

The long noncoding RNA Inc-FANCI-2 intrinsically restricts RAS signaling in human papillomavirus type 16-infected cervical cancer cells

Haibin Liu^{1†}, Lulu Yu^{1†}, Vladimir Majerciak¹, Thomas J Meyer², Ming Yi³, Peter F Johnson⁴, Maggie Cam², Douglas R Lowy⁵, Zhi-Ming Zheng^{1*}

¹Tumor Virus RNA Biology Section, HIV Dynamics and Replication Program, CCR/NCI/NIH, Frederick, United States; ²Collaborative Bioinformatics Resource, CCR/NCI/NIH, Bethesda, United States; ³NCI RAS Initiative, Cancer Research Technology Program, Frederick National Laboratory for Cancer Research, Frederick, United States; ⁴Mouse Cancer Genetics Program, CCR/NCI/NIH, Frederick, United States; ⁵Laboratory of Cellular Oncology, CCR/NCI/NIH, Bethesda, United States

*For correspondence: zhengt@exchange.nih.gov

†These authors contributed equally to this work

Present address: [†]Center for Emerging Infectious Diseases, Wuhan Institute of Virology, and Center for Biosafety Mega-Science, Chinese Academy of Sciences, Wuhan, Hubei, China

Competing interest: The authors declare that no competing interests exist.

Funding: See page 28

Preprint posted

21 August 2024

Sent for Review

02 September 2024

Reviewed preprint posted

28 November 2024

Reviewed preprint revised

07 July 2025

Version of Record published

29 August 2025

Reviewing Editor: Chang Liu, Johns Hopkins University, Baltimore, United States

© This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the [Creative Commons CC0 public domain dedication](https://creativecommons.org/licenses/by/4.0/).

eLife Assessment

This study reports **important** new insights into the roles of a long noncoding RNA, Inc-FANCI-2, in the progression of cervical cancer induced by a type of human papillomavirus. Through a blend of cell biological, biochemical, and genetic analyses of RNA and protein expression, protein-protein interaction, cell signaling, and cell morphology, the authors provide **convincing** evidence that Inc-FANCI-2 affects cervical cancer outcome by regulating the RAS signaling pathway. These findings will be of interest to scientists in the fields of cervical cancer, long noncoding RNA, and cell signaling.

Abstract Increased expression of Inc-FANCI-2, a newly discovered long noncoding RNA, is associated with cervical lesion progression from cervical intraepithelial neoplasia stage 1 (CIN1, low grade), CIN2–3 (high grade), to cervical cancer. Viral oncoprotein E7 of high-risk human papillomaviruses (HR-HPVs) and host transcription factor YY1 are two major factors promoting Inc-FANCI-2 expression. Using CRISPR-Cas9 technology, we knocked out the expression of Inc-FANCI-2 in the HPV16-positive cervical cancer cell line, CaSki cells. The selected knockout (KO) single-cell clones displayed altered cell morphology and proliferation with changes of cellular soluble receptors, but normal HPV16 E6 and E7 expression. Relative to the parental cells, Inc-FANCI-2 KO cells exhibited significantly increased RAS signaling and epithelial-mesenchymal transition, but decreased response to IFN signaling, along with increased p-Akt and p-Erk1/2 (two RAS signaling effectors), IGFBP3, MCAM, VIM, and CCND2 (cyclin D2) and decreased expression of RAC3. Inc-FANCI-2 in CaSki interacts with cellular proteins H13, HNRH1, K1H1, MAP4K4, and RNPS1. MAP4K4 knockdown led to enhance the expression of p-Erk1/2 and p-Akt. High Inc-FANCI-2 and low MCAM levels in cervical cancer tissues were found to be associated with patients' survival. A key function of Inc-FANCI-2 intrinsically regulates RAS signaling to impact cervical lesion progression and cervical cancer prognosis.

Introduction

Cervical cancer is a leading cause of mortality in women worldwide. It is the second most diagnosed cancer among women, with an estimated 661,000 new cases worldwide each year, and the third most frequent cause of cancer-related death, accounting for 348,000 deaths annually (Bray et al., 2024). Persistent infections in the cervical epithelium with the cancer-associated alpha human papillomaviruses (high-risk HPVs [HR-HPVs]), in particular HPV16 and HPV18 with the sustained expression of viral oncoproteins, E6 and E7, contribute to squamous epithelial cell tumorigenesis (Bosch et al., 2002; Munoz et al., 2003; Walboomers et al., 1999). Approximately 31.3% of high-grade neoplastic cervical lesions progress to invasive cervical cancer (ICC) in 30 years (McCredie et al., 2008), while most lesions resolve spontaneously. The progression to cervical cancer typically takes 15–20 years after initial infection (<https://www.who.int/news-room/fact-sheets/detail/cervical-cancer>). These observations suggest that active host defense systems play important roles in preventing malignant transformation of neoplastic cervical lesions, and additional factors besides E6 and E7 are necessary for cervical cancer development. Indeed, one tumor suppressor network, the Fanconi anemia (FA) pathway (Niraj et al., 2019), can be activated by HPV-induced DNA damage. FA proteins, including FANCA, FANCD2, and FANCI, are elevated upon HPV infection (Hoskins et al., 2009; Liu et al., 2021; Santegoets et al., 2012), and the activated FA pathway restricts HPV replication and guards host genome stability (Hoskins et al., 2012). FANCD2 mutation stimulates HPV16 and HPV31 genome amplification and also promotes cervical and vaginal cancer development in HPV16 E7 transgenic mouse (Park et al., 2010; Park et al., 2014).

HPV-positive cervical cancer tissues in general exhibit wild-type (WT) p53, WT K-RAS, and no overexpression of RAS genes (Hietanen et al., 2000; Tommasino et al., 2003; Prior et al., 2012; Fernández-Medarde and Santos, 2011). Viral oncoprotein E7 plays a major role in the immortalization of primary epithelial cells mainly by inactivation of pRb family of proteins (Roman and Munger, 2013), whereas E6 mediates degradation of tumor suppressor p53 and activation of human telomerase reverse transcriptase transcription (Vande Pol and Klingelutz, 2013; Liu et al., 2009; Klingelutz et al., 1996). However, high-risk E6 and E7 are necessary but not sufficient to transform fully the immortalized cells into malignant cells (Storey et al., 1988; Barbosa and Schlegel, 1989; Hawley-Nelson et al., 1989; Münger et al., 1989; Halbert et al., 1991), and additional oncogenic stress is needed. For example, the HRAS^{G12V}, a constitutively activated RAS GTPase mutation, triggers malignant transformation of E6/E7-expressing keratinocytes (Phelps et al., 1988; DiPaolo et al., 1989; Schreiber et al., 2004; Narisawa-Saito et al., 2008). Normal RAS regulates cell proliferation, cell differentiation, and cell adhesion through cellular signal transduction from growth factor receptors such as insulin-like growth factors (IGFs) and insulin-like growth factor binding proteins (IGFBP1–6) (Conover et al., 2000; Allard and Duan, 2018; Bach, 2018). Thus, overactive RAS signaling might facilitate transformation of E6/E7-expressing keratinocytes. In transgenic mice, HPV16 E6 and E7 can promote the development of spontaneous epithelial skin tumors, but not spontaneous tumors of the reproductive tract (Song et al., 1999; Herber et al., 1996; Song et al., 2000; Riley et al., 2003). Prolonged estrogen treatment is required (Brake and Lambert, 2005). Estrogen receptor (ER) activation stimulates the mitogen-activated protein kinase (MAPK/Erk) and phosphoinositide 3-kinase (PI3K/Akt) pathways, both of which are mediated by RAS (Kato et al., 1995). However, the molecular mechanism underlying estrogen-induced cervical cancer in E6 and E7 transgene mice is not fully understood.

Long noncoding RNAs (lncRNAs) are RNAs over 200 nucleotides (nt) in length lacking a coding capacity for translation into functional proteins. Although several lncRNAs have been proposed to function in diverse biological processes, evidence is still lacking to support the functionality of the majority of lncRNAs (Statello et al., 2021; Liu and Zheng, 2022).

We recently reported an increased level of lnc-FANCI-2 expression in high-risk HPV-positive cervical intraepithelial neoplasia (CIN) and ICC tissues (Liu et al., 2021). At least 14 RNA isoforms of lnc-FANCI-2 are expressed by usage of two alternative promoters, alternative RNA splicing, and alternative selection of two polyadenylation sites from the genomic locus adjacent to the FANCI on Chr15. Ironically, the lnc-FANCI-2 was wrongly annotated by NCBI as *MIR9-3HG* in the recently updated RefSeq database (<https://www.ncbi.nlm.nih.gov/refseq/>) and two alternative lnc-FANCI-2 promoters identified for expression of lnc-FANCI-2 are ~10 kb downstream of the non-expressible *MIR9-3* gene (Liu et al., 2021). Both lnc-FANCI-2 and FANCI are upregulated simultaneously in neoplastic

cervical lesions and cervical cancer by HR-HPV infections. E6, and in particular, E7 is responsible for the enhanced expression of lnc-FANCI-2, the transcription of which is also regulated by YY1 (Liu *et al.*, 2021). HPV infection increases YY1 levels but decreases the expression of p53-dependent miR-29a which targets the YY1 3' UTR. Viral E7 interacts with YY1 and facilitates YY1 transactivation of lnc-FANCI-2 promoter (Liu *et al.*, 2021). In situ hybridization has revealed that lnc-FANCI-2 is preferentially cytoplasmic, but its function in HPV-infected cells has not been characterized. In this report, we demonstrate that lnc-FANCI-2 in HPV16-infected cells controls RAS signaling by interaction with MAP4K4 and other RNA-binding proteins. Ablation of lnc-FANCI-2 in the cells promotes RAS signaling and phosphorylation of Akt and Erk. High levels of lnc-FANCI-2 and low levels of MCAM expression in cervical cancer patients correlate with improved survival, indicating that lnc-FANCI-2 plays a critical role in regulating RAS signaling to affect cervical cancer progression and patient outcomes.

Results

Differential expression of lnc-FANCI-2 in cervical cancer tissues and its derived cell lines

As lnc-FANCI-2 expression is upregulated along with cervical lesion progression by HR-HPV infections (Liu *et al.*, 2021), we further verified the increased expression of lnc-FANCI-2 in cervical cancer tissues by RNAscope single-molecule RNA in situ hybridization (RNA-ISH) using an lnc-FANCI-2 antisense probe spanning over the major isoform of lnc-FANCI-2 nt 359–1713 region (GenBank MT669800.1). We found both cytoplasmic and nuclear locations of the increased lnc-FANCI-2 ISH signals within the tumor nest in HPV16-infected cervical squamous cell carcinoma tissues, but not much so in its adjacent normal tissue areas (Figure 1A/B). The increased expression of lnc-FANCI-2 became obvious in human foreskin keratinocytes with HPV16 or HPV18 infection when compared to the HFK cells without HPV infection (Figure 1C).

However, we subsequently demonstrated the high expression of lnc-FANCI-2 in two HPV16-infected cervical cancer cell lines (SiHa and CaSki) and two HPV16-infected low-grade cervical lesion-derived cervical keratinocyte subclone lines (W12 20,861 and W12 20,863 cells), but not in two HPV18-infected cervical cancer cell lines (HeLa and C4II cells), nor other HPV-negative cell lines of HCT116 (colorectal cancer cells), BCBL-1 (body cavity B lymphoma cells), HEK293 (Ad5 E1/E2-immortalized human kidney cells), and HaCaT (spontaneously immortalized human epidermal cells) (Figure 2A). Interestingly, the high expression of lnc-FANCI-2 was observed in an HPV-negative cervical cancer cell line C33A cells with mutations of both p53 and RB genes (Scheffner *et al.*, 1991; Figure 2A). Northern blot confirmed the increased expression of lnc-FANCI-2 in C33A cells but no expression in HeLa cells (Figure 2B).

Subcellular distribution of lnc-FANCI-2 was characterized by cell fractionation and western blot assays using nuclear SRSF3 and cytoplasmic GAPDH protein as an indication of fractionation efficiency (Figure 2C). By RT-qPCR analysis of the fractionated total RNA, we demonstrated that lnc-FANCI-2 RNA is mainly cytoplasmic in HPV16-positive CaSki cells, but nuclear in HPV16-positive SiHa and HPV-negative C33A cells (Figure 2C). The differential subcellular distributions of lnc-FANCI-2 RNA from CaSki to SiHa and C33A cells (Figure 2D) were further confirmed by RNAscope RNA-ISH using the lnc-FANCI-2 antisense probe described above.

lnc-FANCI-2 regulates proliferation of HPV-transformed cervical cancer cells

lnc-FANCI-2 RNA is transcribed mainly from a proximal promoter TSS2, but also from an alternative minor, distal promoter TSS1. Two highly conserved YY1 binding motifs upstream of the TSS2 are essential for the TSS2 transcriptional activity (Liu *et al.*, 2021; Figure 3A). To elucidate the function of lnc-FANCI-2 in high-risk HPV-infected cervical cancer cells, we knocked out lnc-FANCI-2 expression in HPV16-positive CaSki cells using CRISPR/Cas9 deletion of either a 3 kb promoter region encompassing both TSS1 and TSS2 or an 86 bp region containing two YY1-binding motifs in the TSS2 promoter (Figure 3A). Two tested guide RNAs (gRNAs) with high KO efficiency were selected and cloned into a modified CRISPR/Cas9 expression vector to express both 5'-specific gRNA and 3'-specific gRNA simultaneously for efficient genome editing (BeltCappellino *et al.*, 2019). CaSki cells stably transfected with the dual gRNA expression vector were generated. Through a serial dilution,

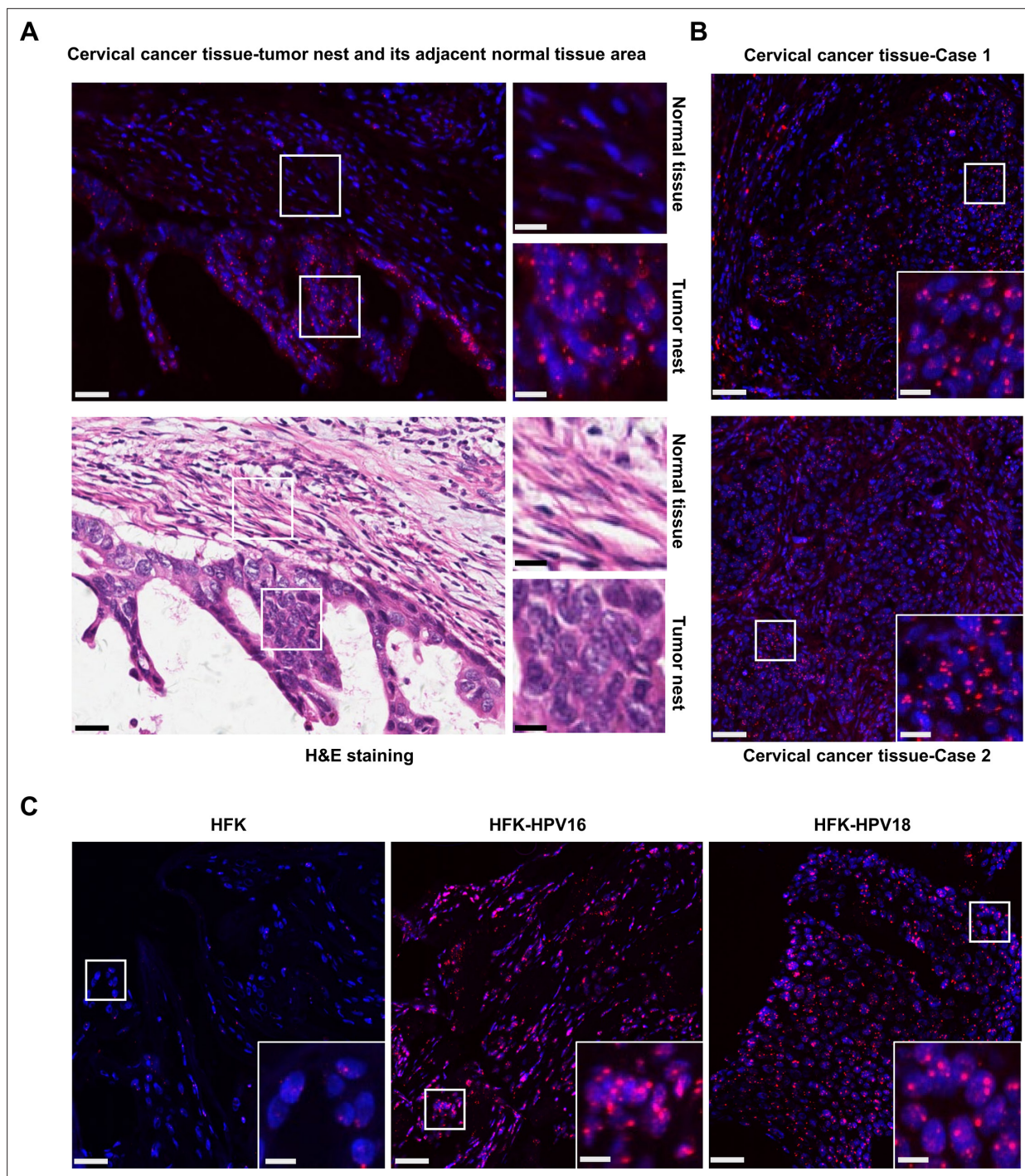


Figure 1. Increased expression of lnc-FANCI-2 in HPV16-infected cervical squamous cell carcinoma tissues and HPV16- and HPV18-infected raft culture tissues. **(A and B)** Expression of lnc-FANCI-2 (red) in HPV16⁺ cervical squamous cell carcinoma tissues was examined by RNAscope RNA in situ hybridization (RNA-ISH) analysis. Nuclei were stained with DAPI (blue). The corresponding regions **(A)** in H&E staining are shown for the adjacent region from the tumor nest and histotype. **(C)** Human foreskin keratinocyte (HFK)-derived raft cultures without (HFK) or with HPV16 or HPV18 infections (HFK-HPV16 or HFK-HPV18) were examined at day 10 for lnc-FANCI-2 RNA by RNAscope RNA-ISH analysis. Scale bars: 25 μ m in the original figures and 10 μ m in the zoomed insets.

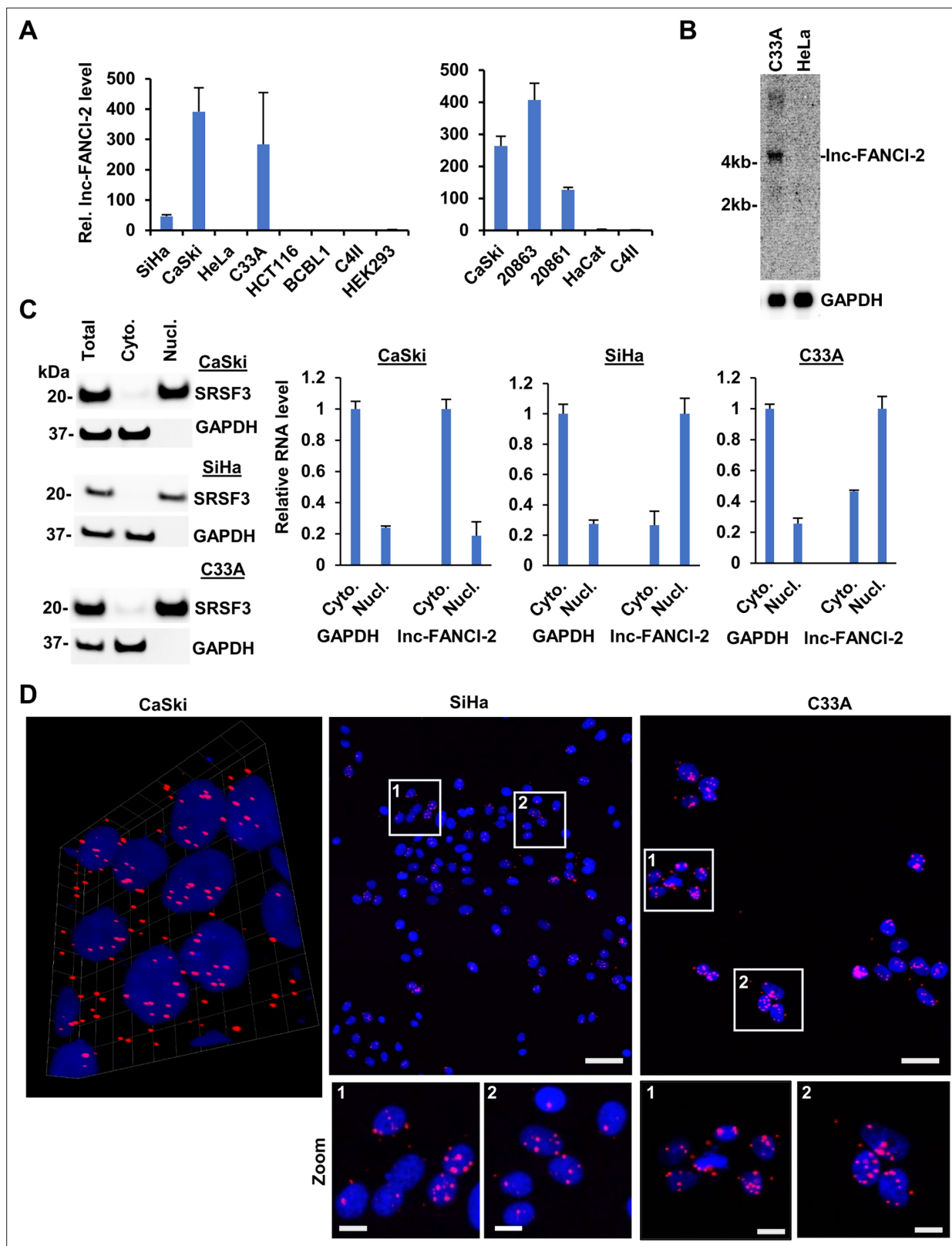


Figure 2. Selective expression of Inc-FANCI-2 RNA in cervical cancer cell lines. **(A)** RT-qPCR detection of Inc-FANCI-2 in HPV16⁺ cervical cancer cell lines SiHa, CaSki, and W12 20861 (integrated HPV16) and 20863 (episomal HPV16), HPV18⁺ cervical cancer cell lines HeLa and C4II, and HPV⁻ cell lines C33A (cervical cancer cells with mutations of p53 and pRb), HCT116 (colorectal cancer cells), BCBL-1 (body cavity B lymphoma cells), HEK293 (Ad5 E1/E2-immortalized human kidney cells), and HaCaT (spontaneously immortalized human epidermal cells) in triplicates. **(B)** HeLa cells express no Inc-FANCI-2

Figure 2 continued on next page

Figure 2 continued

when compared with C33A cells by northern blot. (C) Inc-FANCI-2 is mainly cytoplasmic in CaSki but nuclear in SiHa and C33A cells. Cytoplasmic and nuclear fractionation efficiency was blotted for nuclear SRSF3 (serine- and arginine-rich splicing factor 3) and cytoplasmic GAPDH. Total fractionated cytoplasmic and nuclear RNAs were quantified for Inc-FANCI-2 by RT-qPCR in triplicates, with GAPDH RNA serving as an internal control for RNA fractionation efficiency. (D) Subcellular Inc-FANCI-2 (red) localization in CaSki, SiHa, and C33A cells determined by RNAscope RNA in situ hybridization (RNA-ISH) analysis. Nuclei were stained with DAPI (blue). Scale bars: 25 μ m in the top and 10 μ m in the zoom.

The online version of this article includes the following source data for figure 2:

Source data 1. Northern blot and western blot data for **Figure 2**.

Source data 2. Northern blot and western blot data for **Figure 2**.

several single-cell clones were isolated and verified for homozygous deletion by PCR screening. PCR genotyping indicated successful homozygous deletion of the promoter or YY1-binding motifs, respectively (**Figure 3B**).

Loss of Inc-FANCI-2 expression in the single-cell clones was examined by RT-qPCR (**Figure 3C**). When compared to all control clones transfected and selected from an empty vector containing no gRNA, all single-cell clones with deletion of YY1-binding motifs (Δ YY1) showed 70–80% reduction of Inc-FANCI-2 expression, whereas the single-cell clones with deletion of both TSS1 and TSS2 promoters (Δ Pr) showed >90% reduction (**Figure 3C**). Northern blot analysis confirmed the decrease in levels of the two major Inc-FANCI-2 isoforms (**Figure 3D**), with an abundant 4 kb Inc-FANCI-2 RNA derived from a distal polyadenylation site pA2 and a less abundant 2 kb of Inc-FANCI-2 RNA derived from a proximal polyadenylation site pA1 (Liu et al., 2021; **Figure 3A and D**). Reduced Inc-FANCI-2 RNA expression was also confirmed by RNAscope RNA-ISH (**Figure 3E and F**). The parental (WT) CaSki cells expressed ~10 copies of Inc-FANCI-2 RNA per cell (Liu et al., 2021), whereas the copy number of Inc-FANCI-2 RNA in the Δ YY1-D5 cells dropped to ~5 copies and to ~2 copies per cell in the Δ Pr-A9 cells (each averaged from 200 cells, **Figure 3F**).

Although the WT CaSki cells preferentially grow as distinct cluster cell islands, the Inc-FANCI-2 KO cells displayed a dispersed cell growth pattern, and some cells exhibited an irregular or spindle-like cell morphology (**Figure 3—figure supplement 1**). The KO cells also grew slower than the parental CaSki cells (**Figure 3G**), formed fewer colonies with reduced size (**Figure 3H**), and showed decreased migration capacities (**Figure 3I**), with Δ Pr-A9 cells having a more severe phenotype than Δ YY1-D5 cells. Subsequently, the Δ Pr-A9 and Δ Pr-B3 cells were further examined for their HPV16 E6 and E7 expression and the Δ Pr-A9 for cell senescence. We found both Δ Pr-A9 and Δ Pr-B3 cells, when compared with parental WT CaSki cells, exhibited some small, variable changes in the expression of E6, E7, p53 (E6 downstream target) and E2F1 (E7 downstream target) proteins (**Figure 3E and I**, **Figure 3—figure supplement 2A**), most likely from sampling in the assays, but the Δ Pr-A9 cells displayed significant increase in cell senescence (**Figure 3G and I**, **Figure 3—figure supplement 2B**). Altogether, the data indicate that Inc-FANCI-2 RNA is important for cell growth, colony formation, and cell migration.

Inc-FANCI-2 regulates the expression and secretion of cell soluble receptors

Considering that the altered expression of cell membrane proteins and secreted factors in the Inc-FANCI-2 KO cells might contribute to the observed different cell morphology and growth properties, we performed a Proteome Profiler Human sReceptor Array analysis to examine possible changes in the expression of 105 well-characterized soluble protein receptors being involved in cell signaling using total cell lysates and cell culture supernatants from Δ Pr-A9 and WT CaSki cells.

Using a 30% cutoff (FC $\pm$ 0.3), we identified from the Δ Pr-A9 cell lysate nine proteins with increased expression compared to the WT CaSki cells, including PODXL2, ECM1, NECTIN2, MCAM, ADAM9, ADAM10, CDH5, ITGA5, and NOTCH1, and six proteins with decreased expression, including ITGB6, CDH13, LGALS3BP, TIMP2, ADAM8, and SCARF2 (**Figure 4A and B**). We also found from the Δ Pr-A9 cell culture supernatant five proteins with increased expression, including ADAM9, NECTIN2, ADAM10, CDH5, and ECM1, and the decreased expression of four proteins, including CRELD2, SDC1, SDC4, and TIMP2 (**Figure 4A and B**). By immunoblot assays, we verified selectively the increase in PODXL2, MCAM, and ECM1 and the decrease in ADAM8 and TIMP2 from the Δ Pr-A9 cell lysates and the increase in ECM1 and the decrease in TIMP2 in the Δ Pr-A9 cell culture supernatant (**Figure 4C**).

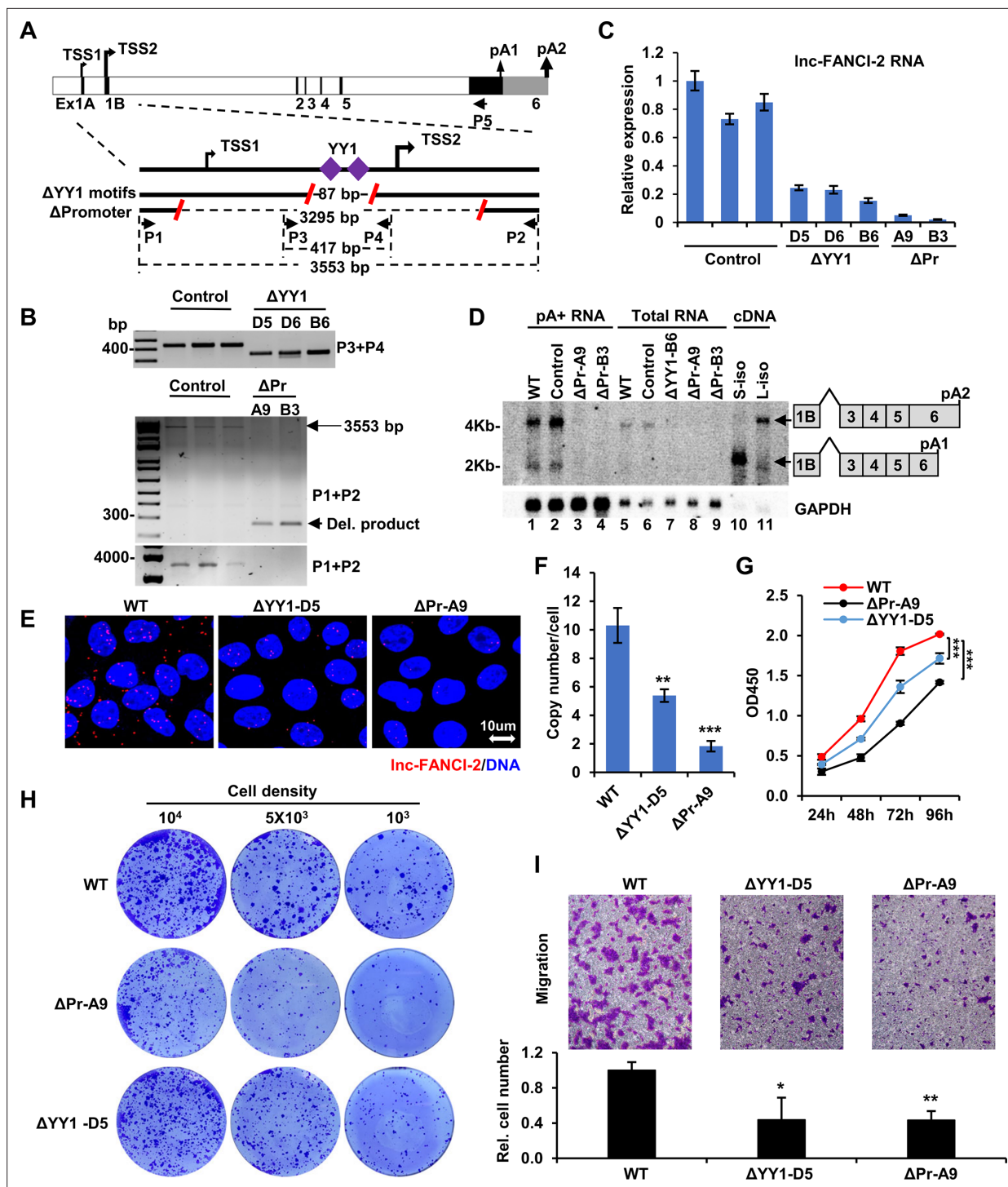


Figure 3. Knockout (KO) of *Inc-FANCI-2* in CaSki cells affects cell proliferation, colony formation, and migration. **(A)** Diagram and KO strategies of *Inc-FANCI-2* gene. On the figure top is the *Inc-FANCI-2* gene structure and its alternative transcription start sites (TSS) and polyadenylation sites (pA). TSS2 or pA2 with a heavier arrow is predominately used for *Inc-FANCI-2* expression. The lower figure part shows KO strategies. Red slashes represent guide RNA (gRNA)-targeted sites to create genomic DNA deletions by CRISPR-Cas9 technology. Deletion of two YY1-binding motifs (Δ YY1) led to a deletion of 86 bp DNA fragment. Deletion of *Inc-FANCI-2* promoter (Δ Pr) led to deleting an ~3.3 kb promoter region. Primers (P1–P4) used for PCR screening were shown as black arrows. **(B)** PCR screening of single-cell clones with homozygous *Inc-FANCI-2* KO, with indicated primer sets diagrammed **(A)**. Single-cell clones selected from the cells transfected with an empty vector served as control (Ctrl) cells. **(C)** Evaluation of *Inc-FANCI-2* KO efficiency in the selected single-cell clones **(B)** by RT-qPCR. **(D)** Northern blot validation of *Inc-FANCI-2* KO efficiency from the individual single-cell clones or the parental (wild-type [WT]) CaSki cells on polyA⁺ RNA (enriched from 100 μ g of total cell RNA, lanes 1–4) or 10 μ g of total cell RNA (lanes 5–9). Total cell Figure 3 continued on next page

Figure 3 continued

RNA (2 µg) of HEK293T cells with ectopic expression of two isoforms (short, S or long, L) of Inc-FANCI-2 cDNA served as a control. Antisense oligo probe P5 (A) labeled with ³²P was used for hybridization. GAPDH RNA served as a loading control and hybridized with a GAPDH-specific oligo probe. (E–F) RNA in situ hybridization (RNA-ISH) validation of Inc-FANCI-2 KO efficiency by RNAscope technology. Two single-cell clones were examined with red color for Inc-FANCI-2 and blue color for the nucleus (E). Bar graphs show the copy number of Inc-FANCI-2 per cell in the WT, ΔYY1-D5, or ΔPr-A9 CaSki cells (each averaged from 200 cells) (F). (G–I) Cell proliferation, colony formation, and migration of WT, ΔYY1-D5, or ΔPr-A9 cells show requirements of Inc-FANCI-2 expression for cell proliferation, colony formation, and cell invasion of CaSki cells. Representative pictures are shown from three separate experiments of cell proliferation by CCK8 assay (G), cell colony formation under 1% methylcellulose solution (H), and cell migration by transwell assay (I) of WT, ΔYY1-D5, or ΔPr-A9 cells. Cell colonies were stained with crystal violet as described in the procedure. Numbers of the invaded cells that crossed the transwell membrane were quantified and shown as bar graphs (I). *, p<0.05; **, p<0.01; ***, p<0.001 by two-tailed Student's t-test.

The online version of this article includes the following source data and figure supplement(s) for figure 3:

Source data 1. PCR screening and northern blot data of Inc-FANCI-2 KO single cell clones for **Figure 3**.

Source data 2. PCR screening and northern blot data of Inc-FANCI-2 KO single cell clones for **Figure 3**.

Figure supplement 1. Inc-FANCI-2 knockout (KO) on characteristic CaSki cell growth behavior and morphology imaged at 24 hr after spreading.

Figure supplement 2. Inc-FANCI-2 knockout (KO) on expression of viral oncoproteins and cell senescence.

Figure supplement 2—source data 1. Inc-FANCI-2 KO in CaSki cells and the expression of HPV16 E6 and E7 and their downstream targets.

Figure supplement 2—source data 2. Inc-FANCI-2 KO in CaSki cells and the expression of HPV16 E6 and E7 and their downstream targets.

As ADAM8 is proteolytically processed into two protein isoforms for cell adhesion (Schlomann et al., 2002) and migration (Conrad et al., 2022), we confirmed by immunoblot the decreased expression of all three sizes of ADAM8 protein in the ΔPr-A9 cell lysate over the WT CaSki cells (Figure 4C).

Inc-FANCI-2 regulates the expression of genes involved in RAS signaling

We next conducted genome-wide RNA-seq analyses of WT and Inc-FANCI-2 KO cells (4 samples/group) to determine the transcriptomic consequences of Inc-FANCI-2 deficiency. We obtained ~120 million mappable RNA reads to the human reference genome hg38 from each sample. Analysis of RNA-seq reads-coverage map by Integrative Genomics Viewer (IGV) showed ~90% reduction of Inc-FANCI-2 expression in ΔPr-A9 cells and ~72% decrease in ΔYY1-D5 cells compared to the WT CaSki cells (Figure 5A), consistent with the results shown in Figure 3C–F.

Hierarchical clustering analysis showed more global transcriptional similarity between ΔYY1-D5 and ΔPr-A9 cells (Figure 5B). By applying a threshold of fold change (FC) ≥1.8 or FC ≤−1.8 with FDR ≤0.01, we found 1230 genes in ΔPr-A9 and 797 genes in ΔYY1-D5 cells that were significantly differentially expressed relative to the WT CaSki cells expressing ~15,890 genes. Nine (*Italic*) of 15 (60%) soluble receptor proteins in cell lysates (PODXL2, ECM1, NECTIN2, MCAM, ADAM9, CDH5, ADAM10, ITGA5, NOTCH1, SCARF2, ADAM8, TIMP2, LGALS3BP, CDH13, and ITGB6) exhibited consistent changes in expression by both RNA-seq and protein array assays. The most significantly affected genes are shown in Volcano plots (Figure 5C and Supplementary file 1). Among these, 211 were upregulated and 189 were downregulated in both ΔPr-A9 and ΔYY1-D5 cells (Figure 5D and Supplementary file 2). By applying more stringent criteria with FPKM ≥7 in at least one of four RNA-seq samples as a cutoff, we profiled the genes with large expression differences in both ΔPr-A9 and ΔYY1-D5 cells. The results displayed in the heatmap of Figure 5E included 52 upregulated and 47 downregulated genes, excluding Inc-FANCI-2. Subsequently, we selectively verified by RT-PCR the decreased expression of ITGB6, NLRP2, PLAC8, PSG4, PSMB9, and SERPINB1 and the increased expression of CFH, CNTN5, EMP3, IGFBP3, PTGS2, and SERPINB2 in ΔPr-A9 cells (Figure 5F), as well as the increased expression of IGFBP3 protein, a RAS signaling driver (Conover et al., 2000; Allard and Duan, 2018; Bach, 2018), in ΔPr-A9 cells by immunoblot (Figure 5G).

By performing the gene set enrichment analysis (GSEA) on the Hallmark gene sets, which provide more refined and concise inputs for GSEA (Liberzon et al., 2015), we found the most significantly upregulated pathways in both ΔPr-A9 and ΔYY1-D5 cells, when compared with the WT CaSki cells, were KRAS signaling and epithelial-mesenchymal transition (EMT). The most significantly downregulated pathways in both ΔPr-A9 and ΔYY1-D5 cells over the WT CaSki cells were interferon gamma (IFN-γ) and interferon alpha (IFN-α) responses (Figure 6A). GSEA plots for ΔPr-A9 show 29 of 111 genes, including IGFBP3 (Conover et al., 2000; Allard and Duan, 2018; Bach, 2018), in RAS signaling

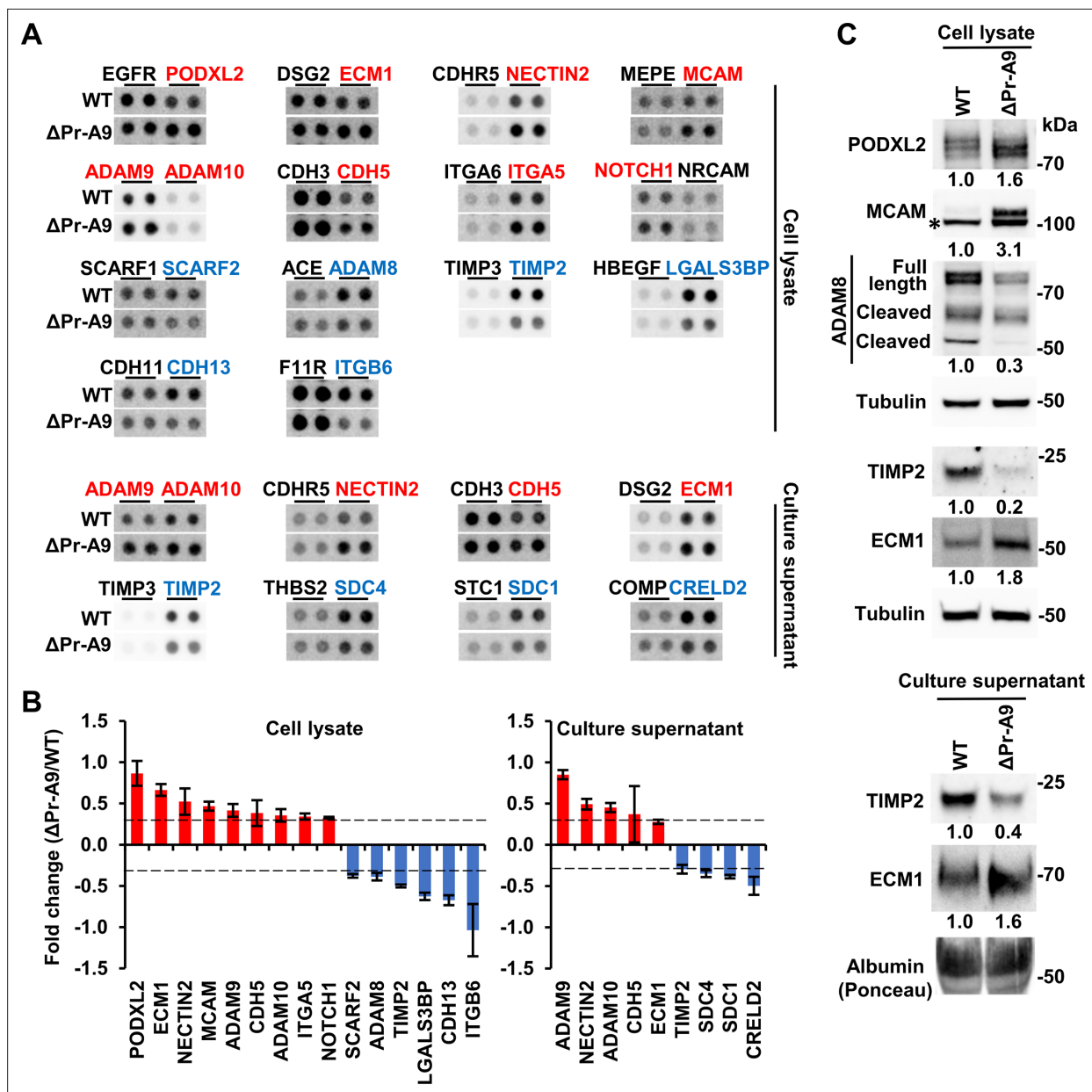


Figure 4. Knockout (KO) of Inc-FANCI-2 in CaSki cells affects expression of cellular soluble receptors. **(A)** Dot blots show differentially expressed soluble receptors in ΔPr-A9 cells when compared to those in the wild-type (WT) CaSki cells. A Proteome Profiler Human sReceptor Array was used to examine 105 cellular soluble receptors. Label in red, upregulated receptors; label in blue, downregulated receptors. **(B)** Quantification of differentially expressed and/or released soluble receptors **(A)** in ΔPr-A9 cells when compared to those in the WT CaSki cells. Error bars represent standard deviation of replicates in each proteome array. The dashed line indicates the threshold of fold change (FC) with a score above or below the threshold (± 0.3 FC) being determined as differentially expressed and/or released receptors. **(C)** Validation of several differentially expressed soluble receptors by immunoblot analysis using specific antibodies. Tubulin served as an internal loading control. *Nonspecific band in MCAM immunoblot.

The online version of this article includes the following source data for figure 4:

Source data 1. Knockout (KO) of Inc-FANCI-2 in CaSki cells affects expression of cellular soluble receptors.

Source data 2. Knockout (KO) of Inc-FANCI-2 in CaSki cells affects expression of cellular soluble receptors.

and 39 of 130 genes in EMT (**Figure 6B and C, Supplementary file 3**) were significantly upregulated, whereas 37 of 139 genes in IFN- γ response and 24 of 71 genes in IFN- α response were significantly downregulated (**Figure 6D and E, Supplementary file 3**). Similar enriched gene sets with increased RAS signaling and EMT and decreased IFN- γ and IFN- α responses were observed in ΔYY1-D5 cells (**Figure 6—figure supplement 1, Supplementary file 3**).

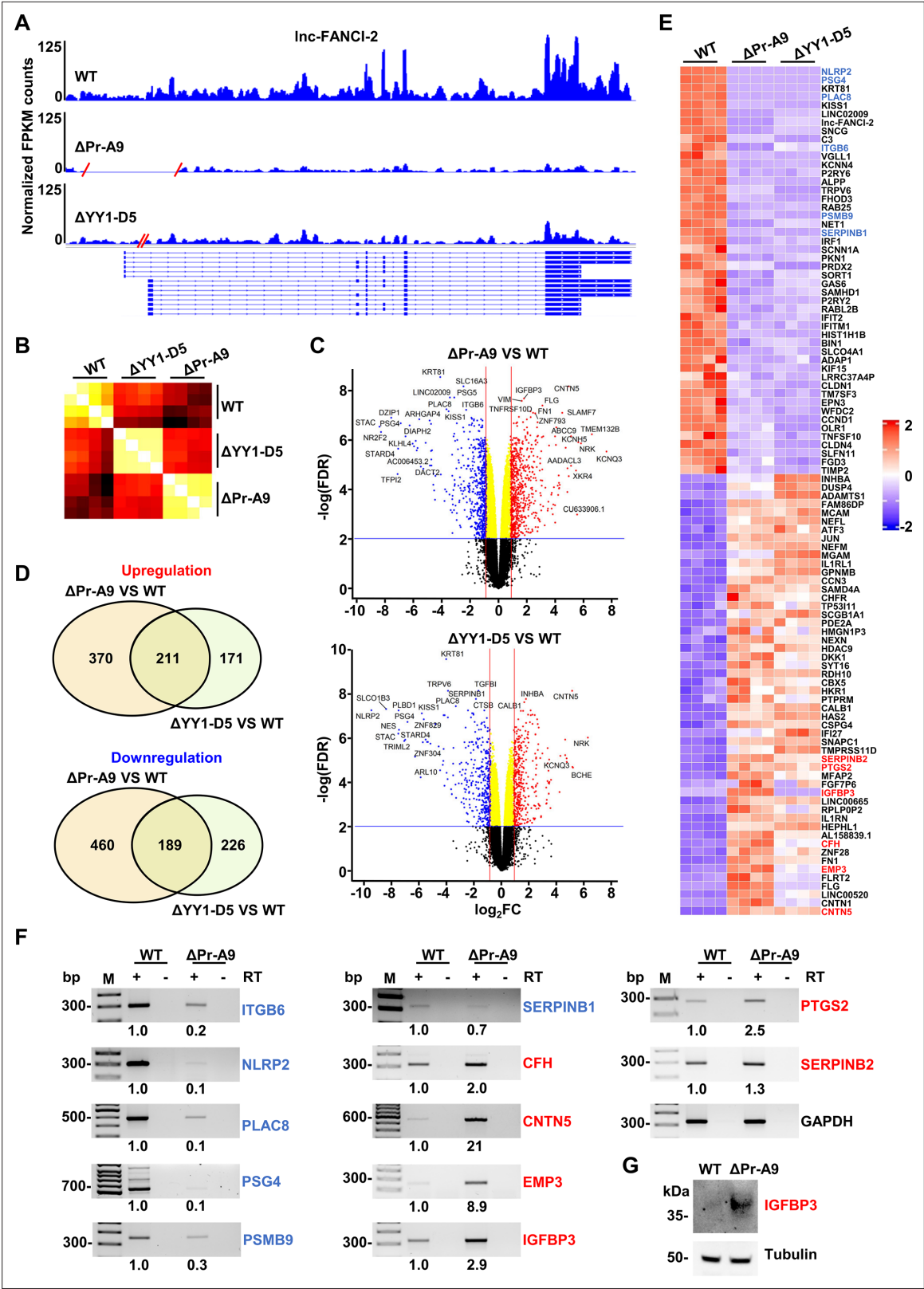


Figure 5. Transcriptomic effect of Inc-FANCI-2 knockout (KO) in CaSki cells by RNA-seq analysis. **(A)** RNA-seq reads-coverage maps by Integrative Genomics Viewer (IGV) showing the expression levels of Inc-FANCI-2 from two KO cell clones, Δ Pr-A9 and Δ YY1-D5, to the wild-type (WT) CaSki cells. One representative coverage profile of four in each type of cells is shown by IGV. FPKM, fragments per kilobase of transcript per million. Red slashes represent the deleted genomic region, with validated 14 Inc-FANCI-2 RNA isoforms shown below (Liu et al., 2021). **(B)** Similarity in heatmap

Figure 5 continued on next page

Figure 5 continued

comparison among Δ Pr-A9, Δ YY1-D5, and WT cells was generated using Limma-normalized counts from each sample by Pearson complete linkage method. (C) Volcano plot visualization of differentially expressed genes (DEGs) in Δ Pr-A9 and Δ YY1-D5. The genes with the most significant change in the increased (red) or decreased (blue) expression are indicated. FC, fold change. (D) Venn diagram depicting the overlapped DEGs between Δ Pr-A9 and Δ YY1-D5 cells over the WT CaSki cells. (E) Heatmap shows overlapped DEGs with FPKM ≥ 7 in Δ Pr-A9 and Δ YY1-D5 cells when compared with the WT CaSki cells. (F) Validation of selective 12 upregulated or downregulated DEGs shown in the heatmap by RT-PCR with gene-specific primers in the presence (+) or absence (-) of reverse transcriptase (RT). GAPDH served as an internal RNA control. Relative expression level of each gene was calculated based on the band density after normalizing to GAPDH RNA band, with the expression level in the WT CaSki cells set as 1. M, a 100 bp DNA marker. (G) Validation of the upregulated expression of IGFBP3 in Δ Pr-A9 cells over the WT CaSki cells by immunoblot analysis.

The online version of this article includes the following source data for figure 5:

Source data 1. Validation of selective 12 upregulated or downregulated DEGs by RT-PCR.

Source data 2. Validation of selective 12 upregulated or downregulated DEGs by RT-PCR.

Since Δ Pr-A9 cells exhibit higher KO efficiency of Inc-FANCI-2 and a higher number of differentially expressed genes (DEGs) and show more severe phenotypic changes in cell proliferation, migration, and colony formation than Δ YY1-D5 cells, the Δ Pr-A9 cells were primarily used for further focused studies on RAS signaling in this report.

Inc-FANCI-2 regulates RAS GTPase activities to phosphorylate RAS signaling effectors Akt and Erk

RAS activation triggers two major downstream signal transduction pathways, Raf/Mek/Erk and PI3K/Akt (**Figure 7—figure supplement 1**), to transduce signals from extracellular stimuli to the cell nucleus where specific effector genes are activated for their corresponding functions (**Conover et al., 2000; Allard and Duan, 2018; Bach, 2018; Downward, 2003; Simanshu et al., 2017**). To further confirm the GSEA data and verify that two major signaling pathways of RAS are activated, we first examined and compared the RAS GTPase activities in Δ Pr-A9 cells and the WT CaSki cells. We found a significant increase in RAS GTPase activity in Δ Pr-A9 cells (**Figure 7A**, top bar graphs), indicating that the endogenous Inc-FANCI-2 RNA in CaSki cells suppresses not only the expression of IGFBP3 (**Figure 5F and G**), the most abundant IGFBP, but also RAS activation (**Figure 6A**, top bar graphs). Since Δ Pr-A9 cells had undergone long-term selection and adaption during single-cell screening, the increased RAS GTPase activity might result from selection pressure for cell survival. However, by transient siRNA knockdown (KD) of Inc-FANCI-2 expression in the WT CaSki cells, we obtained a similar, albeit weaker, increase of RAS GTPase activity (**Figure 7A**, lower bar graphs). This result suggests that the increased RAS GTPase activity in Δ Pr-A9 cells was unrelated to the persistent selection pressure, but rather the deficiency of Inc-FANCI-2.

Next, we examined activation of the PI3K/Akt and Raf/Mek/Erk pathways (**Castellano and Downward, 2011; Asati et al., 2016; Cuesta et al., 2021**). We observed a 3-fold increase in phosphorylated Akt (p-Akt) and 2.5-fold increase of p-Erk1/2 (p44/p42 MAPK), after being normalized to total Akt and Erk levels, in Δ Pr-A9 cells over the WT CaSki cells (**Figure 7B**). These results were confirmed in Δ YY1-D5 cells (**Figure 7—figure supplement 2A**). The increased p-Akt and p-Erk (mostly p-Erk2/p42) was accompanied by elevated expression of MCAM, VIM, and CCND2, and decreased expression of RAC3 (**Figure 7B, Figure 7—figure supplement 2A**). These randomly chosen potential RAS effector proteins facilitate soluble receptor functions, cell proliferation, and EMT. Moreover, transient siRNA KD of Inc-FANCI-2 in the WT CaSki cells also led to the increased levels of p-Akt and p-Erk (**Figure 7C**) and increased expression of MCAM and VIM at 48 hr and 96 hr post-transfection (**Figure 7C**). These observations indicate that a transient loss of Inc-FANCI-2 in CaSki cells is sufficient to trigger RAS signaling. The same siRNA transfection in HeLa cells, an Inc-FANCI-2-negative cell line (**Figure 2A and B**), exhibited no effect on p-Akt or p-Erk1/2 levels (**Figure 7—figure supplement 2B**), but in SiHa cells containing mainly nuclear Inc-FANCI-2 (**Figure 2C and D**), enhanced the expression of p-Akt and p-Erk1/2 (**Figure 7—figure supplement 2B**), verifying the specific effect of Inc-FANCI-2 depletion on RAS signaling pathways independently of predominant cellular distribution of Inc-FANCI-2. The Inc-FANCI-2 KO-mediated increase in p-Akt and p-Erk was sensitive to PI3K inhibitor LY294002 (**Vanhaesebroeck et al., 2021; Figure 7D**, lanes 4–6) and MEK1/2 inhibitor U0126 (**Favata et al., 1998; Figure 7D**, lanes 7–9), respectively. The inhibitors also decreased the expression of VIM and

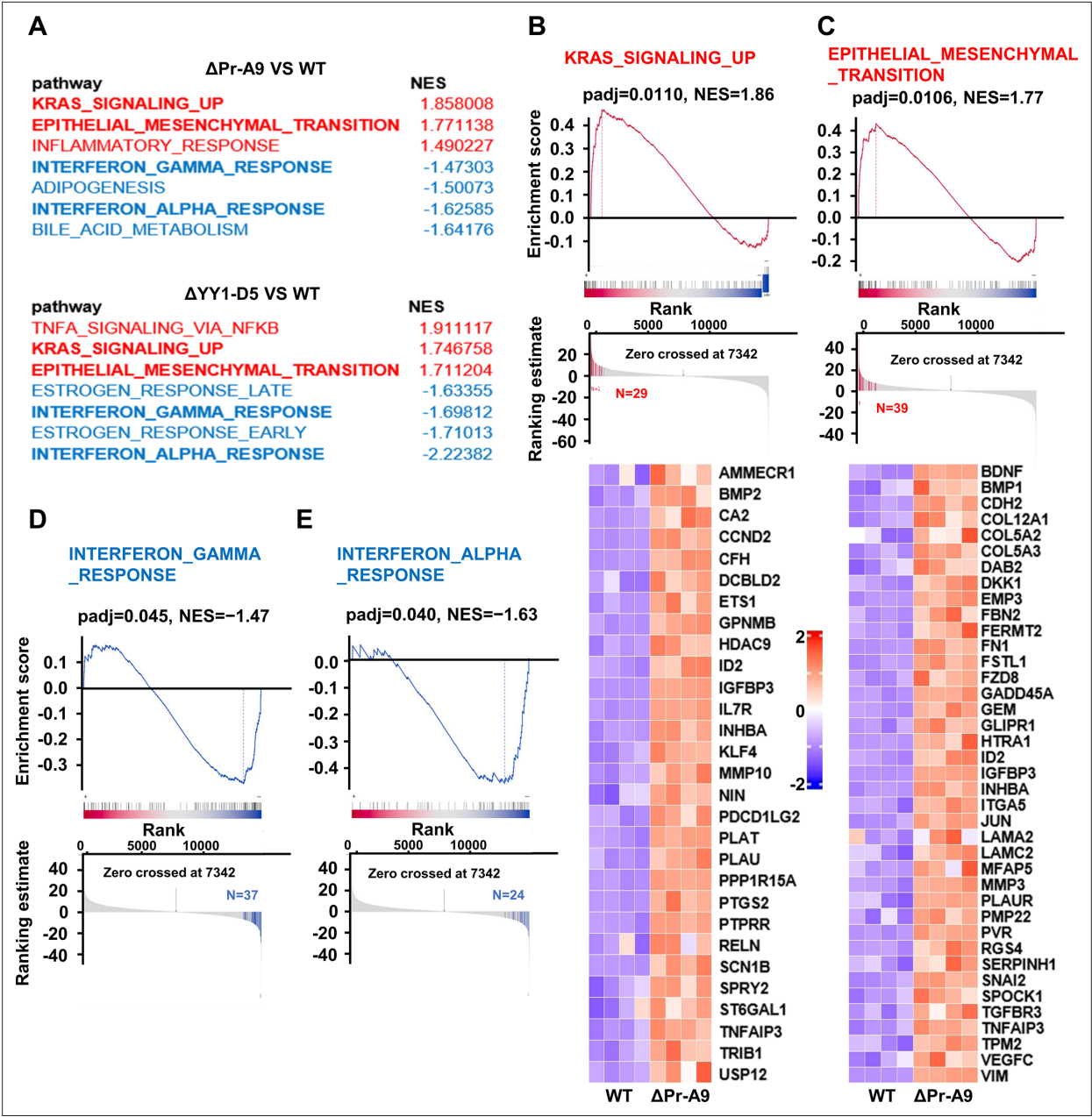


Figure 6. Pathway analyses of differentially expressed genes (DEGs) identified by RNA-seq. **(A)** Top 3 upregulated and top 4 downregulated pathways in ΔPr-A9 and ΔYY1-D5 cells over the wild-type (WT) CaSki cells. Data were generated by gene set enrichment analysis (GSEA) performed with Hallmark gene sets. NES stands for normalized enrichment score. **(B–E)** Each GSEA plot shows the enrichment score and gene hits enriched in ΔPr-A9 cells using Hallmark gene sets, KRAS_SIGNALING_UP **(B)**, EPITHELIAL_MESENCHYMAL_TRANSITION **(C)**, INTERFERON_GAMMA_RESPONSE **(D)**, or INTERFERON_ALPHA_RESPONSE **(E)**. padj, adjusted p-value; Zero crossed 7342, the middle number of total genes in the GSEA at ranking. The heatmaps below the enrichment plots **(B and C)** visualize the genes enriched in respective pathways of KRAS_SIGNALING_UP **(B)** and EPITHELIAL_MESENCHYMAL_TRANSITION **(C)** in ΔPr-A9 cells when compared to the WT CaSki cells.

The online version of this article includes the following figure supplement(s) for figure 6:

Figure supplement 1. Gene set enrichment analysis (GSEA) enrichment plots show enrichment scores and gene hits enriched in ΔYY1-D5 cells using Hallmark gene sets.

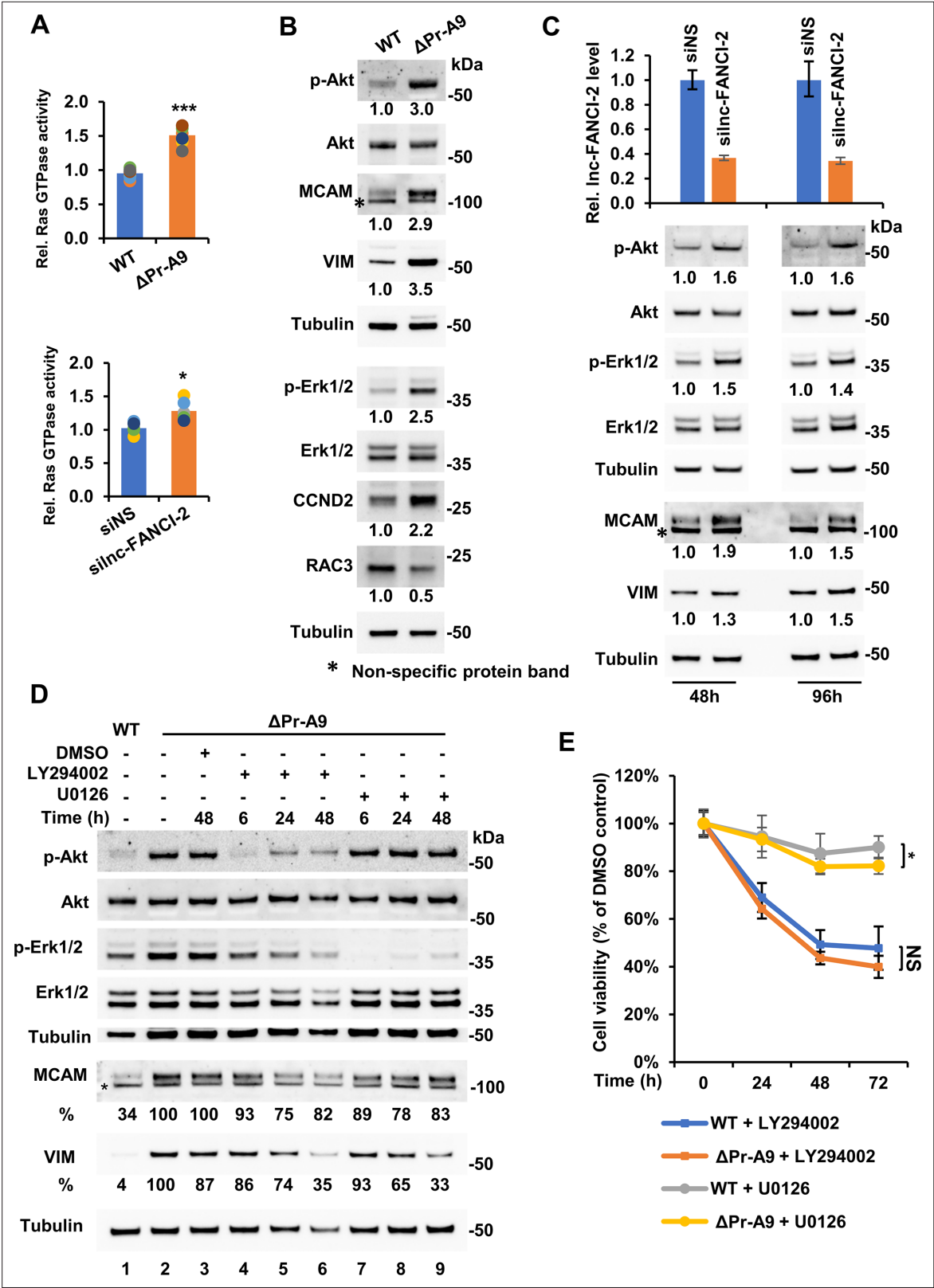


Figure 7. CaSki cells with Inc-FANCI-2 knockout (KO) exhibit activation of RAS signaling pathway. **(A)** CaSki cells with Inc-FANCI-2 KO (top, Δ Pr-A9 cells) or knockdown (KD, lower) by Inc-FANCI-2-specific siRNAs display increased RAS GTPase activity when compared to the wild-type (WT) CaSki cells (top) or the WT cells treated with a nonspecific control siRNA (siNS). The relative RAS GTPase activity was measured by a RAS GTPase Chemi ELISA. *, $p < 0.05$; ***, $p < 0.001$ by two-tailed Student's t-test. **(B and C)** Selective validation of increased expression of RAS signaling-related downstream

Figure 7 continued on next page

Figure 7 continued

genes in Inc-FANCI-2 KO ΔPr-A9 cells (**B**) over the WT CaSki cells or Inc-FANCI-2 KD CaSki cells (**C**) over the WT cells treated with a nonspecific control siRNA (siNS). Relative expression of the indicated genes in ΔPr-A9 cells in comparison to the WT cells (**B**) or in the WT CaSki cells treated for 48 hr and 96 hr with a nonspecific control siRNA (siNS) or a siRNA specifically targeting the Inc-FANCI-2 exon 3 (**C**) was immunoblotted using the corresponding antibodies as indicated. Tubulin served as an internal control for each blot. The level of p-Akt or p-Erk was calculated by normalizing to total Akt or Erk protein and the other proteins by normalizing to tubulin. The protein level in the WT cells or WT cells treated with siNS is set as 1. The KD efficiency of Inc-FANCI-2 RNA (**C**, top bar graphs) was examined by RT-qPCR. (**D**) Effect of blocking RAS signaling on the expression of MCAM and VIM using PI3K inhibitor LY294002 (20 μM) and MEK inhibitor U0126 (10 μM). The protein at each time point was immunoblotted with a corresponding antibody. The level of MCAM or VIM was calculated after normalizing to tubulin. The protein level in ΔPr-A9 cells without the inhibitor was set as 100%. *, Nonspecific protein band. (**E**) The time-dependent cell viability of the WT CaSki and ΔPr-A9 cells in the presence of 20 μM LY294002 or 10 μM U0126. Data was obtained at each time point after normalizing to the cells treated with DMSO. The mean ± SD at each data point was calculated from six samples combined from two independent experiments.

The online version of this article includes the following source data and figure supplement(s) for figure 7:

Source data 1. Inc-FANCI-2 knockout (KO) and activation of RAS signaling pathway.

Source data 2. Inc-FANCI-2 knockout (KO) and activation of RAS signaling pathway.

Figure supplement 1. Pathway map of the RAS Initiative with highlighted differentially expressed genes (DEGs) in ΔPr-A9 cells vs parental wild-type cells.

Figure supplement 2. Knockout or knockdown (KD) of Inc-FANCI-2 affects RAS signaling.

Figure supplement 2—source data 1. Inc-FANCI-2 knockout (KO) and activation of RAS signaling pathway.

Figure supplement 2—source data 2. Inc-FANCI-2 knockout (KO) and activation of RAS signaling pathway.

MCAM and cell proliferation by MEK1/2 inhibitor U0126 (**Figure 7D and E**). The effects of LY294002 on cell proliferation were similar from ΔPr-A9 to the WT CaSki cells (**Figure 7E**).

Inc-FANCI-2 inhibits the expression of IGFBP3 and MCAM (CD146 or MUC18)

Given that IGFBP-3 is the most abundant IGFBP among all six IGFBP members in potentiation of IGF action and PI3K/AKT activities (*Conover et al., 2000*) and a reduced expression of IGFBP-3 mRNA level is associated with progression to cervical cancer (*Serrano et al., 2007*), we further investigated the effect of Inc-FANCI-2 on the expression of IGFBP3 as a Inc-FANCI-2 effector gene. As shown in **Figure 5F and G**, **Figure 8—figure supplement 1A**, the increased RNA and protein expression of IGFBP3 appears in the Inc-FANCI-2 KO ΔPr-A9 cells over the parental WT CaSki cells, indicating a suppressive effect of Inc-FANCI-2 on IGFBP3 expression. This suppressive function of Inc-FANCI-2 was further confirmed in Inc-FANCI-2 rescue experiments in the ΔPr-A9 cells (**Figure 8A and B**). By transient expression of one major isoform of Inc-FANCI-2 RNA (a-PA2, GenBank: MT669800.1) (*Liu et al., 2021*) in the ΔPr-A9 cells, Inc-FANCI-2 (red) and IGFBP3 RNA (green) at 24 hr post transfection were detected by RNAscope RNA-ISH using each specific antisense RNA probe (**Figure 8A**). We demonstrated that the cells with rescued expression of cytoplasmic Inc-FANCI-2 RNA displayed much reduced expression of cytoplasmic IGFBP3 RNA (**Figure 8B**).

As shown in **Figure 5E** and **Figure 8—figure supplement 1B**, the increased RNA expression of MCAM (CD146 or MUC18) (*Wang et al., 2020*) appears to be transcriptional or posttranscriptional in both ΔPr-A9 and ΔYY1-D5 cells. We confirmed by RT-PCR the Inc-FANCI-2 KO-mediated increase of MCAM RNA expression in ΔPr-A9 cells (**Figure 8C**). A rescue experiment was further performed by transient expression of one major isoform of Inc-FANCI-2 RNA (a-PA2, GenBank: MT669800.1) (*Liu et al., 2021*) in ΔPr-A9 cells to determine both nuclear and cytoplasmic MCAM expression (*Kebir et al., 2010*; *Stalin et al., 2016*) in individual Inc-FANCI-2-expressing cells. ΔPr-A9 cells ectopically expressing diffused cytoplasmic Inc-FANCI-2 RNA, as detected by RNAscope RNA-ISH, exhibited a marked reduction in nuclear MCAM protein (**Figure 8D and E**). Quantitative analyses of the ΔPr-A9 cells with or without transient Inc-FANCI-2 RNA expression showed significant reduction of nuclear MCAM protein in the 35 cells with rescued Inc-FANCI-2 expression when compared to the 54 cells expressing no Inc-FANCI-2 (**Figure 8F**). Due to RNAscope procedures, the membrane-bound and cytoplasmic MCAM protein was mostly removed by protease III treatment when RNA-ISH was performed, and thus only the nuclear signal of MCAM protein that remained was detectable by IF staining. Using cell fractionation, we detected a cleaved MCAM of ~46 kDa mainly in the nuclear fraction by western

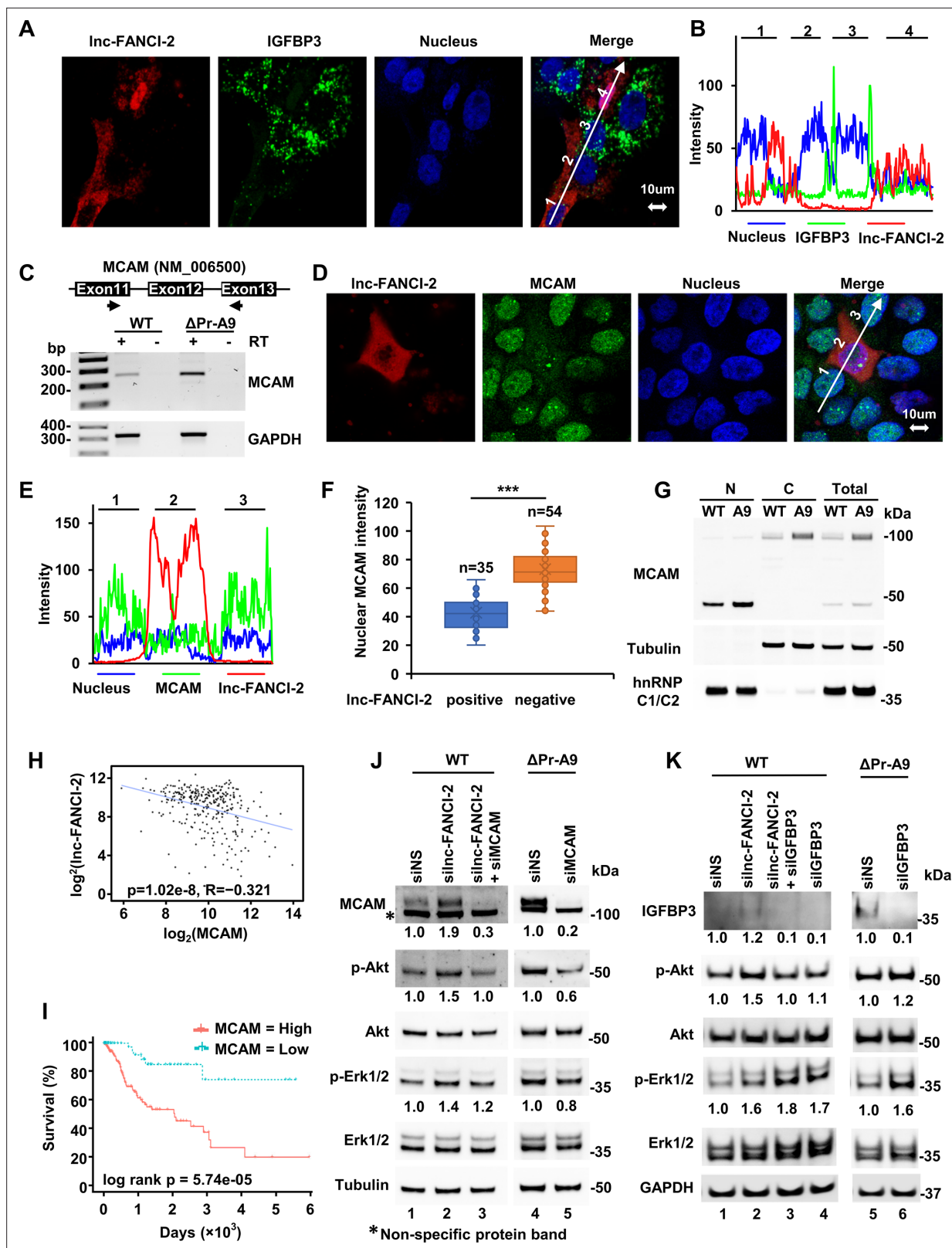


Figure 8. Inc-FANCI-2 is suppressive to the expression of IGFBP3 and MCAM. (A and B) Transient rescue expression of Inc-FANCI-2 in ΔPr-A9 cells inhibits the expression of IGFBP3. ΔPr-A9 cells were transfected with a major isoform Inc-FANCI-2a-PA2 (GenBank ACC. No. MT669800.1) cDNA plasmid. Inc-FANCI-2 in red and IGFBP3 RNA in green were detected by RNAscope RNA in situ hybridization (RNA-ISH) at 24 hr post transfection using each specific antisense RNA probe and imaged by confocal microscopy (A). Expression levels of Inc-FANCI-2 RNA and IGFBP3 RNA in four neighboring

Figure 8 continued on next page

Figure 8 continued

cells (**B**) were measured by signal intensity of a line crossing over the stained cells in **A** (white line arrow). (**C**) Validation of differential expression of MCAM RNA in Δ Pr-A9 cells by RT-PCR in the presence (+) or absence (-) of reverse transcriptase (RT). One pair of primers with one primer at exon 11 and the other at exon 13 of MCAM RNA (NM_006500) were used. GAPDH served as a loading control. (**D**) The major isoform Inc-FANCI-2a-PA2 repressed the expression of MCAM. Δ Pr-A9 cells were transfected with Inc-FANCI-2a-PA2 cDNA plasmid. Inc-FANCI-2 RNA in red was detected by RNAscope ISH and MCAM protein in green was detected by IF with an anti-MCAM antibody. (**E**) Expression levels of Inc-FANCI-2 RNA and MCAM protein in three neighboring cells were measured by signal intensity of a line crossing over the stained cells (**D**, white line arrow). (**F**) Calculation of the expression levels of MCAM in Inc-FANCI-2-positive cells ($n=35$) and Inc-FANCI-2-negative cells ($n=54$) by fluorescent intensity. (**G**) Subcellular MCAM distributions in the nucleus (N) and cytoplasm (C) of WT CaSki cells and Δ Pr-A9 by immunoblot analysis. Fractionation efficiency and sample loading were controlled by cytoplasmic (represented by tubulin) and nuclear (represented by hnRNP C1/C2) proteins. (**H–I**) Correlation and survival analysis of Inc-FANCI-2 and MCAM expression with cervical squamous cell carcinoma (CESC) cases from the cancer genome atlas (TCGA) datasets by GEPIA web server (<http://gepia.cancer-pku.cn/>). The negative correlation at $R=-0.321$ (with $p\text{-value} = 1.02\text{e-}08$) of Inc-FANCI-2 with MCAM in cervical cancer patients was obtained from the RNA-seq data from the TCGA CESC tumors downloaded from the TCGA data portal (<https://portal.gdc.cancer.gov/>). Only primary solid tumor samples ($n=304$ after exclusion of 2 metastatic samples and 3 normal samples) were subjected to analysis, with the data showing as a scatter plot (**H**). Kaplan-Meier plot with log-rank test $p\text{-value}$ at $5.74\text{e-}05$ (**I**) shows MCAM as a biomarker for poor prognosis of cervical cancer survival with a lower quartile group cutoff. RNA-seq and survival data are derived from the TCGA CESC cancer patients. (**J/K**) AKT and ERK phosphorylation are partially regulated by MCAM and IGFBP3 in CaSki cells. KD of MCAM (**J**) and IGFBP3 (**K**) expression in WT parental CaSki or Δ Pr-A9 cells was treated with MCAM siRNA or IGFBP3 siRNA along with or without Inc-FANCI-2 siRNA for 48 hr. Expression levels of individual proteins were immunoblotted using the corresponding antibodies. The level of MCAM, p-Akt, or p-Erk1/2 was calculated after normalizing to tubulin. The protein level in a non-targeting siRNA (siNS) control was set as 1.

The online version of this article includes the following source data and figure supplement(s) for figure 8:

Source data 1. Inc-FANCI-2 is suppressive to the expression of IGFBP3 and MCAM.

Source data 2. Inc-FANCI-2 is suppressive to the expression of IGFBP3 and MCAM.

Figure supplement 1. Expression of IGFBP3, MCAM, MAP4K4, and Inc-FANCI-2.

Figure supplement 1—source data 1. Expression of IGFBP3, MCAM, MAP4K4, and Inc-FANCI-2.

Figure supplement 1—source data 2. Expression of IGFBP3, MCAM, MAP4K4, and Inc-FANCI-2.

Figure supplement 2. Expression levels of Inc-FANCI-2 and MCAM are significantly associated with the cancer genome atlas (TCGA) cervical squamous cell carcinoma (CESC) cancer patients' survival outcome.

blot (**Figure 8G**). The increased nuclear MCAM expression in Δ Pr-A9 cells was proportional to the cytoplasmic and total MCAM levels when compared with the WT CaSki cells (**Figure 8G**). The nuclear MCAM signal appeared as a proteolytic cleavage product presumably by the increased metalloprotease ADAM9 and ADAM10 proteins and decreased metalloprotease inhibitor TIMP2 (**Figure 4**), as observed in other studies (*Stalin et al., 2016*).

The significance of an inverse correlation of Inc-FANCI-2 and MCAM in CaSki cells was further investigated by analyzing their expression in 304 cervical cancer samples from the TCGA dataset. There was a significant negative correlation in Inc-FANCI-2 (being wrongly assigned as LINC00925 by NCBI) (*Liu et al., 2021*) and MCAM RNA levels (**Figure 8H**). The opposite effects of Inc-FANCI-2 and MCAM on cervical cancer survival (**Figure 8I** and **Figure 8H** and **Figure 8—figure supplement 2**) show that the cervical cancer patients with a higher level of Inc-FANCI-2 (*Liu et al., 2021*) but a lower level of MCAM in the cervical tissue (**Figure 8H** and **I** and **Figure 8—figure supplement 2**) exhibited a better survival prognosis.

Regulatory roles of Inc-FANCI-2-mediated increase of MCAM and IGFBP3 on RAS signaling

To further dissect the Inc-FANCI-2-associated expression of MCAM and IGFBP3 on RAS signaling, we examined MCAM and IGFBP3 on phosphorylation of Akt and Erk1/Erk2 in CaSki cells in the presence or absence of Inc-FANCI-2. As shown in **Figure 8J**, we found, although KD or KO of Inc-FANCI-2 promotes phosphorylation of Akt and Erk (**Figure 8J**, lane 2), that KD of MCAM expression in the parental WT CaSki cells in the absence of Inc-FANCI-2 (**Figure 8J**, lane 3) or in Δ Pr-A9 cells (**Figure 8J**, lane 5) led to reduction of Akt and Erk phosphorylation. These data suggest that MCAM is an Inc-FANCI-2 effector but also could be a trigger of signal transduction as reported (*Wang et al., 2020; Joshkon et al., 2020*).

IGFBP3 protein has been viewed as a RAS signaling regulator (*Conover et al., 2000; Allard and Duan, 2018; Bach, 2018*), but recent studies show that IGFBP3 has a variety of intracellular ligands

involved in many unexpected functions (Baxter, 2013; Varma Shrivastav et al., 2020). Thus, KD or KO of Inc-FANCI-2-mediated IGFBP3 expression on RAS signaling in the parental WT CaSki cells or Δ Pr-A9 cells was examined by western blot after siRNA KD of IGFBP3 expression. As shown in Figure 8K, KD of IGFBP3 expression was found to increase phosphorylation of Erk1/2 by ~70% (lane 4) to ~60% (lane 6), but not much so for Akt phosphorylation (lanes 4 and 6). Instead, KD of IGFBP3 expression could prevent Inc-FANCI-2 KD-enhanced Akt phosphorylation in the parental WT CaSki cells (Figure 8K, compare lane 2 and lane 3). These data suggest a separate role of IGFBP3 on phosphorylation of Erk1/2 from Akt in the presence or absence of Inc-FANCI-2.

MAP4K4 association with Inc-FANCI-2 RNA in CaSki cells regulates RAS signaling and phosphorylation of Akt and Erk

We speculated Inc-FANCI-2 restriction on RAS signaling through interactions with cellular proteins. Subsequently, comprehensive identification of Inc-FANCI-2-binding proteins in the parental WT CaSki cells was performed by an isolation of Inc-FANCI-2 RNA-protein complex by RNA purification (IRPCRP) protocol (modified from published ChIRP; Chu and Chang, 2018) in combination with mass spectrometry (Figure 9A). In this protocol, the Inc-FANCI-2-binding proteins in the parental WT CaSki cells were covalently cross-linked to Inc-FANCI-2 RNA via UV irradiation and pulled down by pooled 30 antisense oligos crossing over the entire Inc-FANCI-2. The RNA extracted from the pull-downs was verified to be Inc-FANCI-2 specific by RT-PCR in the absence (-) or presence (+) of reverse transcriptase (RT) (Figure 9B). The Inc-FANCI-2-binding proteins in the pull-downs were then subjected to LC-MS/MS analyses. We identified 32 specific Inc-FANCI-2-binding proteins, including H13, HNRH1, K1H1, MAP4K4, and RNPS1 (Figure 9C, Supplementary file 4).

MAP4K4, a serine/threonine protein kinase, stimulates cancer cell proliferation, invasion, and migration and is recently characterized as a novel MAPK/ERK pathway regulator (Gao et al., 2016; Gao et al., 2017; González-Montero et al., 2023) and negatively regulates RAS signaling by binding to Ras p21 protein activator 1 or RASA1 (Patterson et al., 2023; Roth Flach et al., 2016). Although the exact MAP4K4 binding site on Inc-FANCI-2 was not explored, many enzymes turn out to be an RNA-binding enzyme (Castello et al., 2015; Hentze et al., 2018). Thus, whether MAP4K4 association with Inc-FANCI-2 could regulate PI3K/Akt or MAPK/Erk signal transduction was investigated in the parental WT CaSki cells. We demonstrated that siRNA KD of MAP4K4 expression in the parental WT CaSki cells, as seen for KD of Inc-FANCI-2, led to ~40% or more increase of phosphorylation of both Akt and Erk1/2 and increased expression of MCAM and IGFBP3 (Figure 9D, compare lane 1 to lanes 2 and 3). Interestingly, siRNA KD of MAP4K4 expression in the Inc-FANCI-2 KO Δ Pr-A9 cells had no effect on expression of IGFBP3, only a minimal effect on MCAM and p-Akt, but strongly on pErk1/2 (Figure 8—figure supplement 1C). The data suggest that, through RNA-protein interactions, the increased Inc-FANCI-2 RNA in cells and their association with MAP4K4 and other cellular proteins could be one arm to control RAS signaling and gene expression of its effectors (Figure 9E).

Discussion

HPV oncoproteins E6 and E7 are necessary but not sufficient for development of cervical cancer. Indeed, early studies indicated that other oncogenic stress, such as activated RAS mutant, is needed to trigger malignant transformation of E6- and E7-expressing cells (Storey et al., 1988; Phelps et al., 1988; Crook et al., 1988; Matlashewski et al., 1987) and tumorigenesis (DiPaolo et al., 1989; Schreiber et al., 2004; Greenhalgh et al., 1994; Riou et al., 1988; Eiben et al., 2002; Golijow et al., 1999; Prokopakis et al., 2002). Moreover, E7 has also been reported to repress phosphorylation of Akt and Akt-mediated signaling (Strickland and Vande Pol, 2016). In this study, we show that Inc-FANCI-2, whose expression is highly dependent on E7 and YY1 (Liu et al., 2021), intrinsically restricts RAS GTPase activities and phosphorylation of Akt and Erk presumably by interacting with cellular factors in HPV16-positive CaSki cells. Interestingly, early reports indicated that p-Akt attenuation by E7 could be abolished by introduction of the H73E mutation in the E7 CR3 domain (Strickland and Vande Pol, 2016). This E7 CR3 domain is also essential for interacting with transcription factor YY1 to activate Inc-FANCI-2 transcription (Liu et al., 2021). Thus, it would be presumable that the reported p-Akt attenuation by E7 might be mediated by the increased expression of Inc-FANCI-2. More importantly, these findings also suggest that additional oncogenic stress, such as activated RAS mutant, is

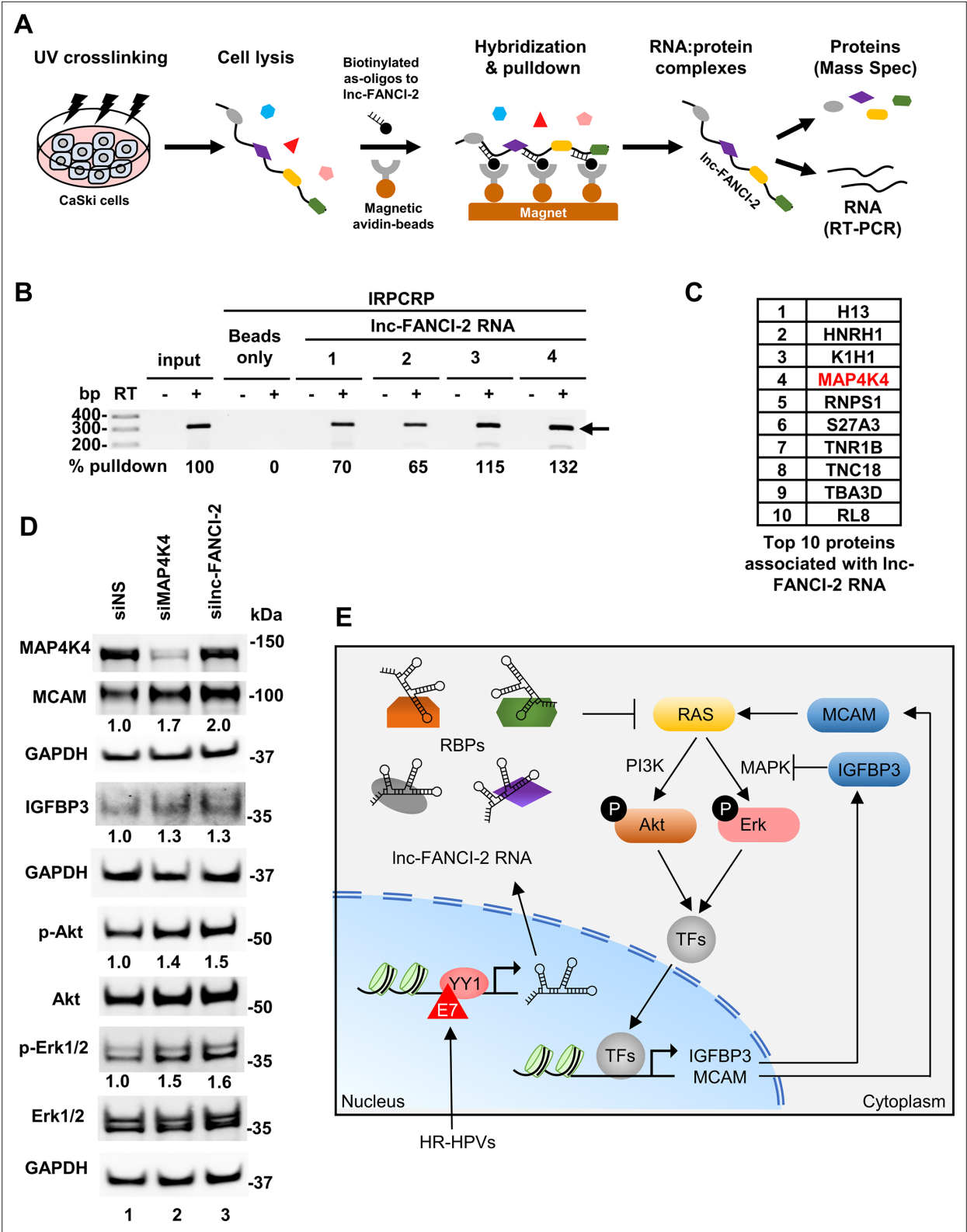


Figure 9. Inc-FANCI-2 interacts with host factors to regulate RAS signaling pathway. **(A)** The Inc-FANCI-2-associated proteins in the wild-type (WT) CaSki cells were identified by isolation of RNA-protein complexes using RNA purification (IRPCR)-mass spectrometry technology. **(B)** Inc-FANCI-2 RNA in the IRPCR-1 and IRPCR-2 pull-downs had the pooled antisense biotinylated oligos (pool 1 with oligos in even numbers and pool 2 with oligos in odd numbers) immobilized to avidin-beads first before mixed with cell lysates, while the IRPCR-3 and IRPCR-4 pull-downs had the oligos pool 1 and 2 separately mixed with cell lysates first before addition to the avidin-beads for the RNA pull-downs. RT-PCR in the absence (-) or presence (+) of

Figure 9 continued on next page

Figure 9 continued

reverse transcriptase (RT) was carried out using the RNA isolated from the individual IRPCR experiments using a primer pair of oHBL5 and oHBL12 (**Supplementary file 5**) specific for Inc-FANCI-2 RNA detection. Beads-only (no oligos) IRPCR experiments served as a negative control. Total RNA from the WT CaSki cells after sonication was used as an input control. The arrow indicates the detected Inc-FANCI-2 RNA. **(C)** Proteins associated with Inc-FANCI-2 RNA identified from Inc-FANCI-2 IRPCR pull-downs. A total of 32 proteins were specifically pulled down from Inc-FANCI-2 IRPCR reactions 1–4 (PSM ≥ 2 from two separate pull-downs, **Supplementary file 4**), with top 10 proteins binding Inc-FANCI-2 shown in the order by the number of identified peptide spectrum match (PSM) in LC-MS/MS. **(D)** Expression of p-Akt and p-Erk from CaSki WT cells 48 hr after siRNA KD of MAP4K4 or Inc-FANCI-2 was immunoblotted by the corresponding antibodies. GAPDH served as a sample loading control. Fold change of the indicated proteins in the cells with KD of MAP4K4 or Inc-FANCI-2 over the cells treated by a non-targeting siRNA (siNS) was calculated after normalizing to GAPDH. **(E)** A proposed model illustrates how Inc-FANCI-2-protein complexes inhibit the RAS signaling pathway to control Akt/Erk phosphorylation and expression of host genes. RAS signaling can be regulated by integrating external and internal factors. In high-risk human papillomavirus (HR-HPV) infected cells, viral oncoprotein E7-YY1 complex transactivates Inc-FANCI-2 expression. By interactions with cellular RBPs, the Inc-FANCI-2-protein complex inhibits RAS signaling. In the absence of Inc-FANCI-2, increased RAS signaling leads to phosphorylation of Akt and Erk and cascaded responses of transcription factors (TFs) and thus regulates the expression of RAS signaling responder genes, such as IGFBP3, MCAM, etc. Consequently, this brings fundamental biochemical and biological processes under control by fine-tuning the RAS pathway.

The online version of this article includes the following source data for figure 9:

Source data 1. Inc-FANCI-2 interacts with host factors to regulate RAS signaling pathway.

Source data 2. Inc-FANCI-2 interacts with host factors to regulate RAS signaling pathway.

required to overcome the Inc-FANCI-2 restriction on RAS signaling for the early observed malignant transformation (*Storey et al., 1988; Phelps et al., 1988; Crook et al., 1988; Matlashewski et al., 1987*) and tumorigenesis (*DiPaolo et al., 1989; Schreiber et al., 2004; Greenhalgh et al., 1994; Riou et al., 1988; Eiben et al., 2002; Golijow et al., 1999; Prokopakis et al., 2002*) of E6- and E7-expressing cells. In CaSki cells, loss of Inc-FANCI-2 in this report was found to promote RAS signaling but reduction of IFN responses. However, high RAS-AKT-ERK signaling induces cell senescence and inhibits tumor cell survival as shown in this report and in a lung cancer study (*Nieto et al., 2017*).

Some lncRNAs function as RAS regulators (*Yang et al., 2019; Saliani et al., 2022*) by their ability to sequester RAS-targeting miRNAs, including MALAT1/miR-217, RMRP/miR-206, and KRAS1P/miR-143/let-7 (*Saliani et al., 2022*). Conceivably, lncRNAs may also regulate RAS signaling through other mechanisms beyond miRNAs. In this report, loss of Inc-FANCI-2 in HPV16-positive CaSki and SiHa cells promotes RAS signaling and expression of RAS signaling effectors (**Figure 9E**). By profiling the proteins associated with Inc-FANCI-2 in CaSki cells, we identified a serine/threonine protein kinase MAP4K4 as one of the major Inc-FANCI-2-binding proteins that may partially mediate the Inc-FANCI-2 restriction on RAS signaling. MAP4K4 is a negative RAS signaling regulator in the context of the early embryo (*Gao et al., 2017; Patterson et al., 2023*) and lymphatic vascular development by interacting with RASA1 (*Roth Flach et al., 2016*). Indeed, silencing of MAP4K4 in parental WT CaSki cells, like KD or KO of Inc-FANCI-2, led to a partial increase of both p-Akt and p-Erk1/2 (**Figure 9D**) as reported (*Patterson et al., 2023; Roth Flach et al., 2016*). Interestingly, KD of MAP4K4 in Δ Pr-A9 cells led to further increased expression of p-Erk1/2 but minimal on p-Akt (**Figure 8—figure supplement 1C**), suggesting a negative correlation of MAP4K4 activity in interaction with Inc-FANCI-2 on Erk1/2 phosphorylation. Thus, our data suggest that MAP4K4 protein may function in coordination with Inc-FANCI-2 RNA in CaSki cells to restrict one arm of RAS signaling, but not the other.

All HR-HPVs are capable of inducing Inc-FANCI-2 expression by experimental infection of keratinocytes (*Liu et al., 2021*). HPV⁺ cervical cancer tissues express high levels of Inc-FANCI-2. As expected, all HPV16⁺ cervical cancer and pre-cancer cell lines examined – such as CaSki, SiHa, W12 subclone cells 20861 (HPV16⁺, integrated) and 20863 (HPV16⁺, episomal) – produce Inc-FANCI-2. Unexpectedly, we found no expression of Inc-FANCI-2 in isogenic HPV18⁺ HeLa and C4II cells, nor in colorectal cancer cell line HCT116, adenovirus 5 DNA-transformed human embryonic kidney cell line HEK293, spontaneously transformed aneuploid immortal keratinocyte cell line HaCaT, and KSHV-infected B cell lymphoma cell line BCBL-1 cells which are all HPV-negative cell lines. It remains to know what negatively regulates Inc-FANCI-2 expression in those cell lines, and more HPV18⁺ cell lines should be examined for Inc-FANCI-2 expression. However, we did find that an HPV-negative cervical cancer cell line, C33A cells expressing mutant p53 and mutant pRB (*Scheffner et al., 1991*), produces a high level of Inc-FANCI-2 (**Figure 2**). Using dual luciferase report assays, we did find Inc-FANCI-2 promoter activity in response to YY1 binding in CaSki and C33A cells, but not in HeLa and HCT116 cells, suggesting

the presence of a repressive factor(s) in the latter two cell lines (data not shown). KD of E2F1 had no effect on lnc-FANCI-2 promoter activity in CaSki cells ([Liu et al., 2021](#)). In the lnc-FANCI-2-producing cells, we noticed that the cellular location of lnc-FANCI-2 also varies from cell types, with lnc-FANCI-2 predominantly in the nucleus in SiHa and C33A cells, but in the cytoplasm in CaSki cells, although both cytoplasmic and nuclear lnc-FANCI-2 restrict RAS signaling. This suggests that a variety of nuclear and cytoplasmic functions of lnc-FANCI-2 remains to be explored.

IGFBP3 is a major IGF carrier and enhances both IGF- and EGF-RAS signaling ([Allard and Duan, 2018](#); [Martin et al., 2003](#)) to activate phosphatidylinositol 3-kinase (PI3K)/AKT and the MAPK/ERK pathways ([Józefiak et al., 2021](#)). Surprisingly, we find a suppressive role of IGFBP3 on Erk phosphorylation, but not much on Akt, in the presence or absence of lnc-FANCI-2 in HPV16-positive CaSki cells. Although ~99% of circulating IGF-1 is bound to IGFBPs, predominantly to IGFBP-3, the most abundant IGFBP in human serum, case-control studies showed significantly lower IGF-1 and IGFBP-3 serum levels in the patients with ICC over the control group ([Józefiak et al., 2008](#); [Serrano et al., 2006](#)). A reduced expression of IGFBP-3 mRNA level appears to be associated with progression to cervical cancer ([Serrano et al., 2007](#)). Consistent with lnc-FANCI-2's suppressive effect on IGFBP3 expression in our study, an increased level of lnc-FANCI-2 expression is associated step-wisely with HR-HPV-positive CIN and ICC tissues ([Liu et al., 2021](#)).

It is obvious from our RNA-seq and human soluble receptor array analysis that KO of lnc-FANCI-2 in CaSki cells affects the expression of a large set of genes, including the expression of increased NECTIN2, ADAM9, ADAM10, ITGA5, MCAM, PODXL2, ECM1, but decreased ADAM8, TIM2, and ITGB6, which are responsible for RAS signaling, EMT pathway, and IFN responses. For example, the dysregulation of RAS signaling and ADAM protein activity is implicated in various cancers. ADAM proteins can modulate RAS signaling by cleaving and releasing ligands that activate or inactivate RAS-related pathways ([Schäfer et al., 2004](#); [Ohtsu et al., 2006](#); [Dang et al., 2011](#); [Kleino et al., 2015](#)). Some ADAM proteins are involved in the migration and invasion of cancer cells, and their loss can promote the degradation of KRAS ([Huang et al., 2024](#)). ADAMs are essential for extracellular signaling and regulation of cell adhesion, and their catalytic activity is strongly correlated to Src-homology 3 (SH3) domain binding of SH3 proteins ([Dang et al., 2011](#); [Kleino et al., 2015](#); [Sun et al., 2010](#)). In addition to the notified activation of PI3K/Akt and Raf/Mek/Erk pathways, one of the major RAS signaling effectors in lnc-FANCI-2 KO or KD cells was found to be MCAM.

MCAM binds various cellular surface receptors or co-receptors to trigger signal transduction, cell proliferation and motility, tumor angiogenesis, and tumor cell metastasis ([Wang et al., 2020](#); [Joshkon et al., 2020](#)). It is an integral, highly glycosylated membrane protein with three common protein isoforms ([Wang et al., 2020](#); [Anfosso et al., 1998](#); [Dye et al., 2009](#)): two membrane-anchored forms with a long (p113) or short (p76) cytoplasmic tail produced by alternative RNA splicing and a proteolytically cleaved soluble form without transmembrane and cytoplasmic regions ([Wang et al., 2020](#); [Dye et al., 2009](#); [Bardin et al., 1998](#)). In addition to its roles in cell signal transduction ([Wang et al., 2020](#); [Joshkon et al., 2020](#); [Anfosso et al., 1998](#); [Dye et al., 2009](#)), the short isoform produced by an intramembrane cleavage may be directed toward the nucleus to regulate gene transcription ([Stalin et al., 2016](#)). We found that CaSki cells express mainly a cytoplasmic form of full-length MCAM (~113 kDa) and a small nuclear form (~46 kDa), but not the short (~76 kDa) isoform. Moreover, lnc-FANCI-2 KO cells exhibit high MCAM levels, which was most likely a result of the increased RAS signaling. Consistently, lnc-FANCI-2 and MCAM levels are negatively correlated in cervical cancer patients, and low levels of MCAM and high levels of lnc-FANCI-2 indicate better prognosis. This suggests that increased MCAM might promote cervical tumorigenesis in vivo. Given that the cells with lnc-FANCI-2 KO exhibit increased levels of IGFBP3 and MCAM, and the ΔPr-A9 cells with rescued lnc-FANCI-2 RNA expression displayed remarkable reduction of IGFBP3 and MCAM expression, our current model ([Figure 9E](#)) highlights how the HPV16-enhanced expression of lnc-FANCI-2 in cervical cancer cells might function as a negative regulator by binding to various RNA-binding proteins to block RAS signaling and reduce the expression of RAS signaling effectors such as IGFBP3 and MCAM.

In conclusion, this study provides the first evidence that one of lnc-FANCI-2 functions is to maintain epithelial functional integrity by repressing RAS signaling in preventing malignant transformation of neoplastic cervical lesions. In addition, more extensive studies are underway to elucidate the role of lnc-FANCI-2 in regulation of IFN responses, which are not covered in this report. Since transactivation of lnc-FANCI-2 expression is largely dependent on host transcription factor YY1, of which expression

is regulated by HPV oncoproteins and host miR-29a, dissecting their transcription network on Inc-FANCI-2 and its effectors may further provide novel insights involved in host homeostasis against HPV infection-induced carcinogenesis. Our observations also highlight additional, unexpected players beyond viral E6 and E7 that drive cells toward malignant transformation.

Materials and methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Cell line (<i>Homo sapiens</i>)	CaSki (HPV16 ⁺)	ATCC	CRL-1550	Cervical cancer cell line
Cell line (<i>Homo sapiens</i>)	C4II (HPV18 ⁺)	ATCC	CRL-1595	Cervical cancer cell line
Cell line (<i>Homo sapiens</i>)	SiHa (HPV16 ⁺)	ATCC	HTB-35	Cervical cancer cell line
Cell line (<i>Homo sapiens</i>)	HeLa (HPV18 ⁺)	ATCC	CCL-2	Cervical cancer cell line
Cell line (<i>Homo sapiens</i>)	C33A (HPV)	ATCC	HTB-31	Cervical cancer cell line
Cell line (<i>Homo sapiens</i>)	HEK293	ATCC	CRL-1573	Kidney epithelial-like cell
Cell line (<i>Homo sapiens</i>)	HCT116	Dr. Bert Vogelstein (Johns Hopkins University)	N/A	Colorectal cancer cell line
Cell line (<i>Homo sapiens</i>)	W12 subclones (20861, 20863)	Dr. Paul Lambert (University of Wisconsin)	N/A	HPV16 ⁺ (integrated/episomal)
Cell line (<i>Homo sapiens</i>)	BCBL-1 (KSHV ⁺)	NIH AIDS Reagent Program	N/A	Body cavity B lymphoma
Cell line (<i>Homo sapiens</i>)	HaCaT	Dr. Carl C Baker (NIH)	N/A	Skin keratinocyte
Antibody	Anti-MCAM (Rabbit polyclonal)	ProteinTech	Cat# 17564-1-AP	Rabbit polyclonal; WB (1:1000), IF (1:200)
Antibody	Anti-E2F1 (Rabbit polyclonal)	ProteinTech	Cat# 12171-1-AP	Rabbit polyclonal; WB (1:1000)
Antibody	Anti-MAP4K4 (Rabbit polyclonal)	ProteinTech	Cat# 55247-1-AP	Rabbit polyclonal; WB (1:1000)
Antibody	Anti-p53 (Rabbit polyclonal)	ProteinTech	Cat# 10442-1-AP	Rabbit polyclonal; WB (1:1000)
Antibody	Anti- α -Tubulin (Mouse monoclonal)	Sigma-Aldrich	Cat# T5201	Mouse monoclonal; WB (1:5000)
Antibody	Anti-hnRNP C1/C2 (Mouse monoclonal)	Abcam	Cat# Ab10294	Mouse monoclonal; WB (1:1000)
Antibody	Anti-RAC3 (Rabbit monoclonal)	Abcam	Cat# ab129062	Rabbit monoclonal; WB (1:1000)
Antibody	Anti-CCND2 (Cyclin D2) (Rabbit monoclonal)	Cell Signaling Technology	Cat# 3741	Rabbit monoclonal; WB (1:1000)
Antibody	Anti-Akt (Rabbit polyclonal)	Cell Signaling Technology	Cat# 9272	Rabbit monoclonal; WB (1:1000)
Antibody	Anti-phospho-Akt (Ser473) (Rabbit polyclonal)	Cell Signaling Technology	Cat# 9271	Rabbit monoclonal; WB (1:1000)
Antibody	Anti-Erk1/2 (p44/42 MAPK) (Rabbit monoclonal)	Cell Signaling Technology	Cat# 4695	Rabbit monoclonal; WB (1:1000)
Antibody	Anti-phospho-Erk1/2 (Thr202/Tyr204) (Rabbit monoclonal)	Cell Signaling Technology	Cat# 4370	Rabbit monoclonal; WB (1:1000)
Antibody	Anti-IGFBP3 (D1U9C) (Rabbit monoclonal)	Cell Signaling Technology	Cat# 25864	Rabbit monoclonal; WB (1:1000)
Antibody	Anti-Vimentin (VIM) (Mouse monoclonal)	Thermo Fisher Scientific	Cat# MA5-11883	Mouse monoclonal; WB (1:1000), IF (1:200)
Antibody	Anti-PODXL2 (Goat polyclonal)	R&D Systems	Cat# AF1524	Goat polyclonal; WB (1:500)
Antibody	Anti-ECM1 (Mouse monoclonal)	R&D Systems	Cat# MAB39371	Mouse monoclonal; WB (1:500)
Antibody	Anti-TIMP2 (Goat polyclonal)	R&D Systems	Cat# AF971	Goat polyclonal; WB (1:500)
Antibody	Anti-ADAM8 (Goat polyclonal)	R&D Systems	Cat# AF1031	Goat polyclonal; WB (1:500)
Antibody	Anti-SRSF3 (Rabbit monoclonal)	Novus Biologicals	Cat# NBP2-76892	Rabbit monoclonal; WB (1:1000)
Antibody	Anti-HPV16 E7 (Rabbit polyclonal)	GeneTex	Cat# GTX133411	Rabbit polyclonal; IF (1:500)

Continued on next page

Continued

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Sequence-based reagent	Inc-FANCI-2 siRNA	Custom (see Supplementary file 5)	N/A	Double-stranded, target-specific siRNA
Sequence-based reagent	MAP4K4 siRNA	Dharmacon, Inc	#M-003971	Human MAP4K4 targeting siRNA
Sequence-based reagent	IGFBP3 siRNA	Dharmacon, Inc	#M-004777	Human IGFBP3 targeting siRNA
Sequence-based reagent	Non-targeting control siRNA	Dharmacon, Inc	#D-001210-01	Negative control siRNA
Chemical compound, drug	LY294002 (PI3K inhibitor)	Cell Signaling Technology	#9901	20 μM treatment
Chemical compound, drug	U0126 (MEK inhibitor)	Cell Signaling Technology	#9903	10 μM treatment
Commercial assay, kit	RAS GTPase Chemi ELISA Kit	Active Motif	#52097	RAS activity measurement
Sequence-based reagent	Inc-FANCI-2 siRNA	Dharmacon	Custom (Supplementary file 5)	Target: Inc-FANCI-2
Sequence-based reagent	Inc-FANCI-2 gRNAs	Zhang Lab CRISPR Designer	Custom (Supplementary file 5)	CRISPR knockout
Software, algorithm	CCBR Pipeliner	GitHub	CCBR/Pipeliner	RNA-seq analysis
Software, algorithm	ZEN 2.3	Zeiss	N/A	Confocal microscopy
Commercial assay, kit	Nuclei EZ Prep Kit	Sigma-Aldrich	#NUC-101	Nuclear/cytoplasmic fractionation
Commercial assay, kit	Superscript First-Strand Synthesis kit	Thermo Fisher Scientific	#11904018	cDNA synthesis
Commercial assay, kit	TaqMan gene expression Master Mix	Applied Biosystems	#4369016	qPCR amplification
Commercial assay, kit	TaqMan Gene Expression Assay (GAPDH)	Applied Biosystems	Hs02758991_g1	Internal control for qPCR
Commercial assay, kit	RNAscope Multiplex Fluorescent V2 Assay	Advanced Cell Diagnostics	#323100	RNA in situ hybridization
Sequence-based reagent	Inc-FANCI-2 custom probe	Advanced Cell Diagnostics	Custom (nt 359–1713 of MT669800.1)	Targets Inc-FANCI-2a-PA2 transcript
Sequence-based reagent	IGFBP3 probe	Advanced Cell Diagnostics	#310351	Channel 1 probe
Sequence-based reagent	Inc-FANCI-2 probe	Advanced Cell Diagnostics	#509061-C3	Channel 3 probe

Cell cultures and treatment

CaSki, a HPV16-positive (HPV16⁺) human cervical cancer cell line with WT p53, pRb, HRAS, KRAS, and NRAS (https://depmap.org/portal/cell_line/ACH-001336?tab=mutation), was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), maintained in Dulbecco’s modified Eagle medium (DMEM) (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and grown in a cell culture incubator at 37°C with 5% CO₂.

SiHa (HPV16⁺), HeLa (HPV18⁺), C4II (HPV18⁺), C33A (no HPV), and HEK293 (Ad5 E1/E2-immortalized human kidney cells) were all obtained from ATCC and grown in DMEM with 10% FBS at 37°C and 5% CO₂. HaCaT (spontaneously immortalized human epidermal cells) was a gift from Dr. Carl C Baker in NIH cultured in DMEM with 10% FBS at 37°C and 5% CO₂. HCT116 cell line, a colorectal cancer cell line, was a gift from Dr. Bert Vogelstein of Johns Hopkins University and was grown in McCoy’s 5A medium with 10% FBS at 37°C and in 5% CO₂. W12 subclones 20861 (HPV16⁺, integrated) and 20863 (HPV16⁺, episomal) were a gift from Dr. Paul Lambert of University of Wisconsin, Madison, and grown on mitomycin C-pretreated NIH3T3 feeder cells in F12 medium at 37°C and in 5% CO₂. BCBL-1 (body cavity B lymphoma cells with KSHV infection) was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH and cultured in RPMI 1640 containing 10% FBS at 37°C and in 5% CO₂. All cell lines used in this study were authenticated by STR profiling and confirmed to be free of mycoplasma contamination.

CaSki cells were treated with 20 μM PI3K inhibitor, LY294002 (Cell Signaling Technology, Danvers, MA, USA #9901) or 10 μM mitogen-activated protein kinase kinase (MEK) inhibitor, U0126 (Cell Signaling Technology, Danvers, MA, USA #9903) and examined for their proliferation and viability by CCK-8 assay and RAS effector expression by western blot.

Raft tissues and cervical tissue sections

Raft tissues with or without HPV16 or HPV18 infection and cervical cancer tissue sections from Zhejiang University Women's Hospital were described in our previous reports ([Liu et al., 2021](#); [Xu et al., 2019](#)).

siRNAs, plasmid constructions, and transfections

Synthetic double-stranded siRNA targeting the Inc-FANCI-2 transcript is listed in **Supplementary file 5**. Human MAP4K4 siRNA (#M-003971), human IGFBP3 siRNA (#M-004777), and non-targeting control siRNA (#D-001210-01) were purchased from Dharmacon, Inc (Lafayette, CO, USA). Each siRNA transfection into CaSki or other type of cells was carried out by LipoJet in vitro transfection reagent (SignaGen, Frederick, MD, USA). Plasmid pHBL03 has a short isoform of Inc-FANCI-2 cDNA (Inc-FANCI-2a-PA1, GenBank Acc. No. MT669801). Overlapping PCR products generated from 5' and 3' RACE PCR products with a primer pair of oHBL 17 and oHBL 18 were digested and inserted into pcDNA3 at Hind III and Xba I sites. The insertion was verified by sequencing. Plasmid pHBL05 has a long isoform of Inc-FANCI-2 (Inc-FANCI-2a-PA2, GenBank Acc. No. MT669800). PCR products generated from genomic DNA with oHBL 19 and oHBL 23 were digested and swapped into pHBL03 to replace Inc-FANCI-2a-PA1 at BsmB I and Xba I sites.

CRISPR/Cas9 system was used to knock out the Inc-FANCI-2 gene from CaSki cells. Two Inc-FANCI-2 specific gRNAs were designed by an online CRISPR design tool developed by the Zhang lab (<https://www.zlab.bio/guide-design-resources>). A modified cloning strategy was used to clone the two gRNA into pSpCas9(BB)-2A-Puro vector (Addgene, Watertown, MA, USA #62988) and described in our previous publication ([BeltCappellino et al., 2019](#)). This strategy allows all three components (two gRNAs and Cas9) to be expressed from the same plasmid to ensure that the transfected cells express two gRNAs simultaneously to knock out the targeted gene ([BeltCappellino et al., 2019](#)). The sequences of primers to make gRNAs were listed in **Supplementary file 5**.

Knockout of Inc-FANCI-2 gene in CaSki cells, PCR screening, and genotyping

Two gRNAs each under control by a U6 promoter in a pSpCas9(BB)-2A-Puro vector were used to create deletion in CaSki cells. Plasmid pHBL25, which contains gRNA 1 and 6, was used to delete the entire promoter region of Inc-FANCI-2, and plasmid pHBL27, which contains gRNA 3 and 4, was used to delete only two YY1-binding motifs spanning over an 87 bp region in the Inc-FANCI-2 promoter. CaSki cells transfected with the gRNA-expressing vectors were then under 2 weeks of puromycin selection (0.1 µg/ml). The puromycin-resistant CaSki cells were diluted to a final concentration of 0.5 cells per 100 µl, plated 100 µl of diluted cells into each well of a 96-well plate, and grew under continuous puromycin selection. The colonies were inspected to ensure the clones were grown from one cell and refed or re-plated as needed in 3 weeks of expansion.

A direct PCR was performed for a number of single-cell clones as described ([BeltCappellino et al., 2019](#)). Briefly, several hundred cells expanded from a single-cell clone were resuspended in 50 µl of phosphate-buffered saline (PBS) and then frozen-thawed on dry ice three times. Cell lysates were treated with 4 µl of QIAGEN protease (QIAGEN, Hilden, Germany, #1017782) at 56°C for 10 min, and the protease was inactivated at 95°C for 5 min. The resulting product was directly used for PCR screening. P1 + P5 were used for verification of promoter deletion and P3 + P4 for YY1 motifs deletion. To ensure the selected single-cell clones displaying homozygous knockout of Inc-FANCI-2, total cell DNA was isolated using a QIAamp DNA Blood Minikit (QIAGEN, Hilden, Germany, #51106) and then the homozygous deletion was screened and the cells with homozygous KO were selected and confirmed by PCR. The primers used for genotyping are listed in **Supplementary file 5**. At least two single-cell clones either with Inc-FANCI-2 promoter deletion or with YY1 motif deletion were selected. Three single-cell clones from the cells transfected with an empty vector, pSpCas9(BB)-2A-Puro, were also selected and served as controls for the genotyping screening.

Cell proliferation and viability assay

Cell proliferation and viability were determined by a CCK-8 assay (Dojindo Molecular Technologies, Rockville, MD, USA). For the proliferation assay, 500 µl of cell suspension (50,000 cells/well) were dispensed into individual wells in a 24-well plate. Cells were then treated with inhibitors (20 µM LY294002 or 10 µM U0126, respectively) after 24 hr culture. At each time point of 24 hr, 48 hr, and

72 hr, 50 µl of CCK-8 solution was added in three wells of each group and incubated for 1 hr in a cell culture incubator at 37°C. Cell proliferation was determined by absorbance at 450 nm, and normal medium was used to subtract background. Percentage of cell viability was calculated by the following formula: cell viability % = OD450 of inhibitors treated sample / OD450 of untreated sample × 100%.

Colony formation, cell migration assay, and senescence analysis

CaSki cells were seeded at 1×10^4 , 5×10^3 , or 1×10^3 cells/well in six-well plates. After 24 hr of cell growth, the culture medium in each well was replaced by DMEM containing 1% methylcellulose and 10% FBS. The plates were fixed with 3.7% formaldehyde and stained with 1% crystal violet in 2 weeks. Transwell cell migration assay was performed as previously described (Justus *et al.*, 2014). Briefly, CaSki cells were resuspended in serum-free DMEM containing 0.1% bovine serum albumin (BSA). 1×10^5 cells in 100 µl were then plated on top of the filter membrane in a transwell insert (SARSTEDT, Nümbrecht, Germany, # 83.3932.800) and incubated for 10 min at 37°C to allow the cells to settle down, then 600 µl of complete media containing 10% FBS were added into the bottom of the lower chamber in a 24-well plate. After 24 hr in culture, the cells on the top of the insert membrane were removed, and cells on the bottom were fixed, stained by 0.2% crystal violet, and then counted.

Cell senescence was determined by Cellular Senescence Assay (Millipore Sigma, Burlington, MA, USA, # QIA117-1KIT). Briefly, CaSki cells were plated at 2×10^5 cells/well in six-well plates and cultured for 2 days to 50% confluence. Cells were washed once with 2 ml PBS and fixed with 0.5 ml of fixative solution at room temperature for 10–15 min. Then, cells were washed twice with 2 ml PBS, and 1 ml staining solution was added to each well. Cells were incubated at 37°C overnight without CO₂ and protected from the light. Blue stained cells were then counted under light microscopy.

Nuclear and cytoplasmic fractionation

CaSki and SiHa cells were fractionated by using Nuclei EZ Prep Kit (Sigma-Aldrich, #NUC-101) following the manufacturer's protocol. The details of the method could be found in our previous publication (Yu and Zheng, 2022). Western blot analysis for nuclear protein SRSF3 and cytoplasmic protein GAPDH was used to determine fractionation efficiency. The fractionated cytoplasmic and nuclear RNAs were used for the detection of lnc-FANCI-2 RNA with human GAPDH RNA serving as a control for RNA fractionation efficiency of the cytoplasmic RNAs.

RT-qPCR

Detection of lnc-FANCI-2 by RT-qPCR was performed as described (Liu *et al.*, 2021). Pre-designed primers for lnc-FANCI-2 are listed in **Supplementary file 5**. Briefly, 2 µg total RNA was converted to cDNA using Superscript First-Strand Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA #11904018). qPCR was performed using TaqMan gene expression Master Mix (Applied Biosystems, Waltham, MA, USA #4369016) on a StepOne Plus Real-Time PCR system (Applied Biosystems). TaqMan Gene Expression Assay (Applied Biosystems, #4331182) for GAPDH (Hs02758991_g1) was served as an internal control. Data were plotted as fold change over the control group using the $2^{-\Delta\Delta C_t}$ method by which the data was normalized first to the values for GAPDH and then to the median value for control samples. Data are presented as a bar graph with mean ± SD for each group.

Northern blot

To validate lnc-FANCI-2 KO in CaSki cells, northern blot was performed using polyA⁺ mRNA isolated from 100 µg of total CaSki RNA with PolyATtract mRNA Isolation Systems (Promega, #Z5310) or 10 µg of total CaSki total RNA as described previously (Liu *et al.*, 2021). Briefly, RNA samples were denatured in NorthernMax Formaldehyde loading dye (Thermo Fisher Scientific, #AM8552) at 75°C for 15 min, then separated in a 1% formaldehyde-containing agarose gel and transferred onto a Gene-Screen Plus hybridization transfer membrane (Perkin Elmer, Waltham, MA, USA #NEF987001PK). RNAs on the membrane were cross-linked by exposing to UV light and then prehybridized with PerfectHyb Plus hybridization buffer (Sigma-Aldrich, St. Louis, MO, USA #H7003) for 2 hr at 42°C. Specific oligos against lnc-FANCI-2 or GAPDH listed in **Supplementary file 5** were labeled with [γ -³²P]-ATP using T4 PNK (Thermo Fisher Scientific, Waltham, MA, USA #18004-010) and added into hybridization buffer for overnight incubation at 42°C. The membrane was then washed with 2× SSPE/0.5% sodium dodecyl

sulfate (SDS) solution for 5 min, followed by two washes with 0.5× SSPE/0.5% SDS each for 15 min at 42°C and then exposed to a PhosphorImager screen.

RNA-seq and data analysis

Total RNA was extracted from WT CaSki, ΔYY1-D5, and ΔPr-A9 cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA #15596018). Total ribo-minus RNA-seq libraries, four samples in each group, were prepared with TruSeq Stranded Total RNA Library Kit and then subjected to paired-end sequencing using Illumina-HiSeq3000/4000 platform.

The obtained reads were processed using the CCBP Pipeliner utility (CCBR, 2023). Briefly, reads were trimmed from adapters and low-quality bases using Cutadapt (version 1.18) (<https://bioweb.pasteur.fr/packages/pack@cutadapt@1.18>) before alignment to the custom reference genome described below. The transcripts were aligned using STAR v2.5.2b in two-pass mode (Dobin et al., 2013). Expression levels were quantified using RSEM (RNA-Seq by Expectation-Maximization) (version 1.3.0) (Li and Dewey, 2011) with a custom gene annotation described below.

The custom reference genome allowing quantification of both HPV16 and host expression used in this alignment consisted of the human reference genome (hg38/Dec. 2013/GRCh38) with an HPV16 FASTA (<https://pave.niaid.nih.gov/>) sequence added as an additional pseudochromosome. The custom gene annotation used for gene expression quantification consisted of a concatenation of the hg38 GENCODE annotation version 30 (Harrow et al., 2012) and the HPV16 genome, with one other notable alteration. The hg38 v30 gene annotation is incorrect at the location of the Inc-FANCI-2 locus in the hg38 genome at chr15:89378104–89398487, which affects quantification of this gene. To correct this annotation and provide accurate quantification, therefore, we first performed BLAST against hg38 using the sequences of the 14 known isoform transcripts of Inc-FANCI-2 (MT669800 for Inc-FANCI-2a-PA2, MT669801 for Inc-FANCI-2a-PA1, MT669802 for Inc-FANCI-2b-PA2, MT669803 for Inc-FANCI-2b-PA1, MT669804 for Inc-FANCI-2c-PA2, MT669805 for Inc-FANCI-2c-PA1, MT669806 for Inc-FANCI-2d-PA2, MT669807 for Inc-FANCI-2d-PA1, MT669808 for Inc-FANCI-2e-PA2, MT669809 for Inc-FANCI-2e-PA1, MT669810 for Inc-FANCI-2f-PA2, MT669811 for Inc-FANCI-2f-PA1, MT669812 for Inc-FANCI-2g-PA2, and MT669813 for Inc-FANCI-2g-PA1). The results were used to determine the precise exon start and stop sites for each of the isoforms. These were then used to manually adjust the default hg38 v30 gene annotations at the locus indicated above to account for the correct structure of the Inc-FANCI-2 locus and its 14 isoforms.

Downstream analysis and visualization were performed within the NIH Integrated Analysis Portal (NIDAP) using R programs developed on the Foundry platform (Palantir Technologies). Briefly, raw counts data produced by RSEM were imported into the NIDAP platform, genes were filtered for low counts (<1 cpm), and the voom algorithm (Law et al., 2014) from the Limma R package (version 3.40.6) (Smyth, 2004) was used for quantile normalization and calculation of DEGs. Pre-ranked GSEA was performed using the Molecular Signatures Database version 6.2 (Liberzon et al., 2011; Subramanian et al., 2005) and the fgsea package (Korotkevich et al., 2021). Raw data and the analyzed RNA-seq data supporting the findings in this study have been deposited in the NCBI GEO database (the accession#: GSE190904).

Human soluble receptor array

To detect the changes of soluble receptors expressed and released from parental WT CaSki cells and Inc-FANCI-2 KO cells, Proteome Profiler Human sReceptor (Soluble Receptor) Array (R&D Systems, Minneapolis, MN, USA # ARY012) was performed according to the manufacturer's protocol. Briefly, 7 × 10⁶ WT CaSki cells and ΔPr-A9 cells were cultured in 15 ml of DMEM supplemented with 10% FBS in a T75 flask for 24 hr. Cell culture supernatants were then collected and centrifuged at 4000 × g for 15 min to remove cell debris. Cells were rinsed by 10 ml of PBS and solubilized in lysis buffer 17 at 4°C for 30 min. The cell lysis was then centrifuged at 14,000 × g for 5 min to remove cell debris. Protein concentration of cell lysis was measured by Micro BCA Protein Assay (PIERCE, Waltham, MA, USA #23235). Next, two sets of N and C membranes were blocked by Buffer 8/1 in 4-Well Multi-dish for 1 hr on a rocking platform. 500 μl of culture supernatant or 100 μg cell lysate generated from the WT CaSki cells or ΔPr-A9 cells were diluted in 3 ml of Buffer 8/1 and then applied to each membrane after three washes with 1× wash buffer. The membranes were incubated overnight at 4°C on a rocking platform and washed three times to remove unbound materials, followed by incubation with their specific

cocktail of biotinylated detection antibodies for 2 hr at room temperature on a rocking platform. 2 ml of diluted Streptavidin-HRP was then applied to each membrane for 30 min at room temperature on a rocking platform after three times of wash. 1 ml of the prepared Chemi Reagent Mix was added onto the membranes after three washings. The signal was then captured by the ChemiDoc Touch Imaging System (Bio-Rad). Quantification of the relative protein expression in Δ Pr-A9 cells compared to the WT cells was determined in Image Lab software (Bio-Rad). The average signal of duplicate spots subtracting background value from negative control spots represented the levels of each protein. The relative change in protein levels was then determined by comparing Δ Pr-A9 cells to the WT cells.

RAS GTPase Chemi ELISA

RAS GTPase activity in cell lysate of the WT CaSki or Δ Pr-A9 cells was determined by RAS GTPase Chemi ELISA Kit (Active Motif, Carlsbad, CA, USA # 52097) according to the manufacturer's protocol. Briefly, 2×10^7 cells were solubilized in 500 μ l of Complete Lysis/Binding buffer at 4°C for 15 min. Protein concentration of cell extract was then measured by Micro BCA Protein Assay (PIERCE, #23235) after cell debris was removed by centrifugation at $14,000 \times g$ for 10 min. 2 μ g of GST-Raf-RBD in 50 μ l of Complete Lysis/Binding buffer was coated in each well of a 96-well plate by incubating for 1 hr at 4°C with 100 rpm agitation. After three washings, 50 μ g of extract in 50 μ l Complete Lysis/Binding buffer was then added to each well. HeLa (EGF-treated) extract was served as a positive control. The plate was incubated for 1 hr at room temperature with 100 rpm agitation. The active RAS protein (GST-Raf-RBD binding) in each well was then detected by incubating with primary RAS antibody and three times of wash, and then, HRP-conjugated secondary antibody and three times of wash. Chemiluminescence in each well was read in a luminometer by adding 50 μ l room temperature chemiluminescent working solution.

RNA-ISH and IF staining

Endogenous Inc-FANCI-2 in cervical cancer tissues and their derived cell lines was examined by RNAscope Multiplex Fluorescent V2 Assay (Advanced Cell Diagnostics, Minneapolis, MN, USA #323100) as described previously (Liu *et al.*, 2021). A custom-designed probe targeting nt 359–1713 of GenBank Acc. No. MT669800.1 transcript for Inc-FANCI-2a-PA2 was utilized. Dual staining of Inc-FANCI-2 and IGFBP RNA was performed according to RNAscope Multiplex Fluorescent v2 Manual part 2 (#323100), and the channel one probe for IGFBP3 (#310351) and channel three probe for Inc-FANCI-2 (#509061-C3) were applied.

For RNA-ISH combined IF staining of MCAM protein, 3×10^5 of CaSki cells were grown on a glass coverslip in a six-well plate for 24 hr before transfection. Plasmid pHBL05 containing a long isoform of Inc-FANCI-2a-PA2 (GenBank Acc. No. MT669800.1) was transfected into cells with LipoD 293 DNA in vitro transfection reagent. Cultured Adherent Cell Sample Preparation for the RNAscope Multiplex Fluorescent v2 was performed according to the manufacturer's protocol (Advanced Cell Diagnostics, Minneapolis, MN, USA MK-50-010). Briefly, the cells were washed with PBS and fixed by 10% neutral buffered formalin at room temperature for 30 min. The cells were washed with PBS three times and dehydrated by 50% ethanol for 5 min, 70% ethanol for 5 min, 100% ethanol for 5 min, and 100% ethanol for 10 min. The cells were then rehydrated by 70% ethanol for 2 min, 50% ethanol for 2 min, and PBS for 10 min. The cells were then applied to hydrogen peroxide at RT for 10 min and washed with distilled water three times. The cells were then applied to Protease III (1:15 dilution with PBS) at RT for 10 min. The cells were ready for RNAscope Multiplex Fluorescent v2 Manual part 2 (#323100). IF was performed after RNA-ISH but before counterstaining with DAPI. The cells were washed with PBS and were then blocked with 2% BSA in PBS for 1 hr at 37°C or overnight at 4°C. The cells were incubated with the MCAM primary antibody (ProteinTech, Rosemont, IL, USA #17564-1-AP, diluted 1:200 in 2% BSA blocking buffer) for 2 hr at 37°C. An Alexa Fluor-conjugated secondary antibody (1:500, Thermo Fisher Scientific) was diluted in a blocking solution and incubated for 1 hr at 37°C. The slides were washed with PBS three times. DAPI was used for nuclei counterstaining before mounting in a Prolong Gold Antifade mounting medium (Thermo Fisher Scientific, #P36934).

Confocal images were collected using a Zeiss LSM710 laser-scanning microscope equipped with a 63 $\times$ Plan-Apochromat (NA 1.4) objective lens. Three-dimensional distributions of Inc-FANCI-2 were generated by Z stacks using ZEN 2.3 software (Zeiss).

IRPCR and LC-MS/MS analysis

To identify the Inc-FANCI-2 RNA-associated proteins, we pulled down Inc-FANCI-2 RNA from WT CaSki cells using IRPCR, a modified ChIRP protocol from the published method (Chu *et al.*, 2012), followed by mass spectrometry (Chu and Chang, 2018). Briefly, a total of 30 Inc-FANCI-2 anti-sense oligo probes (oLLY496-oLLY525), each with biotinTEG at 3' end, across entire Inc-FANCI-2 RNA (GenBank Acc. No MT669800.1) was designed using the online probe designer (singlemoleculefish.com). Two probe pools were used in IRPCR assays: the probe pool 1 was the mixed oligos in even numbers (oLLY496/498/500/502/504/506/508/510/512/514/516/518/520/522/524) and the probe pool 2 mixed with the oligos in odd numbers (oLLY497/499/501/503/505/507/509/511/513/515/517/519/521/523/525) (Supplementary file 5). CaSki cells were seeded and harvested at 24 hr, and the cell lysates from 200 million cells were used for each IRPCR reaction. Instead of formaldehyde cross-link in the standard protocol (Chu *et al.*, 2012), UV cross-linking (254 nm, energy at 480 mJ/cm²) was applied in our study to minimize non-specific binding. After UV cross-linking, the cells were lysed on ice in 1 × radioimmunoprecipitation assay buffer (Boston Bioproducts, Ashland, MA, USA #BP-115) (50 mM Tris base/Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40) supplemented with protease inhibitors (complete mini-EDTA-free protease inhibitor cocktail, Millipore Sigma, Burlington, MA, USA #469315900) and RNase inhibitor (Thermo Fisher Scientific, #AM2694) for 30 min, followed by a brief sonication. The obtained cell lysates were spun for 10 min of centrifugation at 20,000 × g at 4°C, and the collected cell lysate supernatants were pre-absorbed with C-1 magnetic beads (Thermo Fisher Scientific, #65002) for 1 hr at room temperature before proceeding to oligo probe hybridization.

Two different hybridization protocols were used to pull down Inc-FANCI-2 RNA. One had the pre-absorbed cell lysate incubated with two separate probe pools in hybridization buffer (750 mM NaCl, 0.1% SDS, 50 mM Tris-Cl pH 7.0, 1 mM EDTA, 15% formamide) at room temperature overnight, and the pre-washed C-1 magnetic beads were then added and incubated for an additional 4 hr; the other had two separate oligo probe pools immobilized onto pre-washed C-1 magnetic beads first at room temperature for 1 hr and then mixed with the pre-absorbed cell lysates in hybridization buffer overnight at room temperature, along with the negative control-beads only IRPCR reaction without oligo probes serving as a control in the modified protocol. After hybridization, the beads were washed with 1 × wash buffer (2 × NaCl and sodium citrate, 0.1% SDS) for five times and resuspended in 1 ml lysis buffer. 100 µl beads from each IRPCR were set aside for RNA isolation of Inc-FANCI-2 pull-down efficiency, and the leftover beads were sent for mass spectrometry analysis. The input and IRPCR RNA were isolated using a MIRNeasy mini kit (QIAGEN, #217004) following the standard protocol after proteinase K (Millipore Sigma, #71049) digestion.

For mass spectrometry analysis, the IRPCR beads were processed for trypsin/LysC digestion before being submitted to Thermo Scientific Orbitrap Exploris 240 Mass Spectrometer and a Thermo Dionex UltiMate 3000 RSLCnano System for Proteomics. Peptides from trypsin digestion were loaded onto a peptide trap cartridge at a flow rate of 5 µl/min. The trapped peptides were eluted onto a reversed-phase Easy-Spray Column PepMap RSLC, C18, 2 µm, 100 Å, 75 µm × 250 mm (Thermo Scientific) using a linear gradient of acetonitrile (3–36%) in 0.1% formic acid. The elution duration was 110 min at a flow rate of 0.3 µl/min. Eluted peptides from the Easy-Spray column were ionized and sprayed into the mass spectrometer, using a Nano Easy-Spray Ion Source (Thermo Fisher Scientific) under the following settings: spray voltage, 1.6 kV, Capillary temperature, 275°C. Other settings were empirically determined. Raw data files were searched against human protein sequences database using the Proteome Discoverer 2.4 software (Thermo Fisher Scientific, San Jose, CA, USA) based on the SEQUEST algorithm. All the protein peptides identified in the IRPCR pull-downs were summarized in Supplementary file 4.

Acknowledgements

We thank Louise T Chow of the University of Alabama at Birmingham for her critical reading of the manuscript. We thank Craig Meyers of Penn State University Hershey Medical Center for providing HPV16- and HPV18-infected raft cultures, Xing Xie and Yang Li of Zhejiang University Women's Hospital of China for cervical tissue sections, and Johannes G Schweizer from Arbor Vita Corporation for anti-HPV16 E6-specific antibodies. This work was supported by the Intramural Research Program of the National Institutes of Health, the National Cancer Institute, and the Center for Cancer Research (ZIA SC 010357 to ZMZ).

Additional information

Funding

Funder	Grant reference number	Author
The Intramural Research Program of the NIH, NCI, Center for Cancer Research	ZIA SC 010357	Zhi-Ming Zheng

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Author contributions

Haibin Liu, Conceptualization, Data curation, Software, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing – original draft, Writing – review and editing; Lulu Yu, Data curation, Formal analysis, Validation, Visualization, Methodology, Writing – original draft, Writing – review and editing; Vladimir Majerciak, Conceptualization, Visualization, Writing – review and editing; Thomas J Meyer, Software, Visualization, Methodology; Ming Yi, Software, Investigation, Writing – review and editing; Peter F Johnson, Conceptualization, Methodology; Maggie Cam, Software, Formal analysis; Douglas R Lowy, Supervision, Writing – review and editing; Zhi-Ming Zheng, Conceptualization, Resources, Data curation, Formal analysis, Supervision, Funding acquisition, Investigation, Writing – original draft, Project administration, Writing – review and editing

Author ORCIDs

Haibin Liu  <https://orcid.org/0000-0003-3601-6066>

Lulu Yu  <https://orcid.org/0000-0003-0309-9520>

Zhi-Ming Zheng  <https://orcid.org/0000-0001-5547-7912>

Ethics

All clinical cervical tissues were obtained from anonymized excess cervical tissues, with informed consent, that were no longer needed for diagnosis or clinical purposes from a study approved for clinical research by the Institutional Review Board of the Zhejiang University Women's Hospital.

Peer review material

Reviewer #1 (Public review): <https://doi.org/10.7554/eLife.102681.3.sa1>

Reviewer #3 (Public review): <https://doi.org/10.7554/eLife.102681.3.sa2>

Author response <https://doi.org/10.7554/eLife.102681.3.sa3>

Additional files

Supplementary files

Supplementary file 1. Genome-wide effect of Inc-FANCI-2 knockout (KO) in ΔPr-A9 and ΔYY1 cells on gene expression when compared with parental wild-type (WT) CaSki cells.

Supplementary file 2. The genes with altered expression found in both ΔPr-A9 and ΔYY1-D5 cells when compared with parental wild-type (WT) CaSki cells.

Supplementary file 3. The genes with increased expression in KRAS signaling and epithelial-mesenchymal transition (EMT) and with decreased expression in interferon gamma (IFN-γ) and interferon alpha (IFN-α) responses in Inc-FANCI-2 knockout (KO) cells (ΔPr-A9 and ΔYY1-D5) over the parental wild-type (WT) CaSki cells.

Supplementary file 4. CaSki proteins and their peptide spectrum matches (PSMs) identified by Inc-FANCI-2 Isolation of Inc-FANCI-2 RNA-protein complex by RNA purification (IRPCR)-LC-MS/MS.

Supplementary file 5. Sequences of oligonucleotides used in this study.

MDAR checklist

Data availability

RNA Sequencing data have been deposited in GEO under accession code GSE190904.

The following dataset was generated:

Author(s)	Year	Dataset title	Dataset URL	Database and Identifier
Zheng Z, Liu H, Majerciak V, Meyer T	2022	Effect of a long non-coding RNA, lnc-FANCI-2 on host transcriptome	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE190904	NCBI Gene Expression Omnibus, GSE190904

References

- Allard JB, Duan C. 2018. IGF-Binding proteins: why do they exist and why are there so many? *Frontiers in Endocrinology* **9**:117. DOI: <https://doi.org/10.3389/fendo.2018.00117>, PMID: 29686648
- Anfosso F, Bardin N, Francès V, Vivier E, Camoin-Jau L, Sampol J, Dignat-George F. 1998. Activation of human endothelial cells via S-Endo-1 Antigen (CD146) stimulates the tyrosine phosphorylation of focal adhesion kinase p125FAK. *Journal of Biological Chemistry* **273**:26852–26856. DOI: <https://doi.org/10.1074/jbc.273.41.26852>
- Asati V, Mahapatra DK, Bharti SK. 2016. PI3K/Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways inhibitors as anticancer agents: Structural and pharmacological perspectives. *European Journal of Medicinal Chemistry* **109**:314–341. DOI: <https://doi.org/10.1016/j.ejmech.2016.01.012>, PMID: 26807863
- Bach LA. 2018. IGF-binding proteins. *Journal of Molecular Endocrinology* **61**:T11–T28. DOI: <https://doi.org/10.1530/JME-17-0254>, PMID: 29255001
- Barbosa MS, Schlegel R. 1989. The E6 and E7 genes of HPV-18 are sufficient for inducing two-stage in vitro transformation of human keratinocytes. *Oncogene* **4**:1529–1532 PMID: 2556677.
- Bardin N, Francès V, Combes V, Sampol J, Dignat-George F. 1998. CD146: biosynthesis and production of a soluble form in human cultured endothelial cells. *FEBS Letters* **421**:12–14. DOI: [https://doi.org/10.1016/s0014-5793\(97\)01455-5](https://doi.org/10.1016/s0014-5793(97)01455-5), PMID: 9462829
- Baxter RC. 2013. Insulin-like growth factor binding protein-3 (IGFBP-3): Novel ligands mediate unexpected functions. *Journal of Cell Communication and Signaling* **7**:179–189. DOI: <https://doi.org/10.1007/s12079-013-0203-9>, PMID: 23700234
- BeltCappellino A, Majerciak V, Lobanov A, Lack J, Cam M, Zheng ZM. 2019. CRISPR/Cas9-mediated knockout and in situ inversion of the ORF57 gene from all copies of the Kaposi's sarcoma associated herpesvirus genome in BCBL-1 cells. *Journal of Virology* **93**:e00628–e00619. DOI: <https://doi.org/10.1128/JVI.00628-19>, PMID: 31413125
- Bosch FX, Lorincz A, Muñoz N, Meijer CJLM, Shah KV. 2002. The causal relation between human papillomavirus and cervical cancer. *Journal of Clinical Pathology* **55**:244–265. DOI: <https://doi.org/10.1136/jcp.55.4.244>, PMID: 11919208
- Brake T, Lambert PF. 2005. Estrogen contributes to the onset, persistence, and malignant progression of cervical cancer in a human papillomavirus-transgenic mouse model. *PNAS* **102**:2490–2495. DOI: <https://doi.org/10.1073/pnas.0409883102>, PMID: 15699322
- Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, Jemal A. 2024. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA* **74**:229–263. DOI: <https://doi.org/10.3322/caac.21834>, PMID: 38572751
- Castellano E, Downward J. 2011. RAS Interaction with PI3K: more than just another effector pathway. *Genes & Cancer* **2**:261–274. DOI: <https://doi.org/10.1177/1947601911408079>, PMID: 21779497
- Castello A, Hentze MW, Preiss T. 2015. Metabolic enzymes enjoying new partnerships as RNA-Binding proteins. *Trends in Endocrinology and Metabolism* **26**:746–757. DOI: <https://doi.org/10.1016/j.tem.2015.09.012>, PMID: 26520658
- CCBR. 2023. Pipeliner. GitHub. <https://github.com/CCBR/Pipeliner>
- Chu C, Quinn J, Chang HY. 2012. Chromatin isolation by RNA purification (ChIRP). *Journal of Visualized Experiments* **2012**:3912. DOI: <https://doi.org/10.3791/3912>, PMID: 22472705
- Chu C, Chang HY. 2018. ChIRP-MS: RNA-Directed Proteomic Discovery. *Methods in Molecular Biology* **1861**:37–45. DOI: https://doi.org/10.1007/978-1-4939-8766-5_3
- Conover CA, Bale LK, Durham SK, Powell DR. 2000. Insulin-like growth factor (IGF) binding protein-3 potentiation of IGF action is mediated through the phosphatidylinositol-3-kinase pathway and is associated with alteration in protein kinase B/AKT sensitivity. *Endocrinology* **141**:3098–3103. DOI: <https://doi.org/10.1210/endo.141.9.7660>, PMID: 10965879
- Conrad C, Yildiz D, Cleary SJ, Margraf A, Cook L, Schlomann U, Panaretou B, Bowser JL, Karmouty-Quintana H, Li J, Berg NK, Martin SC, Aljohmani A, Moussavi-Harami SF, Wang KM, Tian JJ, Magnen M, Valet C, Qiu L, Singer JP, et al. 2022. ADAM8 signaling drives neutrophil migration and ARDS severity. *JCI Insight* **7**:e149870. DOI: <https://doi.org/10.1172/jci.insight.149870>, PMID: 35132956
- Crook T, Storey A, Almond N, Osborn K, Crawford L. 1988. Human papillomavirus type 16 cooperates with activated ras and fos oncogenes in the hormone-dependent transformation of primary mouse cells. *PNAS* **85**:8820–8824. DOI: <https://doi.org/10.1073/pnas.85.23.8820>, PMID: 2848240
- Cuesta C, Arévalo-Alameda C, Castellano E. 2021. The importance of being PI3K in the RAS signaling network. *Genes* **12**:1094. DOI: <https://doi.org/10.3390/genes12071094>, PMID: 34356110

- Dang M**, Dubbin K, D'Aiello A, Hartmann M, Lodish H, Herrlich A. 2011. Epidermal growth factor (EGF) ligand release by substrate-specific a disintegrin and metalloproteases (ADAMs) involves different protein kinase C (PKC) isoenzymes depending on the stimulus. *The Journal of Biological Chemistry* **286**:17704–17713. DOI: <https://doi.org/10.1074/jbc.M110.187823>, PMID: 21454702
- DiPaolo JA**, Woodworth CD, Popescu NC, Notario V, Doniger J. 1989. Induction of human cervical squamous cell carcinoma by sequential transfection with human papillomavirus 16 DNA and viral Harvey ras. *Oncogene* **4**:395–399 PMID: 2541388.
- Dobin A**, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**:15–21. DOI: <https://doi.org/10.1093/bioinformatics/bts635>, PMID: 23104886
- Downward J**. 2003. Targeting RAS signalling pathways in cancer therapy. *Nature Reviews Cancer* **3**:11–22. DOI: <https://doi.org/10.1038/nrc969>
- Dye DE**, Karlen S, Rohrbach B, Staub O, Braathen LR, Eidne KA, Coombe DR. 2009. hShroom1 links a membrane bound protein to the actin cytoskeleton. *Cellular and Molecular Life Sciences* **66**:681–696. DOI: <https://doi.org/10.1007/s00018-009-8645-1>, PMID: 19137261
- Eiben GL**, Velders MP, Schreiber H, Cassetti MC, Pullen JK, Smith LR, Kast WM. 2002. Establishment of an HLA-A*0201 human papillomavirus type 16 tumor model to determine the efficacy of vaccination strategies in HLA-A*0201 transgenic mice. *Cancer Research* **62**:5792–5799.
- Favata MF**, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *The Journal of Biological Chemistry* **273**:18623–18632. DOI: <https://doi.org/10.1074/jbc.273.29.18623>, PMID: 9660836
- Fernández-Medarde A**, Santos E. 2011. Ras in cancer and developmental diseases. *Genes & Cancer* **2**:344–358. DOI: <https://doi.org/10.1177/1947601911411084>, PMID: 21779504
- Gao X**, Gao C, Liu G, Hu J. 2016. MAP4K4: an emerging therapeutic target in cancer. *Cell & Bioscience* **6**:56. DOI: <https://doi.org/10.1186/s13578-016-0121-7>, PMID: 27800153
- Gao X**, Chen G, Gao C, Zhang DH, Kuan SF, Stabile LP, Liu G, Hu J. 2017. MAP4K4 is a novel MAPK/ERK pathway regulator required for lung adenocarcinoma maintenance. *Molecular Oncology* **11**:628–639. DOI: <https://doi.org/10.1002/1878-0261.12055>, PMID: 28306189
- Golijow CD**, Mouron SA, Gomez MA, Dulout FN. 1999. Differences in K-ras codon 12 mutation frequency between “high-risk” and “low-risk” HPV-infected samples. *Gynecologic Oncology* **75**:108–112. DOI: <https://doi.org/10.1006/gyno.1999.5528>
- González-Montero J**, Rojas CI, Burotto M. 2023. MAP4K4 and cancer: ready for the main stage? *Frontiers in Oncology* **13**:1162835. DOI: <https://doi.org/10.3389/fonc.2023.1162835>, PMID: 37223681
- Greenhalgh DA**, Wang XJ, Rothnagel JA, Eckhardt JN, Quintanilla MI, Barber JL, Bundman DS, Longley MA, Schlegel R, Roop DR. 1994. Transgenic mice expressing targeted HPV-18 E6 and E7 oncogenes in the epidermis develop verrucous lesions and spontaneous, rasHa-activated papillomas. *Cell Growth & Differentiation* **5**:667–675 PMID: 7522035.
- Halbert CL**, Demers GW, Galloway DA. 1991. The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. *Journal of Virology* **65**:473–478. DOI: <https://doi.org/10.1128/JVI.65.1.473-478.1991>, PMID: 1845902
- Harrow J**, Frankish A, Gonzalez JM, Tapanari E, Diekhans M, Kokocinski F, Aken BL, Barrell D, Zadissa A, Searle S, Barnes I, Bignell A, Boychenko V, Hunt T, Kay M, Mukherjee G, Rajan J, Despacio-Reyes G, Saunders G, Steward C, et al. 2012. GENCODE: the reference human genome annotation for The ENCODE Project. *Genome Research* **22**:1760–1774. DOI: <https://doi.org/10.1101/gr.135350.111>, PMID: 22955987
- Hawley-Nelson P**, Vousden KH, Hubbert NL, Lowy DR, Schiller JT. 1989. HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *The EMBO Journal* **8**:3905–3910. DOI: <https://doi.org/10.1002/j.1460-2075.1989.tb08570.x>, PMID: 2555178
- Hentze MW**, Castello A, Schwarzl T, Preiss T. 2018. A brave new world of RNA-binding proteins. *Nature Reviews. Molecular Cell Biology* **19**:327–341. DOI: <https://doi.org/10.1038/nrm.2017.130>, PMID: 29339797
- Herber R**, Liem A, Pitot H, Lambert PF. 1996. Squamous epithelial hyperplasia and carcinoma in mice transgenic for the human papillomavirus type 16 E7 oncogene. *Journal of Virology* **70**:1873–1881. DOI: <https://doi.org/10.1128/JVI.70.3.1873-1881.1996>, PMID: 8627712
- Hietanen S**, Lain S, Krausz E, Blattner C, Lane DP. 2000. Activation of p53 in cervical carcinoma cells by small molecules. *PNAS* **97**:8501–8506. DOI: <https://doi.org/10.1073/pnas.97.15.8501>, PMID: 10900010
- Hoskins EE**, Morris TA, Higginbotham JM, Spardy N, Cha E, Kelly P, Williams DA, Wikenheiser-Brokamp KA, Duensing S, Wells SI. 2009. Fanconi anemia deficiency stimulates HPV-associated hyperplastic growth in organotypic epithelial raft culture. *Oncogene* **28**:674–685. DOI: <https://doi.org/10.1038/onc.2008.416>
- Hoskins EE**, Morreale RJ, Werner SP, Higginbotham JM, Laimins LA, Lambert PF, Brown DR, Gillison ML, Nuovo GJ, Witte DP, Kim M-O, Davies SM, Mehta PA, Butsch Kovacic M, Wikenheiser-Brokamp KA, Wells SI. 2012. The fanconi anemia pathway limits human papillomavirus replication. *Journal of Virology* **86**:8131–8138. DOI: <https://doi.org/10.1128/JVI.00408-12>, PMID: 22623785
- Huang YK**, Cheng WC, Kuo TT, Yang JC, Wu YC, Wu HH, Lo CC, Hsieh CY, Wong SC, Lu CH, Wu WL, Liu SJ, Li YC, Lin CC, Shen CN, Hung MC, Lin JT, Yeh CC, Sher YP. 2024. Inhibition of ADAM9 promotes the selective degradation of KRAS and sensitizes pancreatic cancers to chemotherapy. *Nature Cancer* **5**:400–419. DOI: <https://doi.org/10.1038/s43018-023-00720-x>

- Joshkon A**, Heim X, Dubrou C, Bachelier R, Traboulsi W, Stalin J, Fayyad-Kazan H, Badran B, Foucault-Bertaud A, Leroyer AS, Bardin N, Blot-Chabaud M. 2020. Role of CD146 (MCAM) in physiological and pathological angiogenesis-contribution of new antibodies for therapy. *Biomedicines* **8**:633. DOI: <https://doi.org/10.3390/biomedicines8120633>, PMID: 33352759
- Jozefiak A**, Pacholska-Bogalska J, Myga-Nowak M, Kedzia W, Kwasniewska A, Luczak M, Kedzia H, Gozdicka-Jozefiak A. 2008. Serum and tissue levels of insulin-like growth factor-I in women with dysplasia and HPV-positive cervical cancer. *Molecular Medicine Reports* **1**:231–237. DOI: <https://doi.org/10.3892/mmr.1.2.231>, PMID: 21479402
- Józefiak A**, Larska M, Pomorska-Mól M, Ruszkowski JJ. 2021. The IGF-1 signaling pathway in viral infections. *Viruses* **13**:13081488. DOI: <https://doi.org/10.3390/v13081488>
- Justus CR**, Leffler N, Ruiz-Echevarria M, Yang LV. 2014. In vitro cell migration and invasion assays. *Journal of Visualized Experiments* **88**:51046. DOI: <https://doi.org/10.3791/51046>, PMID: 24962652
- Kato S**, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P. 1995. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270**:1491–1494. DOI: <https://doi.org/10.1126/science.270.5241.1491>, PMID: 7491495
- Kebir A**, Harhour K, Guillet B, Liu JW, Foucault-Bertaud A, Lamy E, Kaspi E, Elganfoud N, Vely F, Sabatier F, Sampol J, Pisano P, Kruihof EKO, Bardin N, Dignat-George F, Blot-Chabaud M. 2010. CD146 short isoform increases the proangiogenic potential of endothelial progenitor cells in vitro and in vivo. *Circulation Research* **107**:66–75. DOI: <https://doi.org/10.1161/CIRCRESAHA.109.213827>, PMID: 20448216
- Kleino I**, Järviluoma A, Hepojoki J, Huovila AP, Saksela K. 2015. Preferred SH3 domain partners of ADAM metalloproteases include shared and ADAM-Specific SH3 interactions. *PLOS ONE* **10**:e0121301. DOI: <https://doi.org/10.1371/journal.pone.0121301>
- Klingelhutz AJ**, Foster SA, McDougall JK. 1996. Telomerase activation by the E6 gene product of human papillomavirus type 16. *Nature* **380**:79–82. DOI: <https://doi.org/10.1038/380079a0>, PMID: 8598912
- Korotkevich G**, Sukhov V, Budin N, Shpak B, Artyomov MN. 2021. Sergushichev A: Fast Gene Set Enrichment Analysis. *bioRxiv*. DOI: <https://doi.org/10.1101/060012>
- Law CW**, Chen Y, Shi W, Smyth GK. 2014. Voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biology* **15**:R29. DOI: <https://doi.org/10.1186/gb-2014-15-2-r29>, PMID: 24485249
- Li B**, Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**:323. DOI: <https://doi.org/10.1186/1471-2105-12-323>, PMID: 21816040
- Liberzon A**, Subramanian A, Pinchback R, Thorvaldsdóttir H, Tamayo P, Mesirov JP. 2011. Molecular signatures database (MSigDB) 3.0. *Bioinformatics* **27**:1739–1740. DOI: <https://doi.org/10.1093/bioinformatics/btr260>, PMID: 21546393
- Liberzon A**, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP, Tamayo P. 2015. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Systems* **1**:417–425. DOI: <https://doi.org/10.1016/j.cels.2015.12.004>, PMID: 26771021
- Liu X**, Dakic A, Zhang Y, Dai Y, Chen R, Schlegel R. 2009. HPV E6 protein interacts physically and functionally with the cellular telomerase complex. *PNAS* **106**:18780–18785. DOI: <https://doi.org/10.1073/pnas.0906357106>, PMID: 19843693
- Liu H**, Xu J, Yang Y, Wang X, Wu E, Majerciak V, Zhang T, Steenbergen RDM, Wang H-K, Banerjee NS, Li Y, Lu W, Meyers C, Zhu J, Xie X, Chow LT, Zheng ZM. 2021. Oncogenic HPV promotes the expression of the long noncoding RNA Inc-FANCI-2 through E7 and YY1. *PNAS* **118**:e2014195118. DOI: <https://doi.org/10.1073/pnas.2014195118>
- Liu H**, Zheng ZM. 2022. Linking a nuclear lncRNA to cytoplasmic lysosome integrity and cell death. *PNAS* **119**:e2123082119. DOI: <https://doi.org/10.1073/pnas.2123082119>
- Martin JL**, Weenink SM, Baxter RC. 2003. Insulin-like growth factor-binding protein-3 potentiates epidermal growth factor action in MCF-10A mammary epithelial cells. Involvement of p44/42 and p38 mitogen-activated protein kinases. *The Journal of Biological Chemistry* **278**:2969–2976. DOI: <https://doi.org/10.1074/jbc.M210739200>, PMID: 12433918
- Matlashewski G**, Schneider J, Banks L, Jones N, Murray A, Crawford L. 1987. Human papillomavirus type 16 DNA cooperates with activated ras in transforming primary cells. *The EMBO Journal* **6**:1741–1746. DOI: <https://doi.org/10.1002/j.1460-2075.1987.tb02426.x>, PMID: 3038534
- McCredie MRE**, Sharples KJ, Paul C, Baranyai J, Medley G, Jones RW, Skegg DCG. 2008. Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: a retrospective cohort study. *The Lancet. Oncology* **9**:425–434. DOI: [https://doi.org/10.1016/S1470-2045\(08\)70103-7](https://doi.org/10.1016/S1470-2045(08)70103-7), PMID: 18407790
- Münger K**, Phelps WC, Bubbs V, Howley PM, Schlegel R. 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *Journal of Virology* **63**:4417–4421. DOI: <https://doi.org/10.1128/JVI.63.10.4417-4421.1989>, PMID: 2476573
- Munoz N**, Bosch FX, Sanjose S, Herrero R, Castellsague X, Shah KV, Snijders PJ, Meijer CJ. 2003. International Agency for Research on Cancer Multicenter Cervical Cancer Study G: Epidemiologic classification of human papillomavirus types associated with cervical cancer. *The New England Journal of Medicine* **348**:518–527. DOI: <https://doi.org/10.1056/NEJMoa021641>

- Narisawa-Saito M, Yoshimatsu Y, Ohno S, Yugawa T, Egawa N, Fujita M, Hirohashi S, Kiyono T. 2008. An in vitro multistep carcinogenesis model for human cervical cancer. *Cancer Research* **68**:5699–5705. DOI: <https://doi.org/10.1158/0008-5472.CAN-07-6862>, PMID: 18632622
- Nieto P, Ambrogio C, Esteban-Burgos L, Gómez-López G, Blasco MT, Yao Z, Marais R, Rosen N, Chiarle R, Pisano DG, Barbacid M, Santamaría D. 2017. A Braf kinase-inactive mutant induces lung adenocarcinoma. *Nature* **548**:239–243. DOI: <https://doi.org/10.1038/nature23297>, PMID: 28783725
- Niraj J, Färkkilä A, D’Andrea AD. 2019. The fanconi anemia pathway in cancer. *Annual Review of Cancer Biology* **3**:457–478. DOI: <https://doi.org/10.1146/annurev-cancerbio-030617-050422>, PMID: 30882047
- Nissley DV, McCormick F. 2022. RAS at 40: update from the RAS initiative. *Cancer Discovery* **12**:895–898. DOI: <https://doi.org/10.1158/2159-8290.CD-21-1554>, PMID: 35046096
- Ohtsu H, Dempsey PJ, Eguchi S. 2006. ADAMs as mediators of EGF receptor transactivation by G protein-coupled receptors. *American Journal of Physiology. Cell Physiology* **291**:C1–C10. DOI: <https://doi.org/10.1152/ajpcell.00620.2005>, PMID: 16769815
- Park JW, Pitot HC, Strati K, Spardy N, Duensing S, Grompe M, Lambert PF. 2010. Deficiencies in the Fanconi anemia DNA damage response pathway increase sensitivity to HPV-associated head and neck cancer. *Cancer Research* **70**:9959–9968. DOI: <https://doi.org/10.1158/0008-5472.CAN-10-1291>, PMID: 20935219
- Park JW, Shin MK, Lambert PF. 2014. High incidence of female reproductive tract cancers in FA-deficient HPV16-transgenic mice correlates with E7’s induction of DNA damage response, an activity mediated by E7’s inactivation of pocket proteins. *Oncogene* **33**:3383–3391. DOI: <https://doi.org/10.1038/ncr.2013.327>, PMID: 24013229
- Patterson V, Ullah F, Bryant L, Griffin JN, Sidhu A, Saliganan S, Blaile M, Saenz MS, Smith R, Ellingwood S, Grange DK, Hu X, Mireguli M, Luo Y, Shen Y, Mulhern M, Zackai E, Ritter A, Izumi K, Hoefele J, et al. 2023. Abrogation of MAP4K4 protein function causes congenital anomalies in humans and zebrafish. *Science Advances* **9**:eade0631. DOI: <https://doi.org/10.1126/sciadv.ade0631>, PMID: 37126546
- Phelps WC, Yee CL, Mürger K, Howley PM. 1988. The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. *Cell* **53**:539–547. DOI: [https://doi.org/10.1016/0092-8674\(88\)90570-3](https://doi.org/10.1016/0092-8674(88)90570-3), PMID: 2836062
- Prior IA, Lewis PD, Mattos C. 2012. A comprehensive survey of Ras mutations in cancer. *Cancer Research* **72**:2457–2467. DOI: <https://doi.org/10.1158/0008-5472.CAN-11-2612>, PMID: 22589270
- Prokopakis P, Sourvinos G, Koumantaki Y, Koumantakis E, Spandidos DA. 2002. K-ras mutations and HPV infection in cervicitis and intraepithelial neoplasias of the cervix. *Oncology Reports* **9**:129–133.
- Riley RR, Duensing S, Brake T, Mürger K, Lambert PF, Arbeit JM. 2003. Dissection of human papillomavirus E6 and E7 function in transgenic mouse models of cervical carcinogenesis. *Cancer Research* **63**:4862–4871 PMID: 12941807.
- Riou G, Barrois M, Sheng ZM, Duvillard P, Lhomme C. 1988. Somatic deletions and mutations of c-Ha-ras gene in human cervical cancers. *Oncogene* **3**:329–333.
- Roman A, Munger K. 2013. The papillomavirus E7 proteins. *Virology* **445**:138–168. DOI: <https://doi.org/10.1016/j.virol.2013.04.013>, PMID: 23731972
- Roth Flach RJ, Guo CA, Danai LV, Yaw JC, Gujja S, Edwards YJK, Czech MP. 2016. Endothelial mitogen-activated protein kinase kinase kinase kinase 4 is critical for lymphatic vascular development and function. *Molecular and Cellular Biology* **36**:1740–1749. DOI: <https://doi.org/10.1128/MCB.01121-15>, PMID: 27044870
- Saliani M, Mirzaiebadizi A, Javadmanesh A, Siavoshi A, Ahmadian MR. 2022. KRAS-related long noncoding RNAs in human cancers. *Cancer Gene Therapy* **29**:418–427. DOI: <https://doi.org/10.1038/s41417-021-00381-x>, PMID: 34489556
- Santegoets LAM, van Baars R, Terlouw A, Heijmans-Antonissen C, Swagemakers SMA, van der Spek PJ, Ewing PC, van Beurden M, van der Meijden WJ, Helmerhorst TJM, Blok LJ. 2012. Different DNA damage and cell cycle checkpoint control in low- and high-risk human papillomavirus infections of the vulva. *International Journal of Cancer* **130**:2874–2885. DOI: <https://doi.org/10.1002/ijc.26345>
- Schäfer B, Marg B, Gschwind A, Ullrich A. 2004. Distinct ADAM metalloproteinases regulate G protein-coupled receptor-induced cell proliferation and survival. *The Journal of Biological Chemistry* **279**:47929–47938. DOI: <https://doi.org/10.1074/jbc.M400129200>, PMID: 15337756
- Scheffner M, Mürger K, Byrne JC, Howley PM. 1991. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *PNAS* **88**:5523–5527. DOI: <https://doi.org/10.1073/pnas.88.13.5523>
- Schlomann U, Wildeboer D, Webster A, Antropova O, Zeuschner D, Knight CG, Docherty AJP, Lambert M, Skelton L, Jockusch H, Bartsch JW. 2002. The metalloprotease disintegrin ADAM8. Processing by autocatalysis is required for proteolytic activity and cell adhesion. *The Journal of Biological Chemistry* **277**:48210–48219. DOI: <https://doi.org/10.1074/jbc.M203355200>, PMID: 12372841
- Schreiber K, Cannon RE, Karrison T, Beck-Engeser G, Huo D, Tennant RW, Jensen H, Kast WM, Krausz T, Meredith SC, Chen L, Schreiber H. 2004. Strong synergy between mutant ras and HPV16 E6/E7 in the development of primary tumors. *Oncogene* **23**:3972–3979. DOI: <https://doi.org/10.1038/sj.onc.1207507>, PMID: 15077191
- Serrano ML, Romero A, Cendales R, Sánchez-Gómez M, Bravo MM. 2006. Serum levels of insulin-like growth factor-I and -II and insulin-like growth factor binding protein 3 in women with squamous intraepithelial lesions and cervical cancer. *Biomedica* **26**:258–268 PMID: 16925098.
- Serrano ML, Sánchez-Gómez M, Bravo MM. 2007. Insulin-like growth factor system gene expression in cervical scrapes from women with squamous intraepithelial lesions and cervical cancer. *Growth Hormone & IGF Research* **17**:492–499. DOI: <https://doi.org/10.1016/j.gthir.2007.07.001>, PMID: 17709267

- Simanshu DK, Nissley DV, McCormick F. 2017. RAS proteins and their regulators in human disease. *Cell* **170**:17–33. DOI: <https://doi.org/10.1016/j.cell.2017.06.009>, PMID: 28666118
- Smyth GK. 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology* **3**:1027. DOI: <https://doi.org/10.2202/1544-6115.1027>, PMID: 16646809
- Song S, Pitot HC, Lambert PF. 1999. The human papillomavirus type 16 E6 gene alone is sufficient to induce carcinomas in transgenic animals. *Journal of Virology* **73**:5887–5893. DOI: <https://doi.org/10.1128/JVI.73.7.5887-5893.1999>, PMID: 10364340
- Song S, Liem A, Miller JA, Lambert PF. 2000. Human papillomavirus types 16 E6 and E7 contribute differently to carcinogenesis. *Virology* **267**:141–150. DOI: <https://doi.org/10.1006/viro.1999.0106>, PMID: 10662610
- Stalin J, Harhour K, Hubert L, Garrigue P, Nollet M, Essaadi A, Muller A, Foucault-Bertaud A, Bachelier R, Sabatier F, Pisano P, Peiretti F, Leroy AS, Guillet B, Bardin N, Dignat-George F, Blot-Chabaud M. 2016. Soluble CD146 boosts therapeutic effect of endothelial progenitors through proteolytic processing of short CD146 isoform. *Cardiovascular Research* **111**:240–251. DOI: <https://doi.org/10.1093/cvr/cvw096>, PMID: 27170199
- Statello L, Guo CJ, Chen LL, Huarte M. 2021. Gene regulation by long non-coding RNAs and its biological functions. *Nature Reviews. Molecular Cell Biology* **22**:96–118. DOI: <https://doi.org/10.1038/s41580-020-00315-9>, PMID: 33353982
- Storey A, Pim D, Murray A, Osborn K, Banks L, Crawford L. 1988. Comparison of the in vitro transforming activities of human papillomavirus types. *The EMBO Journal* **7**:1815–1820. DOI: <https://doi.org/10.1002/j.1460-2075.1988.tb03013.x>, PMID: 2458921
- Strickland SW, Vande Pol S. 2016. The human papillomavirus 16 E7 oncoprotein attenuates AKT signaling to promote internal ribosome entry site-dependent translation and expression of c-MYC. *Journal of Virology* **90**:5611–5621. DOI: <https://doi.org/10.1128/JVI.00411-16>
- Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP. 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *PNAS* **102**:15545–15550. DOI: <https://doi.org/10.1073/pnas.0506580102>, PMID: 16199517
- Sun C, Wu MH, Guo M, Day ML, Lee ES, Yuan SY. 2010. ADAM15 regulates endothelial permeability and neutrophil migration via Src/ERK1/2 signalling. *Cardiovascular Research* **87**:348–355. DOI: <https://doi.org/10.1093/cvr/cvq060>
- Tommasino M, Accardi R, Caldeira S, Dong W, Malanchi I, Smet A, Zehbe I. 2003. The role of TP53 in Cervical carcinogenesis. *Human Mutation* **21**:307–312. DOI: <https://doi.org/10.1002/humu.10178>, PMID: 12619117
- Vande Pol SB, Klingelutz AJ. 2013. Papillomavirus E6 oncoproteins. *Virology* **445**:115–137. DOI: <https://doi.org/10.1016/j.virol.2013.04.026>, PMID: 23711382
- Vanhaesebroeck B, Perry MWD, Brown JR, André F, Okkenhaug K. 2021. PI3K inhibitors are finally coming of age. *Nature Reviews Drug Discovery* **20**:741–769. DOI: <https://doi.org/10.1038/s41573-021-00209-1>
- Varma Shrivastav S, Bhardwaj A, Pathak KA, Shrivastav A. 2020. Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3): Unraveling the role in mediating IGF-Independent effects within the cell. *Frontiers in Cell and Developmental Biology* **8**:286. DOI: <https://doi.org/10.3389/fcell.2020.00286>, PMID: 32478064
- Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Muñoz N. 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *The Journal of Pathology* **189**:12–19. DOI: [https://doi.org/10.1002/\(SICI\)1096-9896\(199909\)189:1<12::AID-PATH431>3.0.CO;2-F](https://doi.org/10.1002/(SICI)1096-9896(199909)189:1<12::AID-PATH431>3.0.CO;2-F), PMID: 10451482
- Wang Z, Xu Q, Zhang N, Du X, Xu G, Yan X. 2020. CD146, from a melanoma cell adhesion molecule to a signaling receptor. *Signal Transduction and Targeted Therapy* **5**:148. DOI: <https://doi.org/10.1038/s41392-020-00259-8>, PMID: 32782280
- Xu J, Liu H, Yang Y, Wang X, Liu P, Li Y, Meyers C, Banerjee NS, Wang HK, Cam M, Lu W, Chow LT, Xie X, Zhu J, Zheng ZM. 2019. Genome-wide profiling of cervical RNA-Binding proteins identifies human papillomavirus regulation of RNASEH2A expression by viral E7 and E2F1. *mBio* **10**:e02687. DOI: <https://doi.org/10.1128/mBio.02687-18>
- Yang J, Nie J, Ma X, Wei Y, Peng Y, Wei X. 2019. Targeting PI3K in cancer: mechanisms and advances in clinical trials. *Mol Cancer* **18**:0954. DOI: <https://doi.org/10.1186/s12943-019-0954-x>
- Yi M, Zhu R, Stephens RM. 2018. GradientScanSurv-An exhaustive association test method for gene expression data with censored survival outcome. *PLOS ONE* **13**:e0207590. DOI: <https://doi.org/10.1371/journal.pone.0207590>, PMID: 30517129
- Yu L, Zheng ZM. 2022. Human papillomavirus type 16 circular RNA is barely detectable for the claimed biological activity. *mBio* **13**:e0359421. DOI: <https://doi.org/10.1128/mbio.03594-21>