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Review

Long noncoding RNAs: Lessons from genomic imprinting☆

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ABSTRACT

Genomic imprinting has been a great resource for studying transcriptional and post-transcriptional-based gene regulation by long noncoding RNAs (lncRNAs). In this article, I overview the functional role of intergenic lncRNAs (*H19*, *IPW*, and *MEG3*), antisense lncRNAs (*Kcnq1ot1*, *Airn*, *Nespas*, *Ube3a-ATS*), and enhancer lncRNAs (IG-DMR eRNAs) to understand the diverse mechanisms being employed by them in cis and/or trans to regulate the parent-of-origin-specific expression of target genes. Recent evidence suggests that some of the lncRNAs regulate imprinting by promoting intra-chromosomal higher-order chromatin compartmentalization, affecting replication timing and subnuclear positioning. Whereas others act via transcriptional occlusion or transcriptional collision-based mechanisms. By establishing genomic imprinting of target genes, the lncRNAs play a critical role in important biological functions, such as placental and embryonic growth, pluripotency maintenance, cell differentiation, and neural-related functions such as synaptic development and plasticity. An emerging consensus from the recent evidence is that the imprinted lncRNAs fine-tune gene expression of the protein-coding genes to maintain their dosage in cell. Hence, lncRNAs from imprinted clusters offer insights into their mode of action, and these mechanisms have been the basis for uncovering the mode of action of lncRNAs in several other biological contexts. This article is part of a Special Issue entitled: Clues to long noncoding RNA taxonomy, edited by Dr. Tetsuro Hirose and Dr. Shinichi Nakagawa.

1. Introduction

In mammals, during early gametogenesis, nearly 1% of protein-coding genes undergo epigenetic marking in such a way that their expression occurs in a parent of origin-specific manner, and this parent-dependent epigenetic marking is termed genomic imprinting. Currently, there are more than 150 imprinted genes that have been identified in mouse. Imprinted genes are often located in clusters, ranging in size from a few kilobases to two to three megabases. All the well-characterized imprinted clusters have been shown to contain at least one or two long noncoding RNAs (lncRNAs) as their partners, and they show an inverse expression pattern to their protein-coding counterparts [1–3]. The promoters of lncRNAs map to differentially methylated regions (DMRs), and their deletion in mice often results in loss of imprinting of protein-coding genes. Since DMRs control imprinting of neighboring genes, they are known as imprinting control regions (ICRs), which are typically 1–3 kb in size.

Genomic imprinting has been a paradigm for understanding cis-acting-based long-distance gene regulatory mechanisms, and these mechanisms have been characterized as chromatin insulators [4–6] and lncRNAs [7,8]. The chromatin insulator-based mechanism primarily regulates functional interaction between enhancers and gene promoters, and this has been exemplified in the case of *H19-Igf2* imprinted gene cluster [6,9,10]. lncRNAs, on the other hand, work by repressing flanking gene promoters in cis, as has been demonstrated in the case of the *Kcnq1ot1* and *Airn* lncRNAs [11]. Neither of these mechanisms alone can explain the reasons underlying the parent-of-origin-expression of genes, but together, they provide a greater understanding of cis-acting-based gene regulatory mechanisms among the imprinted clusters. Nevertheless, accumulating evidence over the last decade indicates that lncRNAs have evolved as a major force in the regulation of parent-of-origin-specific expression.

It is now very clear from the ultra RNA deep sequencing studies that the human genome harbors more than double the number of lncRNA expressed genes, 58,648, compared to protein-coding genes, 21,313 [12]. Evidence accumulated over the last decade also demonstrates that lncRNAs are certainly not the result of transcriptional noise and, like protein-coding RNAs, they perform a myriad of functions impacting on development, differentiation, and disease [13–15]. lncRNAs employ diverse mechanisms at the transcriptional and post-transcriptional level to control gene expression in a spatio-temporal fashion [16]. The

Abbreviations: lncRNA, long noncoding RNA; DMR, Differentially methylated region; ICR, Imprinting control region; PWS, Prader–Willi syndrome; AS, Angelmann syndrome; MEGs, Maternally expressed genes; TTS, Triplex target sites; TFO, Triplex forming oligo; IG-DMR, Intergenic DMR; RNAPII, RNA polymerase II; IGN, Imprinted gene network; MBD1, Methyl-binding domain 1 protein; KSRP, K homology-type splicing regulatory protein; BWS, Beckwith–Wiedemann sydrome; H3K27ac, Histone H3 lysine 27 acetylation; H3K9me3, Histone H3 lysine 9 trimethylation; H3K4me2, Histone H3 lysine 4 trimethylation; DNMT1, DNA methyltransferase1; PRC2, Polycomb repressive complex 2; E13.5, Embryonic 13.5; ES cells, Embryonic stem cells; eRNA, Enhancer RNA; R3C, RNA-guided chromosome conformation capture; IC, Imprinting center; DID, DNA interacting domain; ASO, Antisense oligonucleotides; CeRNA, Competing endogenous RNA; iPS cells, Induced pluripotent stem cells.

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majority of the lncRNAs implicated in transcriptional silencing have been shown to interact with chromatin-modifying complexes such as PRC2, G9a, hnRNPK, and SWI/SNF and recruit them in a sequence-specific manner to silence genes in cis or trans [17–20]. On the other hand, lncRNAs with cytoplasmic localization have been implicated in gene regulation at the post-transcriptional level by regulating Stau1-mediated mRNA decay [21] or acting as sponges for microRNA (miRNA) [22].

Genomic imprinting has been an excellent model system to learn how antisense lncRNAs regulate transcriptional gene silencing in cis [3,23]. Apart from antisense lncRNAs, recent evidence also implicated intergenic lncRNAs, located between two protein-coding genes, and noncoding transcripts derived from enhancer regions, also known as enhancer RNAs (eRNAs), in the parent-of-origin-specific expression of genes. Hence this review, by focusing on antisense, intergenic, and enhancer-derived imprinted lncRNAs, will discuss the novel functional roles of these lncRNAs in the establishment of parent-of-origin-specific gene expression. In addition, this review will provide an update of mechanisms by which intergenic imprinted lncRNAs participate in diverse biological functions by regulating non-imprinted genes in cis or trans. Emerging roles of imprinted lncRNAs as potential therapeutic targets for human conditions arise as a result of imprinting defects will also be reviewed.

1.1. Intergnic lncRNAs in genomic imprinting

Based on their location, lncRNAs can be classified into intergenic lncRNAs, antisense lncRNAs, intronic lncRNAs, and enhancer lncRNAs. With the exception of intronic lncRNAs, all of the remaining lncRNAs have been functionally implicated in the parent-of-origin-specific expression of genes. *H19*, *IPW*, and *MEG3* lncRNA are among the well-investigated intergenic lncRNAs.

1.2. H19

1.2.1. H19 lncRNA controls embryonic growth through regulating the imprinted gene network

The *H19* gene, which encodes a 2.3 kb lncRNA, is one of the first imprinted genes to be identified [24]. *H19* maps to a well-investigated imprinted gene cluster *H19*/*Igf2* on mouse chromosome 7 and its orthologous region in humans is located on chromosome 11. *H19* is only expressed from the maternal allele due to silencing of the paternal allele by promoter CpG methylation. Using transgenic mouse models, the *H19* gene was extensively investigated for its role in genomic imprinting [24]. Deletion of *H19* had no effect on imprinting of the neighboring gene *Igf2* in endodermal tissues, but a partial relaxation of imprinting of *Igf2* was detected in mesodermal tissues, indicating possible lineage-dependent functions for the *H19* lncRNA. Though *H19* is significantly expressed during embryogenesis, its deletion does not result in embryonic lethality. However, deletion of the *H19* transcription unit has modest growth effects, which is consistent with its lineage-specific effects on *Igf2* imprinting [25]. In addition, it has been shown that *H19* is a part of an imprinted gene network (IGN) comprising 16 coexpressing imprinted genes, including well-known growth regulators *Igf2*, *Igf2r*, and *Cdkn1c* [26,27]. By overexpressing the *H19* gene in a transgenic mouse model, it was proposed that *H19* controls growth via regulating members of the IGN network in trans. Moreover, in a recent investigation, it was demonstrated that *H19* interacts with methyl-CpG-binding protein MBD1, and this complex recruits H3K9 methyltransferase to the DMRs of some of the IGN network members, including *Igf2*, *Slc38a4*, and *Peg1* [26]. This full-length *H19* lncRNA-dependent recruitment of MBD1-H3K9 HMT to DMRs on both parental alleles does not involve parent-of-origin-specific expression of IGN members; rather, it fine-tunes their expression from both parental alleles through establishing repressive chromatin modification of H3K9me3. These observations suggest that the *H19* lncRNA controls embryonic growth through epigenetic regulation of IGN network members in trans.

1.2.2. H19 lncRNA and/or its processed products regulates differentiation

H19 is expressed at a higher level during mouse embryogenesis, but after birth, it is expressed at a lower level in all tissues except in muscle tissue where *H19* remains highly expressed. Recent investigations have implicated pro- and anti-myogenic functions for *H19* [28–30]. Interestingly, these contrasting functions of the *H19* gene in myogenic differentiation have been investigated using undifferentiated multipotent mesenchymal stem cells C2C12 as a model system. Two of the recent investigations have shown that *H19* downregulation promotes myogenic differentiation using diverse molecular mechanisms (Fig. 1). In one of the investigations, it was reported that *H19* lncRNA acts as a competing endogenous RNA (CeRNA) to control the levels of let-7 and downregulation of *H19* results in let-7-dependent myogenic differentiation of C1C12 cells [28] (Fig. 1). The other recent investigation has shown that *H19* acts as a scaffold for RNA-processing protein K homology-type splicing regulatory protein (KSRP), and this in turn facilitates the functional interaction between exosome and labile transcripts such as myogenin. Thus, the *H19* lncRNA by acting as a scaffold for KSRP promotes the degradation of myogenin to restrict the differentiation of C2C12 mesenchymal stem cells into myocytes. However, downregulation of *H19* or phosphorylation of KSRP by AKT, which prevents the interaction phosphorylated KSRP with *H19*, leads to differentiation of C2C12 cells into myocytes [30] (Fig. 1). Together, these observations suggest that *H19* restricts the myogenic differentiation of C2C12 cells through acting as a molecular scaffold or CeRNA to control the levels of miRNA let-7. However, in contrast to the above investigations, another recent investigation favored a pro-myogenic function for *H19*, which is mediated by miR-675-3p and miR-675-5p, processed from the exon1 of the *H19* transcript [29]. According to this study, both *H19* and miR-675-3p/miR-675-5p induce C2C12 differentiation into myocytes and downregulation of *H19* or blocking the action of miR-675-3p/miR-675-5p prevents the C2C12 differentiation. Furthermore, a *H19*-depletion-mediated myogenic differentiation block can be rescued by overexpression of miR-675-3p/miR-675-5p. These miRNAs promote myogenic differentiation by downregulating anti-differentiation factors such as *Smad1*, *Smad5*, and *CDC6*, a DNA replication initiation factor (Fig. 1).

As well as its pro-differentiation functions during muscle differentiation, *H19* has been shown to harbor tumor suppressor [31,32] and oncogenic properties [33,34]. Although the functional role of *H19* in tumor initiation and progression is still debated, recent investigations implicate *H19* as an oncogenic lncRNA in several cancer types, and its overexpression is also linked to promotion of metastasis in several cancers. Collectively, these recent observations indicate that the *H19* lncRNA takes part in several important biological functions; however, it remains to be seen which of these functions are regulated by *H19* per se and/or microRNAs miR-675-3p and miR-675-5p.

Taken together, the *H19* lncRNA harbors multiple functions such as CeRNA, miRNA precursor, tumor suppressor/oncogenic, pro- and anti-myogenic properties, and epigenetic regulation of transcription.

1.3. IPW

Ipw is a paternally expressed lncRNA that maps to the *Snurf*/*Snrpn* imprinted cluster on mouse chromosome 7, and its human homologue *IPW* is located in a region on chromosome 15 that is deleted in more than 70% of Prader–Willi syndrome (PWS) patients [35]. The mouse *Ipw* expression is mostly restricted to the brain, while its human homologue is expressed in all tissues. The *IPW* transcript is widely expressed, spliced, and polyadenylated, but without any apparent coding potential [36]. The 5′ end of both mouse and human *Ipw* homologues contain conserved 147 bp tandem repeats. *Ipw* is thought to arise from a read-through transcript of more than one megabase encompassing *SnoRD* cluster and *Ube3a* genes [37].

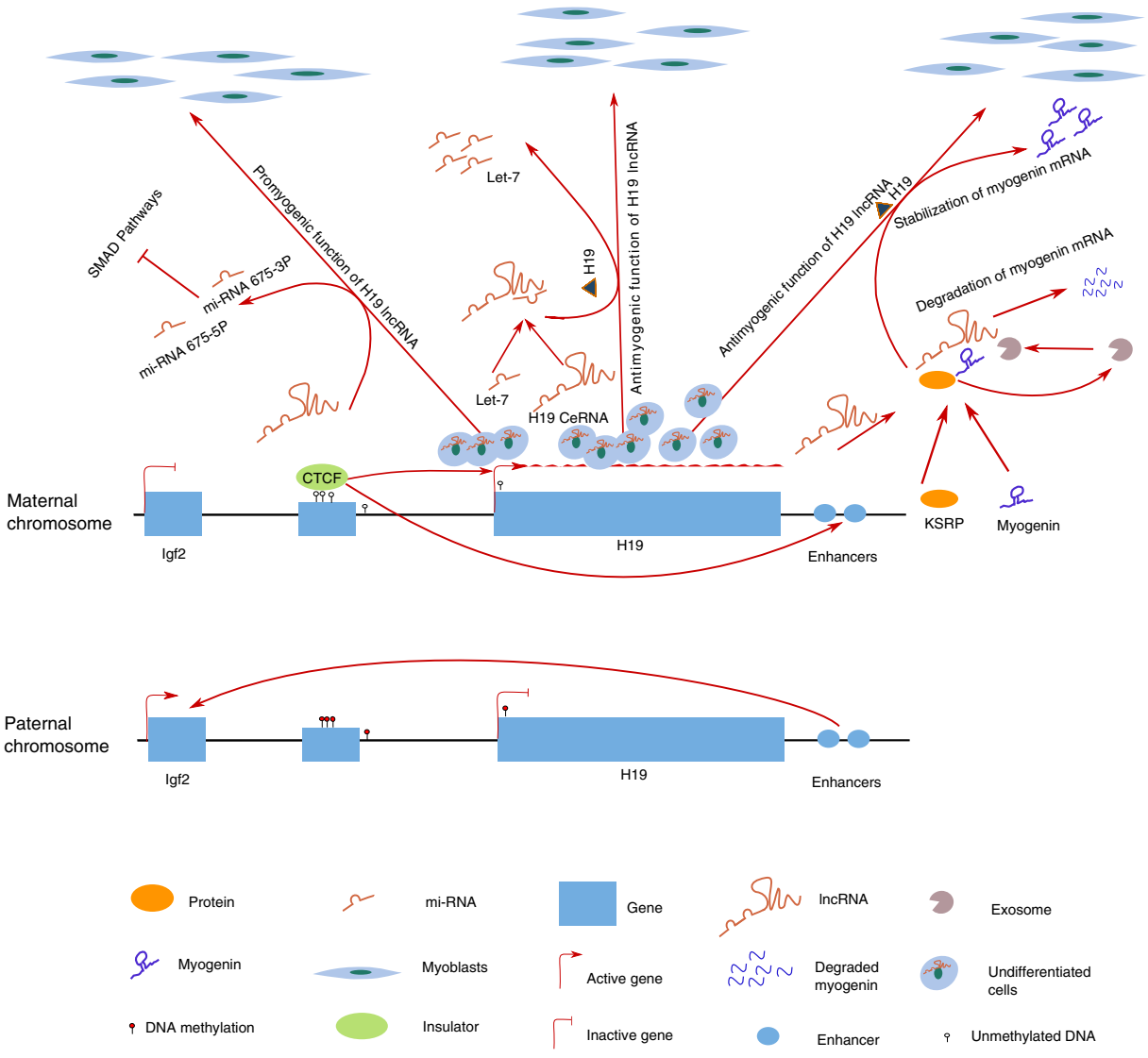

Fig. 1. Schematic showing pro- and anti-myogenic functions of *H19* intergenic lncRNA. CTCF-dependent chromatin insulator mechanism regulates the parent-of-origin-specific expression of the *H19* lncRNA by controlling the activity of the downstream enhancers. The maternally expressed *H19* lncRNA controls myogenic differentiation by acting as CeRNA for let-7 miRNA or scaffold for KSRP, which promotes the degradation of myogenin, to restrict the differentiation of C2C12 mesenchymal stem cells into myocytes. Promyogenic function of *H19* lncRNA is mediated by miR-675-3p/miR-675-5p.

1.3.1. IPW fine-tunes the expression of maternally expressed transcripts from the DLK1-DIO3 locus

Though the *Snurf/Snrpn* imprinted cluster has been relatively well studied from a mechanistic perspective in the pathogenesis of PWS/AS, the functional role of *IPW* has not been investigated. A recent investigation has documented an important role for *IPW* in fine-tuning the expression of maternal expressed transcripts on human chromosome 14. Global comparative gene expression analysis of iPS cells derived from normal and PWS patient fibroblasts indicated that *IPW* is expressed at very low level in PWS iPS cells compared to wild-type iPS cells [38]. Interestingly, several maternally expressed genes *MEG3*, *MIR370*, *MIR409* (MEGs) in the *DLK1-DIO3* region from chromosome 14 showed elevated expression in PWS iPS cells but not in normal iPS cells. This elevated expression of MEGs is not due to relaxation of silencing from their paternal alleles, rather it is from the already expressed maternal alleles. *IPW* has been shown to interact with G9a methyltransferase and sequence-specifically targets G9a to IG-DMR to modify its chromatin structure through methylation of lysine 9 of H3 (H3K9) (Fig. 2). Since IG-DMR is a master controller of gene expression at the *DLK1-DIO3* imprinted cluster, formation of *IPW*-dependent heterochromatin at the IG-DMR affects its ability to promote the gene expression of MEGs, thus affecting their levels. Of note, this observation stands out from the earlier observations that imprinted lncRNAs act exclusively in cis [1]. More importantly, this is the first example wherein a lncRNA has been shown to promote a cross-talk between two imprinted clusters through regulating the chromatin structure of an imprinting control region.

1.3.2. MEG3 lncRNA and its functional connection to pluripotency

Maternally Expressed Gene 3 (*MEG3*) is an imprinted lncRNA that maps to the *DLK1-DIO3* locus on chromosome 14 in humans and its mouse counterpart *Gtl2* (*MEG3*) is located on chromosome 12. The imprinting control region IG-DMR located within the *DLK1-DIO3* locus controls the maternal-specific expression of *MEG3* [39]. *MEG3* as well as other noncoding RNAs in the *Dlk1-Dio3* locus have been identified as the critical players in the reprogramming of mouse fibroblasts cells into iPS cells [40]. *MEG3* expression acts as a marker for iPS cells with fully pluripotent state capable of supporting embryonic development upon germline transfer. It has been shown that *MEG3*-expressing iPS cells support full-term embryonic development upon germline transfer but not the iPS cells lacking *MEG3* expression, which failed to support

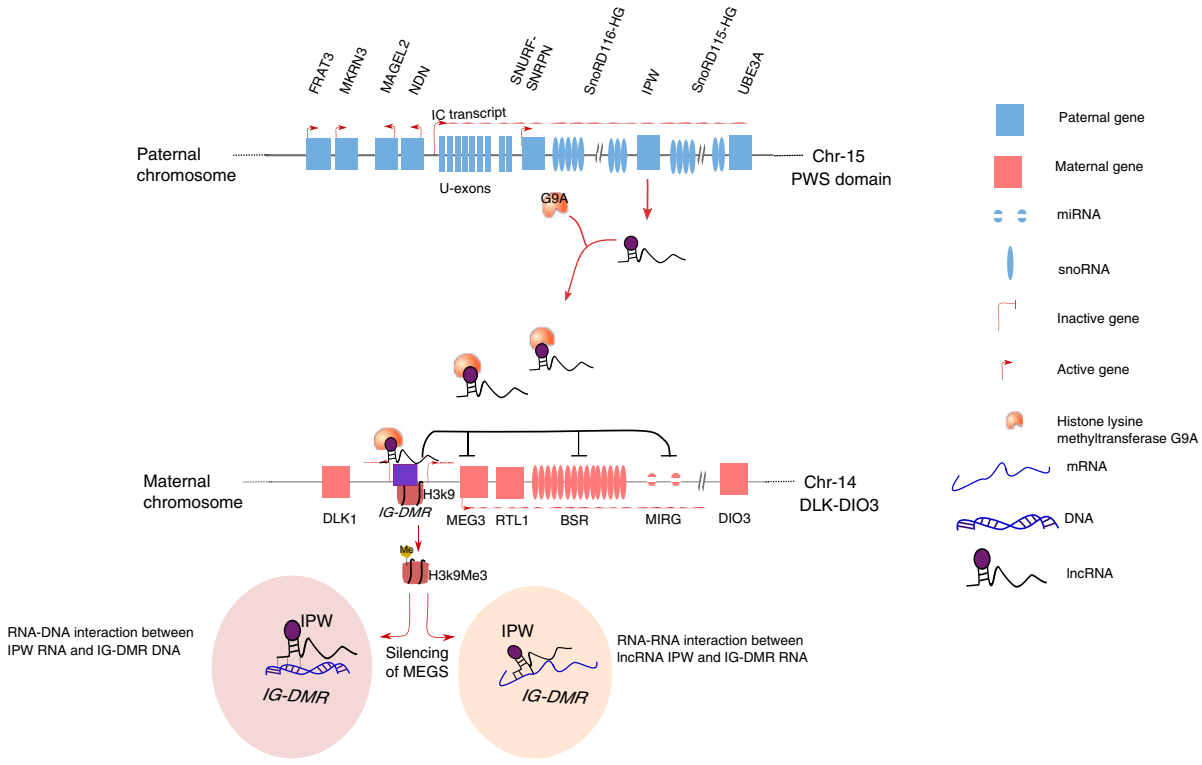

Fig. 2. *IPW* intergnic lncRNA promotes cross-talk between two imprinted clusters. *IPW* from the *SNURF/SNRPN* locus has been shown to interact with G9a methyltransferase and sequence-specifically targets G9a to the IG-DMR to modify its chromatin structure through methylation of lysine 9 of H3 (H3K9) thereby promoting the repression of maternally expressed genes (MEGs). Schematic also proposes that functional interaction between *IPW* and IG-DMR-specific enhancer RNAs could regulate the activity of MEGs.

embryonic development. However, it is not clear how *MEG3* and other members of *Dlk1-Dio3* contribute to the development of iPS cells with a fully pluripotent state. Accumulating evidence indicates that *MEG3* promotes the interaction of the PRC2 with JARID2 and targets PRC2/JARID2 complex across the genome [41]. Moreover, the JARID2/PRC2 complex has been shown to contribute to proper differentiation of embryonic stem cells (ES cells). Thus, *MEG3*'s functional role in recruitment and assembly of PRC2 on target genes in ES cells could underlie the fully pluripotent state of ES cells.

1.3.3. Enhancer RNAs regulate genomic imprinting by modulating replication timing and subnuclear positioning

Enhancers play a very important role in spatio-temporal gene regulation by acting as landing sites for transcription factors and co-activator complexes that in turn activate or boost transcription from distal promoters. Some of the characteristic features of enhancers include DNase I hypersensitive sites and a myriad of post-translational histone modifications H3K4me1, H3K4me2, and H3K27ac [42]. From the literature, it is evident that the majority of enhancers have bidirectional transcription and the enhancer-generated transcripts are present in low copy number and are non-polyadenylated. Enhancer RNAs have been shown to promote target gene expression through recruiting and/or stabilizing binding of the basal transcription machinery at the target promoter regions via establishing higher-order chromatin contacts between enhancer and target gene promoter regions [42].

Recent evidence demonstrated that bidirectionally transcribed transcripts from the IG-DMR control the expression of maternally expressed transcripts at the *Dlk1-Dio3* locus. IG-DMR is differentially methylated on parental alleles: methylated on the paternal chromosome while unmethylated on the maternal chromosome [43]. The unmethylated IG-DMR is critical for the expression of several small and long noncoding RNAs, including *MEG3*, on the maternal chromosome. The unmethylated IG-DMR is a putative enhancer with characteristic enhancer-specific histone markers and encodes bidirectionally transcribed noncoding RNAs. Disruption of the IG-DMR-encoded noncoding RNAs resulted in accumulation of repressive chromatin modification over the IG-DMR and loss of expression of maternally expressed genes. On the maternal chromosome, bidirectional noncoding transcription at the IG-DMR in ES cells correlates with early replication and inner subnuclear positioning of the *Dlk1-Dio3* locus. Downregulation of IG-DMR-encoded noncoding RNA transcripts on the maternal chromosome leads to a change in replication timing from early to late and repositioning of the *Dlk1-Dio3* domain toward nuclear periphery, a nuclear sub-space linked to gene silencing [43]. These results indicate that bidirectionally transcribed IG-DMR transcripts may promote higher-order chromatin organization at the *Dlk1-Dio3* locus on the maternal chromosome that enables early replication and inner subnuclear positioning of the *Dlk1-Dio3* locus, thereby maintaining the expression of maternal expressed genes.

1.3.4. Cross-talk between intergenic IPW lncRNA and IG-DMR enhancer RNAs fine-tunes the maternal expressed genes at the Dlk1-Dio3 genes

As discussed above, the intergenic lncRNA *IPW* is expressed at very low levels, and maternally expressed genes from the *DLK1-DIO3* locus are expressed at higher levels in iPSCs derived from PWS patient's fibroblasts [38]. Overexpression *IPW* transcript in PWS fibroblasts led to increased H3K9me3 at the IG-DMR and reduced expression of MEGs. Considering that expression of MEGs at the mouse *Dlk1-Dio3* locus depends on the bidirectionally transcribed enhancer RNAs from the IG-DMR and that *IPW* lncRNA epigenetically modifies the IG-DMR through the recruitment of G9a HMT in human iPSs, I am tempted to speculate that the functional interaction between *IPW* and bidirectionally transcribed IG-DMR transcripts could regulate the epigenetic status of the IG-DMR, thereby affecting the expression of maternally expressed genes (Fig. 2). This functional interaction between *IPW* lncRNA and IGDMR transcripts could occur either by RNA (*IPW* lncRNA)-RNA (IG-DMR transcripts) interaction or RNA (IPW lncRNA)- double strand DNA (IG-DMR sequence) interaction through triplex structure formation (Fig. 2).

1.3.5. Antisense lncRNA and genomic imprinting

Antisense lncRNAs have been very well investigated for their role in the initiation and maintenance of genomic imprinting [1]. Their modes of action in genomic imprinting are being used as paradigms to understand the lncRNA-dependent epigenetic-based gene regulation across the genome. Antisense lncRNAs epigenetically regulate expression of multiple genes in imprinted clusters by acting as scaffolds to interact with and recruit chromatin-modifying machinery in a sequence-specific fashion. *Kcnq1ot1*, *Airn*, *Nespas*, and *Ube3a-ATS* are some of imprinted long antisense transcripts whose functions have been investigated in a greater detail.

1.4. Kcnq1ot1

1.4.1. Kcnq1ot1 employs lineage-specific transcriptional silencing mechanisms in the initiation and maintenance of silencing

Kcnq1ot1, which is also referred to as *Lit1*, is a 91 kb RNA Polymerase II (RNAPII) transcribed, nuclear localized, long antisense transcript [19]. It maps to *Kcnq1/Cdkn1c* chromosomal domain at the distal end of mouse chromosome 7 and its orthologous region in humans is located on chromosome 11p22. *Kcnq1/Cdkn1c* domain is about one megabase, encompassing 10–12 imprinted genes. All protein-coding imprinted genes, both ubiquitously and placental specific, are expressed from the maternal chromosome, whereas the expression of an antisense lncRNA *Kcnq1ot1* is restricted to the paternal chromosome due to methylation of its promoter on the maternal chromosome [44]. *Kcnq1ot1* RNA is transcribed in an antisense orientation with respect to its host gene *Kcnq1* (Fig. 3).

By using cell culture and transgenic mouse as model systems, it has been demonstrated that *Kcnq1ot1*, upon transcription from the paternal chromosome, interacts with chromatin (EZH2 and G9a) and DNA (DNMT1) modifiers and recruit them in cis to silence both ubiquitously and placental-specific imprinted genes [19,45–47]. This silencing pathway thought to establish higher-order chromatin compartment enriched with repressive chromatin modifications such as H3K27me3, H3K9me3, H2AK119ub, and DNA methylation but devoid of active chromatin marks and RNAPII [45] (Fig. 3). Mouse *Kcnq1ot1* RNA has been shown to contain 890 bp silencing domain at the 5′ end, which harbors several conserved repeats [48]. Previous evidence documented that 890 bp silencing domain is required for chromatin localization and also contribute towards maintaining CpG methylation of somatic DMRs through the recruitment of DNMT1[46]. Somatic DMRs are a group of CpG rich sequences which acquire methylation during post-implantation development, This RNA-dependent DNMT1 recruitment appears to have functional role only in the maintenance of silencing of ubiquitously imprinted genes [49]. The imprinting of placental-specific imprinted genes, on the other hand, was shown to be controlled by repressive histone modifications [50]. Though *Kcnq1ot1* has been shown to be required for the initiation of silencing of both ubiquitously and placental-specific imprinted genes, it is also required for maintaining the silencing of the ubiquitously imprinted genes but not for the silencing of plancentally imprinted genes [49]. Based on these observations, it was proposed that *Kcnq1ot1* employs lineage-specific transcriptional

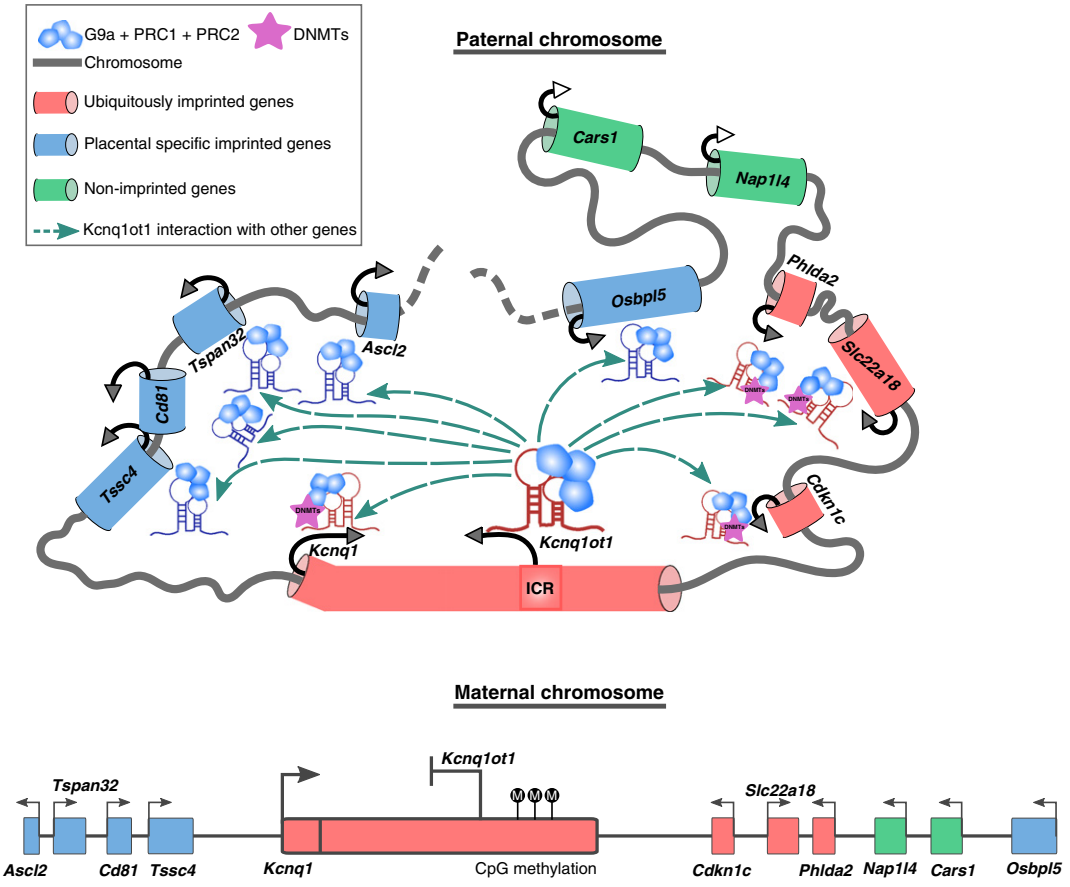

Fig. 3. *Kcnq1ot1* lncRNA silences multiple genes by establishing long-range higher-order intra-chromosomal interactions in cis. *Kcnq1ot1* RNA, upon transcription, is retained on the paternal chromosome and promotes long-range chromosomal interactions through recruitment of chromatin and DNA modifiers thereby establishing higher-order repressive chromatin compartment. Based on *in silico* predictions, it was proposed that *Kcnq1ot1* interacts with target genes via triplex formation. On the maternal chromosome, however, all target imprinted protein-coding genes are active due to the silencing of the *Kcnq1ot1* promoter by DNA methylation.

silencing mechanisms to initiate and maintain silencing of ubiquitously and placental-specific imprinted genes.

1.4.2. Kcnq1ot1 silences multiple genes by promoting long-range intra-chromosomal interactions in cis

A recent investigation devised an elegant approach of RNA-guided chromosome conformation capture (3C; R3C) to uncover the role of *Kcnq1ot1* in long-range gene silencing through promoting higher-order intra-chromosomal interactions [51]. In this approach, the chromatin-associated RNA is first reverse transcribed into double-stranded cDNA with biotin labeling, followed by restriction enzyme digestion and ligation of the double-stranded cDNA and associated DNA with T4 DNA ligase. The biotinylated *Kcnq1ot1* cDNA–DNA complex is purified using streptavidin beads and RNA–DNA interactions are analyzed by PCR using RNA–DNA interaction-specific primers. Using this approach, it was demonstrated that the *Kcnq1ot1* promotes higher-order intra-chromosomal interactions between the Kcnq1 ICR (also known as KVDMR1) and the *Kcnq1* promoter, specifically on the paternal chromosome, thereby inducing *Kcnq1* silencing. These higher-order intra-chromosomal interactions require continuous presence of *Kcnq1ot1* RNA, as downregulation of *Kcnq1ot1* RNA using RNA interference or targeted *Kcnq1ot1* promoter methylation using Zinc Finger technology results in loss of long-range intra-chromosomal interactions. Interestingly, ectopic expression of the 2 kb DNA interaction domain (DID) from the 5′end of *Kcnq1ot1* RNA was sufficient to establish long-range intra-chromosomal interactions in cells carrying targeted CpG methylation at the *Kcnq1ot1* promoter. Taken together, these observations support the previous suggestion that *Kcnq1ot1* RNA silence multiple target genes in cis through establishing higher-order repressive chromatin compartment [1] (Fig. 3).

1.4.3. In silico predictions for the mode of action of Kcnq1ot1

One of the most outstanding questions that has not been addressed in lncRNA research has been how a lncRNA recognizes its target genes. Previously, it has been shown that pyrimidine-rich RNAs (T and C) can bind to purine (A and G)-rich DNA via Hoogsteen-type hydrogen bonding to form RNA:DNA triplexes. To understand more about this phenomenon, a recent investigation has devised a computational method to predict DNA-binding motifs in lncRNAs and also their binding sites across the genome [52]. By using *Kcnq1ot1*/*Kcnq1* imprinted gene cluster as one of the model systems, DNA-binding motifs (TFO) as well as their binding sites (TTS) have been identified for *Kcnq1ot1*. Interestingly, *Kcnq1ot1* TFO has been shown to identify TTS distributed at the promoter regions of both ubiquitously and placental-specific imprinted genes, suggesting that *Kcnq1ot1* makes chromatin contacts at the target gene promoters via triplex formation. By extending *in silico* predictions to other loci across the genome, this investigation further suggests that this mode of lncRNA-dependent DNA recognition is not restricted to *Kcnq1ot1* but also applicable to all well-known lncRNAs implicated in epigenetic modulation of gene expression. It would be interesting to see whether the latter *in silico* predictions can be proven experimentally, demonstrating that this mechanism could underlie the mode of action of chromatin regulatory RNAs.

1.4.4. Enhancers override Kcnq1ot1-mediated transcriptional silencing in a spatio-temporal manner

Some of the *Kcnq1ot1* target genes undergo relaxation of imprinting in a tissue and development-specific manner. However, the mechanism(s) underlying this has not been addressed. A recent investigation has addressed how one of *Kcnq1ot1* target genes *Kcnq1* escapes silencing mediated by *Kcnq1ot1* in a tissue and development-specific fashion [53]. During cardiac development, the *Kcnq1* gene is mono-allelically expressed from the maternal allele until E13.5 but transitions to biallelic expression by E14.5. This transition from mono-allelic to biallelic gene expression occurs due to activation of an upstream cardiac-specific enhancer between E13.5 and E14.5 and its subsequent contacts with the *Kcnq1* promoter through higher-order chromatin looping as has been demonstrated using 3C technique. This clearly indicates that tissue- and developmentally regulated enhancers can override the silencing effects of lncRNAs by making long-range contacts with gene promoters. Non-imprinted genes within the imprinted domain and escapee X-linked genes on the inactive X chromosome seem to employ similar mechanisms to resist RNA-mediated silencing [49]. It has been shown that both the non-imprinted genes and the escapee X-linked genes are enriched enhancer-specific chromatin markers such as H3K4me1 (Histone H3 lysine 4 mono methylation) and H3K27ac, suggesting that enhancer-specific marks restrict RNA-mediated silencing.

1.5. Airn

The *Igf2r* locus harbors three maternally expressed protein-coding genes *Igf2r*, *Slc22a2*, and *Slc22a3* and a paternally expressed lncRNA *Airn* (antisense to *Igf2r* RNA non-coding). It is transcribed in the antisense orientation to its host gene *Igf2r*. Like the *Kcnq1* locus, the imprinting of genes in the *Igf2r* locus on chromosome 17 is controlled by the lncRNA *Airn*. *Airn* is a cis-acting silencer, silences both overlapping *Igf2r* and non-overlapping *Slc22a2* and *Slc22a3* [54].

1.5.1. Transcription- and RNA-dependent functions of Airn in cis-mediated silencing

Evidence from the last 15 years demonstrates that unlike *Kcnq1ot1*, *Airn* employs different mechanisms in the silencing of *Igf2r* and *Slc22a3* genes. By using ES cells inserted with transcription termination signals at various distances from the transcriptional start site of *Airn*, it was demonstrated that the act of *Airn* transcription rather than transcript per se determines the silencing of *Igf2r*. Interestingly, *Airn* transcription-dependent silencing of *Igf2r* is reversible and requires *Airn* transcription continuously, but this transcription becomes dispensable once the *Igf2r* promoter is irreversibly silenced by CpG methylation [55]. Whereas *Airn* itself has been shown to silence the non-overlapping genes via recruitment of chromatin modifiers in a sequence-specific fashion to the non-overlapping gene promoters [56] (Fig. 4). Thus, uniquely both *Airn*'s transcript and the act of its transcription regulate the transcription of overlapping and non-overlapping genes (Fig. 4). This raises an important question of what determines whether target gene silencing undergoes transcript- or transcription-dependent silencing? Does the target gene promoter architecture play any role in dictating the mode of action of a lncRNA? It would be interesting to functionally dissect the *Igf2r* promoter to identify mechanisms that would elucidate the mode of action of lncRNA. Another interesting aspect that needs attention is how DNMTs are attracted to the *Igf2r* promoter while it is being actively transcribed. Further insights into these outstanding questions will further improve our understanding of lncRNA transcription-dependent silencing.

1.6. Nespas

1.6.1. Nespas transcriptional process regulates overlapping gene expression through transcriptional occlusion

Nespas is a paternally expressed antisense lncRNA that belongs to a well-investigated imprinted cluster *Gnas*. The *Nespas* promoter maps to a differentially methylated region *Nespas-Gnaxl* (Nesas-Gnaxl DMR), which also contains a promoter for Gnasxl transcript and the ICR of the Gnas cluster. *Nespas* is derived from the ICR part of the Nespas-Gnaxl DMR and transcribes through the *Nesp* promoter in the antisense direction.

On the paternal chromosome, *Nespas* transcription inversely correlates with the transcription of its sense counterpart *Nesp* [57,58] (Fig. 5). By generating a hypomorph mutant through insertion of a PolyA cassette which activates the normally silent *Nespas* promoter on the maternal chromosome, it has been shown that a low level of *Nespas* transcription is sufficient to suppress maternally expressed *Nesp* [59].

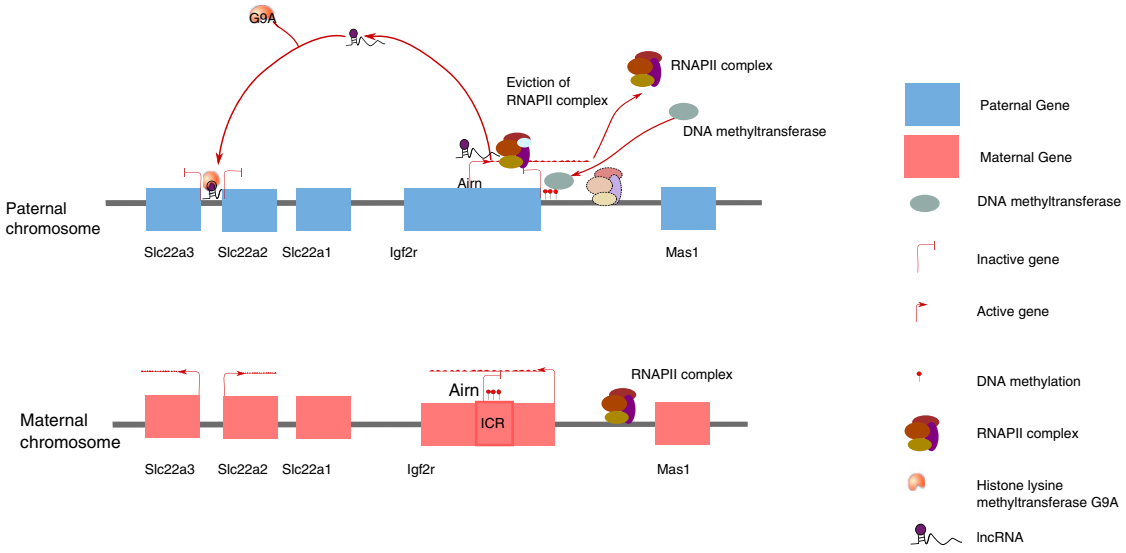

Fig. 4. Transcription- and RNA-dependent functions of *Airn* in transcriptional gene silencing. The act of *Airn* transcription silences the activity of overlapping *Igf2r* promoter through the occlusion of the RNAPII pre-initiation complex (RNAPII-PIC) from the *Igf2r* promoter. While the silencing of *Slc22a3*, a gene on the non-overlapping side, occurs through *Airn*-mediated recruitment of histone methyltransferase G9a.

On the other hand, truncation of the *Nespas* transcript through insertion of a PolyA cassette or deletion of the *Nespas* promoter on the paternal chromosome activates *Nesp* expression, indicating that *Nespas* transcription or transcript is responsible for *Nesp* repression [59]. Interestingly, *Nespas* transcription on the paternal chromosome correlates with reduced H3K4me3 levels and increased CpG methylation at the *Nesp* promoter, whereas loss of *Nespas* transcription correlates with increased H3K4me3 and loss of CpG methylation at the *Nesp* promoter (Fig. 5). This suggests that *Nespas* antisense transcription through the *Nesp* promoter results in demethylation of H3K4, which in turn promotes CpG methylation. Based on these observations, it has been proposed that the act of *Nespas* transcription recruits histone demethylases such as KDM1B, thereby demethylating H3K4me3 to allow the *Nesp* promoter to become methylated [59] (Fig. 5).

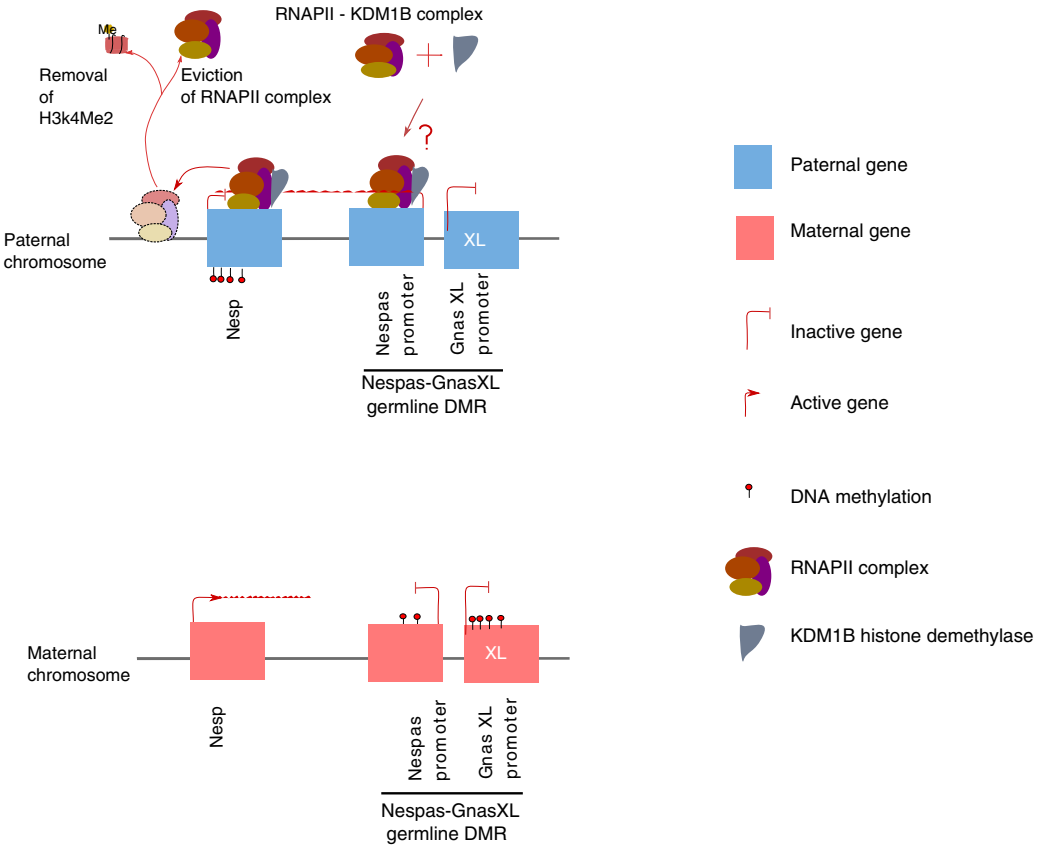

Fig. 5. *Nespas* transcriptional act regulates *Nesp* promoter activity through transcriptional occlusion. *Nespas* antisense transcription through the *Nesp* promoter results in the occlusion of the RNAPII-PIC and demethylation of H3K4, which in turn promotes CpG methylation of the *Nesp* promoter.

1.7. UBE3A-ATS

1.7.1. Transcriptional collision underlies the Ube3a-ATS-dependent silencing in cis

UBE3A-ATS maps to the Prader–Willi (PWS)/Angelman (AS) imprinted region, which is located on chromosome 15q11-q13. The PWS/AS region spans about 2 Mbp, contains paternally expressed *MAGEL2*, *NDN*, *SNRPN*, *SNORD115*, and *SNORD116* genes and a maternally expressed *UBE3A* gene [60]. The PWS/AS region is conserved in mouse and is located in an orthologous region on chromosome 7C. Imprinting in the PWS/AS region is regulated by a bipartite imprinting center (IC) comprising PWS-IC/AS-IC. PWS-IC functions by activating its neighboring genes, whereas AS-IC acts by suppressing PWS-IC, thereby establishing distinct DNA and histone modifications over the PWS genes. The activity of AS gene *UBE3A* on the paternal chromosome is controlled by the lncRNA *Ube3a-ATS*, a paternally expressed lncRNA [60–62].

Selective deletion of the *Snrpn* promoter or truncation of *Ube3a-ATS* using a transcription termination signal leads to loss of *Ube3a-ATS* expression and activation of *Ube3a*, indicating that there is an inverse functional correlation between the expression patterns of *Ube3a-ATS* and *Ube3a* genes [62,63]. *Ube3a-ATS* is a nuclear localized transcript, and combined RNA-DNA *in situ* hybridization revealed that the mature transcript is located proximate its transcription unit. Although the transcript colocalizes to its transcription unit, it does not seem to have functions at the RNA level as has been demonstrated in the case of *Kcnq1ot1* and *Airn*. For example, chromatin structure analysis over the silent and active parental alleles of the *Ube3a* promoter revealed that active promoter-specific histone markers such as H3K4me3 and occupancy of RNAPII preinitiation complex remained the same on both the parental alleles, indicating that the *Ube3a* promoter maintains an active chromatin status despite the absence of active transcription (Fig. 6). Moreover, equal amount of pre-mRNA was detected from both parental alleles, which rules out RNA or transcription-dependent occlusion of PIC complex [63]. Based on the observations that equal amount of PIC enrichment and the absence of allele-specific epigenetic modifications at the *Ube3a* promoter on both parental alleles, it was suggested that transcriptional collision could underlie the *Ube3a-ATS*-dependent silencing of *Ube3a* [63] (Fig. 6). However, the activation of *Ube3a* upon treatment with topoisomerase inhibitors and antisense oligonucleotides (ASO) in murine AS mouse models suggests an alternative mode of action for *Ube3a-ATS* in *Ube3a* silencing. Activation of *Ube3a* upon ASO treatment favors an RNA-dependent function for *Ube3a-ATS* in *Ube3a* silencing and it is not clear how topoisomerase inhibitor rescues *Ube3a* activation. It is possible that *Ube3a-ATS* transcription covering a megabase region, including the *Ube3a* gene, generates high levels of torsional stress leading to stalling of transcriptional elongation complexes and silencing of *Ube3a*. Treatment with topoisomerase inhibitors could be dissipating this torsional stress thereby activating *Ube3a*. Thus, further studies are required to ascertain the exact mechanism of action of *Ube3a-ATS* in the silencing of *Ube3a*.

1.7.2. Imprinted lncRNAs in disease etiology

H19 is one of the first imprinted lncRNAs implicated in disease etiology. Several lines of accumulated evidence suggest that *H19* lncRNA harbors both tumor suppressor [31,32] and oncogenic functions [33,34]. It is highly expressed in several cancers and its higher expression correlates with tumor progression and a poor clinical outcome, thus *H19* expression acts as a potential biomarker in the assessment of tumors. As discussed above, the *H19* lncRNA serves as a precursor of miRNA-675 [64]. Like *H19*, its mature product miRNA-675 is also highly expressed in several cancers. Tumor suppressor genes such as retinoblastoma *RB*, *Runx1*, and *Cadherin 13* are the direct targets of miRNA-675, overexpression of miRNA-675 promotes tumor progression via silencing of tumor suppressor genes [65–67]. Hence, it would be interesting to functionally dissect the pathological role of the *H19* lncRNA and its mature product, miRNA-675, in tumor initiation and progression. Another lncRNA *MEG3* is aberrantly expressed in several cancers, including gliomas, colorectal cancers, and pancreatic neuroendocrine cancers [68]. In the majority of the cancers, *MEG3* is expressed at low levels. Its overexpression in cancer cell lines inhibits cell proliferation and promotes apoptosis. These anti-tumorigenic properties of *MEG3* characterize it as a tumor suppressor and thus serves as a potential drug target in the treatment of various cancers.

Molecular defects at the 11p 15.5-imprinted regions result in two fetal growth disorders with opposite phenotypes: Beckwith–Wiedemann syndrome (BWS) and Silver–Russell syndrome (SRS) [69,70]. BWS is characterized by pre-and post-natal overgrowth and often predisposes children to childhood tumors, whereas SRS is characterized by pre-and post-natal growth retardation. More than 60% of the BWS

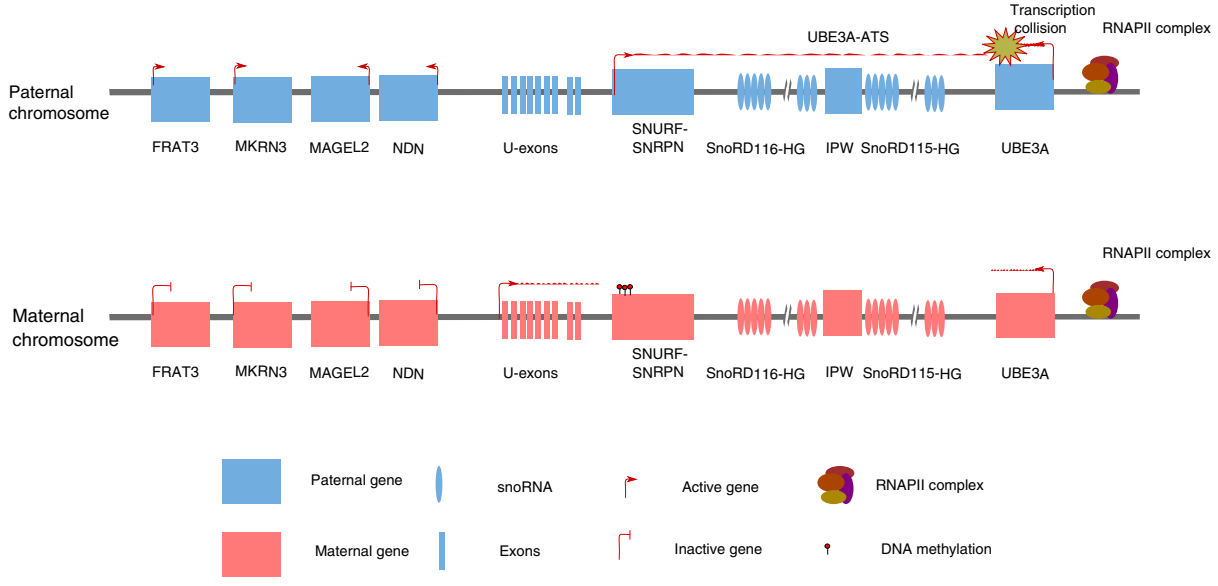

Fig. 6. *Ube3a-ATS*-dependent silencing of *Ube3a* involves *transcriptional collision*. On the paternal chromosome, *Ube3a-ATS* transcription across the *Ube3a* promoter does not result in the occlusion of the RNAPII-PIC and demethylation of H3K4me3. Detection of pre-mRNA but not the mature form of the *Ube3a* transcript from the paternal allele indicates that the transcriptional collision could underlie the *Ube3a-ATS*-mediated silencing of *Ube3a*.

and SRS patients harbor epigenetic abnormalities at the 11p15.5. For example, about 50%–60% of the sporadic BWS patients show the loss of maternal-specific methylation of the KCNQ1 ICR, which harbors the *KCNQ1OT1* promoter. This results in biallelic activation of *KCNQ1OT1* transcription and *KCNQ1OT1*-dependent silencing of cell cycle inhibitor *CDKN1C* on both parental chromosomes [71,72].

Angelman syndrome (AS) is a severe neurodevelopmental disorder with intellectual disability and speech impairment caused by biallelic activation of *UBE3A-ATS* and biallelic silencing of *UBE3A* [73]. Loss of *UBE3A*, an E3 ubiquitin ligase, affects very important neural-related functions such as synaptic development, signal transduction, and plasticity. Several murine models, with either *UBE3A-ATS* promoter deletion or pre-mature termination of its transcript, have been generated to understand the role of *UBE3A-ATS* in AS [37,74]. In addition, pharmacological interventions such as treatment with topoisomerase inhibitors or antisense oligonucleotides (ASO) in murine AS mouse models led to silencing of *Ube3a-ATS* and activation of *Ube3a* [74,75]. Of note, the ASO treatment appears to be very promising, as it has achieved specific inhibition of *Ube3a-ATS* and significant restoration of *Ube3a* activity. This partial restoration of *Ube3a* caused recovery from cognitive deficits associated with the AS.

1.8. Conclusions and future prospects

Genomic imprinting is a wonderful model system to understand lncRNA-dependent epigenetically controlled phenomena. Through combined use of cell culture and transgenic mouse models, involving studies on various imprinted clusters has authenticated the functional role of lncRNA in mammalian development. Moreover, the majority of imprinted lncRNAs are relatively highly conserved at the functional and primary sequence level, thus becoming one of the most tractable systems to study lncRNAs in development and disease. Importantly, lncRNAs from well-investigated imprinted clusters offer insights into their mechanism of action in regulating target genes, and these mechanisms have formed the underlying basis for uncovering the mode of action of lncRNAs in several other biological contexts.

As outlined in the review, it is clear that there is no common mechanism being employed by the imprinted lncRNAs and that they employ diverse molecular mechanisms to control epigenetically regulated transcription across imprinted clusters. From the recent literature, it appears that the choice of mechanism being employed by lncRNA is dependent on the molecular interplay involving the lncRNA and its target gene promoter DNA sequence and chromatin structure. Further investigations are required to understand how functional RNA domains in lncRNA interpret the DNA sequence and chromatin structure of the target gene promoter. Characterization of the latter phenomenon would immensely help us to understand how some genes in a locus are responsive to lncRNA while the others do not. The use of ASOs in the treatment of AS cognitive deficits in a mouse model is a first step towards use of lncRNA-based therapies in the treatment of cancer. From recent evidence, it is apparent that lncRNAs are aberrantly expressed in several cancers, and this establishes an aberrant functional nexus between lncRNAs and protein-coding RNAs to promote tumor initiation and progression. Development and use of novel stable ASOs for the treatment of cancers would be potential option in the future investigations.

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