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Genomic imprinting of the type 3 thyroid hormone deiodinase gene: Regulation and developmental implications☆

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Abstract

Background—In recent years, findings in a number of animal and human models have ignited renewed interest in the type 3 deiodinase (D3), the main enzyme responsible for the inactivation of thyroid hormones. The induction of D3 in models of illness and injury has raised critical questions about the physiological significance of reduced thyroid hormone availability in those states. Phenotypes in transgenic mice lacking this enzyme also point to important developmental roles for D3. A critical determinant of D3 expression is genomic imprinting, an epigenetic phenomenon that regulates a small number of dosage-critical genes in the mammalian genome. The D3 gene (Dio3) is imprinted and preferentially expressed from one of the alleles in most tissues.

Scope of review—In the context of the physiological significance of D3 and the characteristics and purported origins of genomic imprinting, we review the current knowledge about the epigenetic mechanisms specifying gene dosage in the Dio3 locus.

Major conclusions—Altered Dio3 dosage is detrimental to development, suggesting that the level of thyroid hormone action needs to be exquisitely tailored in a timely fashion to the requirements of particular tissues. An appropriate Dio3 dosage is the result of the coordinated action of certain genomic elements and epigenetic marks in the Dlk1-Dio3 domain.

General significance—The imprinting of Dio3 prompts intriguing questions about why the level of thyroid hormone signaling should be regulated in this rare epigenetic manner, and to what extent altered Dio3 expression due to aberrant imprinting may be implicated in human conditions. This article is part of a Special Issue entitled Thyroid hormone signalling.

Keywords

Genomic imprinting; Type 3 deiodinase; Dio3; Thyroid hormone; Mouse chromosome 12; Uniparental disomy of chromosome 14

1. Introduction

Thyroid hormones exert broad biological effects in all mammalian species. Their critical roles in the regulation of growth, development and metabolism are particularly remarkable, but most tissues express some form of thyroid hormone receptor and, thus, are a target for thyroid hormones. As a result, alterations in the mechanisms regulating the availability of

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thyroid hormone or its signaling have the potential to affect many physiological systems and, in humans, be the cause of developmental abnormalities and adult patho-physiological states.

Multiple factors, as reviewed in this special issue, influence the degree of thyroid hormone action that occurs in a given cell. One of them is the type 3 deiodinase (D3), a selenoenzyme that converts the two main hormones produced by the thyroid gland, the pro-hormone thyroxine (T4) and the active hormone triiodothyronine (T3), into metabolites with little or no biological activity [1–3]. Given this enzymatic activity, D3 is positioned to decrease T3 availability in the tissues in which it is expressed and, therefore, to reduce the local level of thyroid hormone action. The ample tissue profile of D3 expression, especially during development, suggests roles for D3 in multiple systems. This notion is being increasingly confirmed in animal models, adding physiological significance to the mechanisms regulating D3 expression.

In this regard, a puzzling observation about the D3 gene (Dio3) is that it is subject to genomic imprinting [4–6], an epigenetic phenomenon affecting a small number of genes that results in the preferential expression of one of the alleles [7]. This characteristic sets the D3 apart from other determinants of thyroid hormone action, and raises the possibility that the epigenetic mechanisms governing the allelic expression of Dio3 are an important determinant of thyroid hormone levels in tissues, and are critical to ensure normal development and physiology.

Here we briefly summarize past and recent observations about the physiological significance of D3, review the current knowledge about the genomic imprinting of Dio3 and the potential implications for human disease. We also briefly discuss its potential role in developmental plasticity, evolution and in the epigenetic inheritance of biological traits.

2. Pathophysiological significance of D3

Although a detailed account of this topic is not the focus of the present review, it is important to fully understand the impact that alterations in Dio3 expression could have on development and physiology. This will provide a context in which to evaluate the significance of Dio3 imprinting in health and disease.

The existence of the D3 (also called type III, 5- or inner-ring deiodinase) has been known for more than 30 years. Initially, D3 garnered less attention than the other deiodinase enzymes of the family, the type 1 (D1) and the type 2 (D2), as investigators were more interested in the activation of thyroid hormones, a process of particular importance in preventing the devastating effects that the lack of thyroid hormones exert on the central nervous system. At the time, it was simply – and rightly – assumed that D3 played a role in protecting tissue under circumstances of thyroid hormone excess, i. e., as a result of hyperthyroidism due to pathology of the thyroid gland, or during fetal development, a time when thyroid hormone levels are much lower than those in the mother.

Partially consistent with this hypothesis was the expression profile of D3, which exhibits a marked developmental pattern (Fig. 1A). D3 activity can be found in most tissues during fetal and early neonatal life [8]. In contrast, during adult life, high D3 expression is limited to the central nervous system [9–11], with lower expression levels found in the skin [12] and certain endocrine organs including the uterus, ovary and adrenal gland [13]. Most other tissues feature very little or no D3 expression in adulthood. For most tissues, D3 activity decreases one to two orders of magnitude from fetal life to adulthood (Fig. 1A). These observations, together with the very high expression of D3 in the placenta and pregnant

uterus [14,15], suggested an important role for this enzyme during development and in the regulation of neural and endocrine functions.

As fetal development is a time of rapid cell proliferation and timely differentiation, it had been postulated that the high D3 expression at these stages was aimed at preventing tissues form undue exposure to thyroid hormones, a hypothesis consistent with the known roles of thyroid hormones in cell differentiation.

In this regard, a review on the role of D3 and other deiodinases in cell proliferation and differentiation and in signaling pathways related with these processes can be found in this issue [16].

2.1. D3 induction in fasting, illness and models of inflammation and injury

In the rodent, most adult tissues exhibit very low or undetectable levels of D3 activity. However, a number of physiological challenges, injuries and inflammatory processes can lead to remarkable inductions of D3 in certain tissues that would not express any D3 in a healthy state [17]. Thus, in humans and rodents, D3 is induced in the liver and skeletal muscle during critical illness [18–20] and after fasting [21,22]. These physiological states are typically associated with the “euthyroid sick syndrome”, which is characterized by low levels of serum T3 and T4 despite the absence of any pathology in the hypothalamic–pituitary–thyroid axis [23]. Although the mechanisms underlying this syndrome are not fully understood, it is possible that an induction of D3 in certain tissues may contribute to this phenomenon.

In certain models of injury and inflammation, D3 expression can also be induced in tissues and cell types which would not normally express D3 in healthy conditions [17]. This type of D3 induction has been found in the heart when subjected to physiological insults such as myocardial infarction and pulmonary hypertension [24,25]. Induction of local D3 has also been observed in infiltrating cells during lung infection [26], after sciatic nerve lesion [27], and during chronic hind limb inflammation [28].

The clinical significance of this re-induction of D3 in adult tissues after an injury or physiological insult is largely unknown. The return of D3 expression in some tissues to levels that resemble those present early in development may reflect the need for new cell proliferation and differentiation processes to supply the cell needs and required endocrine microenvironment of the healing tissue. The increased D3 expression and the subsequently low local levels of thyroid hormones may be an integral component of the response to certain physiological challenges or necessary for optimal recovery. However, D3 induction in some of these models could just be a by-product of inflammation and potentially detrimental for health outcomes.

More studies are needed to determine in each of these models whether the role of D3 induction is adaptive or maladaptive, but this phenomenon may have important implications for human conditions, as it raises the possibility of clinical manipulation of the local or systemic thyroid hormone environment to foster recovery from illness and injury. In addition, the mechanism/s by which these conditions can induce D3 are unknown, but the susceptibility of this gene to expression dosage alteration in particular patho-physiological states may relate to its regulation by epigenetic mechanisms (see below) and warrants further investigation.

2.2. Observations in D3KO mice

An important body of evidence about the physiological significance of D3 comes from studies in D3-deficient (D3KO) mice. As expected from the high D3 expression during fetal

and neonatal life, D3KO mice are overexposed to T3 at those stages [29] (Fig. 1B), which are critical to the maturation of the hypothalamic–pituitary–thyroid (HPT) axis. As a result, D3KO animals present in adulthood with a moderate degree of hypothyroidism that varies slightly between genetic backgrounds, but that is characterized by a reduced serum T4, normal or slightly reduced serum T3 and slightly elevated or unaltered serum thyrotropin [29]. This hypothyroidism is the result of abnormal regulation and/or programming of the HPT axis, as physiological defects in hormonal responses are found in the hypothalamus, pituitary and thyroid gland [30].

D3KO mice manifest phenotypes affecting many other systems (Fig. 1C). Their immune response is deficient, as they show reduced bacterial clearance in a model of lung infection [31]. Their hearing and vision are impaired, as a result of abnormalities in cochlear and retinal development [32,33]. They are markedly growth retarded and their fertility and neonatal viability are reduced [29]. Insulin secretion and glucose homeostasis are impaired in D3KO mice [34]. The level of T3 action in most areas of the central nervous system is altered in developing, adult and aged animals, significantly altering gene expression patterns [35] and potentially affecting many aspects of brain development and function. D3KO mice also exhibit a complex phenotype affecting energy balance (A. Hernandez, unpublished observations). In addition, preliminary results from our laboratory suggest that defects in craniofacial structures and brain morphology, lifespan, adiposity and energy balance, behavior and heart physiology are associated with loss of D3 function.

Overall, the picture emerging from all these studies suggests that Dio3 expression is critical to protect many tissues and cell types from excessive thyroid hormone action. This seems particularly true for tissues regulating neurological and endocrine functions, developing tissues, and those tissues that have been subjected to injury, inflammatory processes or specific physiological challenges. Moreover, the dynamic expression profile of D3 during embryonic and early postnatal development suggests that its appropriate expression dosage is critical for regulating local thyroid hormone action. Although the precise role of D3 and limited thyroid hormone action in all these scenarios is not fully understood, the evidence so far indicates that appropriate Dio3 expression is of importance for functional and developmental outcomes. It is in this context that the genomic imprinting and epigenetic regulation of Dio3 acquire its full relevance.

3. General characteristics of genomic imprinting

In contrast to the majority of genes that are expressed from both parental chromosomes, imprinted genes are expressed from a single allele based on their parental origin. The discovery of imprinted genes in the early 1980s [36,37] provided some of the first evidence of epigenetic regulation, since in order for two alleles of identical sequence to be differentially expressed in a heritable manner, they must be marked with further modifications to the gene that direct their expression. To date approximately 100 imprinted genes have been described in the mouse [38] and a similar number are thought to exist in other mammalian species including humans. Of these ~100 genes, approximately half are expressed from the maternally inherited chromosome, and the remainder are expressed from the paternally inherited chromosome. The majority of imprinted genes are located in genomic clusters rather than randomly scattered across the genome. Imprinting clusters can contain genes that are mono-allelically expressed from either parental chromosome, and genes within a given cluster are regulated in a coordinated manner by a common imprinting control region (ICR) (reviewed by Ferguson-Smith [39]).

3.1. Properties of ICRs

Control of gene expression by imprinting involves several stages; firstly, an epigenetic mark must be established in the gametes to distinguish the two parental alleles. Secondly, this mark must be maintained throughout development, specifically during global epigenetic reprogramming events that occur in early embryogenesis, but must be erased in the germ line to allow for the imprint to be reset according to the sex of the individual. Finally, in somatic cells the imprint must control gene expression *in cis*.

ICRs were initially identified as CG-rich DNA sequences that exhibited differences in the levels of 5-methyl cytosine according to their parental origin. ICRs are differentially methylated between sperm and oocyte, and they retain these methylation differences during fertilization and preimplantation development, and in the majority of somatic cells. During gametogenesis this methylation is erased and reset before gamete maturation (reviewed in Ferguson-Smith [39]).

In addition to DNA methylation, all ICRs are marked by signature modifications to histone proteins, specifically trimethylation of lysine 4 (H3K4me3) and lysine 9 (H3K9me3) of histone H3 and trimethylation of lysine 20 of histone H4 (H4K20me3) [40].

3.2. Imprinting establishment

In both male and female germ lines the establishment of methylation relies on the *de-novo* methyltransferase DNMT3A and its cofactor DNMT3L [41–43]. The mechanism by which methylation is targeted to ICRs is still unknown, but may involve recruitment of repressive chromatin by small RNAs [44], sequence periodicity [45], or other epigenetic modifications. In addition, ICR sequences may be selectively shielded from *de-novo* methylation in the germ line by transcription and histone modifications (reviewed by Smallwood [46]).

3.3. Post-fertilization maintenance of imprinting marks

Following fertilization the chromatin from both parents is subject to reprogramming by the oocyte cytoplasm. Both parental genomes are demethylated in the embryo before implantation by both active and replication-dependent mechanisms [47]. However, ICRs are not demethylated during this period and this protection from genome-wide epigenetic remodeling is dependent upon the Kruppel-associated box domain (KRAB)-containing zinc finger protein ZFP57 [48] and the developmental pluripotency-associated protein 3 (DPPA3/STELLA) [49]. ZFP57 binds to all known ICRs at a hexanucleotide motif when this sequence is methylated [50]. Binding of ZFP57 to the methylated ICR recruits binding *in cis* of the KAP1 complex and subsequent recruitment of DNA methyltransferases and histone H3K9 methyltransferases, and thus maintenance of the ICR [50,51]. Loss of Zfp57 *in vitro* or *in vivo* results in failure of ICRs to maintain their methylation and consequently misexpression of imprinted genes in both the mouse and human [48,50–52].

3.4. Control of gene expression

Two types of ICR have been described; those that are methylated in oocytes and unmethylated in sperm, which form the majority of ICRs, and those methylated in sperm but not oocytes. ICRs that are methylated in oocytes all overlap a CpG island promoter for either a protein coding gene or a non-protein coding macro RNA ([39]). In this case silencing is associated with promoter methylation. In loci containing macro RNAs there is another level of complexity since transcription of the macro RNA prevents *cis* expression of protein encoding genes by recruitment of repressive chromatin, transcriptional interference or by coating the chromosome *in cis* (reviewed by Koerner [53]). Methylation of the promoter on the maternally inherited allele prevents macro RNA expression and thus allows transcription of neighboring genes.

ICRs that are methylated in sperm are all located in intergenic regions that are not CpG island promoters. The mechanism by which these ICRs control the transcription of neighboring genes is probably not identical in each case, but in several clusters the ICR overlies a binding site for an insulator or enhancer-blocking element. When insulator proteins bind DNA they are able to prevent *cis* interactions between neighboring regions, such as between promoter and enhancer regions, and thus repress transcription. Methylation of the ICR prevents insulator binding, and thus parental allele-specific gene expression is achieved (reviewed by Ferguson-Smith [39]).

Some imprinted genes exhibit tissue-specific absence of imprinting. Biallelic expression is achieved by a variety of mechanisms such as the utilization of enhancer elements that spatially bypass the imprinting machinery [54], or by selective biallelic methylation of the ICR [55]. In addition some imprinted genes can employ alternative promoters that are methylated in a tissue-specific and parental specific manner to achieve opposite imprinting in different tissues [56,57].

3.5. Phenotypes affected by imprinted genes

Chromosomal domains in the mouse containing imprinted genes were initially identified in the 1980s by screening for phenotypes associated with aberrant chromosome segregation [58]. Many of these phenotypes were associated with embryonic growth, and subsequently it has become clear that imprinted genes encode proteins and non-coding RNAs (ncRNAs) that are critical for the control of placentation, embryonic growth and for perinatal phenotypes associated with growth, suckling and thermogenesis. In addition, modulation of imprinted gene dosage can affect postnatal behavior and metabolism (Reviewed in [59–61]).

In humans, genetic defects affecting imprinted genes lead to severe phenotypes. Conditions such as the Prader-Willi, Angelman, and Beckwith-Wiedemann syndromes are the result of an abnormal dosage of imprinted genes [62–66] and usually present with abnormalities in growth, development, metabolism, behavior and endocrine and neurological functions. Abnormal expression of the imprinted gene GNAS, which is critical for the signal transduction pathway of many hormones, may cause endocrine pathologies including pseudohypoparathyroidism and McCune-Albright syndrome [67–70].

4. The Dlk1-Dio3 imprinted cluster in chromosome 12

4.1. Genes in the cluster

The existence of imprinted genes on mouse chromosome 12 was predicted by the phenotypes of mice carrying Robertsonian or reciprocal translocation in chromosomes resulting in either two paternal chromosomes and no maternal chromosomes or the inverse [71,72]. Subsequently imprinted genes have been mapped to distal chromosome 12, comprising of a cluster that spans approximately 1.5 Mb (Fig. 2A, [73]). A highly conserved imprinting cluster exists on human chromosome 14 [74].

The genes within the cluster have a variety of functions; Begain encodes a peptide specific to the nervous system, one variant of which is imprinted (Begain 1b, [75]), the delta-like homologue 1 (Dlk1) encodes a non-canonical Notch ligand which controls maturation in a number of stem cell populations [76]. Rtl1 is a retrotranposon-like gene that is essential for placental morphogenesis [77], and Dio3 marks the telomeric end of the domain (Fig. 2A). Several ncRNA genes are also present, though their functions are less well understood; the Gtl2 transcript has no assigned function to date, however its misexpression has been associated with tumorigenesis [78,79]; Rtl1 antisense (Rtl1AS) encodes microRNAs that target Rtl1 [80], though they may have additional targets, Mirg encodes a primary transcript

for a large number of microRNAs [81], and the CD-box containing small nucleolar RNA products (snoRNAs) that are thought to catalyze RNA editing [82].

Four of these genes are expressed from the paternally inherited chromosome and encode proteins (Begain 1b, Dlk1, Rtl1 and Dio3). These genes are silenced on the maternally inherited chromosome, from which there is expression of several ncRNAs, which initiate at the promoter of the Gtl2 gene (Gtl2, Rtl1AS, Mirg and C/D snoRNAs) and form a long polycistronic transcript (Fig. 2A).

4.2. Control of imprinting in the Dlk1-Dio3 cluster

Imprinting of the cluster is controlled by an intergenic ICR (also known as the intergenic differentially methylated region or IG-DMR) which is methylated in sperm, unmethylated in oocytes, and retains paternally-derived methylation in the majority of somatic cells after fertilization (Fig. 2A, [73]). Further regions of differential methylation (DMRs) are found within the Dlk1 gene and at the promoter CpG island of Gtl2, both of which are hyper-methylated on the paternally-inherited chromosome (Fig. 2A) [73]. Deletion of the ICR on the maternally-inherited allele results in an imprinting switch occurring *in cis* to the deletion; the ncRNAs, which are expressed from the maternal allele in normal conditions, are now silenced, and the protein-encoding genes – normally repressed – are now expressed. This leads to bi-allelic expression of Dio3 (overexpression) and presumably to a decrease in thyroid hormone action. In contrast, paternal inheritance of the ICR deletion has no effect on gene expression, the ncRNAs remain silent and the protein-encoding genes retain expression (Fig. 2B) [83].

It is not currently known how the ICR acts to control gene expression across the entire 1.5 Mb domain, though several models have been proposed [84]. However, it is clear that one important action of the ICR is to direct the methylation status of the Gtl2 promoter. Loss of the ICR on the maternally inherited chromosome causes the Gtl2 promoter to gain methylation, which is associated with silencing of all of the downstream ncRNA transcripts (Fig. 2B, [83]). In addition, disrupting the action of the ICR by insertion of a transgene between it and the Gtl2 promoter results in loss of Gtl2 promoter methylation and expression of Gtl2 and associated transcripts (Fig. 2C, [85,86]. In both cases changes in the activity of the Gtl2 promoter have a reciprocal effect on the expression of the protein-coding genes, including Dio3, suggesting that transcription across this region has a regulatory function. However, it is unclear whether transcription is continuously required in all somatic cells, or if there is a critical developmental window in which transcription imposes a stable epigenetic state on the maternally inherited chromosome. To date there is no evidence that either parental chromosome acquires a domain-wide coating of epigenetic marks associated with repressed chromatin [87], but there is some evidence that the paternally-inherited chromosomal domain may occupy a different subnuclear localization than the maternally-inherited domain [88].

4.3. The Dio3 gene

The Dio3 gene is located at the distal end of the chromosome 12 imprinted region [89] and forms the telomeric boundary of the domain, since the neighboring gene Ppp2a5c is biallelically expressed [75]. The Dio3 mRNA is 2.1 kb in length and transcribed from a single 1.85 kb exon. This is the most abundant transcript in developing tissues and placenta. It includes an in-frame TGA triplet that codes for selenocysteine. However the insertion of this amino acid into the peptide sequence is dependent upon a sequence within the 3′UTR, the selenocysteine insertion sequence (SECIS, Fig. 3). Human DIO3 maps to the syntenic imprinted region 14q3, and is highly conserved. A high degree of nucleotide identity is also noted in the SECIS-containing portion of the 3′UTR and in the overall 3′UTR including the

presence of a consensus polyadenylation sequence at the 3′end (Fig. 3) [90]. The promoter for both the human and mouse genes is located in a CpG island [90], which is unmethylated on both parental chromosomes (Fig. 3) [91].

However, additional, larger Dio3 transcripts (approximately 2.6 and approximately 3.2 kb) have been detected in the adult and hyperthyroid rat brain [92,93]. Furthermore, a similar 3.2-kb D3 transcript is present in various adult human tissues [94]. There is evidence that these larger Dio3 transcripts contain additional 5′-untranslated sequence [95], but their imprinting status is unknown and the promoter(s) responsible for their transcription has not been identified.

A second gene is present at the Dio3 locus, and encodes a spliced non-coding RNA that is transcribed in the opposite orientation, Dio3 opposite strand (Fig. 3, Dio3os). Based on the analysis of Dio3os cDNA clones, Dio3os encodes at least 5 transcripts, and their most 5′ end lies within the CpG island that overlaps the Dio3 promoter [94]. The Dio3os promoter has not been identified. Exon 1 of DIO3OS in humans includes the sequence for the pre-microRNA hsa-mir1247, and the homologous sequence in the mouse encodes mmir1247. This microRNA is expressed in human ES cells and induced ~13× upon their induction to embryoid bodies [96]. Dio3os is not imprinted in the mouse fetus [97] and it is not known whether it plays any mediatory role in regulating the expression or imprinting of Dio3. An enhancer that is highly conserved between mouse and human can activate the Dio3 and Dio3os promoters *in vitro* [94,98], but its specific role *in vivo* is unclear.

The Dio3 promoter is regulated by the polycomb group of proteins that mediate tissue-specific gene silencing during development [99,100]. Evidence for this is provided by high throughput assays that measure enrichment of DNA binding factors and histone modifications. In such a study the Dio3 CpG island promoter was found to be enriched for histone H3K27me3 in embryonic stem cells, which is catalyzed by the polycomb 2 complex [99]. The active modification H3K4me3 was also enriched at this region [100]. The juxtaposition of these two histone marks in ES cells is thought to mark promoters that are ‘poised’ for expression. Upon restriction of cell fate the status of the promoter is then resolved as active or repressed depending on cell type. Interestingly, the neighboring Dlk1 gene also has an unmethylated CpG island promoter that is ‘poised’ for transcription in ES cells [100].

In common with the other paternally-expressed protein encoding genes on chromosome 12, Dio3 expression is controlled by the ICR, since maternal inheritance of the ICR deletion results in biallelic expression of Dio3 (Fig. 2B, [83]). The ICR therefore acts over several hundred kilobases to affect the Dio3 promoter. Dio3 is frequently reported to have an imprinting bias since its expression from the maternally-inherited chromosome is not completely silenced [91]. Dio3 imprinting is somewhat relaxed in the placenta [101] and its expression can be biallelic or even maternally-expressed in certain areas of the brain [102]. It is possible that this observation represents a different imprinting status in different tissues or cell types, or they may be due to different allelic activity of alternative Dio3 promoters, given the presence of larger Dio3 transcripts in the brain. Recently, neighboring Dlk1 was shown to have a selective loss of imprinting in the neurogenic niche, and this was associated with biallelic methylation of the ICR [55]. Since Dio3 imprinting is also critically controlled by this element it would be interesting to determine if Dio3 follows the same imprinting pattern as Dlk1 in the context of neurogenesis. It will be also helpful to determine to what extent the methylation status of the ICR is consistent with Dio3 imprinting in those tissues with bi-allelic or maternal expression of Dio3.

5. The implications of Dio3 imprinting for development and disease

5.1. Developmental consequences of altered imprinting in the Dlk1-Dio3 domain

A consequence of genomic imprinting is that it renders its targets functionally haploid. Since only one parental copy is expressed in an individual, any mutations inherited at this locus may not be compensated for by a second allele. In addition, many functions performed by imprinted genes are exquisitely sensitive to gene dosage, such that doubling the level of mRNA expression by loss of imprinting can cause significant phenotypic consequences (reviewed in [59]).

Phenotypes arising from both loss of gene function, and over-expression of dosage-sensitive genes in the Dlk1-Dio3 domain have been observed when imprinting is perturbed as a result of uniparental disomy (UPD) of chromosome 12 [72]. In such models, both copies of chromosome 12 are inherited from the same parent. Mice with paternal UPD12 (associated with loss of ncRNA expression and double expression of Begain 1b, Dlk1, Rtl1 and Dio3) manifest placentomegaly and die late in gestation, while those with maternal UPD12 (which do not express the protein-encoding genes including Dio3, but overexpress the ncRNAs) are growth retarded and die perinatally. Skeletal defects are also noticed in mice with either type of parental disomy [72].

Mice with deletion of the ICR in the maternal allele also exhibit perinatal lethality as a consequence of overexpression of the paternally-expressed genes [83]. Although it is not possible to discern the contribution of particular genes to those phenotypes, the results indicate that appropriate genomic imprinting in the Dlk1-Dio3 domain is necessary for normal viability, growth and development.

5.2. Pathological implications for humans

To date, no direct mutations on the D3 gene with functional consequences have been reported in humans. Relevant evidence for the potential role of DIO3 and the DLK1-DIO3 imprinted domain in humans comes from a number of patients with UPD of chromosome 14 [103–105]. As the human homolog of the Dlk1-Dio3 domain is located in the distal arm of chromosome 14 [89], these patients' symptoms are presumably the consequence of misexpression of the imprinted genes in the domain. Chromosomal UPD in humans is relatively rare, and usually the result of aberrant chromosomal translocations, or the resolution of chromosomal trisomies very early in development, as evidenced in some cases by placental mosaicism of cells with chromosomal trisomy [106]. A number of common features have been shown in these patients. Maternal UPD14 is associated with growth retardation, hypotonia, small hands and feet, neonatal feeding problems, facial dysmorphisms, hydrocephalus, early puberty, mild mental retardation and slightly increased adiposity [103,107,108]. Patients with paternal UPD14 exhibit severe mental retardation, skeletal abnormalities ("coat-hanger" ribs) and facial dysmorphisms [105,109–112]. As no other imprinted genes have been described on this human chromosome, these observations indicate that aberrant expression of imprinted genes in the DLK1-DIO3 domain is likely responsible for the abnormalities associated with these conditions. This is further supported by reports of deletions and epimutations in the domain in association with characteristics typical of UPD14 [113–115], and by the recapitulation of UPD14 phenotypes in patients in which the UPD affects only a chromosomal segment in which the imprinted domain is located [116].

Aberrant expression of DLK1, DIO3 and other genes in the imprinted domain has been associated with some cellular malignancies in humans, suggesting that the cluster may also play a role in cellular transformation [95,117–119].

5.3. The contribution of Dio3 to phenotypes of abnormal imprinting

The phenotype of the D3KO mouse is very rich and surprisingly insightful about the developmental roles of Dio3, but it is the consequence of a constitutive, extremely low Dio3 dosage. However, the degree of Dio3 expression from the paternal allele may vary in some tissues, like placenta and brain. Thus, although in general Dio3 is preferentially expressed from the paternal allele, most tissues still exhibit significant residual expression from the maternal copy of the gene. In this context, it is likely that certain tissues, and the phenotypes associated with them, will be more sensitive than others to abnormal imprinting of Dio3.

5.4. Similarities between D3KO and maternal UPD phenotypes

All imprinted genes in the Dlk1-Dio3 domain are likely contributing to the UPD syndromes. However, we can estimate the individual contribution of Dio3 by comparing the abnormalities of the D3KO mouse to those observed in mouse and human maternal UPD12 and 14, respectively. The perinatal lethality and growth retardation of maternal UPD12 mice are also observed in D3KO mice. In the latter, neonatal lethality is partial (30–70%) in most genetic backgrounds [29], but it is close to 100% in commonly used strains such as C57Bl/6, suggesting that abnormal Dio3 expression is partly responsible for this outcome. Mice lacking the ICR on the maternal allele also exhibit perinatal lethality [83]. This phenotype is not rescued by normalization of the Dlk1 or Rtl1 expression [120], suggesting that aberrant expression of other imprinted genes in the domain, including Dio3, are contributing to this phenotype.

In humans, several genes of the DLK1-DIO3 domain are imprinted and exhibit similar parent-of-origin allelic expression as the mouse homologs [74], but no information about the imprinting status of the human DIO3 has been reported. A preliminary observation in tissue from adult skin indicates that human DIO3 is imprinted [121]. If this is the case and human DIO3 exhibits a similar tissue and developmental imprinting pattern as that in the mouse, one would expect that patients with maternal UPD14 are D3-deficient and should exhibit characteristics similar to that in D3KO mice. Published and unpublished observations reveal that some maternal UPD14 phenotypic features are also present in D3KO mice (Fig. 1C), including growth retardation [29], hydrocephalus, facial dysmorphisms and age of puberty onset. These similarities suggest that deficient D3 expression could be partly responsible for the phenotypes associated with abnormal imprinting of the Dlk1-Dio3 domain in mouse and humans.

To our knowledge, no data on the thyroid status of UPD14 patients have been published. In three case reports of maternal UPD14 [122–124], the authors state that endocrine and thyroid function tests were performed in the patients with normal results, although no specifics about the exact parameters determined are mentioned. If thyroid hormone abnormalities exist in these individuals and are similar to those in D3KO mice, they are unlikely to be revealed by normal tests, as thyroid hormone parameters in humans that correspond to those in D3KO mice will mostly be within the range of normal clinical values. The identification of a potential HPT axis phenotype in maternal UPD14 subjects may require a physiological challenge.

5.5. Dio3 imprinting from an evolutionary perspective

The reason why genomic imprinting occurs in the first place is still controversial. At first glance, genomic imprinting does not make sense from an evolutionary perspective, since the repression of one copy of a given gene leaves an organism more susceptible to the deleterious effects of genetic mutations in the copy that is normally expressed.

Based on available data, a number of theories have been postulated on the subject [125–127], and they are continually tested and modified by new research. Genomic imprinting evolved in a period of rapid speciation during mammalian divergence and, among the animal kingdom, it is essentially limited to mammals [128]. Interestingly, imprinted gene functions appear to converge on mammalian-specific traits such as invasive placentation, suckling and postnatal adaptations to independent life [59,61,129]. Since these processes are also concerned with the allocation of resources from the mother to offspring, it has been postulated that imprinting may have evolved as a result of a conflict between maternal and paternal genomes [126]. This 'parental conflict' theory postulates that in species with multiple paternities, offspring are unequally related to one another. Thus, genes from the father should act to maximize their resource usage, whereas genes from the mother would seek the survival of all her offspring, regardless of paternity, without compromising her own future reproductive function. Consequently paternally expressed imprinted genes are predicted to promote growth and resource allocation, and maternally-expressed genes to restrain it. While this theory fits well for some imprinted genes [126], the phenotypes of some imprinted gene deletions are consistent with a role for imprinting in the co-adaptation of mother and fetus [130].

Environmental factors, potentially driving natural selection and adaptation, are also speculated to influence genomic imprinting. Given the increasing evidence of epigenetic effects exerted by environmental factors, the unique regulatory mechanisms governing the expression of imprinted genes could be particularly susceptible to the environment [131]. In this regard, as iodine is a critical component of thyroid hormones, there is the intriguing possibility that, as mammals colonized other iodine-poor, land-based ecosystems, a deficiency in this element could have played a role in the genomic imprinting of Dio3. This would have reduced Dio3 expression and increased the efficiency of thyroid hormones. Advances in our understanding of the evolutionary reasons of genomic imprinting may provide new insights into the physiological roles of Dio3, and *vice versa*.

Comparative genomic analysis indicates that Dlk1 and Dio3 are the ancestral genes in the chromosome 12 cluster, and they display tight linkage and strong conservation in all vertebrates. The two genes are approximately 10 kb apart in the fish *Takifugu rubripes* and 370 kb apart in chicken, compared to ~800 kb apart in the human and mouse [132]. The linkage of the genes precedes the evolution of imprinting of the locus, which occurred after the divergence of the marsupial and eutherian mammals, and suggests that both genes may share important physiological roles. The acquisition of imprinting at the cluster is coincident with transcription of the ncRNAs and maintenance of an active Rtl1 gene [132].

5.6. Transgenerational epigenetic effects of thyroid hormones

More than forty years ago, several investigators used a rat model to analyze the effects of excessive exposure to thyroid hormones during development [133,134]. Rat neonates were rendered thyrotoxic by the injection of pharmacological doses of T4 for a few days after birth. As adults, these animals showed growth and HPT axis phenotypes that, although less severe, strikingly resemble those in D3KO mice [135]. Additional studies showed that the untreated offspring and descendants of those animals, as well as that of animals subjected to other thyroid hormone manipulations, exhibited modest but significant abnormalities [136,137]. As these effects could be transmitted for two more generations through the paternal line, they indicate that the degree of exposure to thyroid hormones can produce transgenerational epigenetic effects in the descendants. This raises the possibility that alterations in the genomic imprinting of Dio3, and thus in the level of exposure to thyroid hormone, may do likewise.

6. Summary

Many questions remain and much knowledge is yet to be gained on the topics covered here, but the data available is sufficiently indicative of the importance of the mechanisms regulating the genomic imprinting of Dio3 and associated genes may have for proper development, normal biological functions in adulthood and adequate responses to physiological challenges. It is possible that the epigenetic dysregulation of Dio3 may not be of appreciable consequence for certain tissues or biological processes. However, given the broad physiological influence of D3, it is reasonable to expect that important aspects of development and physiology will be markedly affected by an altered dosage of Dio3. It is also likely that epigenetic abnormalities in the regulation of DIO3 will be relevant to human conditions.

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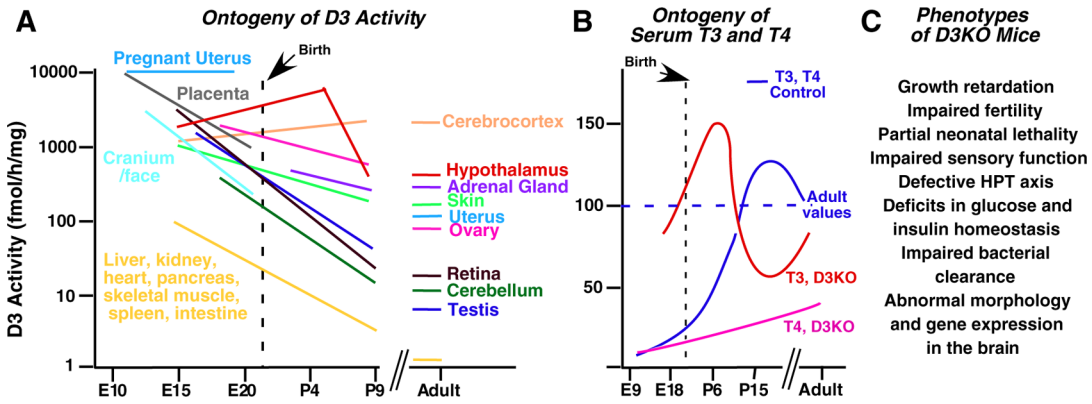

Fig. 1.

Physiological significance of D3. A, Ontogeny and relative level of D3 activity in rodent tissues (patterns are approximate, based on cited literature). Note that D3 activity is reduced by one or two orders of magnitude in most tissues from fetal life to adulthood. B, Ontogeny of serum thyroid hormone levels in normal animals and in D3KO mice. During development D3KO mice are exposed to excessive levels of T3. Values are expressed as a percentage of normal adult values (horizontal dotted line). C, Summary of reported phenotypes in D3KO mice.

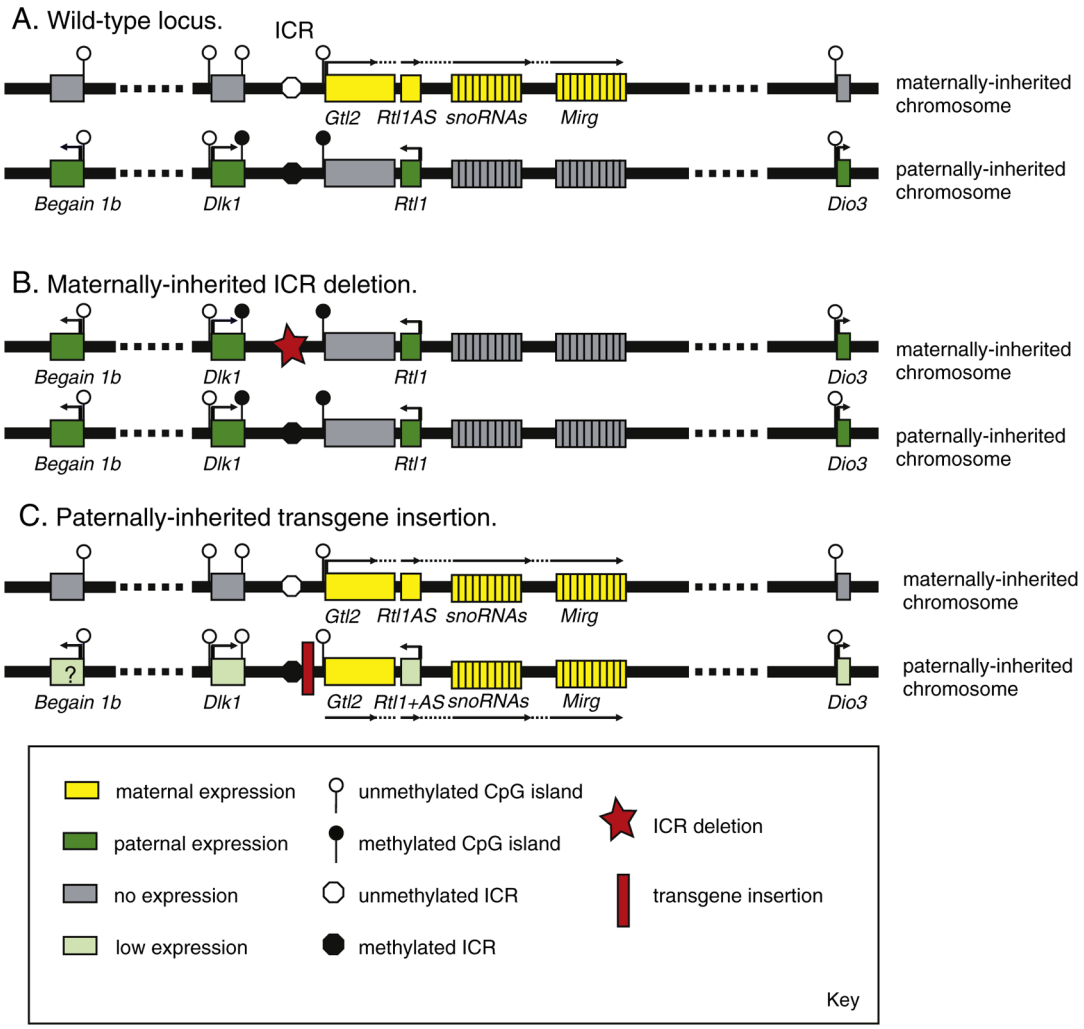

Fig. 2.

The mouse chromosome 12 imprinted cluster. A, The wild type locus. The Dlk1-Dio3 imprinted domain is shown (1.5 Mb, not to scale) on the two parental chromosomes. Following maternal transmission (top) there is expression of a long non-coding RNA (yellow) containing transcripts for Gtl2, Rtl1AS, snoRNAs and Mirg. This transcript arises from a non-methylated Gtl2 promoter (open lollipop), which is *in cis* to an unmethylated ICR (white circle). On the same chromosome Begain 1b, Dlk1, Rtl1 and Dio3 are not expressed (gray boxes). On the paternally-inherited chromosome (bottom) the ncRNAs are silent (gray boxes) *in cis* to a methylated Gtl2 promoter and ICR. The protein-encoding Begain 1b, Dlk1, Rtl1 and Dio3 genes are all expressed from this chromosome (green boxes). Dlk1 and Dio3 are driven by unmethylated CpG island promoters (open lollipops at start of genes) on both parental chromosomes, and there is an additional region of methylation in exon 5 of Dlk1 which is hypermethylated on the paternally-inherited chromosome. B, The maternally inherited ICR deletion. When a deletion of the ICR (red star) is transmitted through the female germline there is an epigenotype switch on this chromosome such that it resembles the paternally-inherited chromosome. The gtl2 promoter becomes methylated, the ncRNAs are silenced, and the protein encoding genes are reactivated *in cis* to the deletion. The paternally-inherited chromosome is unaffected by the deletion. C, Paternally-inherited transgene insertion. When a transgene insertion between the

ICR and Gtl2 promoter (red box) is inherited of the paternal chromosome the Gtl2 promoter fails to become methylated *in cis*. This results in activation of the (normally silenced) ncRNAs and partial silencing of the protein-encoding genes.

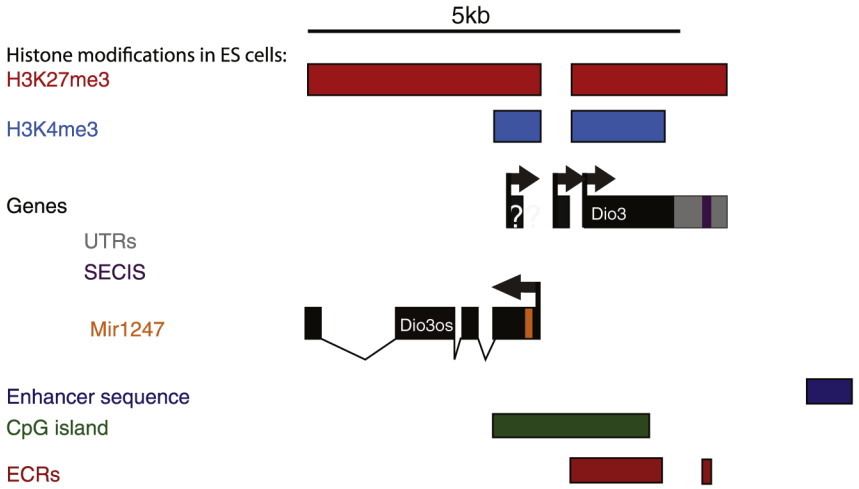

Fig. 3.

Features associated with the Dio3 gene. The major fetal and placental isoform of Dio3 is transcribed from a single 2.1 kb exon with a long 3′UTR (gray) containing the SECIS sequence (purple). The sequence containing the coding region of the gene and the 3′UTR is highly conserved in vertebrates (red boxes). Other transcripts have been described in the brain that may arise from alternative promoters (?), but have not yet been mapped. Dio3os is transcribed in the opposite direction to Dio3, and gives rise to at least 5 alternatively spliced transcripts. The 5′ end of these reported transcripts all originate within the same CpG island as the Dio3 promoter (green box). Exon 1 of Dio3os contains a conserved microRNA, mir1247 (orange box). Approximately 5 kb telomeric to the Dio3 promoter there is a conserved enhancer that can drive Dio3 and Dio3os expression *in vitro* (blue box). In ES cells the Dio3 promoter is decorated with modified histones, H3K27me3 (red bar) and H3K4me3 (blue bar), indicative of a ‘poised’ chromatin state.