Published in final edited form as:

Nat Struct Mol Biol. 2015 May ; 22(5): 370–376. doi:10.1038/nsmb.3005.

A lncRNA regulates alternative splicing via establishment of a splicing-specific chromatin signature

Inma Gonzalez¹, Roberto Munita², Eneritz Agirre¹, Travis A Dittmer³, Katia Gysling², Tom Misteli^{#3}, and Reini F Luco^{#1}

¹ATIP-AVENIR team, Institute of Human Genetics, CNRS UPR 1142, Montpellier, France.

²Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Santiago, Chile.

³National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA.

These authors contributed equally to this work.

Abstract

Alternative pre-mRNA splicing is a highly cell type-specific process essential to generating protein diversity. However, the mechanisms responsible for the establishment and maintenance of heritable cell-specific alternative-splicing programs are poorly understood. Recent observations point to a role of histone modifications in the regulation of alternative splicing. Here we report a new mechanism of chromatin-mediated splicing control involving a long noncoding RNA (lncRNA). We have identified an evolutionarily conserved nuclear antisense lncRNA, generated from within the human *FGFR2* locus, that promotes epithelial-specific alternative splicing of *FGFR2*. The lncRNA acts through recruitment of Polycomb-group proteins and the histone demethylase KDM2a to create a chromatin environment that impairs binding of a repressive chromatin-splicing adaptor complex important for mesenchymal-specific splicing. Our results uncover a new function for lncRNAs in the establishment and maintenance of cell-specific alternative splicing via modulation of chromatin signatures.

The majority of human genes are alternatively spliced in a highly tissue- and cell type-specific manner^{1,2}. The outcome of alternative splicing is controlled by combinatorial use of multiple *cis*-acting RNA elements along the precursor mRNA (pre-mRNA)³. In addition, recruitment of regulatory splicing factors can be influenced by chromatin modifications, transcriptional regulators and the RNA polymerase II elongation rate^{4–11}. An example of the contribution of chromatin to tissue-specific alternative splicing is the *FGFR2* gene, encoding human fibroblast growth factor receptor 2 (ref. 12). In human mesenchymal stem cells

Correspondence should be addressed to R.F.L. (reini.luco@igh.cnrs.fr) or T.M. (mistelit@mail.nih.gov).

AUTHOR CONTRIBUTIONS

I.G. performed ChIP experiments, RNase protection assays and biotinylated-RNA pulldowns; R.M. performed RACE experiments under K.G.'s supervision; E.A. performed the bioinformatic analysis; T.A.D. performed coimmunoprecipitations and western blots; R.F.L. designed the study; R.F.L. and T.M. discussed and wrote the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper

Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

(hMSCs), *FGFR2* is enriched in dimethylated (me2) and trimethylated (me3) histone H3 K36 (H3K36me2,3), which inhibits inclusion of the alternatively spliced exon IIIb¹². This effect occurs via a chromatin-splicing adaptor system in which the chromatin-binding protein MRG15 recognizes H3K36me2,3 (ref. 13) and, through protein-protein interaction, recruits the negative splicing regulator PTB to exon IIIb¹². In contrast, in epithelial PNT2 cells, in which exon IIIb is included, *FGFR2* contains lower levels of H3K36me2,3 and is instead enriched in H3K27me3, a hallmark of Polycomb group-dependent gene silencing¹², suggesting a nontranscriptional role of this histone modification in *FGFR2* regulation. Here we sought to elucidate the role of H3K27me3 in regulation of *FGFR2* splicing and to delineate how cell type-specific splicing patterns are established and maintained.

RESULTS

Polycomb and KDM2a modulate *FGFR2* alternative splicing

To delineate the mechanism of epithelial-specific regulation of *FGFR2* splicing by H3K27me3, we first probed for the enrichment of H3K27-associated chromatin proteins by chromatin immunoprecipitation (ChIP). Consistently with elevated levels of H3K27me3 and H3K9me3 along the *FGFR2* locus in PNT2 cells (Supplementary Fig. 1a-g), EZH2 and SUZ12 of the H3K27 methyltransferase Polycomb repressive complex 2 (PRC2), together with the H3K27me3-binding protein CBX8 and the H3K9me3-binding protein HP1 γ , were enriched along *FGFR2* in PNT2 cells (Fig. 1a-h and Supplementary Fig. 1r). In addition, the H3K36 demethylase KDM2a, which coimmunoprecipitated with SUZ12 but did not bind to CBX8 (Supplementary Fig. 1s), accumulated over *FGFR2* in PNT2 cells in which H3K36me2,3 levels were low (Fig. 1i,j and Supplementary Fig. 1d-i). In contrast, several other members of the Polycomb PRC1 (Bmi1 and Ring1B) and PRC1-like BCOR complexes (BCOR, HP1 α and the H3K36 demethylase KDM2b) were not differentially enriched along *FGFR2* in PNT2 cells compared to hMSCs (Fig. 1k,l and Supplementary Fig. 1j-r). We conclude that several Polycomb-related proteins and KDM2a are differentially recruited along *FGFR2* in a cell type-specific manner, and this differential recruitment correlates with *FGFR2* splicing outcome.

Given their accumulation across *FGFR2* and their correlation with splicing outcome, we tested whether the chromatin regulators EZH2 and KDM2a were functionally relevant for splicing. Induced expression of EZH2 (alone or in combination with PHF1, an enhancer of EZH2 trimethylase activity¹⁴) or expression of KDM2a increased exon IIIb inclusion in hMSCs by ~2.2-fold but did not affect exon IIIc usage (Fig. 1m-v and Supplementary Figs. 2 and 3). Conversely, down-regulation of KDM2a, and to a lesser extent EZH1 and EZH2, moderately reduced inclusion of exon IIIb in favor of exon IIIc usage (Supplementary Fig. 4). The effect on *FGFR2* splicing was not due to changes in expression of *FGFR2*-regulatory splicing factors (Supplementary Figs. 2–4) and was gene specific because splicing of other PTB-dependent genes (*PKM2* (official symbol *PKM*) and *TPM2*) and alternatively spliced control genes (*CD44* and *HMGA1*) were not altered (Fig. 1p,q,u,v and Supplementary Figs. 2–4).

EZH2 recruits KDM2a to FGFR2 and favors exon IIIb inclusion

In order to uncover the molecular mechanisms by which EZH2 and KDM2a bring about changes in *FGFR2* splicing, we analyzed their downstream effects in hMSC cells. As expected, expression of EZH2 and PHF1 in hMSCs increased levels of EZH2 and H3K27me3 along *FGFR2* but not *CD44* or *HMGA1* alternatively spliced control genes (Fig. 2a-d and Supplementary Fig. 5a,b). Consistently with SUZ12-mediated interaction of KDM2a with PRC2 (Supplementary Fig. 1s), EZH2 also induced recruitment of KDM2a along the *FGFR2* locus to an extent similar to that of the observed increase in H3K27me3 levels (Fig. 2e,f and Supplementary Figs. 2p-r and 5c,d). In agreement with this result, H3K36me2 and H3K36me3 levels were reduced along *FGFR2*, but not along the control genes *CD44* or *HMGA1*, upon expression of EZH2 and PHF1 (Fig. 2g,h and Supplementary Fig. 5e,f). In turn, KDM2a expression in hMSCs reduced H3K36me2 levels along *FGFR2*, as did expression of EZH2 and PHF1, and it also reduced recruitment of the H3K36me2,3-binding protein MRG15, which has previously been shown to act as an adaptor between chromatin and PTB, the repressor of *FGFR2* exon IIIb splicing¹² (Fig. 2i-l and Supplementary Figs. 3p,q and 5g-j). To directly test whether H3K27me3 and EZH2 exert their effect on *FGFR2* alternative splicing via KDM2a, we expressed EZH2 in hMSCs after knockdown of KDM2a by RNA interference (RNAi). EZH2 failed to induce exon IIIb inclusion in the absence of KDM2a (Fig. 2m-s), thus suggesting that PRC2's effect on splicing was dependent on KDM2a. These results indicate that EZH2 promotes exon IIIb inclusion by maintaining low H3K36me2,3 levels via recruitment of KDM2a, which impairs binding of the exon IIIb-inhibitory chromatin-splicing adaptor complex MRG15-PTB. The antagonistic effect of H3K36me3 and H3K27me3 on *FGFR2* splicing points to cross-talk between these histone modifications, thus suggesting a combinatorial role of histone marks on alternative-splicing regulation¹⁵.

An antisense FGFR2 lncRNA is expressed when exon IIIb is included

A key question in chromatin-mediated splicing control is how the regulatory chromatin domains are established and maintained¹⁵. Recent observations have pointed to a prominent role of lncRNAs in establishing chromatin domains¹⁶. These lncRNAs act via recruitment of chromatin-modifying complexes, including Polycomb-related proteins, to specific genomic locations^{16–22}. ChIP mapping of histone modifications around the *FGFR2* gene locus in epithelial PNT2 cells revealed several features of a transcriptionally active promoter, including enrichment of H3K4me3 and a peak of the transcription initiation-competent phospho-Ser5 RNA polymerase II along the alternatively spliced *FGFR2* IIIb-IIIc intragenic region (Supplementary Fig. 6a-c). 5' and 3' rapid amplification of cDNA ends (RACE) in MCF7 and PNT2 epithelial cells identified an antisense transcript of 875 nt in length and starting 282 bp upstream of exon IIIc (Fig. 3a, Supplementary Fig. 6d). Northern blotting with three consecutive probes complementary to the antisense *FGFR2* transcript (asFGFR2), combined with a sense intronic probe and two antisense probes downstream of the putative antisense lncRNA to exclude nonspecific or artifactual detection of sense-strand *FGFR2* transcripts, confirmed the existence of the antisense lncRNA in MCF7 and PNT2 cells, in which *FGFR2* exon IIIb is included (Fig. 3a,b and Supplementary Fig. 6e). This lncRNA was absent in hMSC, myofibroblast PRC30 and skin fibroblast CRL cells, which do not include exon IIIb (Fig. 3c and Supplementary Fig. 6f). We confirmed the presence and cell

specificity of asFGFR2 by strand-specific quantitative reverse-transcription PCR (qRT-PCR) in MCF7, PNT2 and hMSC cells (Supplementary Fig. 6g). Northern blotting with exonic and intronic probes indicated that the antisense lncRNA is not spliced, and 3' RACE demonstrated that asFGFR2 is not polyadenylated (Supplementary Fig. 6d,e). As expected for a splicing regulator, we found asFGFR2 predominantly in the nuclear fraction by strand-specific qRT-PCR (Supplementary Fig. 6h). Finally, we identified three mouse ESTs with more than 89% similarity to asFGFR2; this suggests that asFGFR2 is evolutionary conserved (Supplementary Fig. 6d).

asFGFR2 lncRNA modulates *FGFR2* alternative splicing

To test whether asFGFR2 regulates exon IIIb-IIIc splicing, we downregulated it with locked nucleic acid (LNA) antisense oligonucleotides targeting antisense exon IIIc and asFGFR2 5' untranslated regions (UTRs). Knockdown of asFGFR2 to background levels reduced exon IIIb inclusion more than 40% and almost doubled exon IIIc inclusion in PNT2 cells without affecting *CD44* splicing, *FGFR2* transcript levels or expression of several well-established regulators of *FGFR2* splicing (Fig. 3d-i and Supplementary Fig. 7a-k). Importantly, the use of strand-specific short interfering RNAs (siRNAs) against antisense exon IIIc gave similar results, whereas siRNAs against antisense IIIb did not affect exon IIIb-inclusion levels, thus supporting a specific role for asFGFR2 in exon IIIb inclusion (Supplementary Fig. 7l-o and data not shown). Conversely, ectopic expression of asFGFR2 in hMSCs induced inclusion of exon IIIb in favor of exon IIIc by approximately three-fold (Fig. 3j-n and Supplementary Fig. 7p-z), which was similar to the splicing change observed upon downregulation of the splicing repressor PTB (Supplementary Fig. 6m-n). It is important to note that, unlike the splicing-activator protein ESRP1 (ref. 23), asFGFR2 does not activate exon IIIb inclusion but inhibits its repression by interfering with PTB recruitment. We therefore did not expect a larger effect on *FGFR2* splicing upon expression of asFGFR2 than that caused by the downregulation of PTB itself²⁴. As a control, we expressed a *GAPDH* antisense transcript (asGAPDH), which had no effect on *FGFR2* splicing (Fig. 3j-n and Supplementary Fig. 7p-z). The asFGFR2 splicing effect was gene specific because exons with high sequence complementarity to FGFR2-IIIb in *FGFR1* and *FGFR3* were not affected (Supplementary Fig. 7a-f). Furthermore, sequence analysis of the lncRNA did not identify any RNA-binding motif for known regulators of *FGFR2* splicing in asFGFR2, thus making it unlikely that the effect of asFGFR2 is due to suppression of *FGFR2*-regulatory factors. Finally, unlike small RNAs that have previously been reported to alter alternative splicing by inducing Argonaute recruitment^{4,25,26}, the asFGFR2 effect was RNAi independent because downregulation of Argonautes 1 and 2 or Dicer affected neither *FGFR2* splicing in PNT2 cells nor induction of exon IIIb inclusion in hMSCs upon asFGFR2 expression (Fig. 4). We conclude that an antisense lncRNA expressed from within the *FGFR2* gene modulates *FGFR2* alternative splicing.

asFGFR2 modulates *FGFR2* splicing by recruiting PRC2 and KDM2a

To explore the mechanisms of asFGFR2 splicing regulation, we examined the consequences of expressing asFGFR2 in hMSCs. Ectopic expression of asFGFR2 lncRNA, but not control asGAPDH, induced recruitment of the PRC2 component SUZ12, but not the PRC1 components Ring1b or KDM2b, to the *FGFR2* locus (Fig. 5e,f and Supplementary Fig. 8a-

h). It also increased H3K27me3 levels along the *FGFR2* alternatively spliced region (e7 to e9), by approximately two-fold, but not in downstream constitutively spliced exons (i13–e15) (Fig. 5g,h). In contrast, downregulation of asFGFR2, which is immunoprecipitated by SUZ12 in PNT2 cells, reduced SUZ12 and H3K27me3 levels, thus supporting a role for asFGFR2 in tethering the Polycomb complex to *FGFR2* (Fig. 5a-d,p). In agreement with the observed recruitment of KDM2a to PRC2- and H3K27me3-rich chromatin, asFGFR2 expression in hMSCs also enhanced KDM2a binding along *FGFR2* by approximately two-fold, and this in turn lowered H3K36me2 levels and binding of the repressive chromatin-adaptor MRG15 along the *FGFR2* alternatively spliced region. Consequently, binding of the splicing regulator PTB to exon IIIb pre-mRNA was severely reduced by asFGFR2 expression, which promoted exon IIIb inclusion (Fig. 5i-o and Supplementary Fig. 8e-k). Deletion mutants of asFGFR2 demonstrated that antisense exon IIIc (asIIIc), lacking the intronic regions, was sufficient to induce exon IIIb inclusion in hMSC cells, whereas expression of a truncated antisense exon IIIc transcript (asIIIc^{1/2}) or an antisense sequence containing *FGFR2* exon IIIb, but not exon IIIc (del(IIIc)), was not sufficient (Fig. 6a-d). These results suggest that the antisense IIIc exonic region is necessary and sufficient for the function of asFGFR2. In support of this, an antisense sequence lacking exon IIIc was unable to induce recruitment of SUZ12 to *FGFR2* and failed to increase H3K27me3 levels in hMSCs (Fig. 6e-h), thus confirming that this region drives asFGFR2-dependent recruitment of the PRC2 complex to the gene locus. Of note, expression of asFGFR2 in mesenchymal cells favored exon IIIb inclusion relative to IIIc to a greater extent than increased expression of EZH2 or KDM2a and to a similar level as inhibition of PTB itself, suggesting that asFGFR2 induces recruitment of chromatin regulators to *FGFR2* more efficiently than upon global expression of the chromatin modifiers themselves (Figs. 1m-v and 3j-n and Supplementary Figs. 2, 3, 6m,n and 7p-z). These results demonstrate that asFGFR2 promotes epithelial-specific splicing of *FGFR2* by creating a cell type-specific chromatin environment, along the alternatively spliced region, that is unfavorable to the recruitment and binding of the splicing repressor PTB to its specific RNA-binding sites along exon IIIb. In support of a role for asFGFR2 in maintaining the *FGFR2* IIIb splicing isoform in a physiological context, asFGFR2 expression levels were severely downregulated during epithelial-to-mesenchymal transition, concomitantly with a splicing switch from *FGFR2* exon IIIb to exon IIIc (Supplementary Fig. 6i-l).

asFGFR2 effect on FGFR2 splicing is chromatin dependent

To finally test whether asFGFR2 acts through remodeling of chromatin rather than interference with *FGFR2* pre-mRNA, we expressed asFGFR2 in hMSCs after siRNA knockdown of KDM2a and probed *FGFR2* splicing patterns. Whereas asFGFR2 induced exon IIIb inclusion in the presence of KDM2a, it had no effect on *FGFR2* splicing in the absence of KDM2a, thus suggesting a chromatin-dependent function of the lncRNA (Fig. 6i-m and Supplementary Fig. 8m). Furthermore, asFGFR2 lacked sequence homology with exon IIIb or its PTB-binding sites, and expression of only antisense IIIc exonic sequence, without intronic regions, affected *FGFR2* splicing, thus ruling out potential interference of asFGFR2 with PTB binding or recruitment of the spliceosome to the *FGFR2* pre-mRNA. In addition, RNase A digestion failed to protect the asFGFR2 sequence, and chromatin pulldown of a biotinylated asFGFR2 RNA sequence showed that upon overexpression of the

lncRNA, asFGFR2 was targeted to the *FGFR2* locus precisely around the IIIc region (Fig. 6n and Supplementary Fig. 8l). When taken together, these observations support a mechanism by which the expression of asFGFR2 lncRNA in epithelial cells creates, via direct or indirect recruitment of the Polycomb complex PRC2 and KDM2a to the *FGFR2* locus, a splicing-specific chromatin signature that favors exon IIIb inclusion by interfering with recruitment of inhibitory splicing regulators, such as PTB (Fig. 6o).

DISCUSSION

Here we report regulation of alternative splicing by a lncRNA. Our data suggest a mechanism in which an antisense lncRNA establishes and maintains cell type-specific splicing patterns via epigenetic mechanisms involving histone modifications and the Polycomb complex. Our finding that H3K36me_{2,3} and H3K27me₃ have antagonistic effects on *FGFR2* splicing outcome and that the H3K36 demethylase KDM2a is recruited to H3K27me₃-rich chromatin suggests cross-talk between histone modifications rather than independent action of these epigenetic marks in the regulation of alternative splicing¹⁵. The lncRNA generated from within *FGFR2* is necessary to maintain the epithelial-specific splicing pattern of *FGFR2* in PNT2 cells, and, upon experimental overexpression, it is sufficient to establish an epithelial-specific chromatin signature that promotes exon IIIb inclusion in mesenchymal cells. The asFGFR2 effect on splicing is comparable in size to downregulation of the splicing repressor PTB or downregulation of the splicing regulator IWS1, which was recently shown to induce cell migration and invasiveness in lung tumor cells via modulation of *FGFR2* IIIb-IIIc splicing through recruitment to FGFR2 of the H3K36 methyltransferase SETD2 and the chromatin-adaptor complex MRG15-PTB¹¹. Even though antisense lncRNAs generally act in *cis* by staying attached to their transcriptional units and locally recruit chromatin-remodeling complexes²⁷, we found that exogenously expressed asFGFR2, albeit at considerably higher levels than its endogenous counterpart, can also affect its target locus in *trans*, by targeting the *FGFR2* locus precisely around the IIIc region, possibly by forming RNA-DNA heteroduplexes via sequence complementarity²⁸. We suggest that chromatin-based regulatory mechanisms of alternative splicing, such as the one described here, act in concert with other known alternative-splicing mechanisms, including elongation-dependent splicing-factor recruitment and cis-acting RNA elements, to optimize and fine-tune cell type- and tissue-specific alternative-splicing patterns.

We have here used the well-characterized *FGFR2* gene as a model system to study the role of an antisense lncRNA in cell-specific alternative splicing. The identification of highly homologous asFGFR2 lncRNAs in mice suggests an evolutionary conservation of this mechanism of splicing regulation. Furthermore, computational analysis of expression data from 176 human lymphoblastoid cell lines has shown that ~75% of the identified areas of sense-antisense gene overlap are in alternatively spliced regions²⁹, and we have identified 29 antisense transcripts that are expressed within PTB-dependent alternatively spliced genes, among which 55% are also MRG15 dependent, thus suggesting that lncRNAs may have a more universal role in chromatin-dependent alternative-splicing control. Given the highly tissue-specific and developmentally regulated expression of lncRNAs³⁰, we propose antisense lncRNAs as a new mechanism in determining cell- and tissue-specific alternative-splicing patterns.

METHODS

Methods and any associated references are available in the online version of the paper.

ONLINE METHODS

Cell lines and plasmids.

Human normal prostate epithelial PNT2 cells (Sigma) and immortalized human mesenchymal hMSC stem cells³¹ were grown at 37 °C, 5% CO₂ in RPMI (Invitrogen) and DMEM (Invitrogen), respectively, both supplemented with 10% fetal bovine serum. Human mammary epithelial MCF10a-Snail-ER cells, which stably express the epithelial repressor Snail fused to the estrogen receptor, were kindly provided by D. Haber (Massachusetts General Hospital) and cultured as previously described³². Briefly, to induce the epithelial-to-mesenchymal transition, MCF10a-Snail-ER cells were treated with 100 nM tamoxifen (Sigma) for 6 d. Importantly, parental MCF10a cells did not show any differences in cell morphology or transcriptional and splicing patterns upon the same treatment. All cell lines were regularly tested for mycoplasma. Plasmids were nucleoporated in PNT2 and hMSC cells according to the manufacturer's recommendations. Briefly, 3–5 µg of endo-free plasmid prepared by Maxi prep (Qiagen) was electroporated with an Amaxa nucleoporator into 1×10^6 cells resuspended in 100 µl of either solution V (T30 program) for PNT2 cells or solution hMSC (C17 program) for hMSC cells. 55%–65% of cells typically survived, thus allowing for ~60%–65% transfection efficiency. For transfection of higher numbers of cells, 20 µg of plasmid was electroporated (220 V, 5×1 -ms pulses) into 4×10^6 hMSCs in 100 µl of DMEM. After 72 h in culture, transfected cells were used for experimentation. pCMV-Flag-hKDM2a was provided by R. Klose (University of Oxford); pEBB-Flag-hEZH2 was provided by C.S. Duckett (University of Michigan); and pCMV4-Flag-hPHF1 was provided by D. Reinberg (NYU Langone Medical Center). pCDNA3.1 (+) (Clontech) was used as a negative control (mock). Human antisense *FGFR2* (asFGFR2) was amplified by PCR, on the basis of 5' and 3' RACE results, and cloned in the mammalian expression vector pCDNA3.1 (+). It is 875 nt long, starts right before antisense exon IIIc and ends in intron i8 in the antisense direction. asFGFR2 mutants expressing different exons from *FGFR2* in the antisense direction were synthesized by GenScript and subcloned in pCDNA3.1 (+). As a control, 300 bp of human *GAPDH* 3' UTR were cloned in an antisense direction in pCDNA3.1 (+) (pCDNA3-asGAPDH).

Quantitative RT-PCR and strand-specific RT-PCR.

Total RNA was extracted with the RNeasy Plus Mini kit (Qiagen), and 500 ng of RNA was retrotranscribed (RT) by random priming into cDNA with a High Capacity cDNA RT kit (Applied Biosystems), according to the manufacturers' instructions. Quantification of the amount of cDNA was done with SsoFast EvaGreen Supermix for real-time qPCR (CFX96, Bio-Rad) with oligonucleotide sequences that specifically recognize *FGFR2*-IIIb, *FGFR2*-IIIc, *TPM2*-e6, *TPM2*-e7, *PKM2*-e9 and *PKM2*-e10 splicing variants, and *FGFR2*-e4, *FGFR2*-e14 and *FGFR2*-e15 constitutive exons. *CD44* exon variant v6 and *HMGA1* exon e2 were used as controls for PTB-independent alternative-splicing events. The gene encoding cyclophilin A was used as a house-keeping control. The percentage of exon inclusion was

obtained by division of the expression levels of the alternatively spliced exon by the total expression levels of the corresponding transcript by looking at constitutive exons.

The lncRNA asFGFR2 was detected by strand-specific RT. Gene-specific primers (GSPs) that bind to antisense exons IIIc were used to retrotranscribe asFGFR2 with a Transcriptor First-Strand cDNA Synthesis kit (Roche) at 55 °C, according to the manufacturer's instructions. The asFGFR2 was detected by qRT-PCR by amplification of its 5' UTR cDNA fragment, corresponding to the sequence identified by 5' RACE and antisense exon IIIc. To test the strand specificity of the RT, we amplified *FGFR2* exons e4 and e5 (referred to as Ctl) that are not present in the antisense cDNA. An RT with no primers was used as a negative control, and results were represented as the fold increase with respect to the RT with no primers. The oligonucleotide sequences used are listed in Supplementary Table 1.

RNAi knockdown.

Downregulation of EZH1, EZH2, KDM2a, SUZ12, Argonaute 1, Argonaute 2, Dicer and PTB was performed with siGENOME SMARTpool siRNA oligonucleotides (knockdown efficiencies in Supplementary Data Set 2). Downregulation of asFGFR2 was performed with either custom ON-TARGET plus strand-specific siRNA oligonucleotides against antisense exons IIIc or IIIb, to demonstrate specificity (Dharmacon), and strand-specific LNA GapmeRs antisense oligonucleotides against the 5' UTR and exon IIIc regions of asFGFR2 (Exiqon). siGENOME nontargeting siRNA was used as a negative control. A maximum of 50 to 100 nM total of siRNA oligos was delivered to cells for 72 h with the transfection reagent DharmaFECT 1 (Dharmacon), according to the manufacturer's instructions. For cotransfection of plasmids and siRNA oligos, 1 µg of plasmid together with 50 nM total of siRNA was delivered to the cells with DharmaFECT Duo reagent (Dharmacon). Knockdown efficiency was typically 60%–75%, as assessed by qRT-PCR.

Chromatin immunoprecipitation (ChIP).

ChIP was performed as previously described¹², with approximately 2×10^6 cells per sample. Samples were cross-linked for 10–15 min in 1% formaldehyde at room temperature, nuclear extracts were collected and the purified chromatin was sonicated in an ultrasonic bath (Bioruptor Diagenode) to an average length of 200–500 bp. Sonicated chromatin was immunoprecipitated overnight with 40 µl of precoated anti-IgG magnetic beads (Dynabeads M-280; Invitrogen) previously incubated with the antibody of interest for 4–6 h at 4 °C. Antibodies used were: mouse anti-EZH2 (Cell Signaling 3147), rabbit anti-SUZ12 (Cell Signaling 3737), rabbit anti-CBX8 (provided by K. Helin (University of Copenhagen)), rabbit anti-BCOR (provided by V.J. Bardwell (University of Minnesota)), mouse anti-RING1B (provided by H. Koseki (RCAI Riken Institute, Japan)), mouse anti-HP1α (Upstate 05–869), mouse anti-HP1γ (Chemicon MAB3450), rabbit anti-KDM2a (provided by R. Klose (University of Oxford)), rabbit anti-KDM2b (provided by R. Klose (University of Oxford)), rabbit anti-MRG15 (provided by K. Tominaga (Jichi Medical School)), rabbit anti-phospho-Aer5 RNA Pol II (Abcam ab5131), rabbit anti-RNA Pol II (Abcam ab5408), rabbit anti-unmodified histone H3 (Abcam ab1791), rabbit anti-H3K36me2 (Abcam ab9049), mouse anti-H3-K36me2 (Diagenode Mab-182–050), rabbit anti-H3K36me3 (Abcam ab9050), rabbit anti-H3K4me3 (Millipore 04–745), rabbit anti-H2K27me3

(Diagenode pAB-195–050) and mouse anti-K4me3, mouse anti-H3K9me3, mouse anti-H3-K27me3 (all provided by H. Kimura (Osaka University)). Control immunoprecipitations were performed with no antibody. All the antibodies have been well characterized, and the specificity of the most relevant antibodies is shown in Supplementary Data Set 2. Additional validation information is available on the respective manufacturers' websites. Beads were washed and eluted in 1% SDS and 100 mM NHCO_3 buffer for 15 min at 65 °C, and cross-linking was reversed for 6 h. Chromatin was precipitated with ethanol overnight, treated with proteinase K and purified with QiaQuick PCR-purification columns (Qiagen), according to the manufacturer's instructions. Immunoprecipitated DNA (1.5 μl) and serial dilutions of 10% input DNA (1:2, 1:10, 1:50 and 1:250) were analyzed by SYBR-Green real-time qPCR. The percentage input between samples was normalized with rabbit anti-histone H3, when indicated, for better comparison between cell types and experimental conditions. The oligonucleotide sequences used are listed in Supplementary Table 1.

Rapid amplification of cDNA ends (RACE).

5'RACE.—10 μg of MCF7 and PNT2 total RNA, extracted with TRIzol (Invitrogen), was subjected to 5' RACE with a First Choice RLM-RACE kit (Ambion), according to the manufacturer's instructions. The obtained cDNA was subjected to nested touchdown PCR with strand-specific primers designed within *FGFR2* intron 8 (R_5.1) and inner exon IIIc (R_5.2). The resulting PCR product was purified with a GeneJET PCR Purification kit (Fermentas) and cloned into pGEM-T easy vector (Promega) for sequencing. Sequences from different clones were aligned to human genome build 19 (hg19) with the BLAT search engine. A negative control without tobacco acid pyrophosphatase (TAP) treatment was performed. The oligonucleotide sequences used are listed in Supplementary Table 1.

3'RACE.—Nuclear RNA from MCF7 and PNT2 cells was extracted with a Cytoplasmic and Nuclear RNA Purification kit (Norgen). Nuclear RNA was *in vitro* poly(A)-tailed with a poly(A) polymerase (Ambion), according to the manufacturer's protocol. The polyadenylated RNA was ethanol precipitated and treated with a Turbo DNA-free kit (Ambion). 2 μg of poly(A) RNA was reverse transcribed with a RevertAid First Strand cDNA Synthesis kit (Thermo) and RT-poly(T) primer. The obtained cDNA was subjected to nested touchdown PCR with two pairs of gene-specific primers and the poly(T) adaptors. The resulting PCR products were purified with a GeneJET PCR Purification Kit (Fermentas) and cloned into the pGEM-T easy vector (Promega) for sequencing. Sequences from different clones were aligned to human genome build 19 (hg19) with the BLAT search engine. The oligonucleotide sequences used are listed in Supplementary Table 1, and the full asFGFR2 sequence obtained is in Supplementary Table 2.

Northern blotting.

Northern blotting was performed with the Ambion NorthernMax kit, as recommended by the manufacturer. Briefly, 25 μg of DNase I-treated total RNA extracted with TRIzol (Invitrogen) was loaded in a 1% denaturing formaldehyde-agarose gel for separation of the RNA transcripts by electrophoresis. RNA was transferred onto an Ambion BrightStar-Plus membrane by capillarity for less than 2 h at room temperature and was immediately UV cross-linked to reduce RNA degradation. The membrane was prehybridized for 30–60 min at

68 °C in hybridization buffer, while RNA probes were labeled with radioactive [³²P]UTP. 10⁶ c.p.m./mL of column-purified labeled riboprobe was added to the membrane for overnight hybridization at 68 °C in a rotating oven. After several washes at 68 °C and room temperature, the blot was exposed to film for autoradiography. RNA probes complementary to the *FGFR2* antisense exon IIIc (asIIIc), antisense intron i8 (asI8-IIIc), antisense exon IIIb (asIIIb-I8), antisense e7 (asE7), the region detected by 5' RACE (5' UTR), sense exon 7 (E7), sense intron 8 (senseI8) and *GAPDH* 3' UTR as control were *in vitro* transcribed with a MAXIscript kit (Ambion). The size of the lncRNA was estimated with a nonradiolabeled RNA ladder (Ambion) run on the same agarose gel as the RNAs for transfer, detected with BrET and then marked on the blot by taking the wells as a reference.

Coimmunoprecipitation and western blotting.

As previously described¹², cross-linked chromatin was isolated and sonicated for immunoprecipitation with 60 µl of magnetic beads (anti-IgG Dynabeads M-280 Invitrogen) coated with either rabbit anti-CBX8 (provided by K. Helin (University of Copenhagen, Denmark)), rabbit anti-SUZ12 (Cell Signaling 3737) or no antibody, as a negative control, in 750 µl sonication buffer overnight at 4 °C. Immune complexes were washed and eluted in 20 µl SDS sample buffer for 15 min at 95 °C. Proteins were separated on 10% SDS-PAGE and transferred onto nitrocellulose membrane. After 1 h blocking, the membrane was incubated with rabbit anti-KDM2a (provided by R. Klose (University of Oxford)) and rabbit anti-CBX8 or rabbit anti-SUZ12 as positive controls of the immunoprecipitation. Horseradish peroxidase-conjugated secondary antibodies were detected with the ECL Substrate system (Pierce).

RNA immunoprecipitation (RNA-IP).

As previously described¹², approximately 5 × 10⁶ cells per IP were cross-linked with 1% formaldehyde. Nuclear extracts were collected and sonicated for immunoprecipitation with 40 µl of magnetic beads (anti-IgG Dynabeads M-280 Invitrogen) coated with rabbit anti-PTB (provided by M. Garcia-Blanco (Duke University)), rabbit anti-SUZ12 (Cell Signaling 3737) or IgG as a negative control. Antibody specificity is shown in Supplementary Data Set 2. After washes in high-salt RIPA buffer, beads were eluted, and the cross-linking was reversed for 1 h at 70 °C in 200 µl of elution buffer (50 mM Tris-HCl, pH 7.0, 5 mM EDTA, 10 mM dithiothreitol (DTT) and 1% SDS). RNA was extracted with TRIzol (Invitrogen), DNase I treated and retrotranscribed with either random primers or gene-specific primers to detect asFGFR2. Immunoprecipitated RNA and serial dilutions of the 10% input RNA were analyzed by SYBR-Green real-time qPCR. Results were normalized to no-primer RT and IgG RNA-IP. We used the same exon IIIb oligonucleotide sequence as in the ChIP assay and asFGFR2-5UTR as in the strand-specific RT-PCR, to detect FGFR2 pre-mRNA and asFGFR2 cDNA, respectively. All buffers were sterile, RNase free and supplemented with 100 U/ml of RNase inhibitor (RNasin, Promega) and 1× Protease Inhibitor Cocktail Set III (Calbiochem).

UV-RNA-IP.

For a more direct detection of an RNA-protein interaction, we performed UV-cross-linked RNA-IP as described in Cabianca *et al*²². Briefly 3 × 10⁶ cells were UV-cross-linked with

100 mJ/cm² twice on ice and lysed in 0.5% NP-40, 0.5% Na deoxycholate, 300 U/mL of RNasin (Promega) and 1× protease-inhibitor cocktail (Roche) in PBS for 25 min in rotation at 4 °C. Samples were then treated with 30 U DNase I (Promega) for 15 min at 37 °C. After centrifugation at 1,350#, the supernatants were incubated with 75 µl of protein A magnetic beads (Invitrogen) coated with rabbit anti-SUZ12 (Cell Signaling 3737), mouse anti-RING1B (provided by H. Koseki (RCAI Riken Institute)) or IgG as a negative control overnight at 4 °C. Beads were washed three times in washing buffer I (PBS supplemented with 1% NP-40, 0.5% Na deoxycholate, 150 mM NaCl and 1:200 RNasin), treated with 3 U of DNase I for 15 min at 37 °C, washed again three times in washing buffer II (PBS supplemented with 1% NP-40, 0.5% Na deoxycholate, 150 mM NaCl, 10 mM EDTA and 1:200 RNasin) and finally eluted in 100 µl of 100 mM Tris, pH 8.0, 50 mM NaCl, 10 mM EDTA, 100 µg proteinase K and 0.5% SDS for 30 min at 55 °C. RNA was isolated with TRIzol (Invitrogen), DNase I treated and retrotranscribed for qRT-PCR analyses as described above.

RNase A.

DNase I-treated nuclear RNA from PNT2 cells was exposed to RNase A (0.01 µg, Sigma-Aldrich) for 30 min at 37 °C. The samples were heat inactivated for 10 min at 95 °C, and cDNA was analyzed by qRT-PCR for detection of undigested double-stranded RNA. As controls, *FGFR2* exon IIb was amplified to provide evidence of nonprotected RNA, and the overlapping region between the PTENpg1 sense RNA and a divergent asRNA was used as a positive control (oligonucleotide sequences in Supplementary Table 1).

Biotinylated lncRNA pulldown assay.

Biotinylated asFGFR2 and asGAPDH lncRNAs were transcribed with the AmpliScribe T7-Flash Biotin-RNA transcription kit (Epicentre) with biotin-UTPs. After DNase I treatment and purification with an RNeasy Mini kit (Qiagen), 6 µg of either biotinylated asFGFR2 or biotinylated asGAPDH was nucleoporated into hMSC cells with an Amaxa system for 48 h. Cells were then rinsed in PBS and fixed with 1% formaldehyde for 10 min at room temperature. As in the ChIP protocol, cross-linking was quenched with 125 mM glycine for 5 min, cells were rinsed in 5 ml ice-cold PBS and incubated in swelling buffer (25 mM HEPES, pH 8, 1.5 mM MgCl₂, 10 mM KCl, 0.1% NP-40, 1 mM DTT and 1× Roche Protease Inhibitor Mixture) for 10 min at 4 °C, and after centrifugation the pellet was Dounce homogenized for nuclear extraction. Nuclei were further lysed in nuclear lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.0, and 1× Roche Protease Inhibitor Mixture) and sonicated with a Bioruptor (Diagenode) for 15 min, 30 s on, 30 s off, to obtain sheared chromatin of ~100–600 bp. The chromatin was then diluted 1:10 with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.0, 167 mM NaCl, and 1× Roche Protease Inhibitor Mixture) for immuno-precipitation. Streptavidin magnetic C1 beads (Invitrogen) were washed three times in dilution buffer, blocked with 500 ng/µl yeast total RNA and 1 mg/ml BSA for 1 h at room temperature, and washed three times again in dilution buffer before resuspension in their original volume. 50 µl washed/ blocked C1 beads was added to the chromatin and mixed for another 30 min at room temperature. Bead-biotinylated lncRNA-chromatin complexes were washed five times with washing buffer (2× SSC, 0.5% SDS, 1× DTT and fresh PMSF). Beads were

resuspended in 50 µl of DNA elution buffer (50 mM NaHCO₃, 1% SDS, 100 µg/ml RNase A (Sigma-Aldrich) and 0.1 U/µL RNase H (NEB). DNA elution was carried out twice at 37 °C, and eluents were combined before chromatin was reverse cross-linked at 65 °C overnight. After proteinase K treatment (0.2 U/µL, NEB) at 65 °C for 2 h, DNA was purified with QiaQuick PCR purification columns (Qiagen). Immunoprecipitated DNA (1.5 µl) and serial dilutions of 10% input DNA (1:2, 1:10, 1:50 and 1:250) were analyzed by SYBR-Green real-time qPCR. The oligonucleotide sequences used are listed in Supplementary Table 1.

asFGFR2 conservation in vertebrates.

The asFGFR2 sequence was aligned to the human (hg19) genome with BLAT and UCSC Genome Browser default parameters to extract the conservation values with 46 vertebrate species, as described in Siepel *et al.*³³. Briefly, conservation of the *FGFR2* alternative-splicing region and asFGFR2 was extracted from UCSC Genome Browser phastcons scores, which are based on a phylogenetic hidden Markov model (phylo-HMM) that considers *n* species and the phylogeny with statistical models of nucleotide substitution (<http://hgdownload.cse.ucsc.edu/goldenPath/hg19/phastCons-46way/vertebrate/>). In support of evolutionary conservation of asFGFR2, we identified three mouse ESTs (BI854242.1, BG975446.1 and BG863185.1) from the UCSC mm10/(GRCm38) assembly and a human antisense EST (CV310688.1) from hg19/(GRCh37) with 89%, 91.4%, 89% and 99.4% identity to the asFGFR2 sequence region, respectively.

In silico identification of natural antisense transcripts (NATs) expressed along PTB-dependent alternatively spliced events.

Candidate PTB-dependent exon cassettes were recovered from Luco *et al.*¹², and the three sequences that form the triple-exon cassette event were aligned against the human (hg19) genome with BLAT default parameters. Antisense gene annotations were extracted from Ensembl (GRCh37.p12) with Biomart, with selection for antisense gene type. We first intersected the full alternative-splicing exon-cassette event coordinates, from the first exon start to the third exon end, with fjoin³⁴. We then selected the events that showed overlap with antisense transcripts. As a second complementary approach, we first extracted the antisense sequences from Ensembl (GRCh37.p12) and then aligned them against the whole alternative-splicing event (hg19) to recover a percentage of exact complementary sequence match between the two sequences, measured as the percentage of bases from the antisense Ensembl gene with exact complementarity with the alternative event. We selected the events that showed a percentage higher than 40%.

To increase the data set of antisense transcripts, we added the RNAs that were identified in Zhao *et al.*¹⁹ to directly interact with PRC2 by RIP-seq of EZH2 in mouse ESCs. Locations of PRC2-interacting RNAs in the NCBI37/mm9 mouse assembly were converted with the UCSC liftover tool (<http://genome.ucsc.edu/cgi-bin/hgLiftOver/>) to mm10/GRCm38 assembly coordinates and then to human hg19/GRCh37. We recovered the PRC2-interacting RNA sequences in hg19, and we intersected them with PTB-dependent-event genomic regions with fjoin³⁴. We selected the events that showed overlap with PRC2-interacting RNAs from the opposite strand to the parent gene. As a second approximation, we extracted

the PRC2-interacting RNA sequences of the leftover to hg19, and we calculated the percentage of exact complementary sequence match between the alternative-splicing event and the RIP-seq sequence, as explained before. Combining all these strategies, we found that 5.6% of PTB-dependent events overlap with an antisense transcript, among which 55% were also MRG15 dependent.

Statistical analysis.

All values represent means $\pm$ s.e.m. of three to eight biological replicates and follow a normal distribution. Statistical significance of the differences between tissues was estimated with the two-tailed paired Student's *t* test comparing either the two cell types or relative to control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank V.J. Bardwell (University of Minnesota), R. Carstens (University of Pennsylvania), C.S. Duckett (University of Michigan), M. Garcia-Blanco (Duke University), K. Helin (Biotech Research and Innovation Centre, University of Copenhagen), H. Kimura (Osaka University), H. Koseki (RIKEN Center for Integrative Medical Sciences), R. Klose (Oxford University), D. Reinberg (New York University School of Medicine), K. Tominaga (Jichi Medical School) and D. Haber (Massachusetts General Hospital) for reagents; A. Farcas (R. Klose laboratory) for developing the anti-KDM2b antibody; S. Martinez (R.F.L. laboratory) for establishing the epithelial-to-mesenchymal-transition system; and P. Scaffidi, N. Rascovan, K. Meaburn and R. Klose for discussions and critical reading of the manuscript. This work was supported by the Intramural Research Program of the US National Institutes of Health, National Cancer Institute, Center for Cancer Research (T.M., R.F.L. and T.A.D.), the French Fondation de la Recherche Medical starting grant AJE201132 (R.F.L., I.G. and E.A.), the Fondation ARC pour la Recherche sur le Cancer under the program ATIP-AVENIR (I.G.), the European EpiGeneSys network of excellence (E.A.) and the Chilean Millennium Science Initiative grant P10/063-F (R.M. and K.G.).

References

1. Pan Q, Shai O, Lee LJ, Frey BJ & Blencowe BJ Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat. Genet.* 40, 1413–1415 (2008). [PubMed: 18978789]
2. Wang ET et al. Alternative isoform regulation in human tissue transcriptomes. *Nature* 456, 470–476 (2008). [PubMed: 18978772]
3. Barash Y et al. Deciphering the splicing code. *Nature* 465, 53–59 (2010). [PubMed: 20445623]
4. Alló M et al. Control of alternative splicing through siRNA-mediated transcriptional gene silencing. *Nat. Struct. Mol. Biol.* 16, 717–724 (2009). [PubMed: 19543290]
5. Shukla S et al. CTCF-promoted RNA polymerase II pausing links DNA methylation to splicing. *Nature* 479, 74–79 (2011). [PubMed: 21964334]
6. Saint-André V, Batsche E, Rachez C & Muchardt C Histone H3 lysine 9 trimethylation and HP1 γ favor inclusion of alternative exons. *Nat. Struct. Mol. Biol.* 18, 337–344 (2011). [PubMed: 21358630]
7. Pradeepa MM, Sutherland HG, Ule J, Grimes GR & Bickmore WA Psip1/ Ldgf p52 binds methylated histone H3K36 and splicing factors and contributes to the regulation of alternative splicing. *PLoS Genet.* 8, e1002717 (2012). [PubMed: 22615581]
8. Auboeuf D, Honig A, Berget SM & O'Malley BW Coordinate regulation of transcription and splicing by steroid receptor coregulators. *Science* 298, 416–419 (2002). [PubMed: 12376702]
9. Luco RF, Allo M, Schor IE, Kornblihtt AR & Misteli T Epigenetics in alternative pre-mRNA splicing. *Cell* 144, 16–26 (2011). [PubMed: 21215366]

10. Bielli P et al. The transcription factor FBI-1 inhibits SAM68-mediated BCL-X alternative splicing and apoptosis. *EMBO Rep.* 15, 419–427 (2014). [PubMed: 24514149]
11. Sanidas I et al. Phosphoproteomics screen reveals akt isoform-specific signals linking RNA processing to lung cancer. *Mol. Cell* 53, 577–590 (2014). [PubMed: 24462114]
12. Luco RF et al. Regulation of alternative splicing by histone modifications. *Science* 327, 996–1000 (2010). [PubMed: 20133523]
13. Zhang P et al. Structure of human MRG15 chromo domain and its binding to Lys36-methylated histone H3. *Nucleic Acids Res.* 34, 6621–6628 (2006). [PubMed: 17135209]
14. Sarma K, Margueron R, Ivanov A, Pirrotta V & Reinberg D Ezh2 requires PHF1 to efficiently catalyze H3 lysine 27 trimethylation in vivo. *Mol. Cell. Biol.* 28, 2718–2731 (2008). [PubMed: 18285464]
15. Luco RF & Misteli T More than a splicing code: integrating the role of RNA, chromatin and non-coding RNA in alternative splicing regulation. *Curr. Opin. Genet. Dev.* 21, 366–372 (2011). [PubMed: 21497503]
16. Tsai MC et al. Long noncoding RNA as modular scaffold of histone modification complexes. *Science* 329, 689–693 (2010). [PubMed: 20616235]
17. Pandey RR et al. Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. *Mol. Cell* 32, 232–246 (2008). [PubMed: 18951091]
18. Lee JT Lessons from X-chromosome inactivation: long ncRNA as guides and tethers to the epigenome. *Genes Dev.* 23, 1831–1842 (2009). [PubMed: 19684108]
19. Zhao J et al. Genome-wide identification of polycomb-associated RNAs by RIP-seq. *Mol. Cell* 40, 939–953 (2010). [PubMed: 21172659]
20. Yap KL et al. Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. *Mol. Cell* 38, 662–674 (2010). [PubMed: 20541999]
21. Wang KC et al. A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression. *Nature* 472, 120–124 (2011). [PubMed: 21423168]
22. Cabianca DS et al. A long ncRNA links copy number variation to a polycomb/trithorax epigenetic switch in FSHD muscular dystrophy. *Cell* 149, 819–831 (2012). [PubMed: 22541069]
23. Warzecha CC, Sato TK, Nabat B, Hogenesch JB & Carstens RP ESRP1 and ESRP2 are epithelial cell-type-specific regulators of FGFR2 splicing. *Mol. Cell* 33, 591–601 (2009). [PubMed: 19285943]
24. Wagner EJ & Garcia-Blanco MA RNAi-mediated PTB depletion leads to enhanced exon definition. *Mol. Cell* 10, 943–949 (2002). [PubMed: 12419237]
25. Ameyar-Zazoua M et al. Argonaute proteins couple chromatin silencing to alternative splicing. *Nat. Struct. Mol. Biol.* 19, 998–1004 (2012). [PubMed: 22961379]
26. Taliaferro JM et al. Two new and distinct roles for *Drosophila* Argonaute-2 in the nucleus: alternative pre-mRNA splicing and transcriptional repression. *Genes Dev.* 27, 378–389 (2013). [PubMed: 23392611]
27. Koziol MJ & Rinn JL RNA traffic control of chromatin complexes. *Curr. Opin. Genet. Dev.* 20, 142–148 (2010). [PubMed: 20362426]
28. Ratmeyer L, Vinayak R, Zhong YY, Zon G & Wilson WD Sequence specific thermodynamic and structural properties for DNA:RNA duplexes. *Biochemistry* 33, 5298–5304 (1994). [PubMed: 7513557]
29. Morrissy AS, Griffith M & Marra MA Extensive relationship between antisense transcription and alternative splicing in the human genome. *Genome Res.* 21, 1203–1212 (2011). [PubMed: 21719572]
30. Cabili MN et al. Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Genes Dev.* 25, 1915–1927 (2011). [PubMed: 21890647]
31. Okamoto T et al. Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. *Biochem. Biophys. Res. Commun.* 295, 354–361 (2002). [PubMed: 12150956]

32. Javaid S et al. Dynamic chromatin modification sustains epithelial-mesenchymal transition following inducible expression of Snail-1. *Cell Reports* 5, 1679–1689 (2013). [PubMed: 24360956]
33. Siepel A et al. Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. *Genome Res.* 15, 1034–1050 (2005). [PubMed: 16024819]
34. Richardson JE fjoin: simple and efficient computation of feature overlaps. *J. Comput. Biol.* 13, 1457–1464 (2006). [PubMed: 17061921]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.

Polycomb-complex components in *FGFR2* alternative-splicing regulation. (a–l) Recruitment of EZH2 (a,b), SUZ12 (c,d), CBX8 (e,f), HP1γ (g,h), KDM2a (i,j) and KDM2b (k,l) to *FGFR2* and control *CD44* loci in PNT2 and hMSC cells. ChIP values (mean ± s.e.m.; $n = 3$ independent experiments) are depicted as percentage of input, with no-antibody (no Ab) ChIP as negative control. * $P < 0.05$; ** $P < 0.01$ by two-tailed Student's t test relative to hMSCs. TSS, transcription start site. (m–v) Inclusion levels of *FGFR2* exons IIIb (m,r) and IIIc (n,s), and control *CD44*-v6 (p,u) and *HMGA1*-e2 (q,v), in PNT2 and hMSC cells after expression of EZH2 and PHF1 (m–q) or KDM2a (r–v). qRT-PCR values (mean ± s.e.m.; $n = 9$ independent experiments) are normalized to total mRNA of the corresponding gene and are shown as the fold change over transfection with empty vector (mock). ** $P < 0.001$ by two-tailed Student's t test relative to mock transfection. IIIb/IIIc, splicing ratio of exons IIIb and IIIc (o,t).

Figure 2.

EZH2 promotes *FGFR2* exon IIIb inclusion via KDM2a. (a-l) Levels of EZH2 (a,b), H3K27me3 (c,d), KDM2a (e,f), H3K36me2 (g,h,k,l) and MRG15 (i,j) along *FGFR2* and control *CD44* loci after combined expression of EZH2 and PHF1 (a-h) or KDM2a (i-l) in hMSCs. ChIP values (mean $\pm$ s.e.m.; $n = 4$ independent experiments) are shown as the percentage of input or normalized to control H3. * $P < 0.05$; ** $P < 0.01$ by two-tailed Student's t test relative to mock transfections. (m-s) Inclusion levels of *FGFR2* exons IIIb (m) and IIIc (n), and controls *CD44-v6* (p), *HMGA1-e2* (q), *KDM2A* (r) and *EZH2* (s) after expression of EZH2 in the presence or absence of KDM2a in hMSCs. The gene encoding cyclophilin A (CycA; official symbol *PPIA*) is a housekeeping control. qRT-PCR values (mean $\pm$ s.e.m.; $n = 6$ independent experiments) are normalized to total mRNA of the corresponding gene and are shown as fold change over transfection with negative siRNA and empty vector (mock). ** $P < 0.01$ by two-tailed Student's t test relative to mock. IIIb/IIIc, splicing ratio of exons IIIb and IIIc (o).

Figure 3.

A lncRNA antisense to *FGFR2* promotes exon IIIb inclusion. **(a)** UCSC Genome Browser mapping of the *FGFR2* locus (blue) and the full sequence of asFGFR2 lncRNA identified by 5'–3' RACE (orange). Red, northern blot probes that detected as FGFR2; black, negative probes. Chr, chromosome. **(b,c)** Northern blot detection of asFGFR2 by exon IIIc antisense probe (as(IIIc)) in cells that include exon IIIb (PNT2 and MCF7) and cells that do not include exon IIIb (CRL, PRC30 and hMSCs). Detection of *GAPDH* and an *FGFR2* sense intronic sequence (sense(i8)) are used as controls. From a nonradiolabeled RNA ladder (M, marker), the asFGFR2 fragment is estimated to be around 1 kb, in agreement with results obtained by RACE (Supplementary Data Set 1). **(d–i)** Expression levels of *FGFR2* exons IIIb **(d)** and IIIc **(e)**, and constitutive e14 **(g)**, control *CD44-v6* **(h)** and asFGFR2 after knockdown of asFGFR2 (anti-IIIc) with antisense LNA-modified DNA oligonucleotides in PNT2 cells for 72 h. qRT-PCR values (mean $\pm$ s.e.m.; $n = 6$ independent experiments) are normalized to total mRNA and are shown as fold change over transfection with control siRNA. In the case of asFGFR2, strand-specific qRT-PCR values (ssRT **(i)**, mean $\pm$ s.e.m.; $n = 4$ independent experiments) are shown as the fold change over reverse transcription with no primer. Oligo, oligonucleotide. * $P < 0.05$; ** $P < 0.01$ by two-tailed Student's *t* test relative to control. **(j–n)** Inclusion levels of *FGFR2* exons IIIb **(j)**, IIIc **(k)**, e14 **(m)** and *CD44-v6* **(n)** after expression of asFGFR2 or an antisense *GAPDH* sequence in hMSC cells. qRT-PCR values (mean $\pm$ s.e.m.; $n = 8$ independent experiments) are normalized to total mRNA and are shown as the fold change over transfection with empty vector (mock). ** $P < 0.001$ by two-tailed Student's *t* test relative to mock. IIIb/IIIc, splicing ratio of exons IIIb and IIIc **(f,l)**.

Figure 4.

asFGFR2 effect on *FGFR2* splicing is independent of Argonaute and Dicer. (a-g) Inclusion levels of *FGFR2* exons IIIb (a) and IIIc (b), after combined downregulation of Argonautes 1 and 2 (Ago siRNA) for 72 h in PNT2 (red) and hMSC (black) cells. The inclusion levels of the Ago-independent PKM2 (d) and the Ago-dependent PCSK6 (e) alternatively spliced exons are shown as controls. Knockdown validation of *AGO1* (f) and *AGO2* (g) are also shown. qRT-PCR values (mean $\pm$ s.e.m.; $n = 6$ independent experiments) are normalized to total mRNA of the corresponding gene and are shown as the fold change over transfection with a negative-control siRNA (Ctrl). * $P < 0.01$; ** $P < 0.001$ by two-tailed Student's t test relative to control siRNA. IIIb/IIIc, splicing ratio of exons IIIb and IIIc (c). (h-m) Splicing ratio of *FGFR2* exons IIIb and IIIc (h), and expression levels of control *CD44-v6* (i), asFGFR2 (j), *DICER1* (k), *AGO1* (l) and *AGO2* (m) after combined overexpression of asFGFR2 and downregulation of *DICER1* or *AGO1* and *AGO2* (Ago siRNAs) for 72 h in hMSC cells. qRT-PCR values (mean $\pm$ s.e.m.; $n = 6$ independent experiments) are normalized to cyclophilin A (CycA) or total mRNA and are shown as fold change over transfection with a control siRNA and asGAPDH. ** $P < 0.001$ by two-tailed Student's t test relative to cells treated with control siRNA and asGAPDH.

Figure 5.

asFGFR2 induces an epithelial-specific chromatin signature in *FGFR2*. (a-n) Levels of SUZ12 (a,b,e,f), H3K27me3 (c,d,g,h), KDM2a (i,j), H3K36me2 (k,l) and MRG15 (m,n) along *FGFR2* and control *CD44* loci after downregulation of asFGFR2 in PNT2 cells (a-d) or ectopic expression of asFGFR2 in hMSCs (e-n). ChIP values (mean $\pm$ s.e.m.; $n = 3$ independent experiments for downregulation and $n = 4$ for ectopic expression) are shown as the percentage of input or normalized to control H3. * $P < 0.05$; ** $P < 0.01$ by two-tailed Student's t test relative to asGAPDH or control siRNA. (o,p) RNA immunoprecipitation of PTB to exon IIIb pre-mRNA upon asFGFR2 expression in hMSCs (o) or SUZ12 to endogenous asFGFR2 in PNT2 cells (p). Exon e15 and cyclophilin A (CycA) are negative controls. qRT-PCR values (mean $\pm$ s.e.m.; $n = 3$ independent experiments) are the

percentage of input relative to IgG RNA immunoprecipitation. * $P < 0.05$ by two-tailed Student's t test relative to overexpression of asGAPDH.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6.

asFGFR2's effect on alternative splicing is dependent on the IIIc region and KDM2a. (a-d) Inclusion levels of *FGFR2* exons IIIb (a) and IIIc (c), and control *CD44-v6* (d) after expression of deletion mutants of asFGFR2 (b) for 48 h in hMSCs. qRT-PCR values (mean $\pm$ s.e.m.; $n = 9$ independent experiments) are normalized to their corresponding gene and are shown as the fold change over transfection with control asGAPDH. * $P < 0.05$; ** $P < 0.001$ by two-tailed Student's *t* test relative to asGAPDH. (e-h) Enrichment levels of SUZ12 (e,f) and H3K27me3 (g,h) along *FGFR2* (e,g) and control *CD44* (f,h) loci after expression of asFGFR2 mutants that lack antisense exons IIIc (del(IIIc+IIIb)) or that express only antisense IIIc (as(IIIc+IIIb)). Expression of asGAPDH is a control. ChIP values (mean $\pm$ s.e.m.; $n = 3$ independent experiments) are normalized to control H3. * $P < 0.05$; ** $P < 0.01$ by two-tailed Student's *t* test relative to del(IIIc+IIIb). (i-m) Inclusion levels of *FGFR2* exons IIIb (i) and IIIc (j), e14 (l) and control *CD44-v6* (m) after expression of asFGFR2 in the presence or absence of KDM2a in hMSC cells. qRT-PCR values (mean $\pm$ s.e.m.; $n = 6$, independent experiments) are normalized to total mRNA and are shown as the fold change of transfection with control siRNA and asGAPDH. * $P < 0.05$; ** $P < 0.01$ by two-tailed Student's *t* test relative to asGAPDH. IIIb/IIIc, splicing ratio of exons IIIb and IIIc (k). (n) Enrichment levels of the *in vitro*-transcribed biotinylated asFGFR2 along the *FGFR2* locus in hMSC cells transfected with the biotinylated lncRNAs. Pulldown of a biotinylated asGAPDH sequence is a control. lncRNA pulldown values (mean $\pm$ s.e.m.; $n = 4$ independent experiments) are depicted as the percentage of input. (o) Schematic model of how expression of asFGFR2 (thick red line) induces recruitment in cis of PRC2 (EZH2 and SUZ12) to lead to enrichment of the H3K36 demethylase KDM2a, which inhibits

recruitment of the chromatin-adaptor complex MRG15-PTB, thus favoring exon IIIb inclusion.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript