UNIVERSITÀ DEGLI STUDI DI TRIESTE

Dipartimento di Scienze della Vita

BMC

CRISPR-Cas13d screens identify KILR, a breast cancer risk-associated lncRNA that regulates DNA replication and repair

Wang L, Bitar M, Lu X, Jacquelin S, Mol Cancer. 2024 May

Presented by Zamboni Matteo & Balzan Riccardo

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1. Introduction

- lncRNAs
- lncRNAs rules in Cancer
- Genome editing
- RNA-targeting CRISPR

3. Conclusion

- Functional lncRNAs
- Breast cancer risk

2. Results

- Altered proliferation
- Sense intronic
- KILR Expression
- Viability Assay
- KILR Rules

4. Discussion

- CRISPR/Cas issues
- KILR different controls in cancer

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Background & Aim

Breast cancer remains **one of the most prevalent and deadly cancers among the women** worldwide, 1 of 8 women is affected. Beyond genetic mutations, tumor progression is strongly influenced by the Tumor Microenvironment (TME).

- **Long non-coding RNAs** (lncRNAs) act as **key regulators** within the tumor microenvironment (TME), **shaping the different Cancer Hallmark** *(Yao W, Wang L, Liu F, Xia L. Epub Jul 24)*

- The **CRISPR/Cas system** today offers powerful and versatile tools for **precise genome editing**. Its rapidly expanding applications in oncology hold great promise for improving both cancer diagnosis and the development of targeted, personalized treatments. *(Di Carlo E, Sorrentino C. State. 2024 Dec)*

What are lncRNAs?

LncRNAs are RNA **transcripts longer than 200 nucleotides** that do not code for proteins. Although the number of lncRNAs has surpassed protein-coding genes

Once thought to be mere transcriptional "noise", they are now **recognizing as key regulators** of:

- Gene expression
- RNA stability and processing
- Cell proliferation, differentiation, migration, invasion and apoptosis

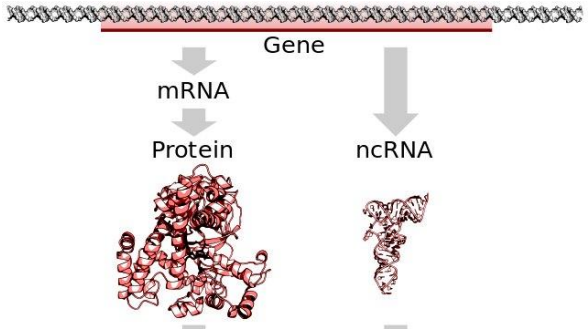


Fig.1 Most lncRNAs likely functional

In cancer, **lncRNAs can act as oncogenes or tumor suppressors**. Their expression is both influenced by and shapers the tumor microenvironment (TME) affecting processes such as immune cell activation, angiogenesis and metastasis.

Type of lncRNAs and their Roles

lncRNAs represent a **highly promising frontier for** novel breast cancer **diagnostics**. Their stable presence in serum and specific expression patterns within the TME **provide a strong basis** for their development as **non-invasive biomarkers.**

Function	LncRNA	Source	Target	Mechanism
Protumor	LINC00514	Tumor cell	Macrophage	STAT3/Jagged1/Notch
	LINC00337	Tumor cell	Macrophage	CD163/ARG1
	XIST	Tumor cell	Macrophage	miR-503
		Tumor cell	Tumor cell	miR-362–5p/UBAP1
		Macrophage	Macrophage	miR-101
	Lnc-BM	Tumor cell	Macrophage	JAK2/STAT3
	SNHG3	Fibroblast	Tumor cell	miR-330–5p/PKM
	SNHG5	Fibroblast	Fibroblast	ZNF281/CCL5/CCL2
	NKILA	T cell	T cell	NF-κB
	SNHG1	T cell	T cell	miR-448/IDO
		Macrophage	Macrophage	STAT6
	SNHG16	T cell	T cell	miR-16–5p/ TGF-β1/SMAD5
	IRENA	Macrophage	Macrophage	NF-κB
	GNAS-AS1	Macrophage	Macrophage	miR-433–3p/GATA3
	MALAT1	Macrophage	Macrophage	CD80/mesothelin
	p21	Macrophage	Macrophage	NF-κB/STAT3
	LncRNA187415.1	Macrophage	Macrophage	CISH
	HISLA	Macrophage	Tumor cell	HIF-1α/ PHD2
	NCAL1	NK cell	NK cell	Gab2-PI3K-AKT
	NEAT1	Tumor cell	Tumor cell	miR-141–3p/KLF12
	THOR	Tumor cell	Tumor cell	MAPK/PI3K-AKT
	NDRG1-OT1	Tumor cell	Tumor cell	miR-875–3p
	HOTAIR	Tumor cell	Tumor cell	TGF-β1
	LINC01133	Tumor cell	Tumor cell	miR-199a/ KLF4
	UCA1	Tumor cell	Tumor cell	AKT
	PCAT6	Tumor cell	Tumor cell	miR-4723–5p/ VEGFR2
Antitumor	BM466146	Tumor cell	T cell	CXCL13

Possible Opportunities:

1. Serum-Based Liquid Biopsies
2. TME-Specific Diagnostic Models Beyond serum

Fig.2 lncRNAs serve as modulators among tumor cells, fibroblasts, and immune cells

lncRNAs and Cancer Hallmarks

Building on their diverse regulatory functions lncRNAs orchestrate complex interactions among tumor, stromal and immune cells within the TME, shaping tumor behaviour and therapy. lncRNAs contribute to multiple cancer hallmarks by modulating:

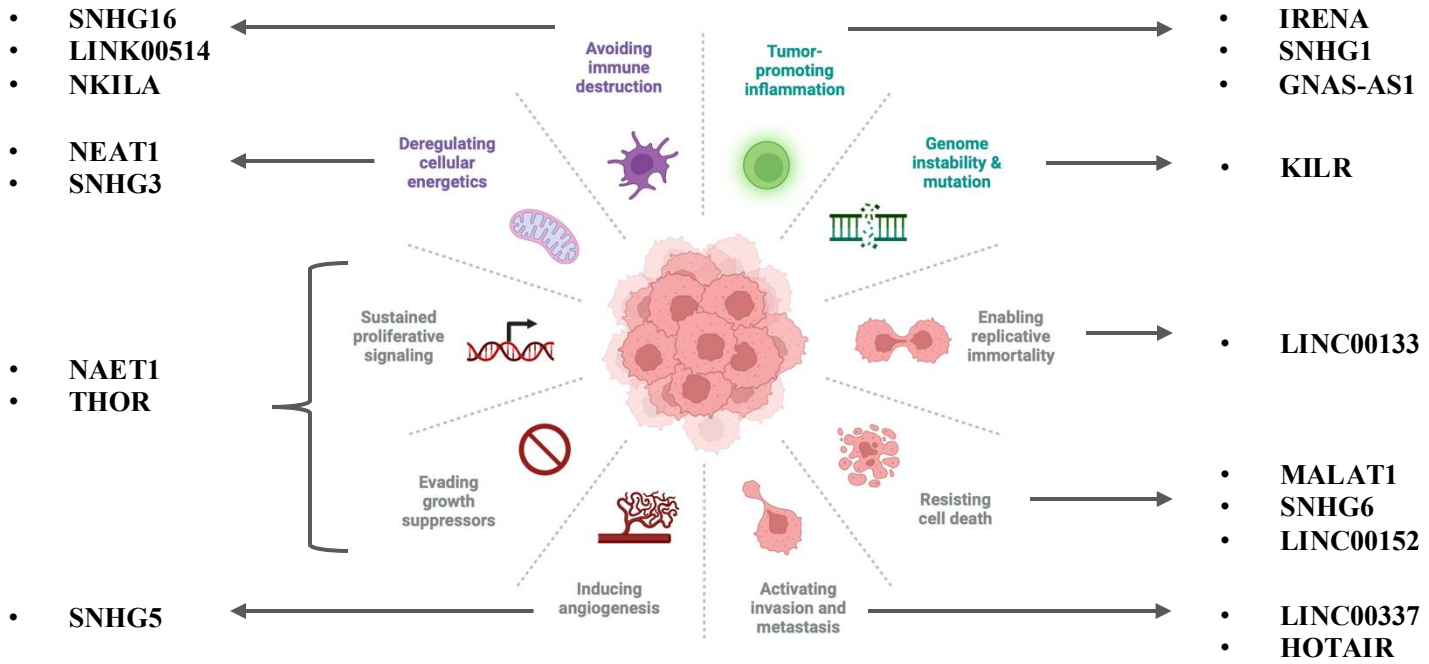
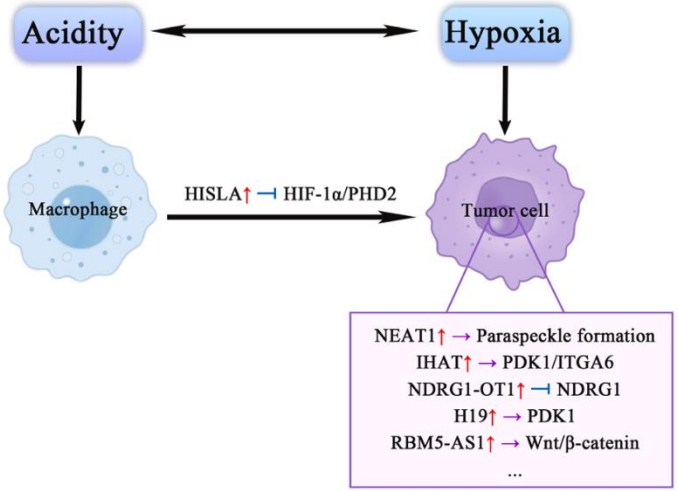


Fig.3 lncRNAs are relate specifically to the different Cancer Hallmarks

Reciprocal regulations: TME & lncRNAs

The tumor microenvironment (TME) is characterized by conditions such as **hypoxia** and **acidity**, which profoundly influence cellular behaviour.



lncRNAs not only regulate the TME but are also **modulated by these stress conditions**, creating a feedback loop that drives **tumor growth and progression**.

Fig.4 A hypoxic microenvironment can induce the expression of lncRNAs in tumor cells by activating or inhibiting downstream signaling and thus affect tumor growth. Meanwhile, an acidic microenvironment can increase the expression of HISLA in macrophages, which stabilize HIF-1α by blocking the interaction between PHD2 and HIF-1α in breast cancer cells and eventually regulate tumor progression. ↑: upregulated; →: activate; ↑: inhibit.

Hypoxia, acidity, and intercellular signaling within the TME form a **reciprocal regulatory network** that controls **lncRNA expression and tumor behaviour**, representing potential **targets for therapy and prognosis**.

NAET1 as a key regulator in breast cancer

NEAT1 is a multifunctional lncRNA highly upregulated in breast cancer and responsive to TME stresses such as hypoxia.

It acts as a molecular regulator of:

- Tumor metabolism
- Gene expression
- Cell survival
- Cell proliferations

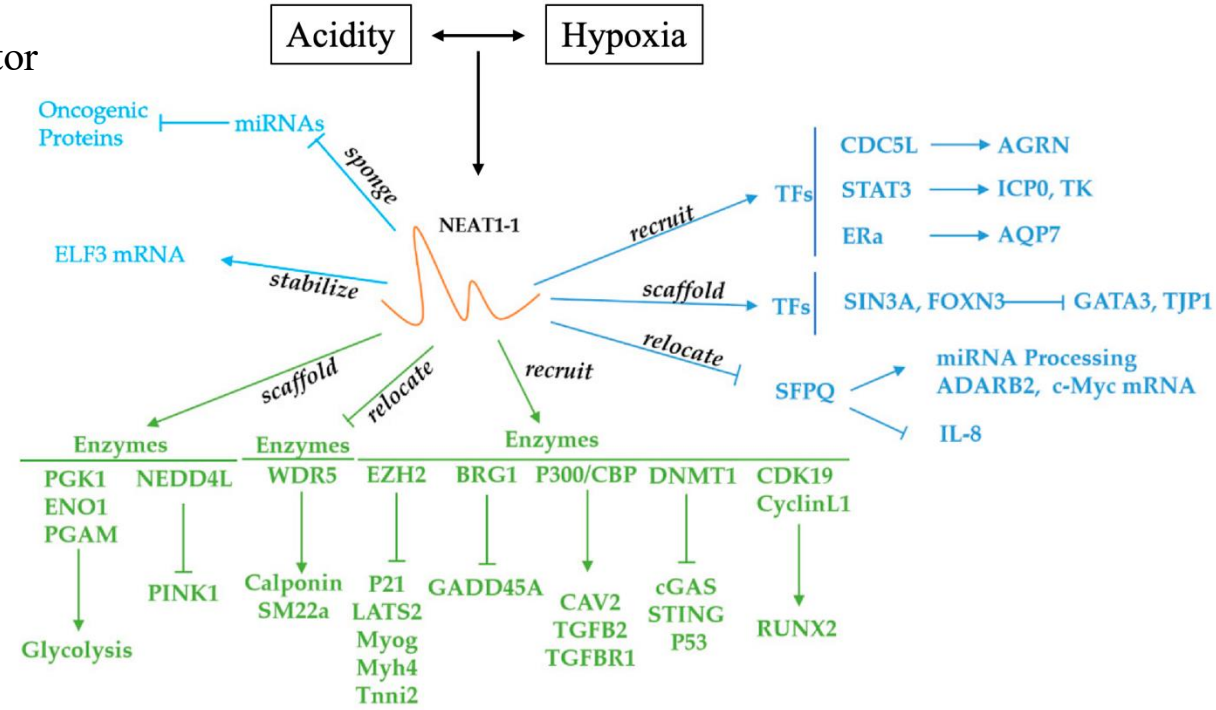


Fig.5 NEAT1 representative scheme as a key regulator in breast cancer progression

Techniques for functional screening

How can we identify which lncRNAs play an important role in tumor progression?

By performing a functional CRISPR/Cas-based knockdown screen, which is:

- High-throughput
- RNA-level knockdown (Cas13d)
- Phenotype-based readout

CRISPR family

- **CRISPR** (clustered regulatory interspaced short palindromic repeats) **family** is a group of DNA sequences found in the genomes of bacteria and archaea and are **part of the adaptive immune system.**

- CRISPR is composed by different components: →
  - CRISPR arrays;
  - Spacer;
  - Cas (CRISPR-associated) proteins.

- CRISPR/Cas system operate in **three stages**: →
  1) Adaptation;
  2) Expression;
  3) Interference.

- CRISPR/Cas system is classified into two main classes which are divided into subtype:
  - **Class 1**, divided in: Type I; Type III; Type IV.
  - **Class 2**, divided also in: Type II; Type V; Type VI.

CRISPR/Cas9 system for cancer therapy & diagnosis

CRISPR/Cas9 effectors are Class 2, Type II nucleases. Guided by a **sgRNA**, Cas9 recognizes complementary **dsDNA** targets next to a specific **PAM** and cleaves them through its catalytic **HNH domains**.

Beyond the canonical SpCas9, other Cas9 orthologs have distinct PAMs and naturally cleave only dsDNA; only some **engineered variants** can act on **ssDNA** or **ssRNA** via artificial **PAMmers**.

These characteristics make CRISPR/Cas9 an attractive **toolkit** for treatment and diagnosis of cancer.

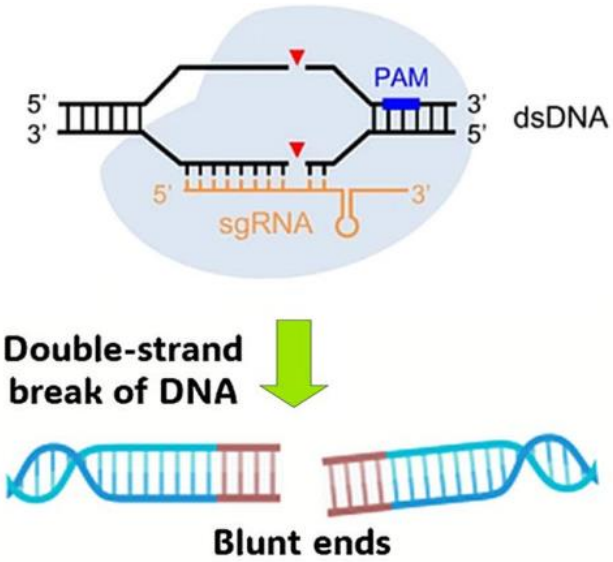


Fig.6 Diagram of the Cas9 function

CRISPR/Cas9 system for cancer therapy & diagnosis

Therapeutic applications	Diagnostic & research applications
Gene editing and repair; inactive oncogenes, restoring tumor suppressor genes, epigenome editing.	**Genome-wide CRISPR screens** to identify cancer drivers and prognostic biomarkers.
Immunotherapy enhancement; engineering T cells by deleting immune checkpoints, or by intoducing CARs.	**Drug resistance studies;** identifying genes involved in resistance and pathways that sensitize cancer cells to therapy.
Overcoming drug resistance by editing resistance genes or sensitizing cancer cells.	**Functional genomics;** understanding gene function at the single-cell level and in the tumor Microenvironment.

New possibility using Cas-13 based CRISPR system

CRISPR/Cas13 effectors belong to the Class 2, Type VI. Guided by a **sgRNA**, Cas13 recognizes complementary **ssRNA** targets next to a specific **PFS** and cleaves them through its catalytic **HEPN** domains.

Cas13 can be safely used in contexts where transient regulation of gene expression is desired **without altering** the host genome, making it a versatile tool for detecting viral/cancer-related RNA sequences in **molecular diagnostics**.

Cas13a is one of the best-characterized and most widely used Cas13 effectors; once activated by binding to its target RNA, it also cleaves nearby non-specific RNAs. This **collateral** (trans) **cleavage activity** underlies several highly sensitive RNA detection platforms.

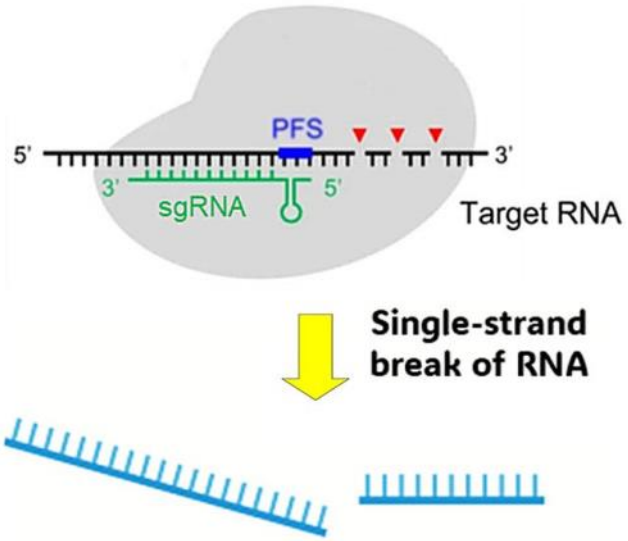


Fig.7 Diagram of the Cas13a function

New possibility using Cas13 based CRISPR system

Application	Details
RNA editing	Cas13 can be used for precise RNA editing in cancer cells.
Knock-down of oncogenes and drug-resistant genes (for **therapeutic** and **discovery purposes**)	• Therapeutic targeting of oncogenes and noncoding RNAs • Identifying cancer driver genes and therapeutic targets • Identifying drug-resistant genes and mutations at the RNA level
Detection of cancer-associated RNA (e.g. **SHERLOCK** platform using CRISPR/Cas13)	• Detection of specific RNA sequences associated with cancer • High sensitivity and specificity • Versatility • Rapid results and cost-effectiveness

Limitation of CRISPR/Cas system for cancer

Limitation	Examples / Possible approaches
Delivery methods	Efficient and targeted delivery of CRISPR components is crucial. Common vectors (AAV, lentivirus, lipid nanoparticles) have limitations. Alternative methods include: • Naturally occurring AAV variants • Polymeric nanoparticles • Naturally occurring exosomes and extracellular vesicles
Off-target effects	Off-target cutting remains a major concern and can be limited by: • Enhancing HDR efficiency • Using cell cycle regulators • Improving sgRNA design • Using high-fidelity Cas variants • Using base editors • Applying prime editing and advanced screening techniques
Other critical issues	• Immune responses to CRISPR/Cas components • Ethical and regulatory issues • Long terminal effect and stability • Patient selection and genetic diversity

Advantages of CRISPR/Cas system for cancer

Category	Oncology-specific advantage
Precision & specificity	Selective editing of oncogenes and tumor suppressor genes.
Efficiency & scalability	Genome-wide screens to find cancer drivers and resistance genes.
Platform versatility	Therapeutic genome editing and CRISPR-based detection of tumor biomarkers
Functional genomics	Identify genes controlling tumor growth and response to therapy
Molecular diagnostics	Sensitive detection of tumor mutations and circulating tumor DNA/RNA.
Toward personalized medicine	Support development of tailored cancer therapies (ex. CAR-T cells).

RESEARCH Open Access

CRISPR-Cas13d screens identify *KILR*, a breast cancer risk-associated lncRNA that regulates DNA replication and repair

Lu Wang[1,2], Mainá Bitar[1,2,3], Xue Lu[1], Sebastien Jacquelin[1,4], Sneha Nair[1], Haran Sivakumaran[1], Kristine M. Hillman[1], Susanne Kaufmann[1], Rebekah Ziegman[1], Francesco Casciello[1], Harsha Gowda[1], Joseph Rosenbluh[5,6], Stacey L. Edwards[1,2,3*†] and Juliet D. French[1,2,3*†]

Abstract

Background Long noncoding RNAs (lncRNAs) have surpassed the number of protein-coding genes, yet the majority have no known function. We previously discovered 844 lncRNAs that were genetically linked to breast cancer through genome-wide association studies (GWAS). Here, we show that a subset of these lncRNAs alter breast cancer risk by modulating cell proliferation, and provide evidence that a reduced expression on one lncRNA increases breast cancer risk through aberrant DNA replication and repair.

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Research-environment

GWAS identifies **thousands of disease-associated variants**, primarily located **in noncoding regions**, modulating risk by altering DNA regulatory elements (enhancers).

1) The **nc-genome is actively transcribed**, generating a diverse set of molecules, mainly Long Noncoding RNAs (lncRNAs).

2) **LncRNAs exhibit** exquisite cell-type **specific expression**, and their aberrant expression is implicated in various diseases, including cancer.

3) GWAS-based analyses reveal **lncRNAs** as **mediators of breast cancer risk**, with risk variants concentrated in their exons.

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Breast cancer-lncRNAs impact proliferation

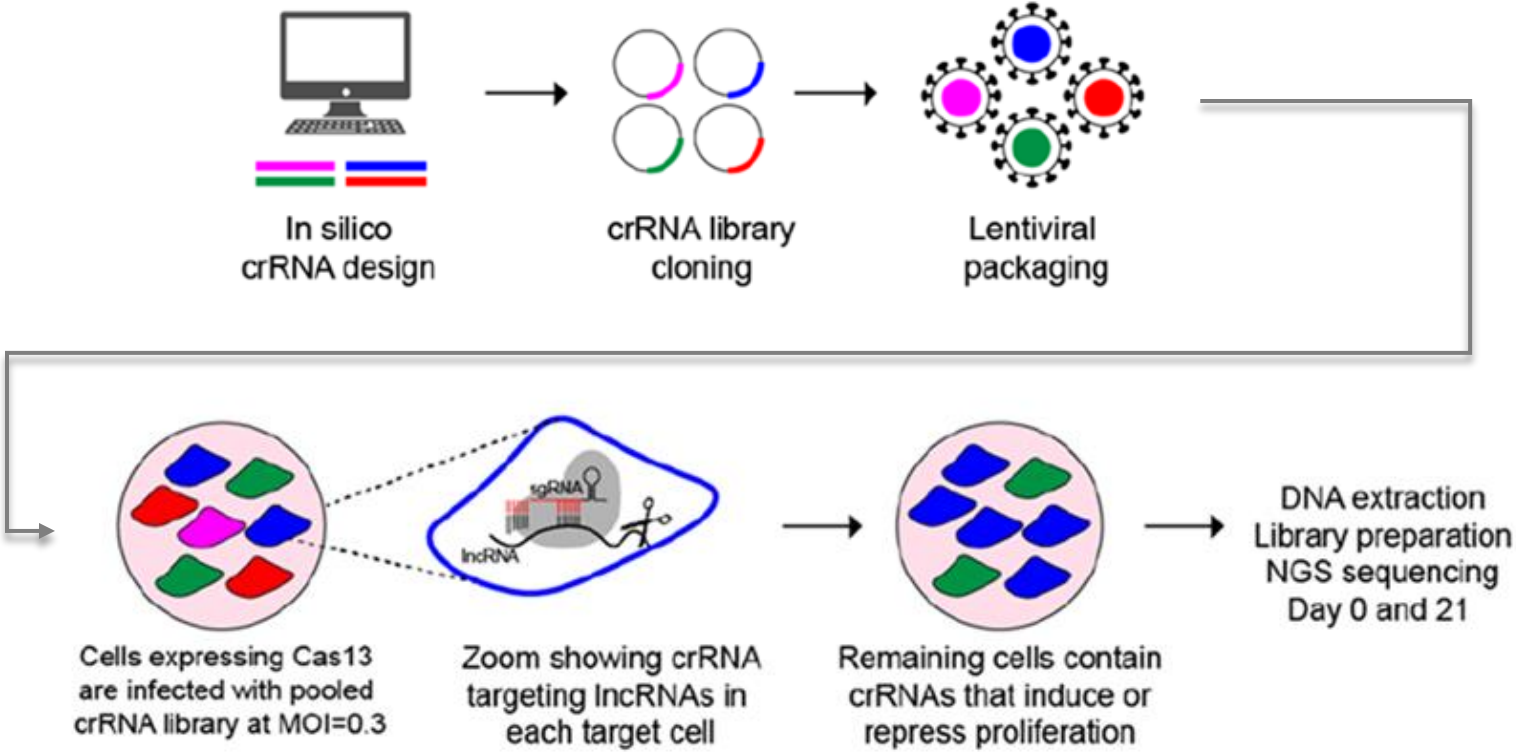

Fig.8 Schematic for CRISPR-Cas13d screens, to identify lncRNAs that modulate breast cell proliferation

Breast cancer-lncRNAs impact proliferation

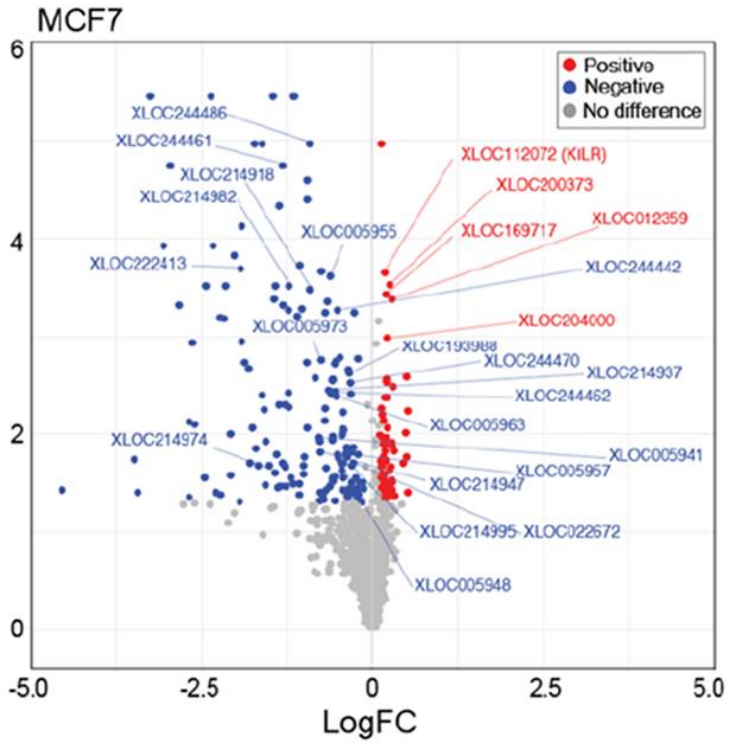

Fig.9 Scatterplots from CRISPR-Cas13d screen data showing differentially represented crRNAs (red/blue dots; log2[fold-change] > 0.1 and p value < 0.05) targeting candidate genes and lncRNAs. Labels are unannotated breast cancer-associated lncRNAs with FDR ≤ 0.3.

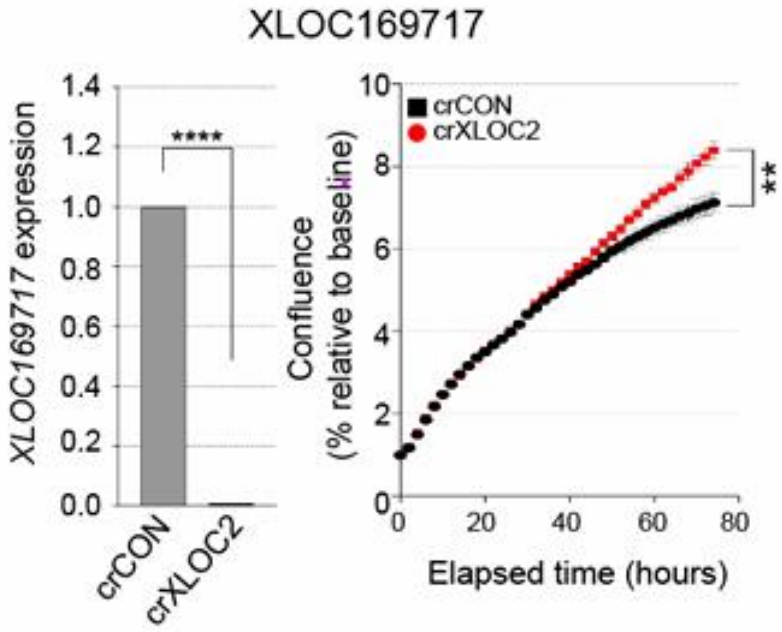

Fig.10 qPCR for lncRNA expression (left panels) and cell confluence measured over time using Incucyte (right panels) in MCF7 cells after CRISPR-Cas13d lncRNA knockdown. The crCON contains a non-targeting control. p values were determined by Student's t-test (**p < 0.01, ****p < 0.0001).

Breast cancer-lncRNAs impact proliferation

The results of the KD are:

- Knockdown of essential genes/oncogenes → ↓ cell proliferation
- Knockdown of tumor suppressors → ↑ cell proliferation
- Knockdown of 39 high-confidence breast cancer–associated lncRNAs **altered proliferation**, 5 shared across all 3 cell lines;
- Top crRNAs for 5 lncRNA hits were validated, confirming **screen reliability**.

GWAS-eQTL-Cas13d pipeline for KILR selection

844 breast cancer-associated lncRNA from GWAS and eQTL analysis
+
59 annotated lncRNAs, **935** coding genes associated with breast cancer
=
1864 targets

↓

Pooled CRISPR–Cas13d screen in K5+/K19+, MCF7, MDAMB231, where each target has at least **5 to 10** crRNAs (**~18.000**)

↓

MAGeCK analysis (FDR ≤ 0.3) that highlight the presence of **39** high-confidence breast cancer–associated lncRNAs whose knockdown alters proliferation

↓

Subset of **7** lncRNAs that were already "strong" eQTLs for risk variants.
After applying the filters, there are **2** clear positive hits remaining, one of wich is KILR.

↓

Prioritization of KILR, for more in-depth studies:

- Has multiple risk variants within the transcript
- Has a solid phenotype in the screen
- Has nuclear/regulatory lncRNA characteristics

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KILR is a sense intronic lncRNA

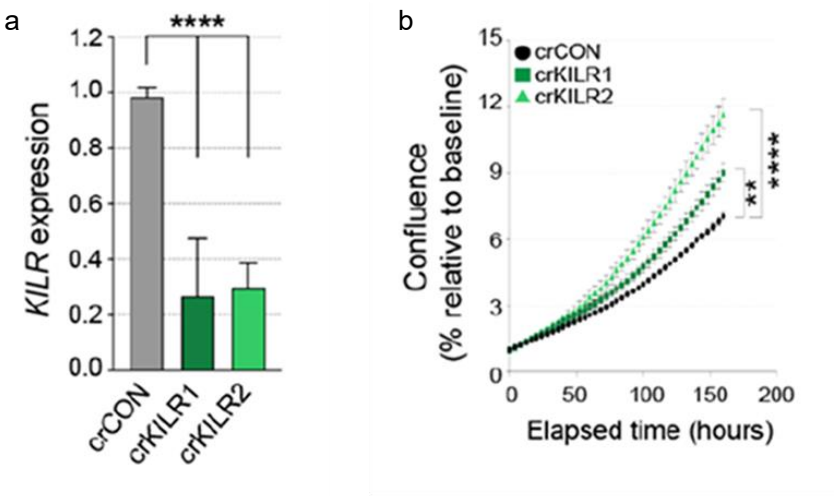


Fig.11 A) qPCR for KILR expression. **B)** cell confluence measured over time using Incucyte in MCF7 cells after Cas13d-KILR knockdown with two independent crRNAs (crKILR1-2). The crCON contains a non-targeting control. p values were determined by one-way ANOVA and Dunnett's multiple comparisons test (**p < 0.01, ****p < 0.0001).

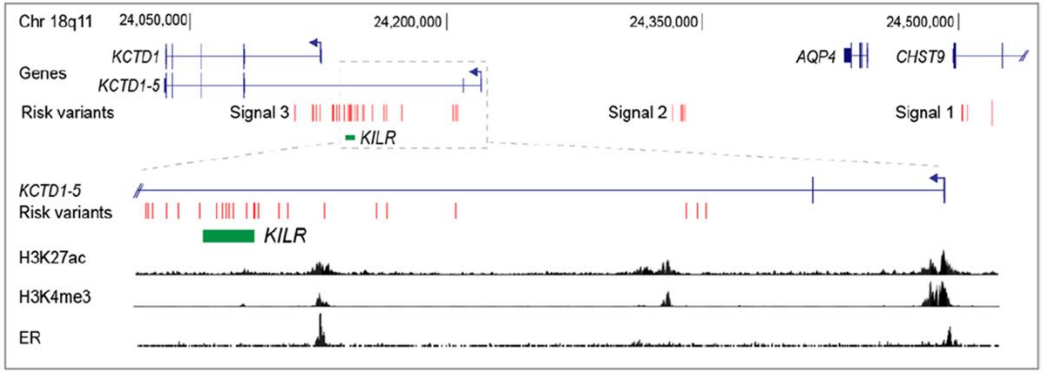
The two most significant crRNAs targeting KILR were **individually validated.**

Both crRNAs efficiently knocked down KILR.

KILR knockdown led to **increased proliferation** of MCF7 breast cancer cells.

KILR is a sense intronic lncRNA

a



b

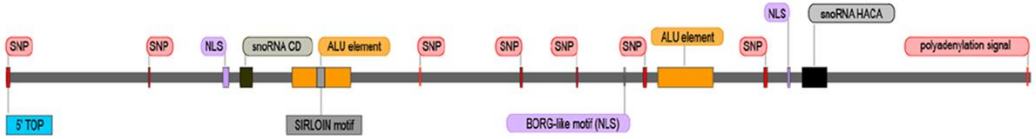


Fig.12 A) WashU genome browser (hg19) showing GENCODE annotated genes (blue) and KILR (green). The breast cancer risk variants are shown as red vertical lines (Signals 1–3). The H3K27ac, H3K4me3 and ER (estrogen receptor) binding tracks from MCF7 cells are shown as black histograms. signal). **B)** A linear schema of KILR. SNP (single nucleotide polymorphism); 5' TOP (terminal oligopyrimidine tract); NLS (nuclear localization signal).

KILR's features:

- Located on chromosome **18q11** within the **first intron** of KCTD1-5 (**sense intronic** lncRNA)
- Transcript variant of KCTD1 generated from an **alternative TSS**
- Contains seven breast cancer **risk variants**
- Displays a **5′ poly(U) tract**
- Includes two predicted **H/ACA box snoRNAs** and one **C/D box snoRNA**
- Contains **BORG-like** and **SIRLOIN** motifs
- Contains multiple predicted **NLSs** and **IRAlu elements**

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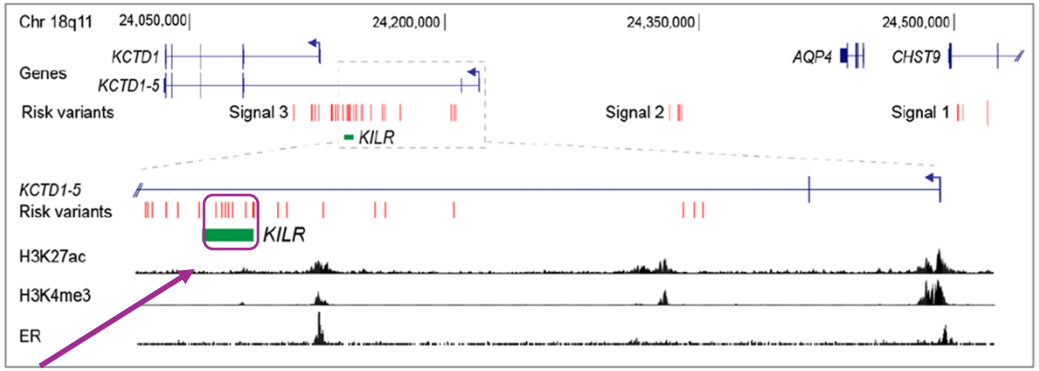
Results

Breast cancer risk variants at 18q11 reduce the half-life



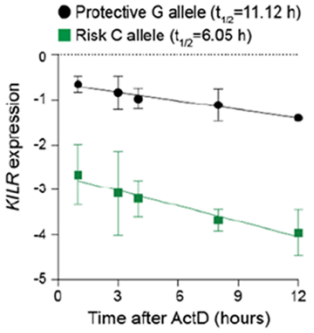
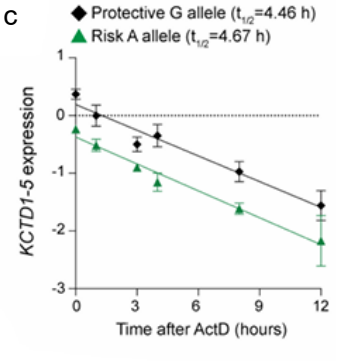


Fig.13 A) WashU genome browser (hg19) showing GENCODE annotated genes (blue) and KILR (green). The breast cancer risk variants are shown as red vertical lines (Signals 1–3). **B)** KILR RNA stability assay in MDAMB361 cells after treatment with actinomycin D (ActD), then qPCR for KILR RNA relative to CDKN2A mRNA levels. KILR mRNA half-life (t1/2) was calculated by linear regression analysis. **C)** KCTD1 5 hnRNA stability assay in MDAMB361 cells after treatment with actinomycin D (ActD), then qPCR for KCTD1-5 hnRNA relative to CDKN2A mRNA levels.

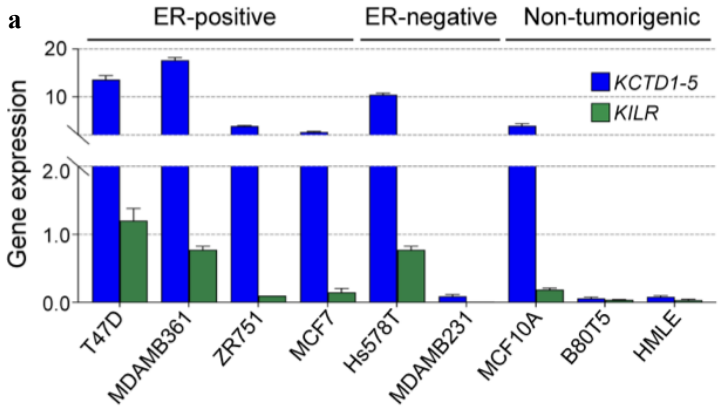
Seven highly correlated breast cancer risk variants are within the KILR transcript.

In heterozygous MDAMB361 cells, KILR with risk alleles has half the **RNA half-life** compared with KILR with protective alleles.

No **allele-specific effect** on RNA stability was observed for KCTD1-5 hnRNA, indicating a **specific destabilizing** effect of risk alleles on KILR transcript that reduced KILR expression.



KILR expression



With a specific focus on the breast cancer, we analyze different cell subtypes to investigate the conditions that lead to a KILR overexpression

The sense lncRNA KILR present a different expression level:

- Normale breast tissue
- Breast tumors
- Normal breast
- Breast cancer
- Other cancer type

} Cell line

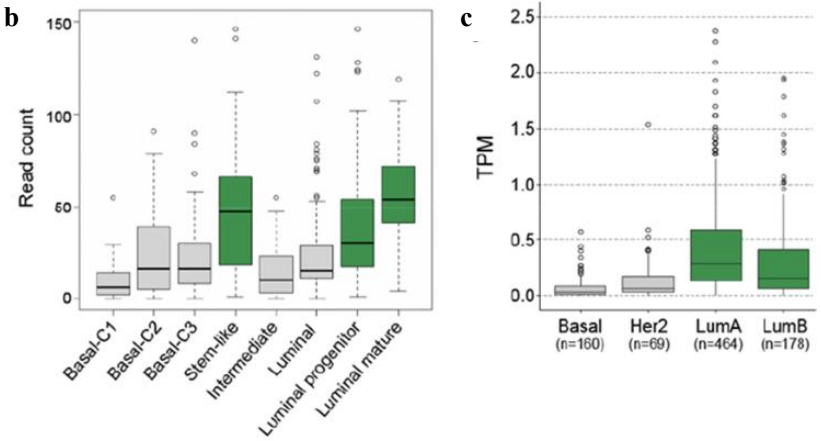


Fig.14 A) qPCR for *KCTD1-5* and *KILR* expression in ER-positive, ER-negative breast cancer and non-tumorigenic breast cell lines **B)** Boxplot of KILR read counts in normal breast tissue from scRNA-seq data clustered based on NB-lncRNA expression **C)** Boxplot of KILR TPM (transcript per million) in breast tumor samples from TCGA RNA-seq data stratified by tumor subtype

KILR Estrogen regulation

Estrogen treatment induces activations of the estrogen binding at KCTD1-5 promoter region.

↓

KCTD1-5 activation after hormone treatment, promote the expression of KILR

↓

KCTD1-5 suggest a potential hormone-responsive regulation of KILR expression in breast cancer.

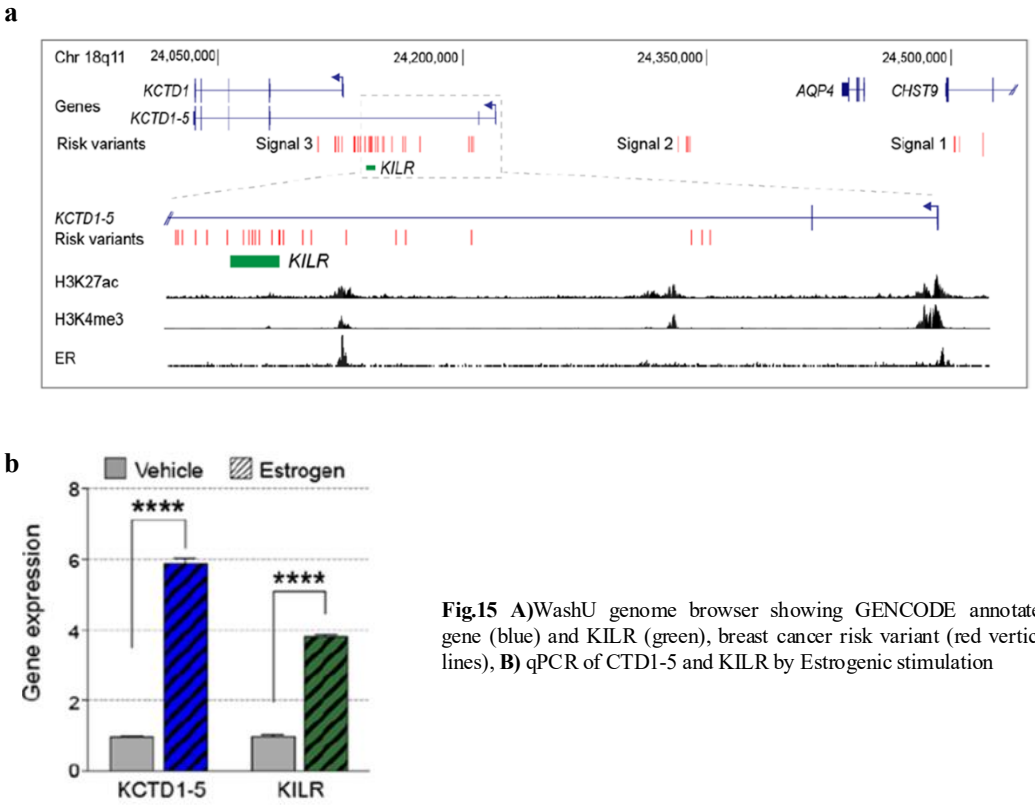


Fig.15 A) WashU genome browser showing GENCODE annotated gene (blue) and KILR (green), breast cancer risk variant (red vertical lines), **B)** qPCR of CTD1-5 and KILR by Estrogenic stimulation

KILR Localization

The **KILR transcript contains** nuclear localization motif:

- NLS
- SIRLOIN
- IRAIu element

Promote nuclear retention

Confirmations:

1. RNA-FISH
2. Subcellular fractionation

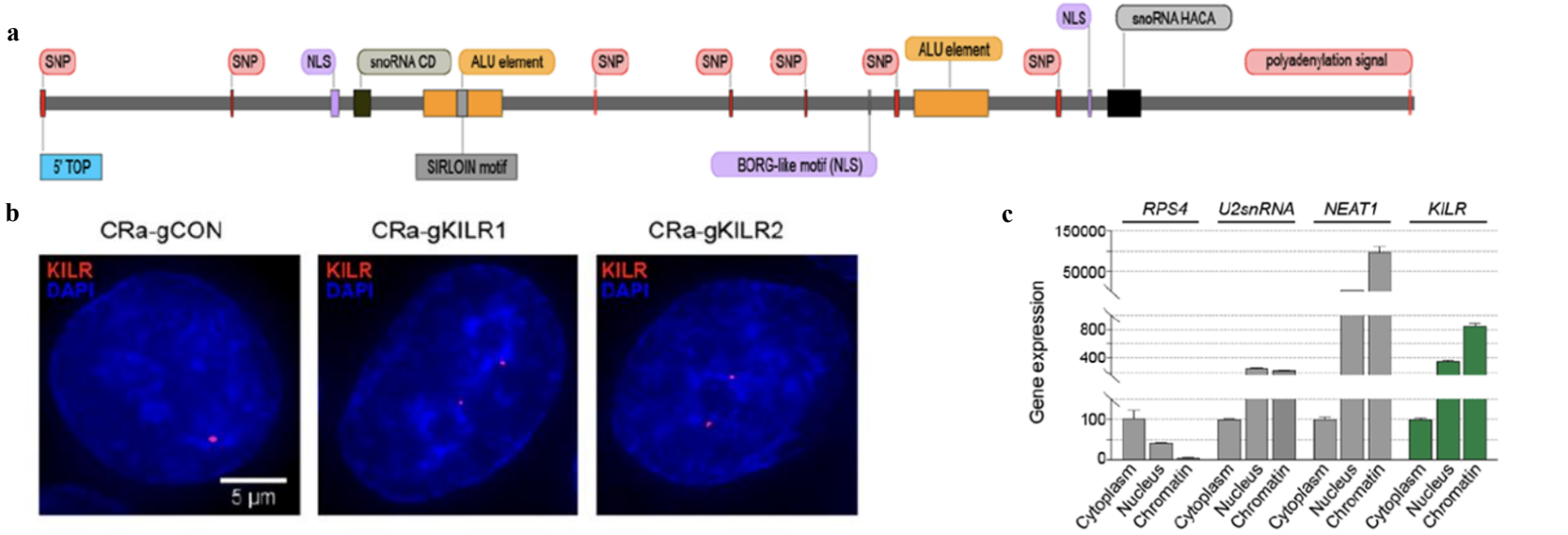


Fig.16 A) linear scheme of KILR, **B)** representative confocal microscopy of KILR in MCF7 cell after CRISPR stained with RNA FISH probes, **C)** qPCR after cell fractionation detecting the distribution of indicating transcripts

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- Sense intronic
- KILR Expression
- **Viability Assay**
- KILR Rules

4. Discussion

- CRISPR/Cas issues
- KILR different controls in cancer

KILR Overexpression suppresses proliferation

Since the Knock-Down of KILR promotes cell proliferation. We perform a CRISPRa-mediated **induction of KCTD1-5 promoter to increase KILR expression** level and observe a cells proliferation suppression.

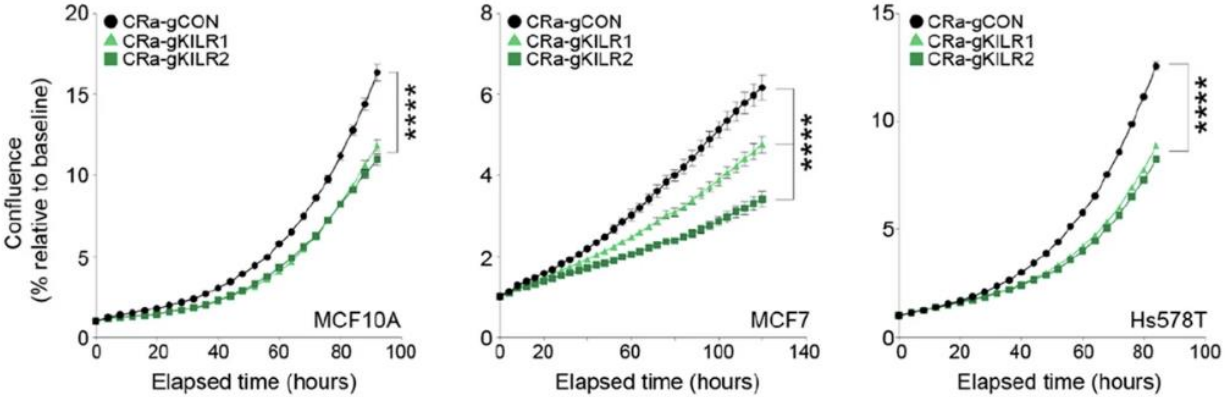
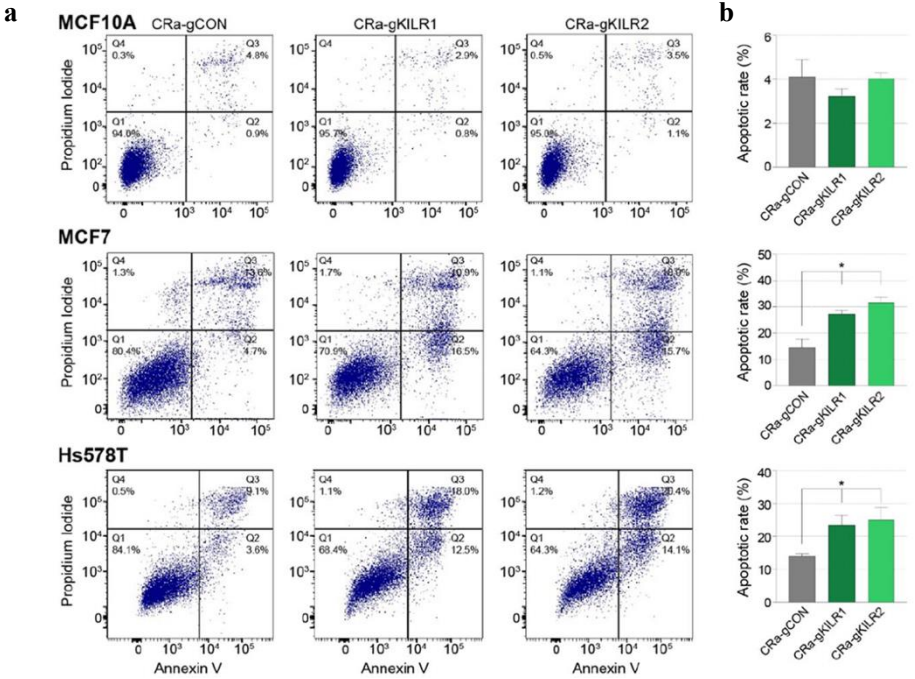


Fig.17 Cell confluence measured over time after CRISPRa activation of the CTD1-5 promoter to overexpress KILR with two independent gRNAs, the Cra-gCON contains a non targeting control

After the treatment cell confluations of normal and cancer lines **demonstrate an increase cell death rate**

KILR Overexpression suppresses proliferation



CRISPRa-mediated induction of KILR and KCDTD1-5 promote apoptosis

↓

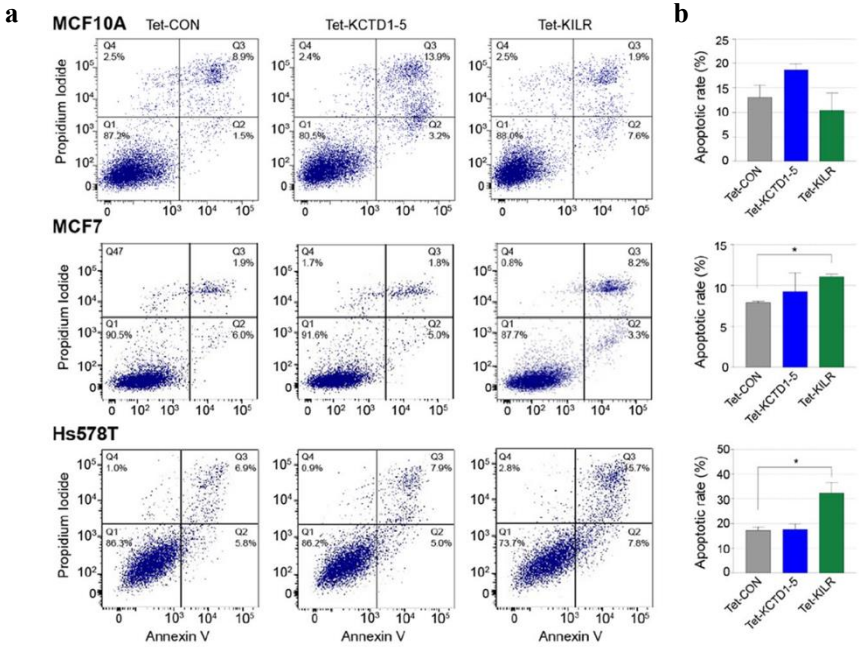
Annexin V assay performed to observe cells death and confirm the hypothesis

- Validate in breast cancer-cell lines
- Non validate in normal breast cell lines

Fig.18 A) Cell confluence measured over time using Incucyte in breast cells after CRISPRa activation of the KCTD1-5 promoter to overexpress KILR with two independent gRNAs (CRa-gKILR1-2). The CRa-gCON contains a non-targeting control **B)** Representative apoptosis analysis of breast cells after CRISPRa (CRa-gKILR1-2) by double staining with annexin V and PI. The CRa-gCON contains a non-targeting control. The quadrants (Q) were defined as Q1 = live (Annexin V- and PI-negative), Q2 = early stage of apoptosis (Annexin V-positive/PI-negative), Q3 = late stage of apoptosis (Annexin V- and PI-positive) and Q4 = necrosis (Annexin V-negative/PI-positive). c The percentage of cells in early and late-stage apoptosis in each group (Q2 + Q3)

KILR drives apoptosis in cancer-cells

To distinguish the roles, of the two genes, inducible Tet-ON overexpression constructs were used.



Only KILR and not KCDTD1-5 triggered apoptosis, again the selectivity in cancer cells and not in normal breast cells

↓

KILR inducing apoptosis confirmed also in several non-breast cancer cell lines

↓

Suggesting a broader tumor-suppressive effect upon overexpression

Fig.19 A) Representative apoptosis analysis of breast cells after doxycycline induction of ectopic KCTD1-5 or KILR expression by double staining with annexin V and PI. The Tet-CON represents an empty vector control. **B)** The percentage of cells in early and late-stage apoptosis in each group (Q2 + Q3)

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- **KILR Rules**

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- KILR different controls in cancer

KILR interactors

KILR interactors are identified using RNA pull-down followed by MS. KILR binding proteins identified by the presence of at least five peptides.

→ RPA1 is most highly enriched protein

- Main single stranded DNA (ssDNA) binding protein in humans
- Member of heterotrimeric RPA complex

Essential for multiple complex in DNA metabolism:

- DNA replication
- DNA damage repair

KILR-RPA1 interaction supported using:

1. RIP
2. RNA-Fishing

Confirming also that KILR-RPA1 are colocalize

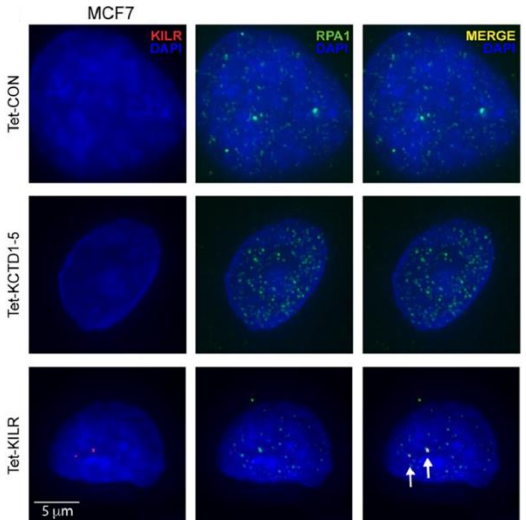
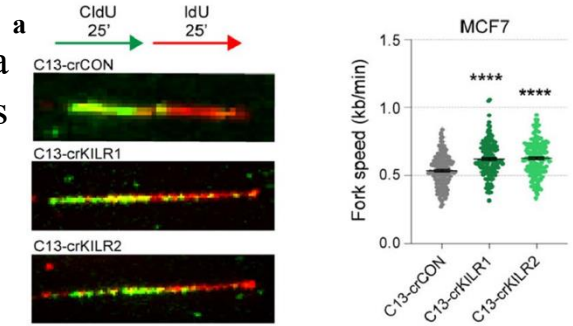
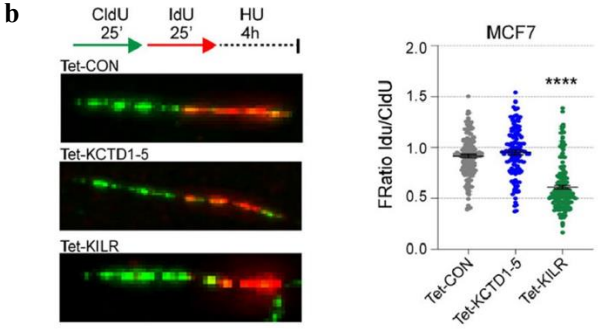


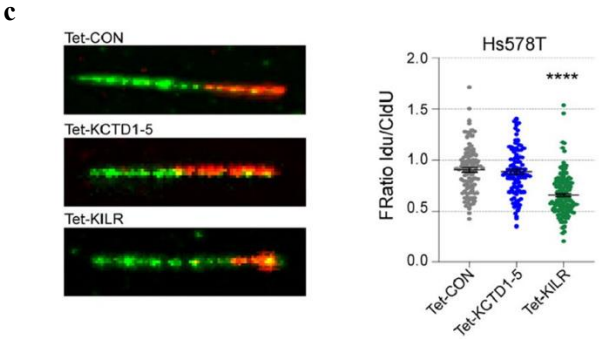
Fig.20 Representative confocal microscopy images of KILR and RPA1 in MCF7 cells after doxycycline induction of ectopic KCTD1-5 or KILR expression stained with Stellaris KILR RNA FISH probes (red) and immunostained with anti-RPA1 (green) (n = 3). The Tet-CON represents an empty vector control. Nuclei were stained with DAPI (blue). White arrows highlight KILR/ RPA1 co-localization

KILR inhibits DNA replications

KILR overexpression sequesters RPA1 into nuclear puncta suggesting that **KILR abrogate RPA1 functions** by reducing its availability levels.







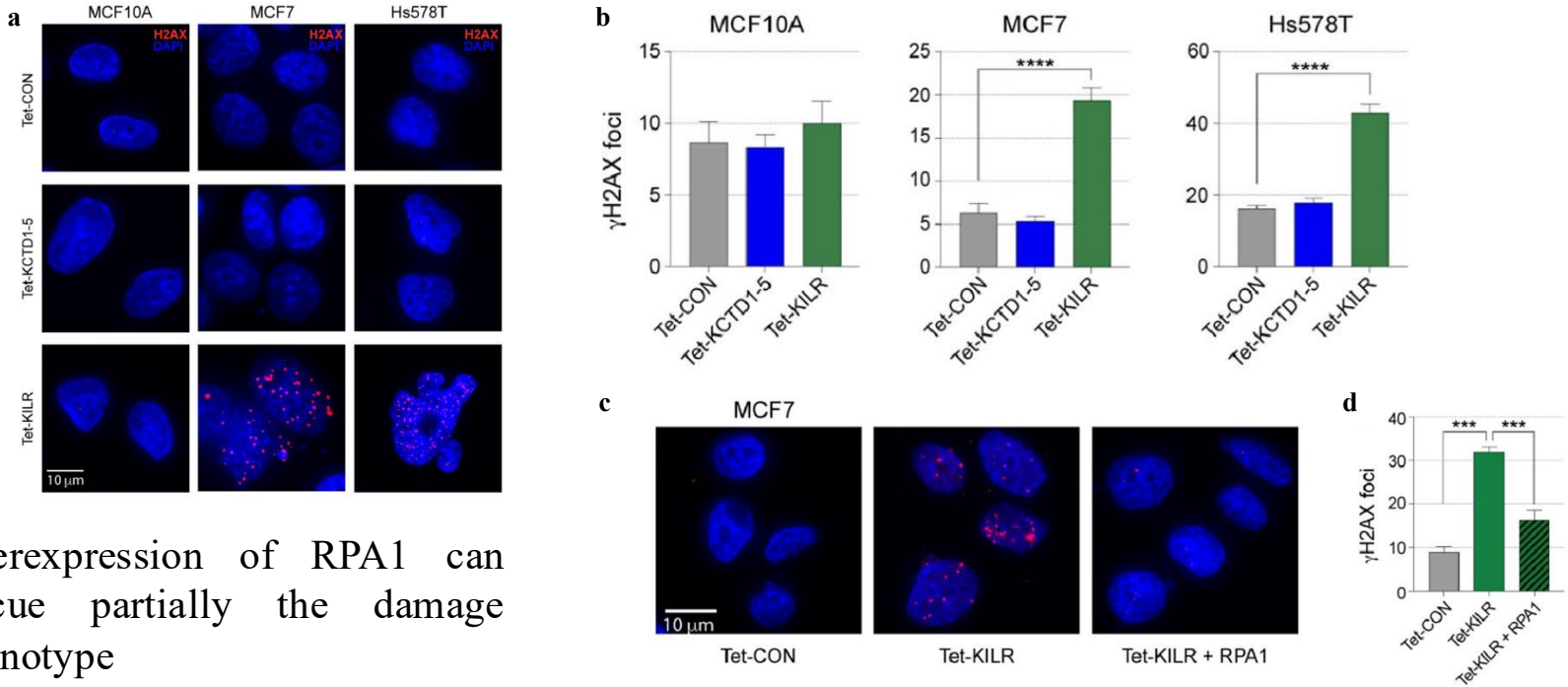
KILR overexpression but not CTDC1-5 **cause degradations of nascent** DNA (under hydroxyurea-induced replications stress)

→ Indicating Forck replication stress

Fig.21 A) Left panels: Representative images of DNA fibers in MCF7 cells after Cas13d-KILR knockdown with two independent crRNAs (crKILR1-2) then labelling with CldU and IdU. Right panel: Replication fork speed was calculated by length of track/time of CIdU pulse. Data are presented from two independent fiber assays. **B-C)** Left panels: Representative images of DNA fibers in MCF7 (b) and Hs578T (c) cells after doxycycline induction of KCTD1-5 or KILR, labelling with CldU and IdU then treatment with 4 mM HU for 4 h. Right panels: Ratio of IdU/CldU. Data are presented from two independent fiber assays.

KILR mediate H2AX phosphorylation

KILR overexpression **increase the DNA damage** by H2AX phosphorylation into **γH2AX**

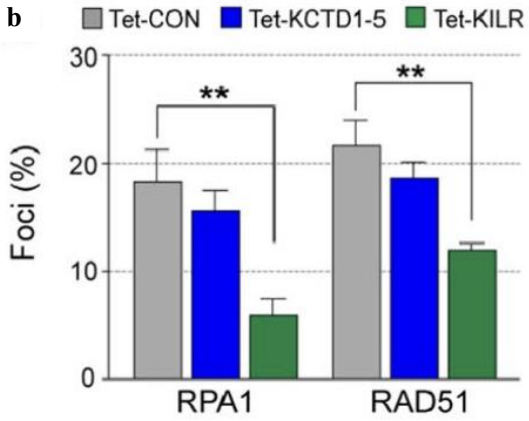


Overexpression of RPA1 can rescue partially the damage phenotype

Fig.22 **A)** Representative confocal microscopy images of breast cells after doxycycline induction of ectopic KCTD1-5 or KILR expression immunostained with anti-γH2AX (red). The Tet-CON represents an empty vector control. Nuclei were stained with DAPI (blue). **B)** Quantification of γH2AX foci in three breast cell lines. A cell with > 10 distinct γH2AX foci in the nucleus was considered as positive. **C)** Representative confocal microscopy images of MCF7 cells after doxycycline induction of ectopic KILR expression with or without RPA1 overexpression immunostained with anti-γH2AX (red). The Tet-CON represents an empty vector control. Nuclei were stained with DAPI (blue). **D)** Quantification of γH2AX foci in MCF7 cells. A cell with > 10 distinct γH2AX foci in the nucleus was considered as positive.

KILR inhibits HR

RPA1 cover an important **rule in Homologues Recombination Repair (HRR)** in DSBs. RPA1 is subsequently replace by RAD51 at ssDNA in response to DNA damage.



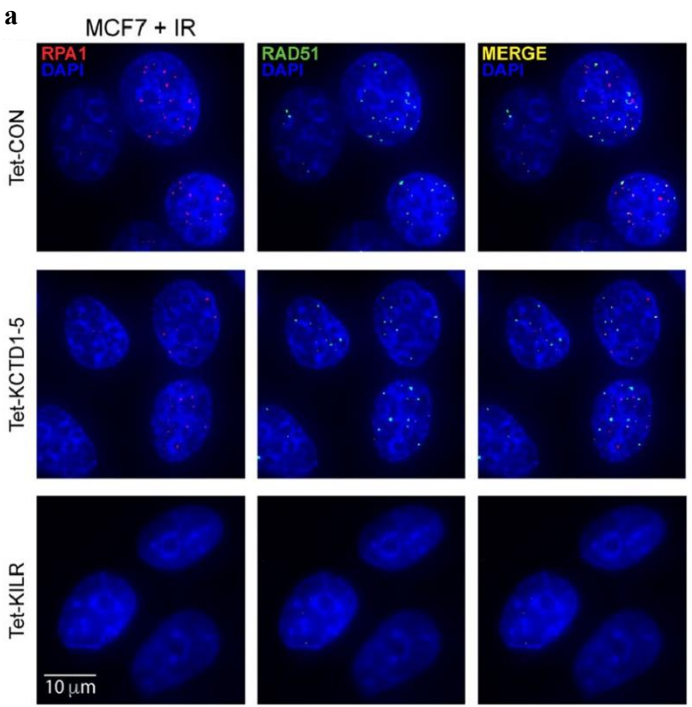
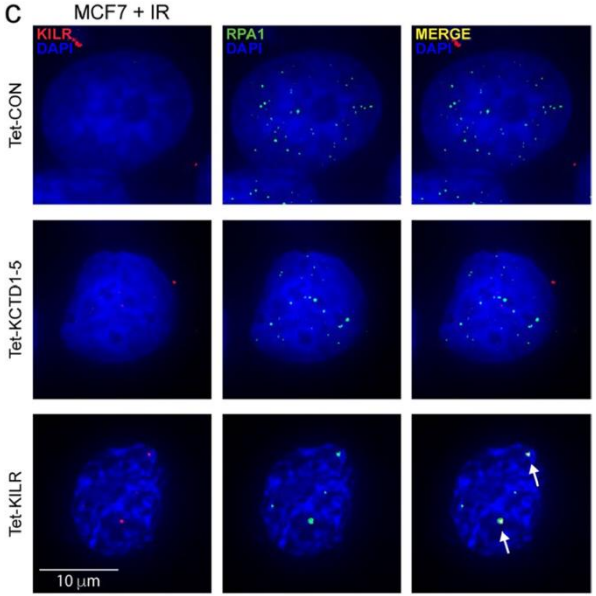


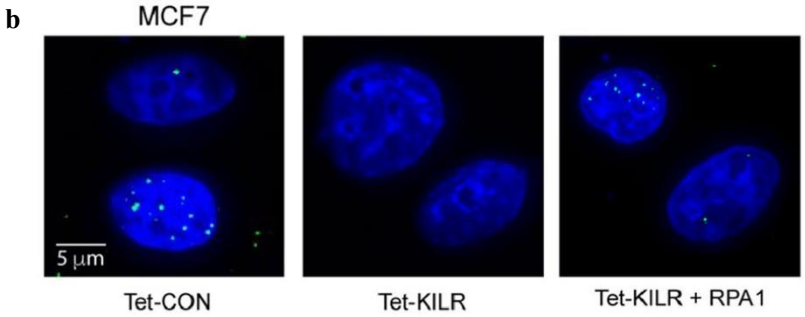
Fig.23 **A)** Representative confocal microscopy images of RPA1 and RAD51 in MCF7 cells after doxycycline induction of ectopic KCTD1-5 or KILR expression and exposure to 6-Gy IR (n = 3). 6 h post-IR, cells were immunostained with anti-RPA1 (red) and anti-RAD51 (green). The Tet-CON represents an empty vector control. Nuclei were stained with DAPI (blue). **B)** Quantification of RPA1 or RAD51 foci in MCF7 cells. A cell with > 5 distinct RPA1 or RAD51 foci in the nucleus was considered as positive.

Impaired RAD51 recruitment



Overexpression of **KILR** but not KCTD1-5 **inhibits the RPA1 and RAD51 recruitment** to DBSs in breast cancer.
→ This effect is mediated by the sequestration of RPA1 into nuclear puncta

Overexpression of RPA1 rescue partially the RAD51 recruitment defect after KILR OE



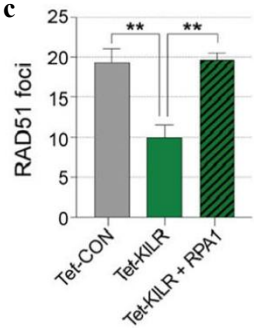


Fig.24 **A)** Representative confocal microscopy images of KILR and RPA1 in MCF7 cells after doxycycline induction of ectopic KCTD1-5 or KILR expression and exposure to 6-Gy IR (n = 3). 6 h post-IR, cells were stained with Stellaris KILR RNA FISH probes (red) and immunostained with anti RPA1 (green). The Tet-CON represents an empty vector control. Nuclei were stained with DAPI (blue). White arrows highlight KILR/RPA1 co-localization. Scale bar, 10 µm. **B)** Representative confocal microscopy images of MCF7 cells after doxycycline induction of ectopic KILR expression with or without RPA1 overexpression and exposure to 6-Gy IR (n = 3). 6 h post-IR, cells were immunostained with anti-RAD51 (green). The Tet-CON represents an empty vector control. Nuclei were stained with DAPI (blue). Scale bar, 5 µm. **C)** Quantification of RAD51 foci in MCF7 cells. A cell with > 5 distinct RAD51 foci in the nucleus was considered as positive.

Impaired RAD51 recruitment

KO of KILR result in fact in an opposite effect in breast cancer cells, resulting in a more efficient DNA damage repair

The same are not observed in Cas13d-control cels where the number of DBSs remain constant

Indicate that **HRR is more efficient in the absence of KILR**, likely due to an increase in the pool of available RPA1

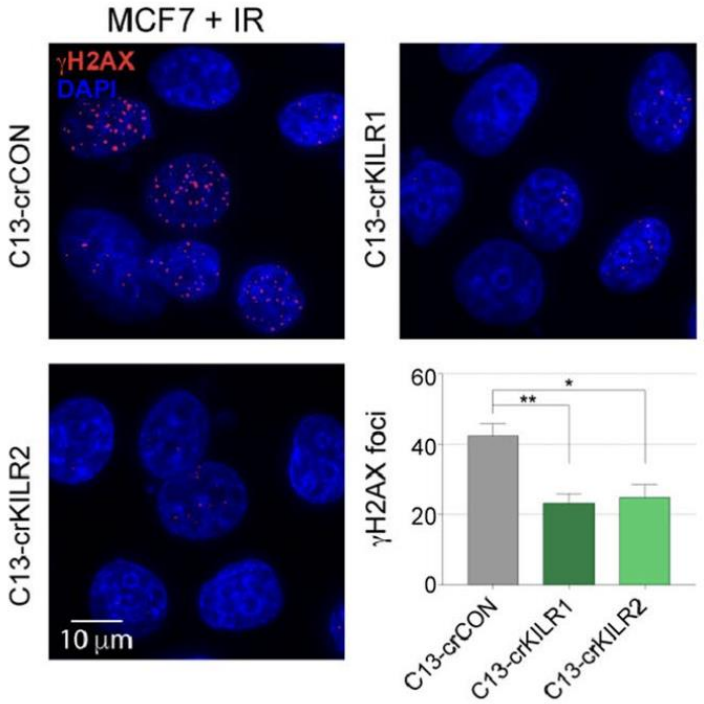


Fig.25 Representative confocal microscopy images of γH2AX in MCF7 cells after Cas13d-KILR knockdown with two independent crRNAs (crKILR1-2) and exposure to 6-Gy IR (n = 3). 6 h post-IR, cells were immunostained with anti-γH2AX (red). The crCON contains a non-targeting control. Nuclei were stained with DAPI (blue). Scale bar, 10 µm. Quantification of γH2AX foci in MCF7 cells. A cell with > 10 distinct γH2AX foci in the nucleus was considered as positive.

KILR mechanism key concept

KILR Overexpression

- RPA1 is trapped
- RPA and RAD51 cannot be recruited to DNA breaks
- Homologous recombinations is impaired

KO of KILR

- RPA pool is freed
- RPA and RAD51 is efficiently assembled in DBSs
- DNA repair accelerate
- HRR become significantly more significative

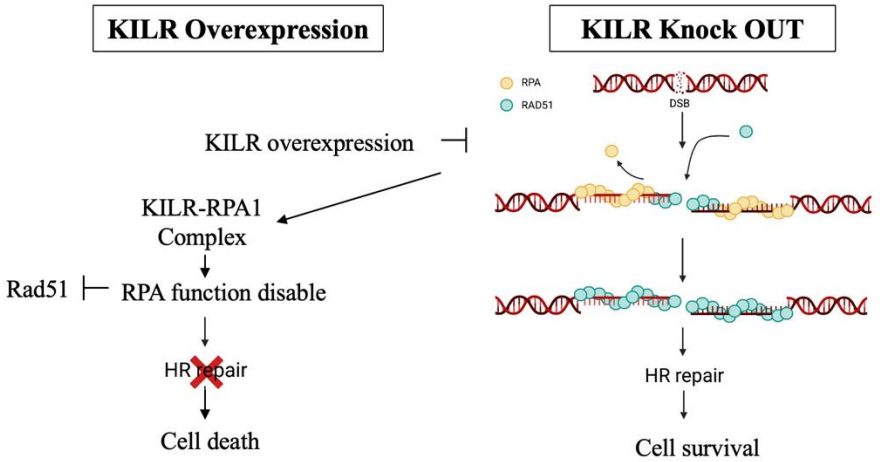


Fig.26 KILR mechanism between overexpression and Knock Down CRISPRa mediated

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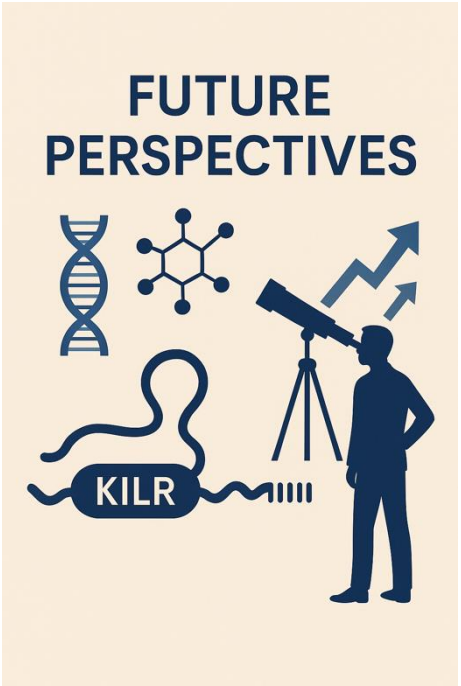
Overall Conclusions

For KILR, GWAS **risk variants destabilize** the transcript and **reduce** its expression, providing a new mechanism by which noncoding variants can **influence cancer risk**.

Reduced KILR **increases** the pool of free RPA1, accelerates DNA replication, and **promotes** breast cancer cell proliferation.

KILR overexpression in breast cancer cells **induces apoptosis** by sequestering RPA1 in **nuclear foci**, impairing DNA replication and repair, suggesting KILR as a **potential novel RPA inhibitor or therapeutic tool**.

Future perspectives



Based on what we have seen in this presentation, future antitumor strategies could aim to increase the level of KILR in breast cancer cells through:

1) **Administration of molecules similar to KILR**

2) **Activation of the endogenous KILR locus**

3) **Enhancement of KILR activity in cancer cells**

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Discussion

Limitations & Challenges of CRISPR/Cas13d

Cas13d screens

- **Off-targeting effects** from crRNAs binding unintended genomic regions.
- **Collateral activity**, where Cas13d **promiscuously cleaves bystander RNAs.**
- Often **ineffective for lncRNAs.**

KILR Controls DNA replication & stability

KILR overexpression

KILR overexpression **sequesters RPA1** (phenocopying RPA1 knockdown) and leading to:

- **Replication stress**
- **Spontaneous DSBs**
- **Apoptosis**.

KILR KO

Reduce KILR expression **increases RPA1** availability:

- **Accelerating replication fork** progression
- **Promotes** breast cancer cell **proliferation**

RPA1 levels are clinically relevant:

1. High RPA1 → tumor aggressiveness & poor survival.
2. Partial RPA1 loss → hypersensitivity to DNA damage.

KILR behaves like an **endogenous RPA inhibitor**, suggesting potential therapeutic applications.

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Thanks for your attention

Zamboni Matteo & Balzan Riccardo

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Questions?

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Supplementary slides

Zamboni Matteo & Balzan Riccardo

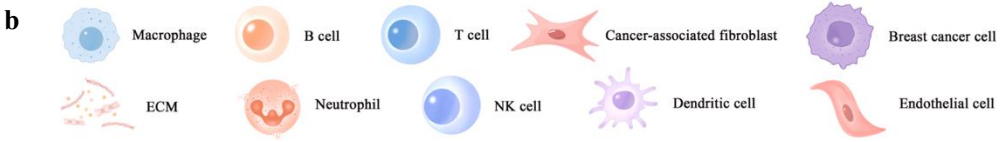
Tumor Microenvironment

The **TME** is a **complex ecosystem** including:

- tumor cells,
- fibroblasts,
- immune cells,
- endothelial cells,
- extracellular matrix.

All dynamically interacting

Cells within the TME can either inhibit or promote tumor progression depending on tumor type and stage.



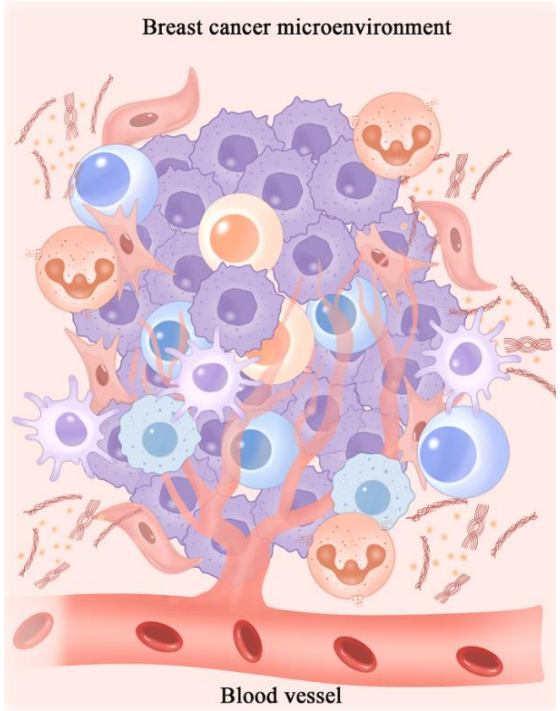


Fig.27 The role of lncRNAs in the breast cancer microenvironment. A-B)The breast cancer microenvironment includes breast cancer cells, cancer-associated fibroblasts, endothelial cells, immune cells, and blood vessels.

Functional Rules lncRNAs in breast cancer TME

Building on their diverse regulatory functions, **lncRNAs orchestrate complex interactions among tumor**, stromal and immune cells within the TME, shaping tumor behavior and therapy response.

Tumor cell-derived lncRNAs	CAF-derived lncRNAs	Immune cells-derived lncRNAs
↑ Proliferations, migrations, EMT drug resistance	↑ Angiogenesis, glycolysis, metastasis through paracrine signaling	T-cell regulate immuno balance: • *SNHG1* promotes **Treg differentiation** • *NKILA* induces **CD8⁺ T-cell apoptosis**
↑ CD8+ T-cell infiltration supporting anti-tumor immunity	Influence tumor metabolism and immunotherapy response	Macrophage polarizations: • *IRENA, SNHG1, GNAS-AS1* → **M2** • *GAS5, HOTTIP* → **M1**
Modulate fibroblast activation and immune cell polarization		B-cell & NK-cell modulate immune-activations and cytotoxicity (XIST, MIAT, NCAL1)

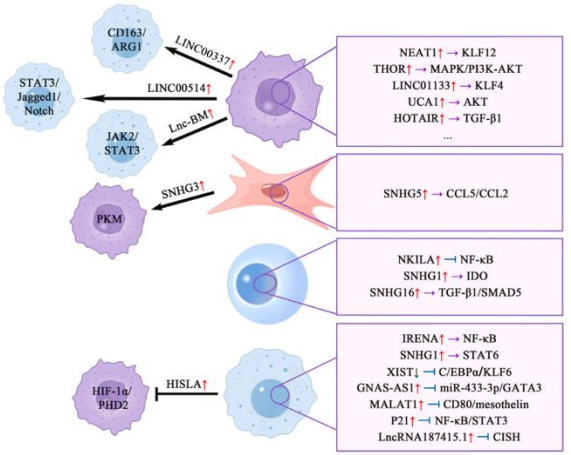


Fig.28 LncRNAs can regulate the phenotype and function of breast cancer cells, cancer-associated fibroblasts, T cells and macrophages in the breast cancer microenvironment by activating or inhibiting downstream signaling and the expression of certain proteins. ↑: upregulated; ↓: downregulated; →: activate; ↑: inhibit.

NEAT1 Rules

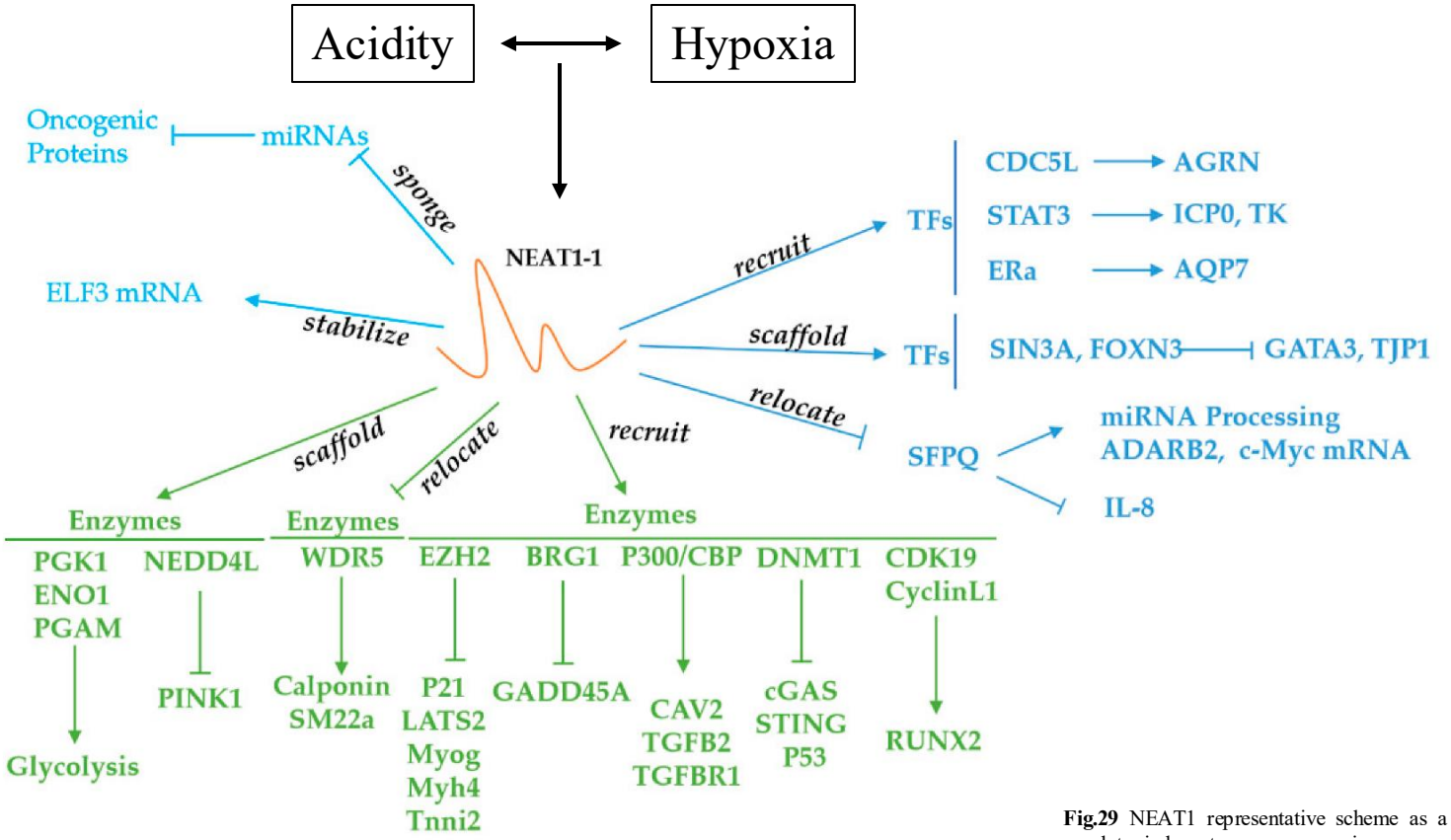


Fig.29 NEAT1 representative scheme as a key regulator in breast cancer progression

Extra

New possibility using Cas-12 based CRISPR system

CRISPR/Cas12 belong to the Class 2, Type V CRISPR-Cas systems. Under the guidance of a crRNA, Cas12 recognizes complementary target dsDNA with a specific PAM sequence and mediates DNA cleavage through a single RuvC structural domain.

Compared with Cas9:

- Cas12 expands the range by recognizing different PAM sequences;
- Can act at several distinct genomic loci;
- Has a non-specific ssDNA cleavage activity after activation trough a trans-clevage activity;
- It generate sticky ends;
- In specific engineered configurations can introduce single-strand nick.

Cas12a (Cpf1) is one of the best-characterized effectors; it controls target genes by recognizing thymine-rich PAM sequences and shows higher sensitivity to mismatches in the guide RNA than Cas9.

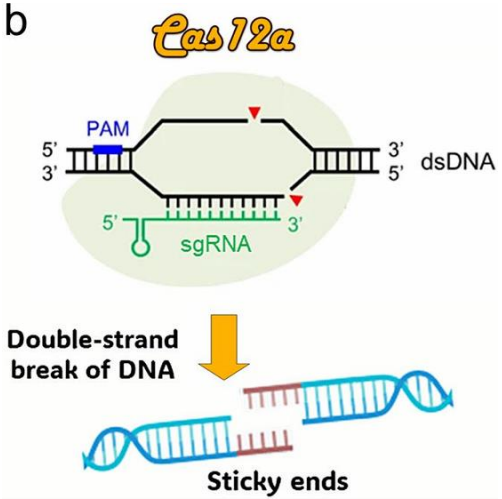


Fig.30 CAS12 scheme

New possibility using Cas-12 based CRISPR system

- The activity of Cas12 is advantageous for certain applications in oncology like:
  - Gene editing;
  - Targeted therapies;
  - Synthetic lethality approaches;
  - Cancer diagnostic.

- The CRISPR/Cas12 system has also been utilized in DETECTR, a diagnostic platform developed for the rapid and accurate detection of specific nucleic acid sequences. It has demonstrated great potential in various applications, such as infectious disease diagnostics, genetic testing, and cancer diagnostics.

  The main feature of DETECR assay are:
  - Single amplification;
  - Specificity for target sequence and programmable specificity;
  - Speed and simplicity;
  - Versatility;
  - Monitoring disease progression and treatment response.

Extra

Breast cancer eQTL lncRNAs alter cell proliferation

Seven breast cancer–associated lncRNAs in the screen were known eQTLs for risk variants.

Four were hits by the screen; **two** (XLOC209276, XLOC022678) **lost significance** after crRNA filtering (FDR $\leq$ 0.3).

Knockdown of XLOC112072 and XLOC169717 **increased proliferation** in MCF7 cells.

Based on its proliferative effect, lack of off-targets, and genetic link to breast cancer, XLOC112072 (KILR; KCTD1 Intronic lncRNA) was **prioritized** for functional studies.

Extra

KILR is a sense intronic lncRNA

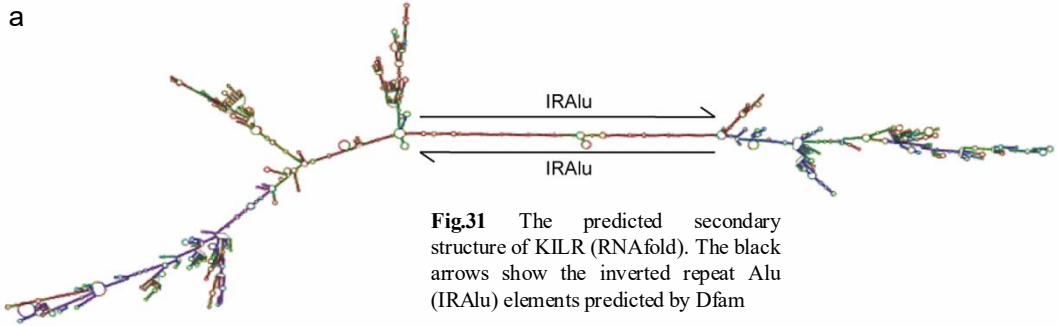


Fig.31 The predicted secondary structure of KILR (RNAfold). The black arrows show the inverted repeat Alu (IRAlu) elements predicted by Dfam

KILR forms a **long double-stranded RNA** region through **pairing** of its **IRAlu elements**, giving it the typical structure of IRAlu-containing lncRNAs.

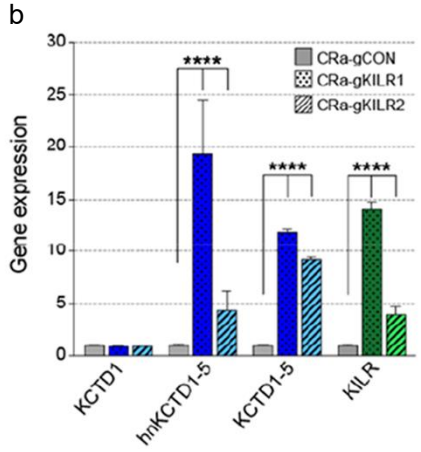


Fig.32 qPCR for KCTD1, KCTD1-5 and KILR expression in T47D cells after CRISPRa activation of the KCTD1-5 promoter to overexpress KILR with two independent gRNAs (CRa-gKILR1-2). The CRa-gCON contains a non-targeting control.

The 5′ end of the KILR transcript does not overlap histone marks typical of active promoters.

CRISPRa activation of the KCTD1-5 promoter **increased expression** of both KCTD1-5 and KILR.

KCTD1 expression was **not affected**, indicating that KILR is generated specifically from the KCTD1-5 promoter.

Extra

Breast cancer risk variants at 18q11 reduce the half-life

Capture **Hi-C** data show that GWAS Signal 1 and Signal 2 at 18q11 **physically interact** with the KCTD1-5/KILR promoter via **chromatin looping**.

These interactions suggest that additional 18q11 GWAS signals may **regulate KILR** (and KCTD1-5) **expression**.

Overall, the data support KILR as a target gene of breast cancer GWAS signals at 18q11.

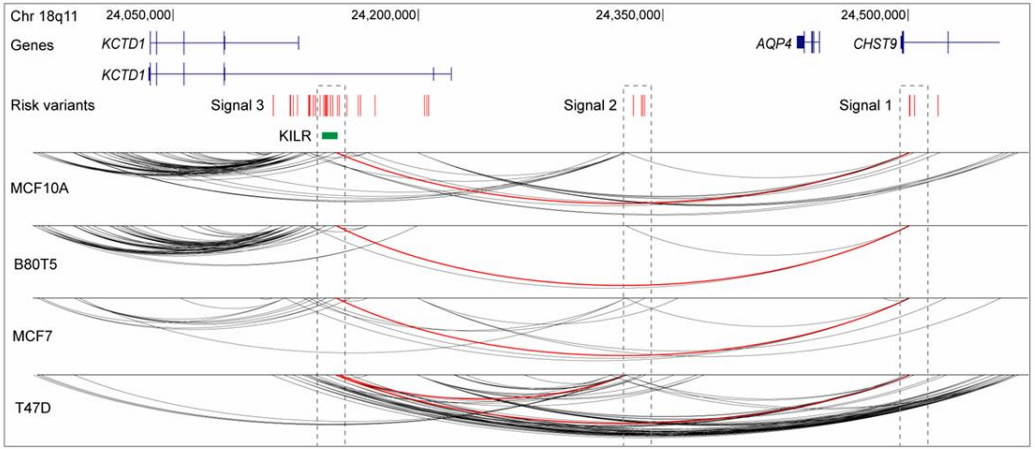


Fig.33 Variant capture Hi-C chromatin interactions in breast cell lines are shown as arcs. The dashed gray outlines and red arcs highlight chromatin interactions between risk variants and KILR in MCF10A, B80T5, MCF7 and T47D cell lines.

KILR Controls DNA replication & stability

Most breast cancer GWAS variants map to **noncoding regions**, often within **lncRNA exons**, but there is still limited functional evidence linking lncRNAs to disease risk.

We identified 43 lncRNAs whose knockdown **alters breast cell proliferation**; among them, KILR is an unannotated **sense intronic lncRNA** within a KCTD1-5 intron, transcribed from an alternative KCTD1 promoter.

KILR has a post-transcriptionally generated 5′ end, likely **stabilized** by an internal snoRNA structure (like a **SPA-lncRNA–like**), and a 3′ poly(A) tail, and it contains several predicted snoRNAs in its sequence.

Complex secondary structures at both ends, together with snoRNA and poly(A) features, are thought to **protect** KILR from **exonuclease-mediated degradation**.

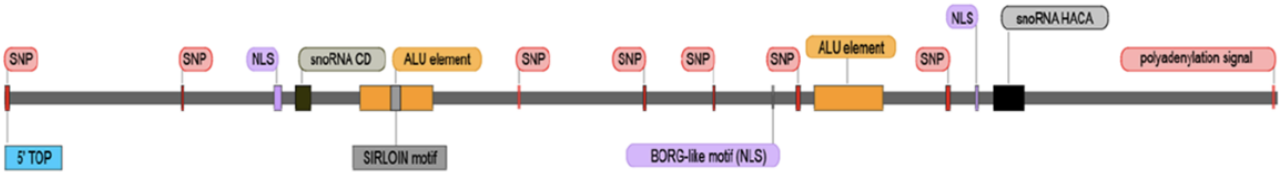


Fig.34 A linear schema of KILR.

DNA Fiber Assay

Understanding the **mechanisms of replication stress response** following genotoxic stress induction is rapidly emerging as a **central theme in cell survival** and human disease.

DNA fiber assay is one of the most **powerful tools** to study **alterations in replication fork dynamics** genome-wide at single-molecule resolution.

This approach **relies on the ability** of many organisms to **incorporate thymidine analogues into replicating DNA** and is widely used to study how genotoxic agents perturb DNA replication.

$$\text{Fork speed} = \frac{lenght\ trak}{time\ of\ staining}$$

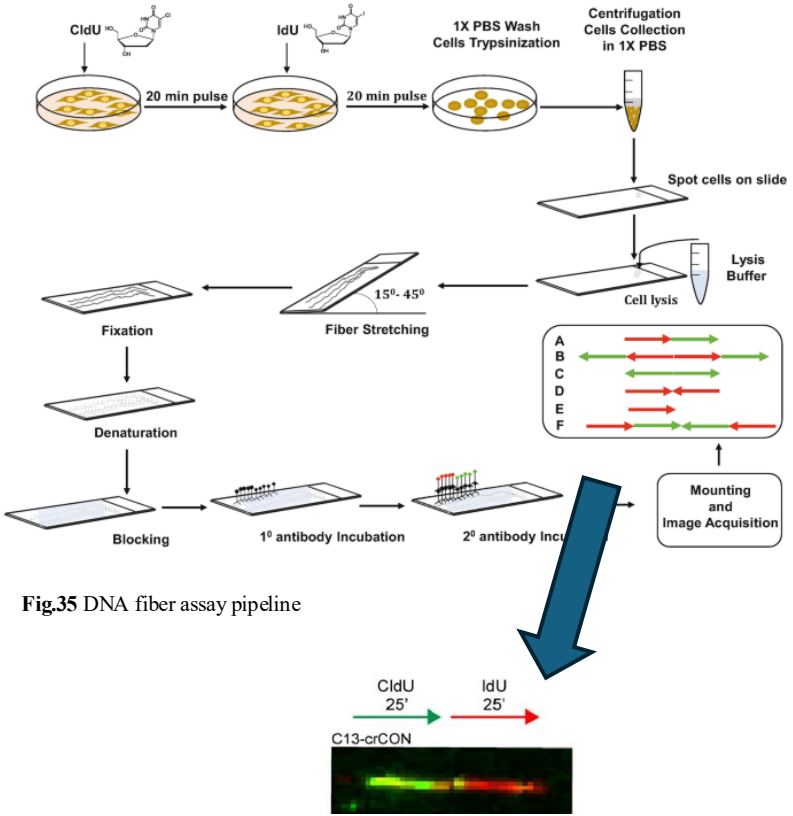


Fig.35 DNA fiber assay pipeline

KILR mechanism key concept

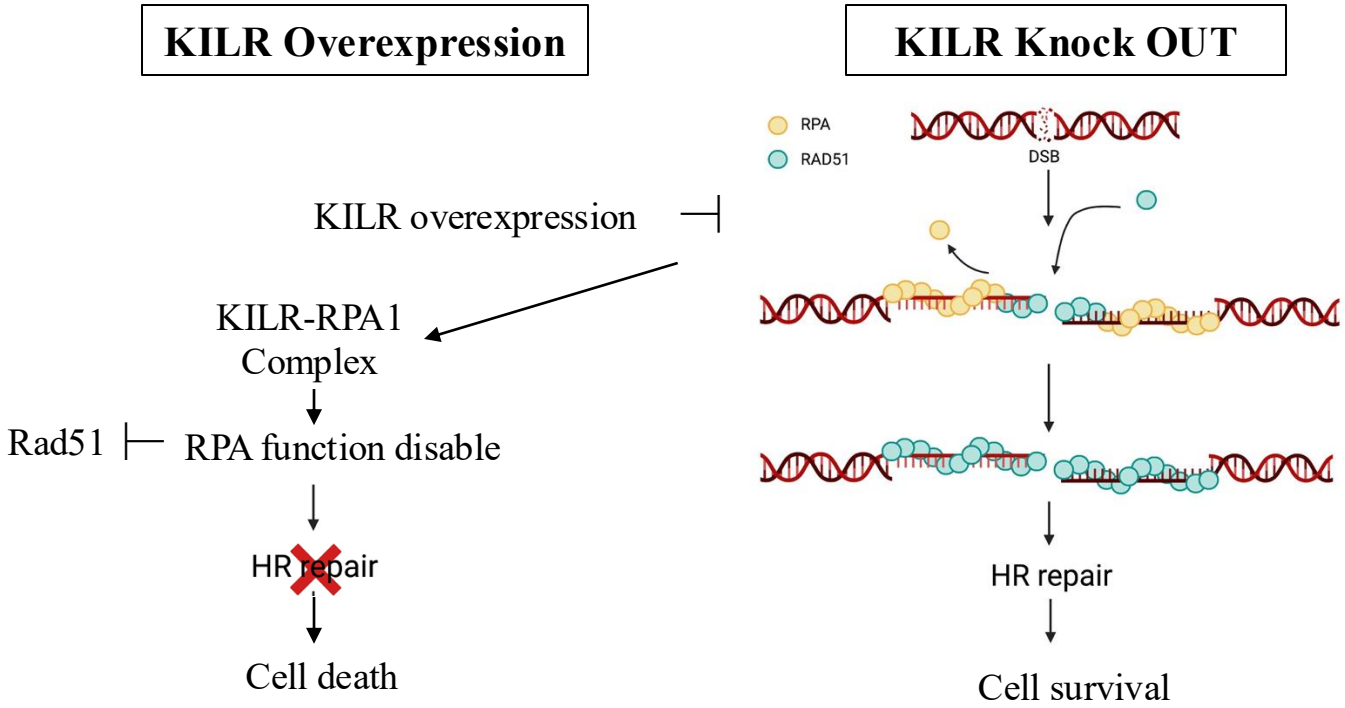


Fig.36 KILR mechanism between overexpression and Knock Down CRISPRa mediated

Extra

What challenges must be overcome?

To move **SHERLOCK** into clinical oncology we have to overcome some issues:

- **Clinical trials** and **validation studies** are needed to standardize SHERLOCK for cancer diagnostics and to ensure its reliability across diverse patient populations.

- For CRISPR/Cas13 system we have other **challenges** to overcome like:
  - Delivery mechanism
  - Off target effect
  - Long terminal effect and stability
  - Immunogenicity
  - Patient selection and genetic diversity

Extra

Limitations & Challenges of CRISPR/Cas13d Screens

High-throughput pooled CRISPR screens **enable unbiased identification** of protein-coding and noncoding genes involved in biological processes.

CRISPR–Cas9 screens work well for protein-coding genes but are often **ineffective for lncRNAs**, because the effect of Cas9-induced indels on lncRNA function is hard to predict.

CRISPRi screens can identify functional lncRNAs, but many are enhancer-derived, so phenotypes may **reflect enhancer repression** rather than loss of the RNA.

Additional experiments are needed to distinguish DNA-mediated effects from those driven by the lncRNA transcript.

Extra

Limitations & Challenges of CRISPR/Cas13d Screens

We used Cas13d RNA knockdown screens to find **lncRNAs linked to breast cancer** and to improve crRNA design.

To **reduce off-target effects**, we removed crRNAs that could also bind other, unwanted regions, or we can also use **high fidelity can proteins**.

Cas13d can also present **collateral activity**, which is a limitation of this system.

To **avoid false results**, we re-tested the key lncRNAs one by one using several independent methods.

Extra

KILR as a selective cancer vulnerability

Why we observe that KILR kills cancer cell and not normal cells?

Cancer cells

Cancer cells have **chronic replication stress** → rely heavily on free RPA1.
KILR overexpression **exhausts RPA1**, leading to:

- Unprotected ssDNA
- Massive DSB accumulation
- **Replication catastrophe → apoptosis**

Normal Cells

Normal cells retain a **free RPA1 pool**, preserving replication & repair even with high KILR.

Therapeutic Implications

1. KILR behaves like an **RNA-based RPA inhibitor**.
2. Could complement or replace **synthetic RPA inhibitors (RPAis)**.
3. Potential combination therapy with:
   - Platinum-based chemo
   - HR-directed treatments (PARPi)