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The long noncoding RNA *PRANCR* is associated with alternative splicing of fibronectin-1 in keratinocytes

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To the editor:

We recently identified a long noncoding RNA (lncRNA), *PRANCR,* that regulates epidermal renewal and stratification in cell culture and organoid models (Cai et al, 2020), but its mechanism of action is not fully known. We initially hypothesized that *PRANCR* may alter the expression of genes associated with keratinocyte cell fate (KCF) (Wu et al, 2012). However, when comparing control vs. *PRANCR*-depleted keratinocytes, we observed no significant expression changes in KCF genes (Figure S1A). This led us to consider post-transcriptional mechanisms. Emerging evidence suggests that lncRNAs can regulate alternative splicing (AS) by controlling the level or activity of splicing factors, forming RNA:RNA duplexes with pre-mRNA molecules (Yang et al, 2014), or altering chromatin remodeling (Romero-Barrios et al, 2018). AS generates multiple isoforms of the same gene and is involved in cell differentiation, development, and diseases of the epidermis (Tanis et al, 2018). However, whether lncRNAs affect AS in the skin remains largely unknown.

To detect AS events associated with *PRANCR*, we compared mRNA isoform expression in RNA-seq of control and *PRANCR*-depleted primary human keratinocytes (Cai et al, 2020). Using replicate Multivariate Analysis of Transcript Silencing (rMATS), we identified 238 AS events associated with *PRANCR* depletion (Figure S1B). These included the splicing of cassette exons in two KCF genes: Fibronectin-1 (*FN1*) and *PTPN12* (Figures S1C-D). *FN1*

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Conceptualization: A.B.C.O., K.Q., B.K.S., Methodology: A.B.C.O., O.A., P.C., K.Q., B.K.S., Software: P.C., K.Q., Validation: A.B.C.O., O.A.. B.C., B.K.S., Formal analysis: A.B.C.O., P.C., Investigation: A.B.C.O., O.A., P.C., B.C., Resources: K.Q., B.K.S., Data Curation: P.C., K.Q., Writing – Original Draft: A.B.C.O., B.K.S., Writing – Review & Editing: P.C., B.C., K.Q., Visualization: A.B.C.O., O.A., B.K.S., Supervision: A.B.C.O., K.Q., B.K.S., Project Administration: A.B.C.O., B.K.S., Funding Acquisition: A.B.C.O., B.K.S.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

regulates keratinocyte proliferation, adhesion, and differentiation (Watt, 2002), sparking our interest to focus on this event. *PRANCR* depletion (Figure S1E) leads to reduced expression of the *FN1*-isoform containing an exon encoding extra-domain A (Figure S1F). Using semi-quantitative RT-PCR, we confirmed that the proportional expression of EDA-containing isoforms (hereafter: *FN1*-EDA+) decreased upon *PRANCR* depletion (Figure 1A-B). At the protein level, *PRANCR* depletion resulted in 70-80% reduction of FN1-EDA+ (Figure 1C-D). The shift in isoform expression mimicked the proportionally reduced expression of FN1-EDA+ that occurs upon calcium-induced keratinocyte differentiation *in vitro* (Figure 1E-H and Figure S2A-B).

We explored how *PRANCR* could affect *FN1* splicing. AS of pre-mRNAs is regulated by the binding of splicing factors to *cis*-exonic elements (Ouyang et al, 2022). Within the *FN1*-EDA exon, an exon splice enhancer (ESE) (Figure S2C) mediates EDA exon recognition and splicing (Buratti et al, 2004). To identify splicing factors interacting with this element, we used the database of molecular interaction associated with alternative splicing (MiasDB) (Xing et al, 2016), which nominated three members of the serine/arginine-rich (SR) family of splicing factors (SRSFs): *SRSF1, SRSF3* and *SRSF7*(Figure S2C). *SRSF1* and *7*expression were reduced in *PRNCR*-depleted keratinocytes (Figure 1I), which we recapitulated using double targeting of *SRSF1* and *SRSF7* using RNA interference (RNAi) (Figure S2D). Dual knockdown led to a reduction of *FN1*-EDA+ (Figure 1J), which phenocopied FN1 splicing changes resulting from *PRANCR* depletion (Figure 1A-B) and led us to consider a preliminary model in which *PRANCR* influences FN1 splicing through SRSF1 and 7. We additionally performed RNA-immunoprecipitation to explore a potential direct interaction between *PRANCR* lncRNA and SRSF1/7 proteins but did not observe an association between them (data not shown).

We next evaluated how AS of *FN1* affected keratinocyte cellular phenotypes. We used RNA interference (RNAi) targeting the *FN1*-EDA+ isoform specifically or that targeted total *FN1* (Figure S3A). RNAi targeting the *FN1*-5'UTR depleted total *FN1* RNA (Figure S3B-C) and protein (Figure 2A-B), while targeting the EDA exon specifically depleted *FN1*-EDA+ mRNA and protein (Figure S3B-C and Figure 2A-B). Approximately 60% of FN1-EDA− (negative) protein remained after targeting FN1-EDA+, indicating ~40% of total FN1 includes the EDA exon in keratinocyte progenitors. We next compared the effect of total *FN1* vs. *FN1*-EDA+ depletion on keratinocyte proliferation. Total *FN1* knockdown inhibited proliferation (Figure 2C) and led to a ~2.25x increase in CDKN1A protein levels (Figure 2D-E). Notably, depletion of FN1-EDA+ resulted in a similar inhibition of proliferation (Figure 2C) and CDKN1A induction (Figure 2D-E), despite substantial residual expression of the FN1-EDA− isoform. This result indicated that *FN1*-EDA+ has a major effect on keratinocyte proliferation that is not compensated by FN1-EDA−. This conclusion is consistent with findings that embryonic stem cells proliferate faster in the presence of EDA (Losino et al, 2013), and that actively growing keratinocytes have the highest *FN1*-EDA+/EDA− ratio (Szell et al, 2004).

FN1 has a role in cutaneous wound healing, a process that requires coordinated keratinocyte proliferation, migration, and differentiation (Wu et al, 2012). We examined the effect of FN1 and *PRANCR* in scratch assays to evaluate proliferation and migration (Figure S3D). Wild-

type keratinocytes closed the scratch gap in ~9 hours (Figure 2F-G). Total *FN1* and selective *FN1*-EDA+ depletion delayed gap closure (Figure 2F-G). FN1-EDA+ depletion resulted in a closure delay comparable to total FN1 depletion, indicating a major role for FN1-EDA+ in this phenotype. Our results are consistent with the observation that FN1-EDA+ is highly expressed in skin wounds (Ffrench-Constant et al, 1989) and that FN1-EDA+ deficient mice have wound healing defects (Muro et al, 2003).

FN1 activates integrin signaling that is required for keratinocyte function (Duperret et al, 2015). In fibroblasts, integrins recognize the EDGIHEL motif in EDA, which phosphorylates focal adhesion kinase (FAK) at Tyrosine-397 (Kohan et al, 2010) and maintains LTBP1 protein levels (Klingberg et al, 2018). Upon total FN1 and FN1-EDA+ depletion, we observed decreased phosphorylation of Tyr-397-FAK (Figure 2H-I), but no change in LTBP1 levels (Figure S2E-F). FAK phosphorylation is required for skin formation (Duperret et al, 2015) and regulates CDKN1A expression (Bryant et al, 2006), suggesting a potential molecular mechanism by which FN1-EDA+ may function. Notably, *PRANCR* depletion also leads to scratch gap closure delays and decreased Tyr-397-FAK phosphorylation (Figures 2H-I), similar to the effect of FN1-EDA+ knockdown.

Viewed together, our data support a hypothetical model by which *PRANCR* regulates keratinocyte cell function, at least in part, through alternative splicing of *FN1* (Figure S2H). Based on their effects on proliferation and scratch gap closure, we speculate that *PRANCR* and *FN1*-EDA+ could have intertwined roles in wound healing. Our findings also support the broader appreciation of diverse lncRNA mechanisms by suggesting a role for the lncRNA *PRANCR* in alternative mRNA splicing in the skin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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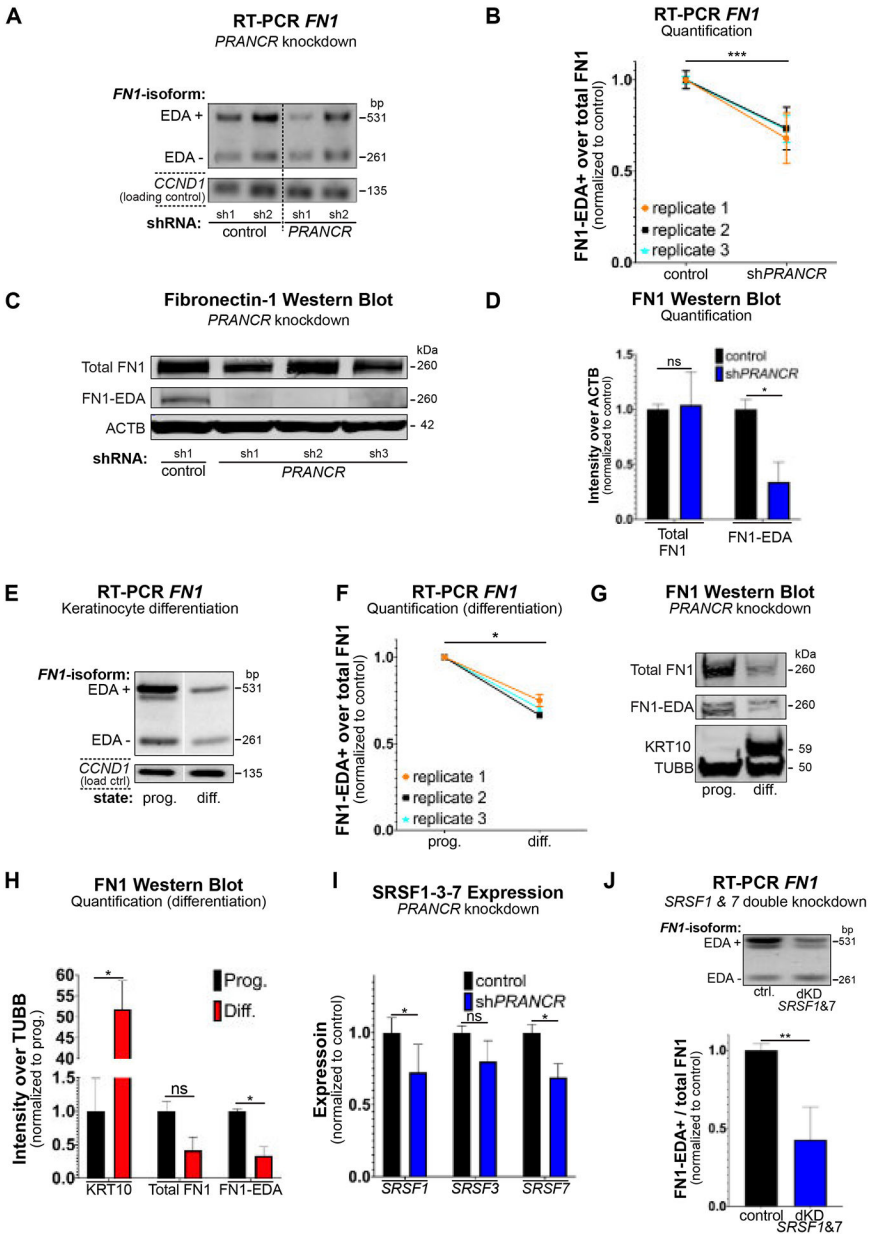

Figure 1 |. Depletion of the long noncoding RNA *PRANCR* alters relative expression levels of Fibronectin-1 (*FN1*) isoforms relevant for keratinocyte differentiation.

A) Reverse transcriptase-polymerase chain reaction (RT-PCR) of *FN1* from RNA from control and *PRANCR*-depleted (Figure S1E) keratinocytes. Two independent non-targeting controls and *PRANCR*-targeting shRNAs (sh1 and sh2) were used. *CCND1* is a loading control. B) Quantification of the relative expression of *FN1* mRNA isoforms in control vs. *PRANCR*-depleted keratinocytes. Each point represents the ratio of *FN1*-EDA+ isoform intensity divided by total *FN1* intensity. n=3, error bars=SEM, two-tailed unpaired t-test; *** p=0.0002. C) Western blot of total-FN1 and FN1-EDA+ protein isoforms in control and *PRANCR*-depleted keratinocytes. Three independent shRNAs were used (sh1, −2, −3). ACTB is a loading control. D) Relative expression of FN1 protein isoforms in control vs *PRANCR*-depleted keratinocytes. n=2, error bars=SEM, 2way ANOVA Sidak's multiple

comparison test, * adjusted p=0.024. E) RT-PCR of *FN1* from RNA from progenitor (prog.) and differentiated (diff.) keratinocytes (non-adapted image in Figure S2B). F) Quantification of the relative expression of *FN1* isoforms in progenitor (prog.) and differentiated (diff.) keratinocytes. Each point represents the ratio of the *FN1*-EDA+ isoform divided by the total *FN1* expression. n=3, error bars = SEM, two-tailed paired t-test; * p=0.01. G) Western blot of total-FN1 and FN1-EDA+ protein isoforms in progenitor (prog.) and differentiated (diff.) keratinocytes. Keratin-10 (KRT10) is a keratinocyte differentiation marker and Beta-tubulin (TUBB) is a loading control. H) Relative expression of KRT10 and FN1 protein isoforms in progenitor (prog.) vs differentiated (diff.) keratinocytes. n=2, error bars=SEM, Holm-Sidak corrected multiple comparison t-tests, * adjusted p=0.026 (KRT10) and 0.041 (FN1-EDA). I) mRNA expression of *SRSF1, 3*, and *7* in control and *PRANCR*-depleted (sh*PRANCR*) cells. Data retrieved from NIH Gene Expression Omnibus GSE125400 (Cai et al, 2020). n=4, error bars=SEM, * FDR=0.039 (SRSF1) and 0.024 (SRSF7). J) *Top*: RT-PCR of *FN1* from control or SRSF1/7-depleted keratinocytes. *Bottom*: Quantification of the relative expression of *FN1* isoforms in control or *SRSF1/7*double-knockdown (dKD; co-transduced with sh*SRSF1* and sh*SRSF7*; Figure S2D) keratinocytes. n=3, error bars=SEM, twosided t-test; ** p=0.0097.

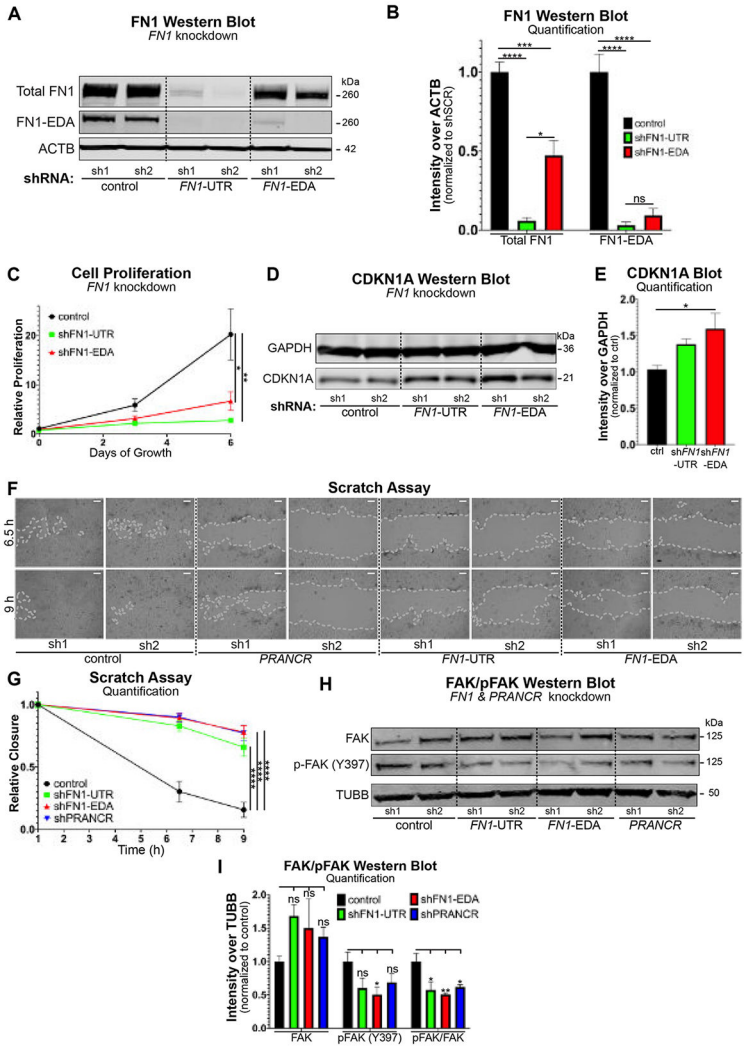

Figure 2 |. *PRANCR* and FN1-EDA regulate keratinocyte proliferation & differentiation.

A) Western blot of total FN1 and FN1-EDA+ in control, total-*FN1*-depleted (*FN1*-UTR) or *FN1*-EDA-depleted keratinocytes. Beta actin (ACTB) is a loading control. Two independent shRNAs were used for each condition (sh1 and sh2). B) Quantitation of relative expression of total-FN1 and FN1-EDA+ protein isoforms in control, total-*FN1*-depleted (shFN1-UTR) or *FN1*-EDA-depleted (shFN1-EDA) keratinocytes. n=3, error bars=SEM, ANOVA with Sidak's multiple comparison test, **** adjusted p<0.0001, *** adjusted p=0.0002, ** adjusted p=0.0036. C) Relative proliferation of control, total *FN1*-depleted (shFN1-UTR) or *FN1*-EDA-depleted (shFN1-EDA) keratinocytes, measured with a fluorescence-based cell quantitation assay. Each dot represents the average of n=2 biological replicates. Error bars=SEM, ordinary one-way ANOVA Sidak's multiple comparison test, ** adjusted p=0.0074, * adjusted p=0.0291. D) Western blot of CDKN1A in control, total-*FN1*-depleted (*FN1*-UTR) or *FN1*-EDA-depleted keratinocytes. GAPDH is a loading control. E) Relative protein expression of CDKN1A in control, total-*FN1*-depleted (shFN1-UTR) or *FN1*-EDA-depleted (shFN1-EDA) keratinocytes. n=2, error bars=SEM, ordinary one-way ANOVA Sidak's multiple comparison test, * adjusted p=0.018. F) *In vitro* scratch assay of control,

PRANCR-depleted, total-*FN1*-depleted (*FN1*-UTR) or *FN1*-EDA-depleted keratinocytes. Images captured at 6.5 or 9 hours after scratching; dotted lines indicate boundaries. Each perturbation was performed with two independent shRNAs (sh1 and sh2). Scale bar=100 μm. G) Quantification of scratch wound closure. Each dot represents the average cell-free area (in pixels^2). n=3, error bars=SEM, ordinary one-way ANOVA Sidak's multiple comparison test, **** all adjusted p<0.0001. H) Western blot of total FAK (FAK) and phosphorylated-FAK (p-FAK, at Tyrosine (Y)-397). Beta-tubulin (TUBB) is a loading control. I) Relative expression of total-FAK, p-FAK (Y397) and pFAK/total-FAK ratio. n=2, error bars=SEM, ordinary one-way ANOVA Sidak's multiple comparison test, * adjusted p=0.028 (pFAK, shFN1-EDA vs control), 0.016 (Ratio, shFN1-UTR vs control) and 0.031 (Ratio, shPRANCR vs control), ** adjusted p=0.0059.