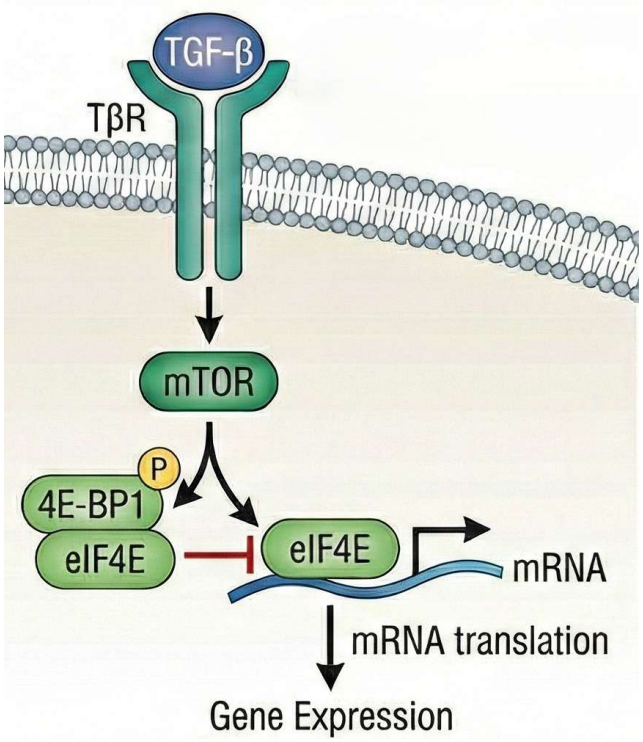


Figure 39. Schematic representation of mTOR signaling mechanism in TNBC cells

Identification of CIP2A-BP interacting proteins:

- Co-IP
- Mass Spectrometry (MS)
- Western Blot analysis

Only CIP2A binds to CIP2A-BP with an RNA indipendent interaction

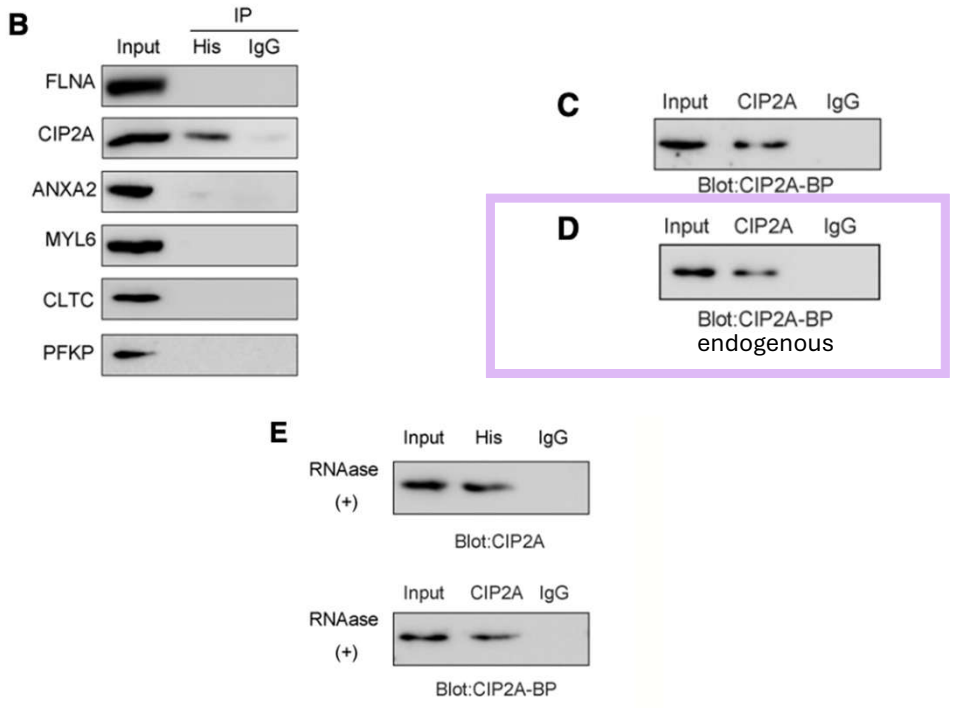


Figure 40. Identification of CIP2A-BP interacting proteins
(B) Co-IP and immunoblotting analysis using anti-His Ab and CIP2A-BP Ab on TNBC cell line transfected with LINC00665 ORF-His plasmid
(D) Immunoblotting of endogenous CIP2A-BP and endogenous CIP2A
E) TNBC cells transfected with LINC00665 ORF-His plasmid were subjected to co-immunoprecipitation, and after RNase A treatment, the interaction with CIP2A-BP was detected.

Cancerous inhibitor of PP2A cancer inhibitory factor - CIP2A

Oncogene that promotes tumor progression through inhibiting PP2A

Sustains proliferative and survival pathways:

↑ AKT signaling

↑ MYC activity

↑ E2F1 activity

Promotes metastasis through:

↑ MMP2 and MMP9 (ECM degradation)

↑ Snail (EMT and cell migration)

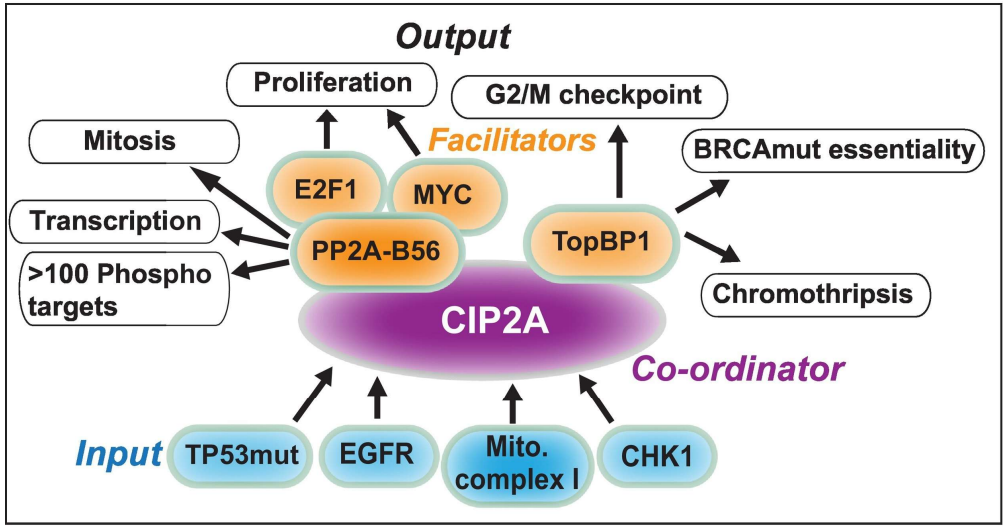


Figure 41. CIP2A's interactions and effects on the different pathways

IDENTIFICATION OF THE BINDING SITE FOR CIP2A-BP:

- MYC-tagged CIP2A truncated protein fragments
- Co-IP
- WB analysis

CIP2A-BP binding site at the N-terminus domain of CIP2A (amino acid 159–245)

PP2A's subunits B56γ and B56α also bind to the N-terminus of CIP2A to maintain the stability of the dimer

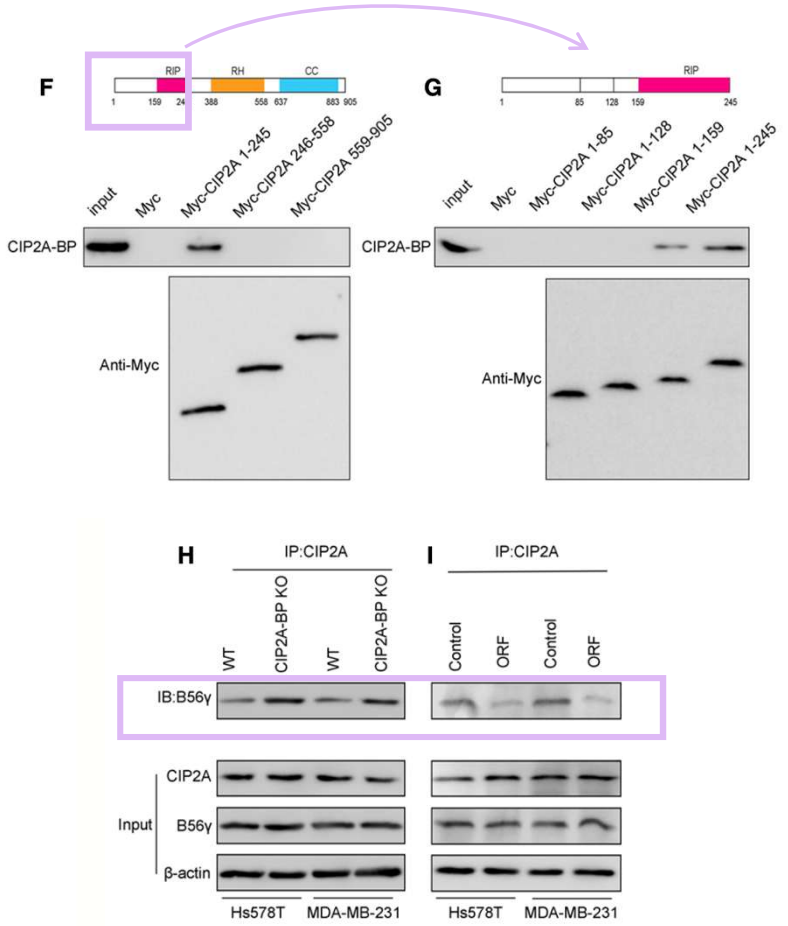


Figure 42. Identification of CIP2A-BP interacting proteins
(F) &(G) co-immunoprecipitation-coupled WB analysis assay in full lenght CIP2A-BP and in a fragment
(H) & (I) co-immunoprecipitation assay to show the interaction between CIP2A and B56γ in TNBC

Does this two subunits compete for the same CIP2A binding site?

The interaction between CIP2A and B56γ in TNBC cells was affected by CIP2A-BP expression

CIP2A-BP competes for B56γ's binding site on CIP2A, on the contrary it has no effect on B56α's binding to CIP2A

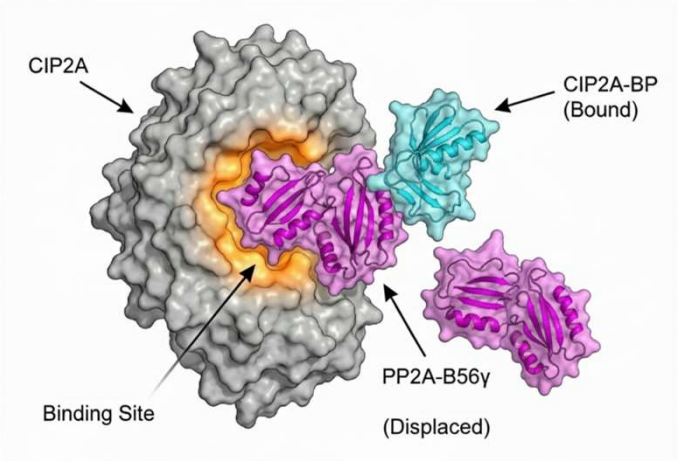
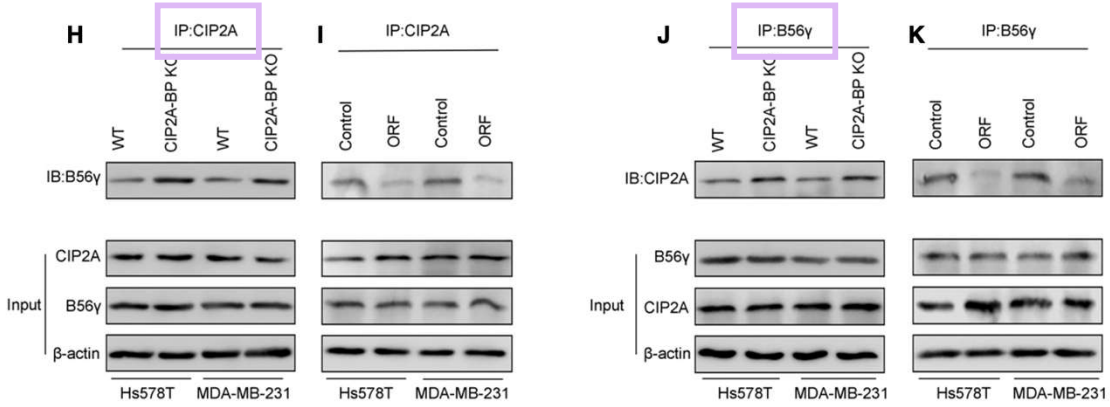


Figure 43. Modello strutturale della competizione biochimica tra CIP2A-BP e PP2A-B56γ per il legame con CIP2A



(H) & (I) co-immunoprecipitation assay to show the interaction between CIP2A and B56γ in TNBC
(J) & (K) co-immunoprecipitation assay to show the interaction between CIP2A and B56γ in TNBC

What's the role of CIP2A-BP in the PI3K/AKT/NF-kB pathway?

- CIP2A-BP OE cell lines ⟶ reduced phosphorylation in PI3K/AKT/NF-kB pathway and further affected the expression of MMP2, MMP9, and Snail*
- CIP2A-BP KD cell lines ⟶ increased AKT Thr308, AKT Ser473 and IkBα Ser32 phosphorylation ⟶ **PI3K/AKT/NF-kB pathway activated**

- **CIP2A-BP overexpression ⟶ phosphorylation downregulated**
- CIP2A-BP KD cell lines ⟶ not effected

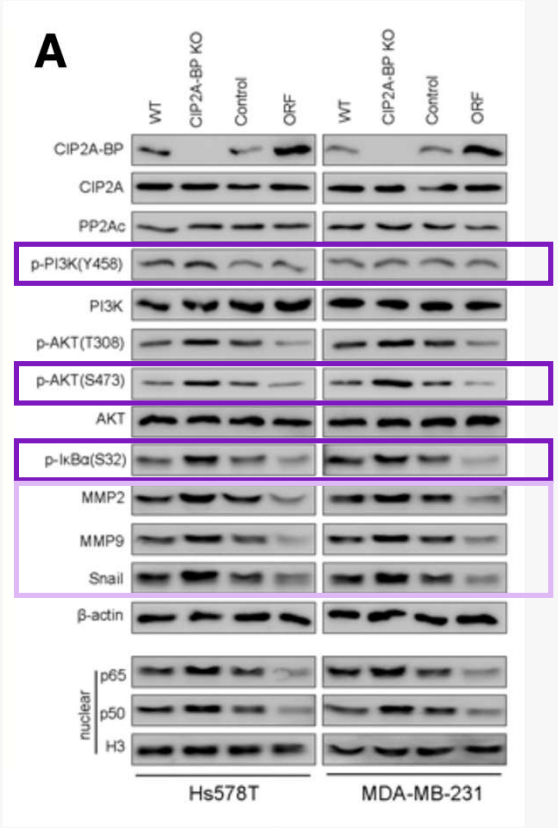


Figure 44. investigating the role of CIP2A-BP in the PI3K/AKT/NF-kB pathway

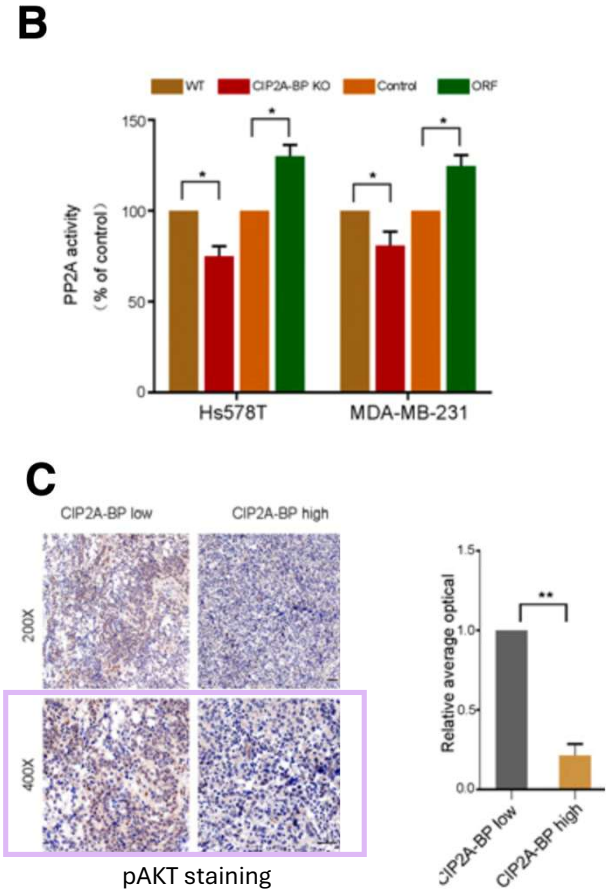
(A) Immunoblotting analysis in CIP2A-BP detecting the expression levels of the indicated proteins in CIP2A-BP KO, LINC00665 ORF overexpressed (OE) and respective controls of TNBC cells

* that transcription of MMP2, MMP9, and Snail was increased, but E-cadherin was decreased

Determination of PP2A activity:

- CIP2A-BP knockdown significantly decreased PP2A activity, while CIP2A-BP overexpression increased it

- Immunohistochemistry analysis on clinical samples: high CIP2A-BP expression was significantly associated with downregulation of p-AKT.



(B) evaluation of PP2A activity in CIP2A-BP KO
(C) Immunohistochemistry (IHC) staining of p-AKT from TNBC primary tumor

CIP2A-BP can effectively inhibit activation of PI3K/AKT/NFkB pathway, and expression of downstream targets, including MMP2, MMP9, and Snail.

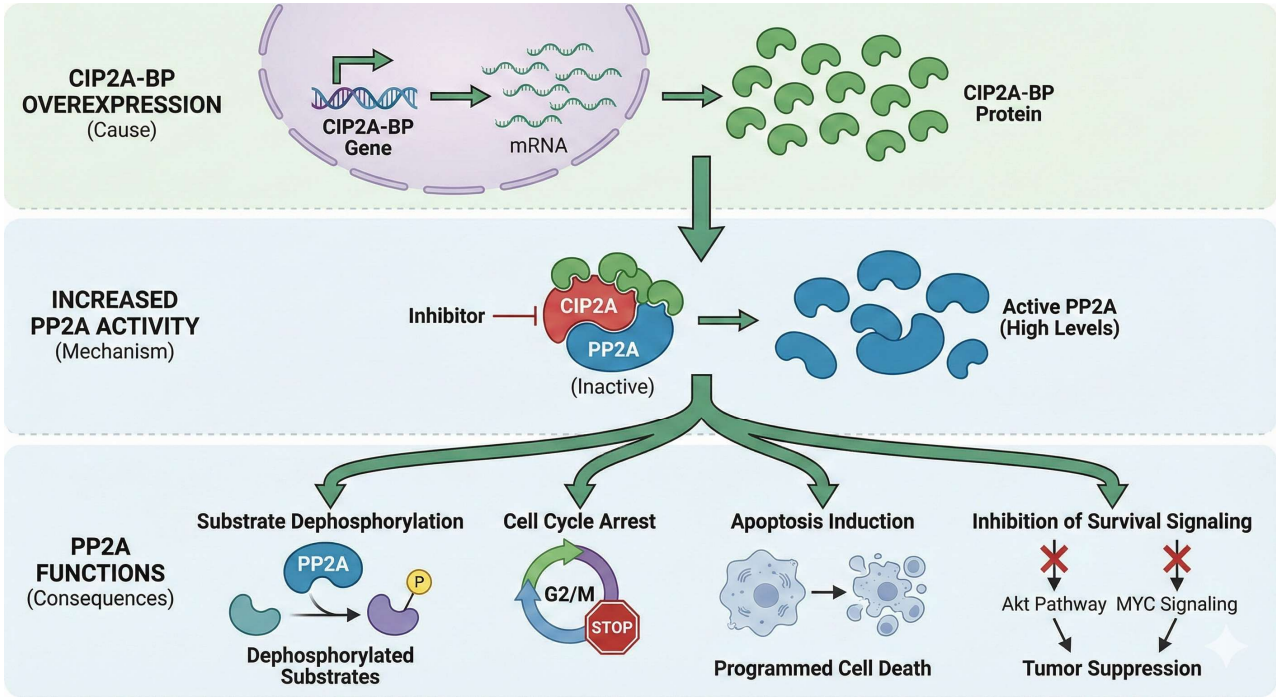


Figure 45. Molecular Mechanism of Tumor Suppression via CIP2A-BP Overexpression

Investigate the effect of exogenous CIP2A-BP treatment on lung metastasis

- Mammary pad injection of CIP2A-BP significantly reduced the number of lung metastasis loci
- Exogenous CIP2A-BP treatment significantly reduced p-AKT level of primary tumor.

CIP2A-BP inhibited AKT phosphorylation in primary tumor and lung metastasis in MMTV-PyMT mouse model.

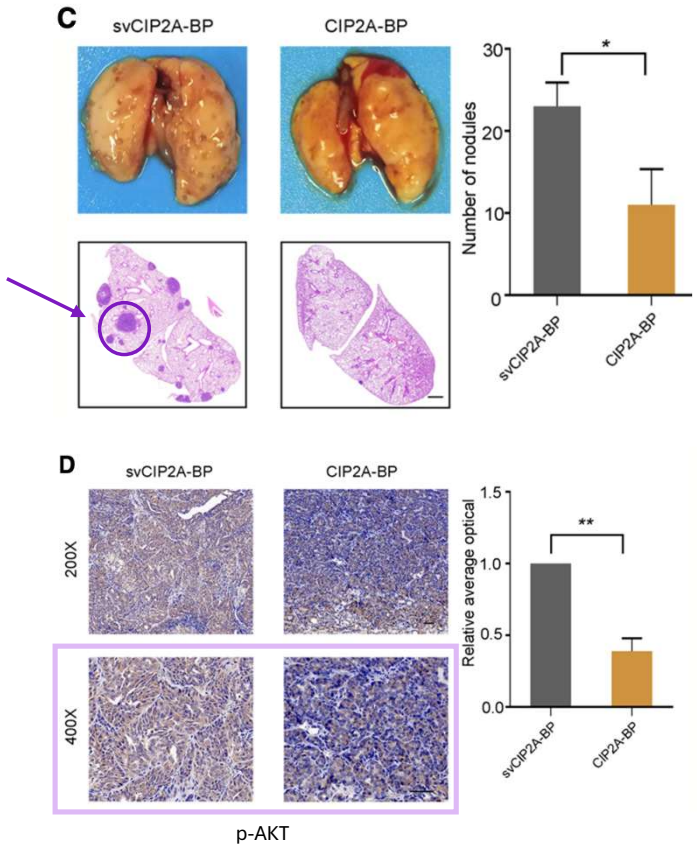


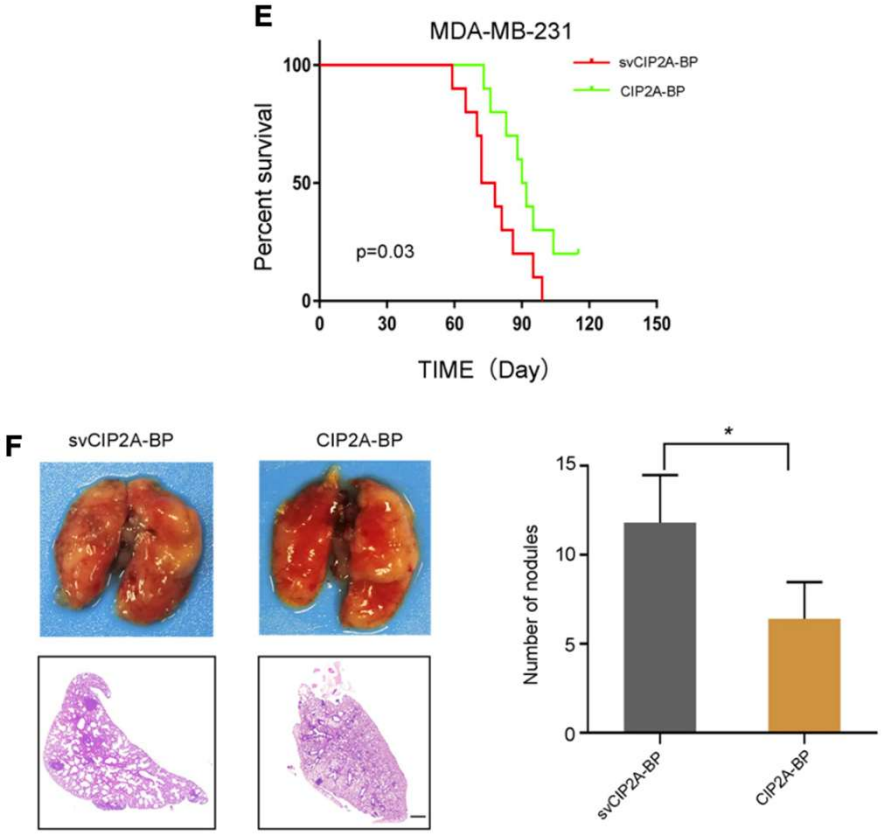
Figure 46. Investigating the effect of CIP2A-BP on metastasis and invasion in TNBC
(C) Macroscopic and histological analysis of lung metastatic nodules visualized 8 weeks post-transplantation. Right panel is the quantification of pulmonary metastases.
(D) IHC images of p-AKT of mammary tumors. Right panel is the quantification of IHC staining p-AKT.

Has the micropeptide CIP2A-BP an antitumor activity?

- Significantly improved survival compared to control mice, and tissue HE staining* indicated that CIP2A-BP also significantly reduced the number of lung metastases

CIP2A-BP can effectively suppress breast cancer metastasis and invasion, therefore improve overall survival.

* Tissue HE staining stands for Hematoxylin and Eosin staining



(E) Kaplan–Meier survival curves of nude mice transplanted with MDA-MB-231 cells and injected with CIP2A-BP or svCIP2A-BP.
(F) Macroscopic and histological analysis of the lungs of nude mice injected with MDA-MB-231 cells and CIP2A-BP or svCIP2A-BP via tail vein; lung metastatic nodules were visualized 8 weeks post transplantation. Right panel is the quantification of pulmonary metastases.

Outline

1. Theoretical framework: Unveiling the Hidden Microproteome;
2. The candidate: LINC00665 and its canonical oncogenic role;
3. Discovery of CIP2A-BP: From translational silencing to molecular decoy;
4. Translational impact: *In Vivo* validation and peptide therapy
5. Discussion and Conclusion

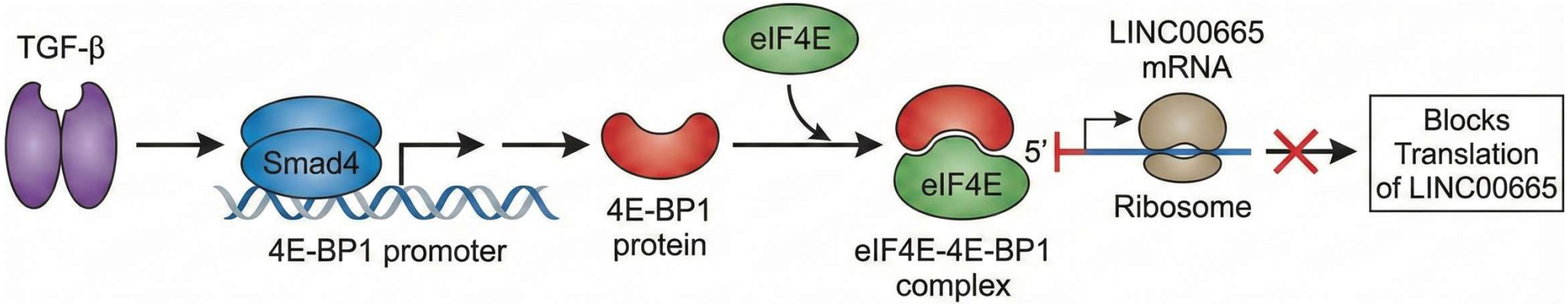


Figure: TGF-β/Smad4 upregulates 4E-BP1, which binds eIF4E to block LINC00665 translation

Discussion: A new layer of regulation by TGF-β

- **Canonical View:** TGF-β is known to induce EMT and metastasis via transcriptional regulation (Smad complex).
- **Novel Finding:** TGF-β regulates metastasis at the **translational level**.
- **The Mechanism:**
  - TGF-β activates Smad4, which acts as a transcription factor for **4E-BP1**.
  - Accumulated 4E-BP1 binds eIF4E, specifically preventing the ribosome from translating the CIP2A-BP micropeptide.

Discussion: The decoy-effect

- **The Players:**
  - **CIP2A (Oncogene):** Inhibits the tumor suppressor PP2A.
  - **PP2A (Tumor Suppressor):** A phosphatase that keeps cell growth in check.
- **The Interaction:**
  - CIP2A-BP binds to the N-terminus of CIP2A (aa 159–245)
  - It acts as a **molecular decoy**, competing with the PP2A regulatory subunit (B56γ)
- **The Result:** CIP2A is sequestered → PP2A is released and reactivated.

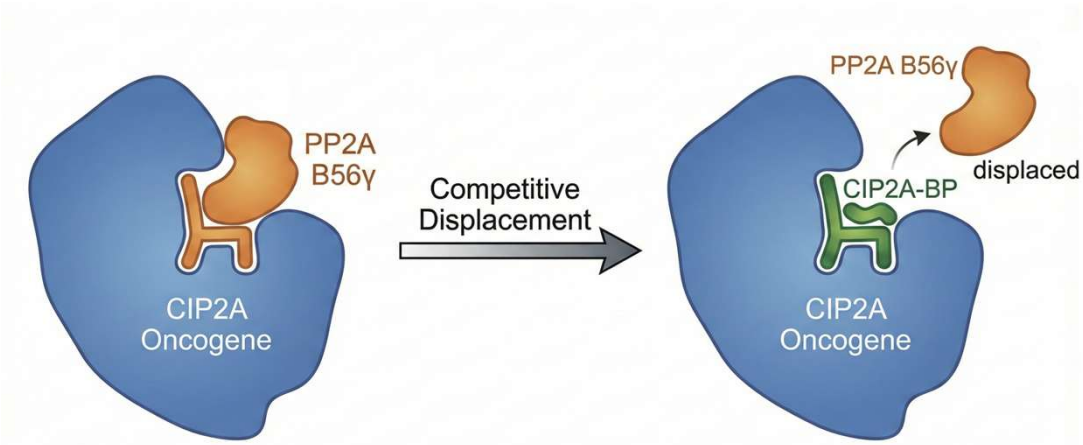


Figure: CIP2A-BP acts as a decoy, displacing B56γ from CIP2A to release and reactivate PP2A

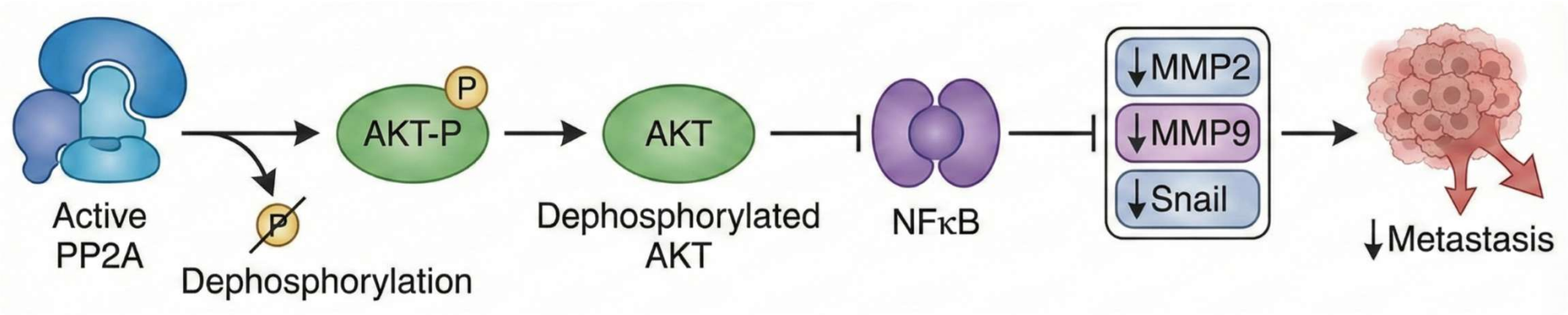


Figure: Reactivated PP2A dephosphorylates AKT, inhibiting NF-κB and metastasis drivers (MMP2/9, Snail)

Discussion: Downstream signaling & phenotype

- **Restoring the Brake:** Reactivated PP2A dephosphorylates **AKT** (at Thr308/Ser473).
- **Stopping the Signal:** Inactive AKT leads to the inhibition of the **NF*k*B pathway**.
- **Phenotypic Outcome:**
  - Downregulation of metastasis drivers: **MMP-2, MMP-9, and Snail**.
  - Result: Suppression of cell migration, invasion, and lung metastasis.

Discussion: The hidden proteome

- **A Growing Class:** LINC00665 joins a growing list of lncRNAs that encode functional peptides (e.g., SPAR, HOXB-AS3) .
- **Paradigm Shift:** Challenges the definition of "non-coding" RNA.
- **Implication:** Many "oncogenic" or "tumor-suppressive" lncRNAs may actually function through overlooked micropeptides.

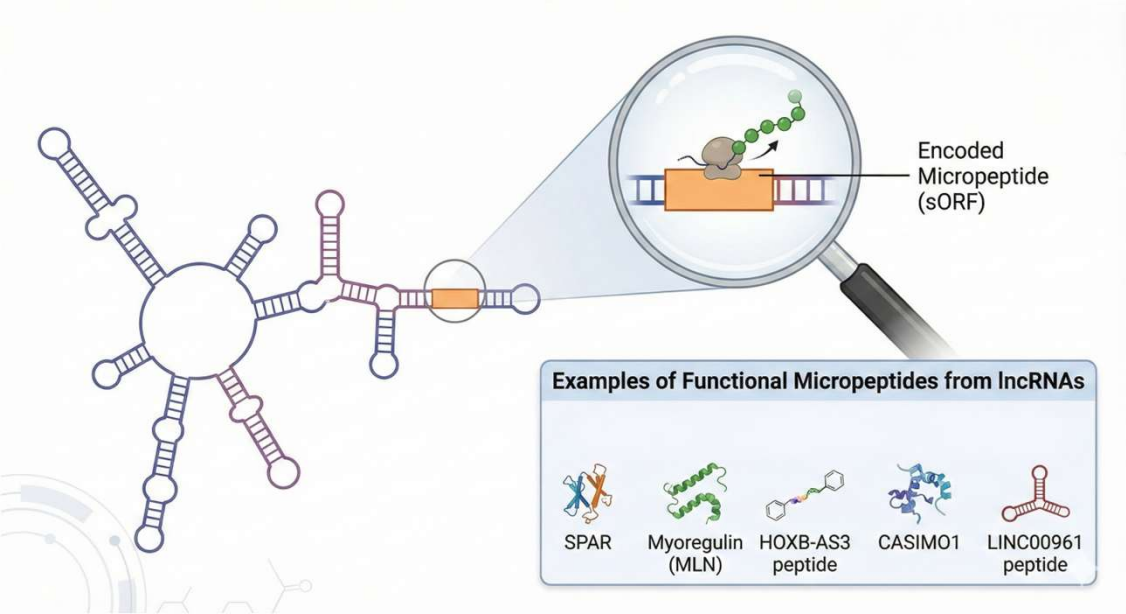
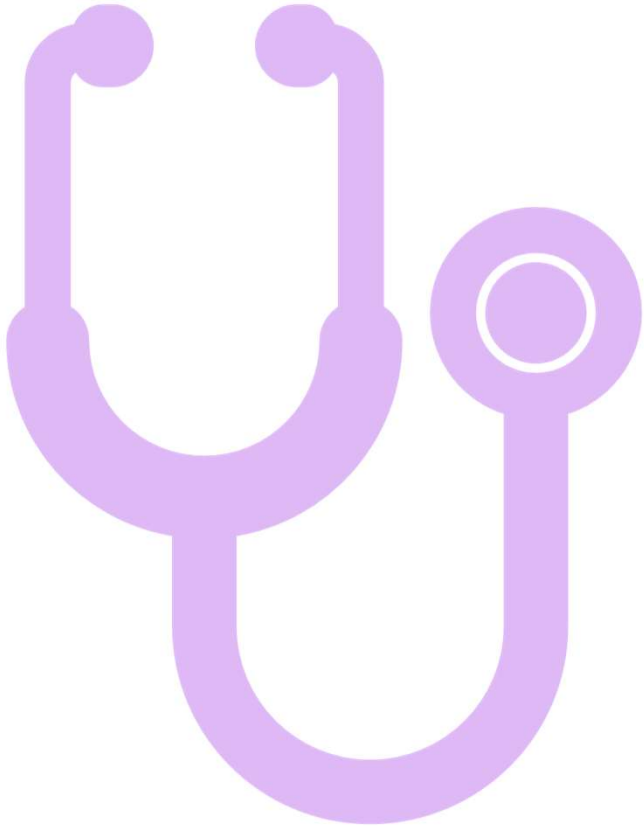


Figure: Different functional micropeptides encoded by lncRNAs

Future perspectives

- Add an **anti-tumor peptide** to the existing literature
- CIP2A-BP expression could be used as a **prognosis marker among TNBC**
- CIP2A-BP represents a **potential therapeutic candidate** to treat TNBC metastasis through augmenting PP2A activity



Conclusion

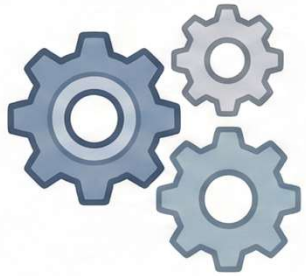
Mechanism: TGF-β drives TNBC metastasis by translationally suppressing the **CIP2A-BP** micropeptide via 4E-BP1.

- **Function:** CIP2A-BP acts as a tumor suppressor by inhibiting the CIP2A oncogene and reactivating **PP2A**.

Clinical Value:

- Low peptide levels predict **poor survival** and metastasis.
- Direct peptide administration inhibits metastasis *in vivo*, offering a **novel therapeutic strategy**.

Mechanism



Prognosis

Therapy

Thanks for your attention!

Supplementary material

Supplementary material

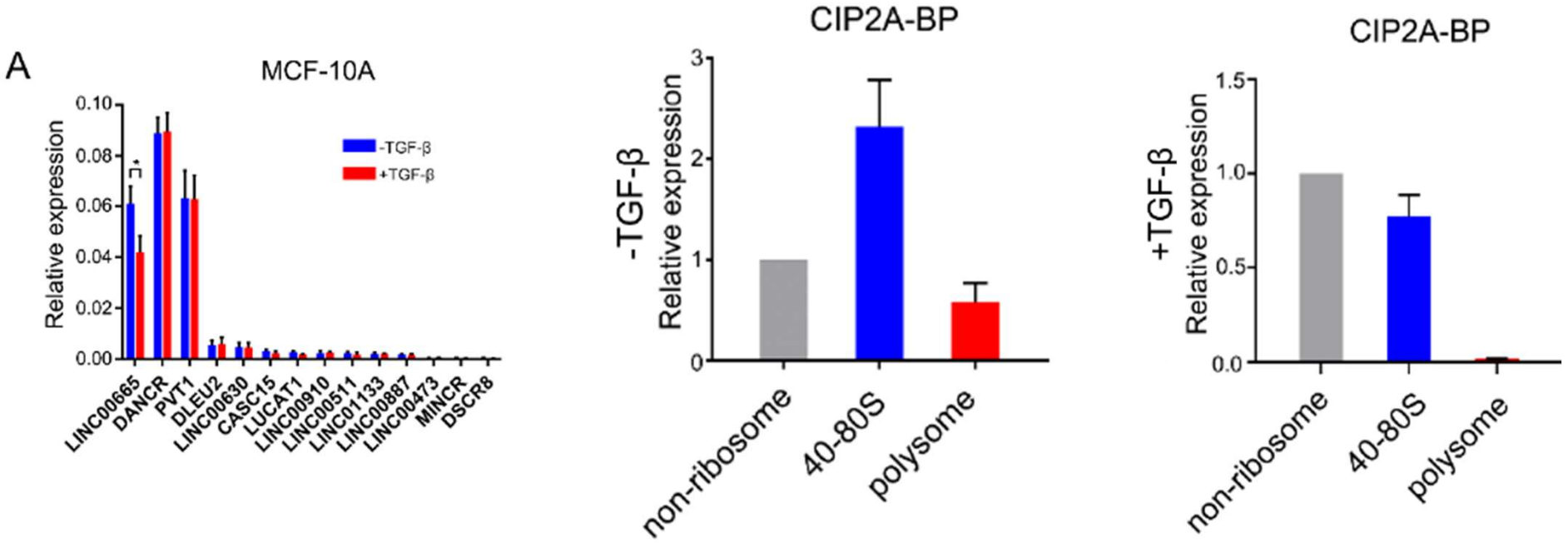


Figure S1: Polysome profiling identifies LINC00665 translational repression by TGF-β despite stable RNA levels [Guo, B. (2020)]

Selection rationale: Why LINC00665?

- **Screening approach:** Integrated analysis of Ribo-seq and RNA-seq in TGF-β trated cells;
- **Candidate identification:** Out of 14 candidates, only LINC00665 showed translational repression with stable transcript levels;
- **Validation:** Polysome profiling confirms that TGF-β shifts LINC00665 from active polysomes to non-translating fractions.

Technical control: Antibody specificity

- **Custom tool: polyclonal antibody generated against CIP2A-BP sequence;**
- **Validation assay:** LINC00665 knockdown (shRNA) performed in MCF-10A and MDA-MB-231 cell lines;
- **Results:** The specific protein band disappears in shRNA-treated cells, confirming the detection of endogenous CIP2A-BP.

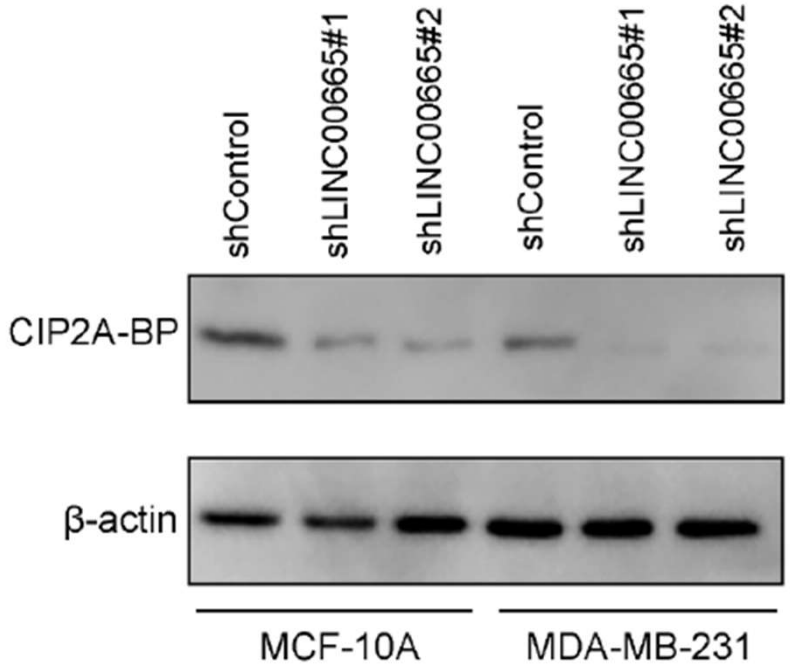


Figure S2: Western blot confirms antibody specificity: the CIP2A-BP band vanishes upon LINC00665 knockdown [Guo, B. (2020)]

Validating the peptide size

- **Confirmation:** Western Blot with anti-GFP and anti-His antibodies;
- **Evidence:** The mutant construct produces **no protein band;**
- **Conclusion:** The signal is not an artifact; it relies on the specific ORF sequence.

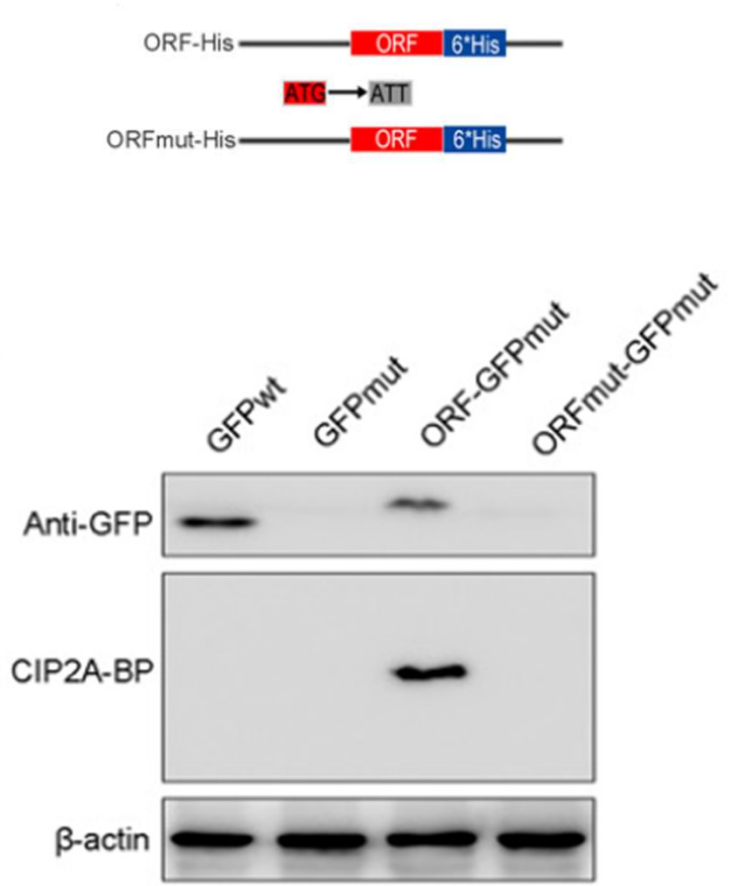


Figure S3: Start codon validation. (A) Mutation strategy (ATG → ATT); (B) Mutation abolishes peptide translation [Guo, B. (2020)]

Polysome Profiling

- **Question:** Is the *endogenous* RNA actually translated?
- **Method:** Polysome Profiling (separating RNA by ribosome density);
- **Result:** *LINC00665* RNA is enriched in the **Polysome fraction** (actively translating);
- **Control:** Puromycin treatment (disassembles ribosomes) shifts the RNA to the non-ribosome fraction.

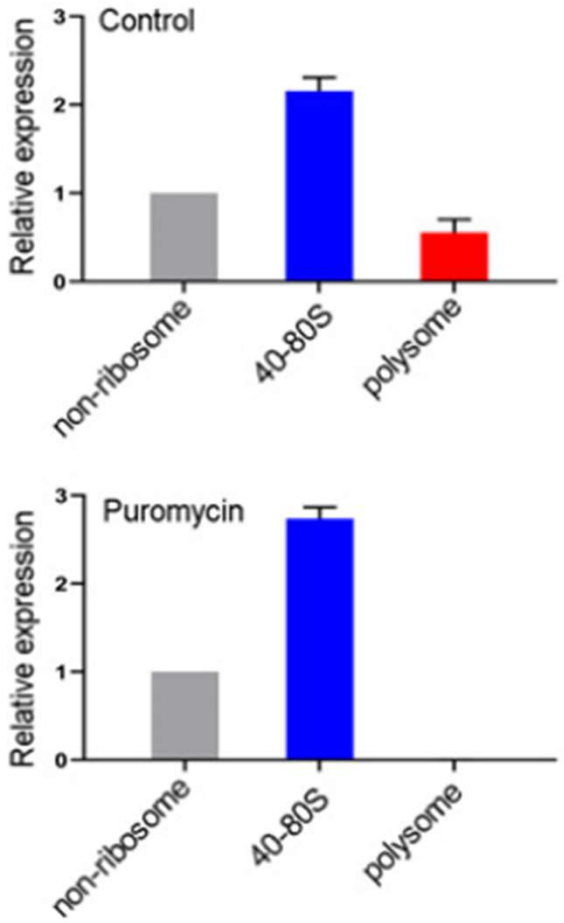


Figure S4: Polysome profiling. CIP2A-BP transcripts detected in the polysome fraction are eliminated by puromycin treatment [Guo, B. (2020)]

In Vitro Results: Wound Healing

- **Assay:** Wound Healing (Scratch Assay).
- **Result:**
  - ➢ CIP2A-BP slows down wound closure (Tumor Suppressor).
  - ➢ Loss of CIP2A-BP accelerates wound closure.

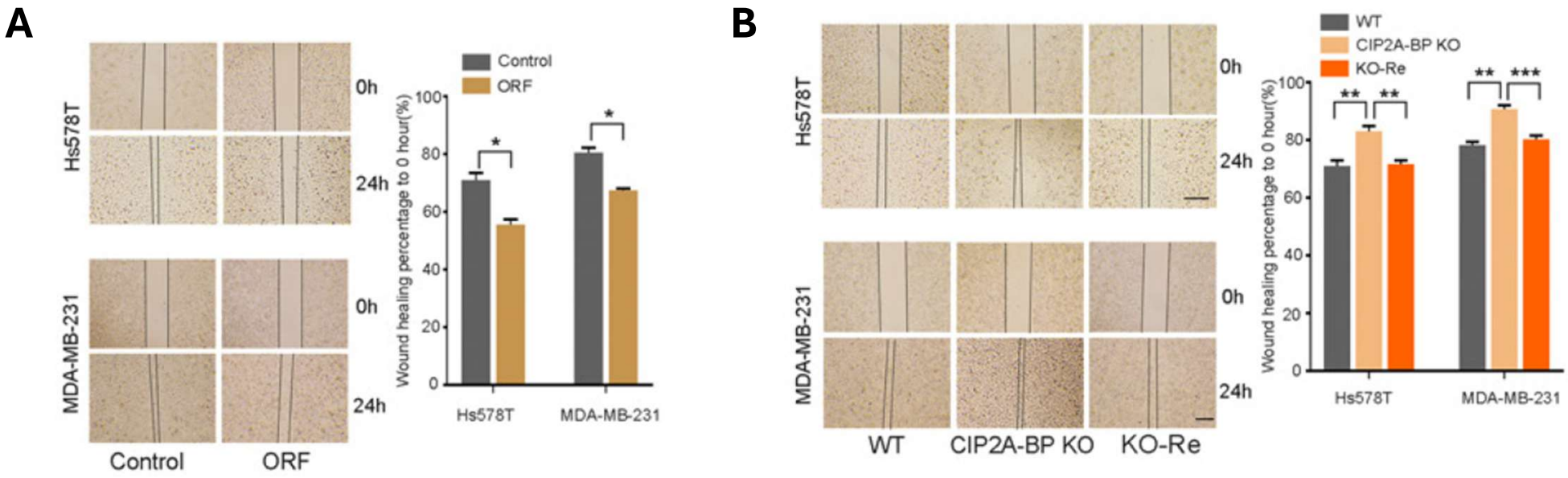


Figure S5: (A) Overexpression inhibits, and (B) knockout promotes wound healing, a phenotype reversed by rescue [Guo, B. (2020)]

Dissociating lncRNA Transcript from Peptide Function

- **Experimental Strategy:** CRISPR/Cas9-mediated mutation of the translation initiation site (**ATG → ATT**).
- **Molecular Validation:**
  - **Transcription Intact:** The *LINC00665* RNA transcript is stable and expressed at Wild-Type levels;
  - **Translation Abolished:** The CIP2A-BP micropeptide is undetectable;
- **Conclusion:** The loss of tumor suppression in KO cells is essentially due to the **absence of the peptide**, ruling out RNA-dependent functions (e.g., "sponge" effects).

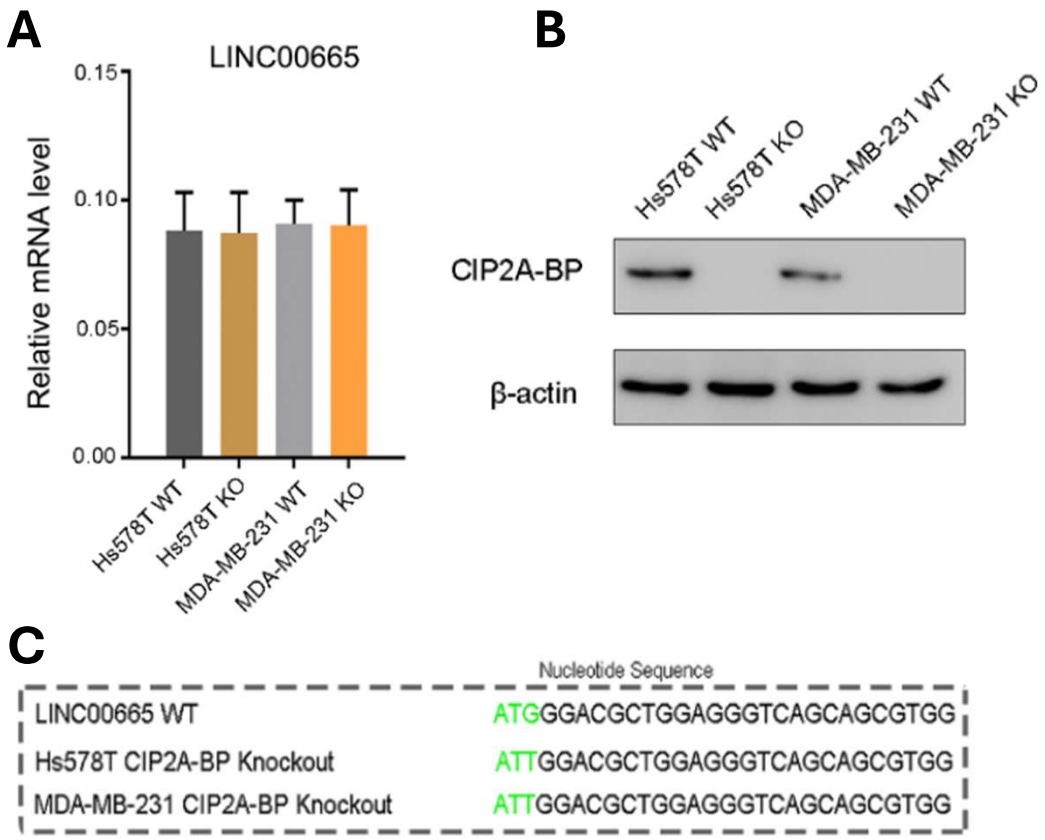
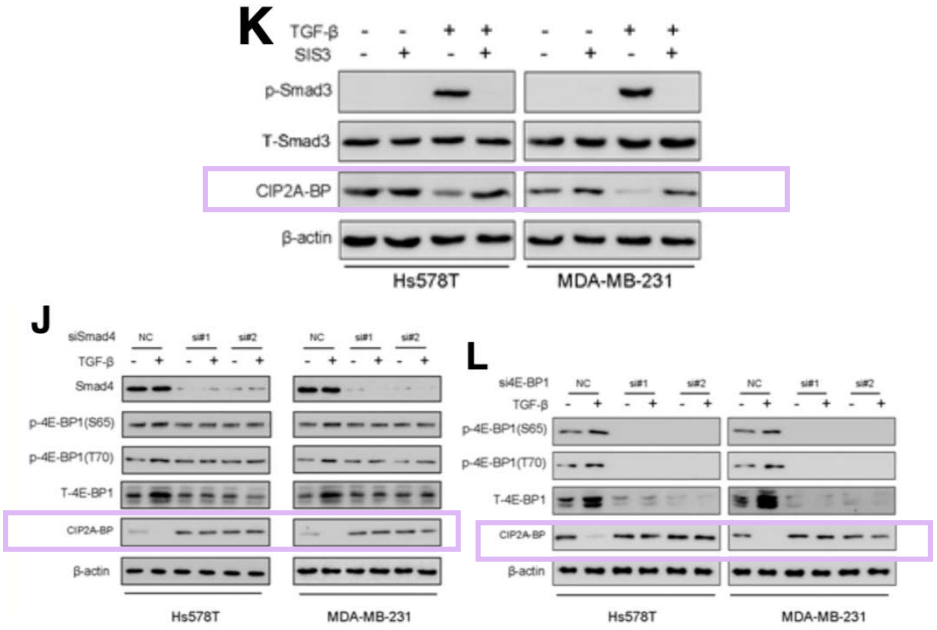


Figure S6: Specific CIP2A-BP peptide knockout validation; (A) LINC00665 mRNA levels remain unchanged; (B) Loss of CIP2A-BP peptide expression; (C) Sequencing confirms the ATG>ATT start codon mutation [Guo, B. (2020)]



(K) TNBC cell lines transfected with SIS3
(J) TNBC cell lines transfected with Smad4 siRNA
(L) TNBC cell lines transfected with 4E-BP1 siRNA
[Guo, B. (2020)]

Further confirm...

Downregulation of CIP2A-BP by TGF-β treatment could be reversed by:

- siRNA knockdown of Smad4
- siRNA knockdown of 4E-BP1
- inhibition of Smad3 phosphorylation by Smad3 phosphorylation inhibitor (SIS3)

↑ CIP2A-BP

Downregulation of CIP2A-BP in TNBC was a direct consequence of activation of TGF-β/Smad signaling pathway.

CIP2A-BP Regulation by mTOR and TGF-β Signaling

When I inhibit mTOR, I observe a downregulation of CIP2A-BP, confirming its involvement in **mTOR-dependent translation**. When I treat cells with TGF-β, I also observe a downregulation of CIP2A-BP even in the absence of an mTOR inhibitor.

- mTOR active: ↑ CIP2A-BP
- **mTOR inhibitor: ↓ CIP2A-BP**
- TGF-β treatment: ↓ CIP2A-BP

- mTOR inhibitor + TGF-β treatment: ↓ CIP2A-BP
- **mTOR active + TGF-β treatment: ↓ CIP2A-BP**

Under high level of TGF-β, LINC00665 translation is insensitive to regulation by mTOR signaling pathway.

Downregulation of CIP2A-BP in TNBC is a direct consequence of activation of TGF-β/Smad signaling pathway.

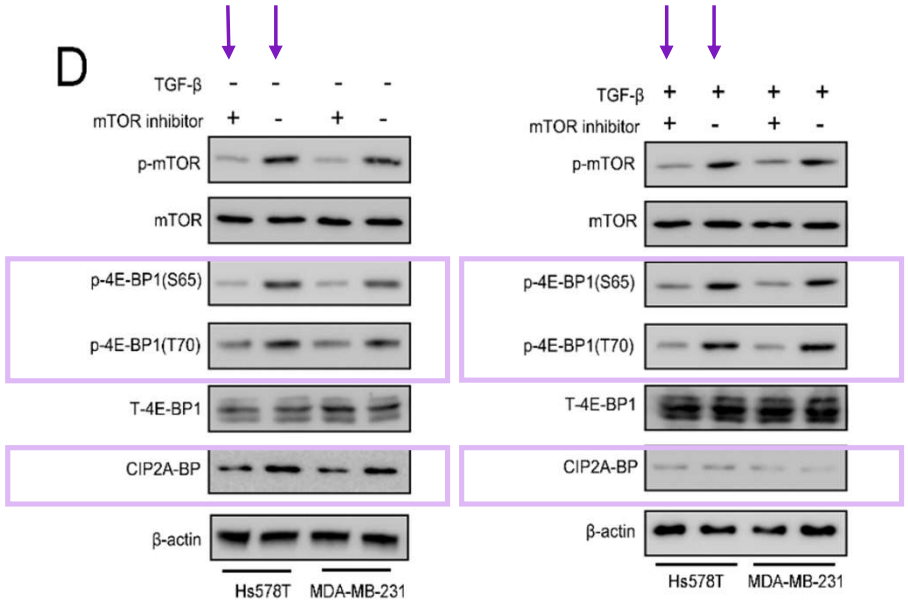


Figure S8. 4E-BP1 affect the translation of multiple mRNAs
(D) TNBC cells were pretreated with specific antagonist against mTOR for 1h and then cultured with or without TGF-β. The indicated proteins were determined by immunoblotting analysis. ***[Guo, B. (2020)]***

LINC00665 and the efficacy of Anticancer Drugs: Cisplatin

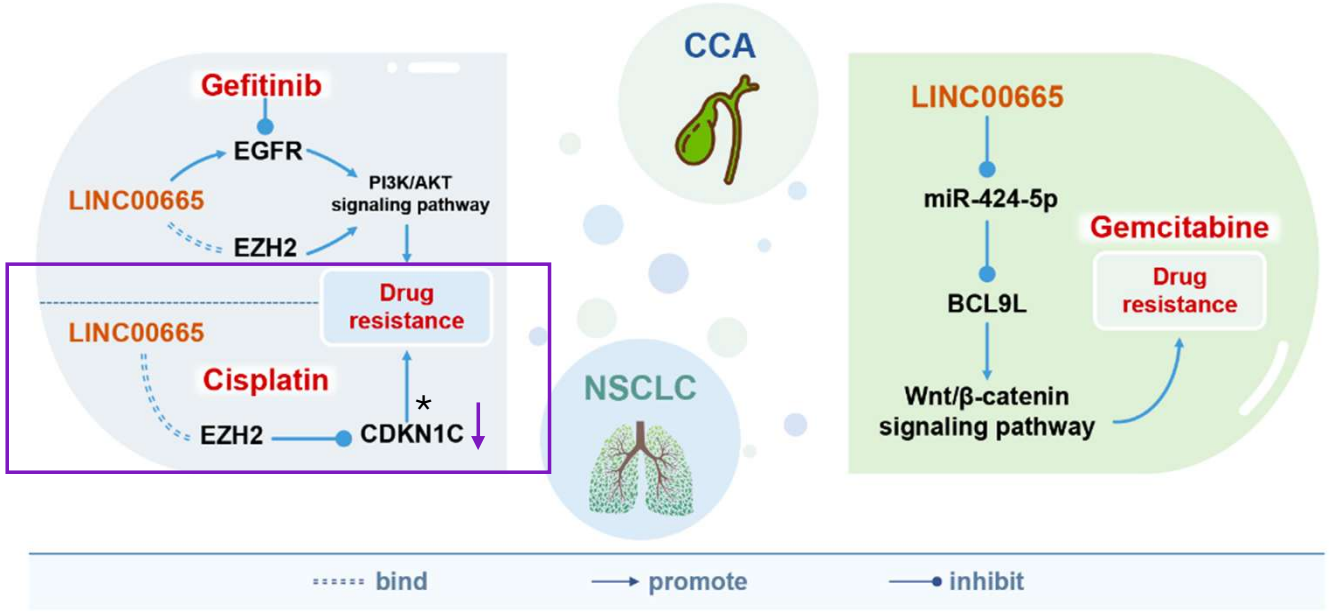


Fig. S9. Role of LINC00665 in resistance to anticancer drugs

* CDKN1C tumor suppressor also knows as p57

Platinum-based chemotherapeutic drug that kills cancer cells by inducing **DNA crosslinks**.
These lesions block DNA replication and transcription, causing **replication stress**, **double-strand breaks**, and ultimately **apoptosis** in rapidly dividing cancer cells.

In TNBC (especially BRCA-mutated subtypes) is more responsive because of defective DNA repair.