Molecular basis of imprinting disorders affecting chromosome 14: lessons from murine models

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Abstract

Uniparental inheritance of chromosome 14q32 causes developmental failure during gestation and early postnatal development due to mis-expression of a cluster of imprinted genes under common epigenetic control. Two syndromes associated with chromosome 14q32 abnormalities have been described, Kagami–Ogata and Temple syndromes. Both of these syndromes are characterised by specific impairments of intrauterine development, placentation and early postnatal survival. Such abnormalities arise because the processes of intrauterine growth and postnatal adaptation are critically modulated by the dosage of imprinted genes in the chromosome 14q32 cluster. Much of our understanding of how the imprinted genes in this cluster are regulated, as well as their individual functions in the molecular pathways controlling growth and postnatal adaptation, has come from murine models. Mouse chromosome 12qF1 contains an imprinted region syntenic to human chromosome 14q32, collectively referred to as the *Dlk1–Dio3* cluster. In this review, we will summarise the wealth of information derived from animal models of chromosome 12 imprinted gene mis-regulation, and explore the relationship between the functions of individual genes and the phenotypic result of their mis-expression. As there is often a considerable overlap between the functions of genes in the *Dlk1–Dio3* cluster, we propose that the expression dosage of these genes is controlled by common regulatory mechanisms to co-ordinate the timing of growth and postnatal adaptation. While the diseases associated with mis-regulated chromosome 14 imprinting are rare, studies carried out in mice on the functions of the affected genes as well as their normal regulatory mechanisms have revealed new mechanistic pathways for the control of growth and survival in early life.

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Genomic imprinting

Genes subject to genomic imprinting are expressed predominantly from a single parental chromosome, with the other copy silenced. The choice of which chromosomal copy is expressed is determined during gametogenesis, and is known to require parental origin-specific modifications to regulatory regions, including cytosine methylation of DNA and covalent modifications to core histone tails (recently reviewed in [Hanna & Kelsey \(2014\)](#)). Imprinted genes often occur in clusters, and their parental origin-specific expression status is commonly conferred by a shared imprinting control region (ICR) that is differentially methylated between parental chromosomes. Experiments carried out in mice that delete these ICRs cause reversal of the imprinting status of all genes in the cluster (reviewed in [Ferguson-Smith \(2011\)](#)). To date, ~150 imprinted genes have been described, many of which are imprinted in both mice and humans. Importantly, uniparental inheritance of many of these imprinted clusters underlies human disease – most notably paediatric syndromes of growth:

Beckwith–Wiedemann, Russell–Silver, Prader–Willi, Kagami–Ogata and Temple syndromes (TSs; [Williamson et al. 2013](#)).

In this review, we will consider the regulation and functions of genes, the *Dlk1–Dio3* cluster, underlying the imprinted syndromes associated with chromosome 14, Kagami–Ogata and TSs.

The *Dlk1–Dio3* imprinted cluster

Gene organisation

The human imprinted cluster on chromosome 14 spans ~1 Mb, and in the mouse 12qF1 spans a similar 1.3 Mb. Both clusters contain a number of genes encoding both proteins and functional RNAs (summarised in [Fig. 1](#)). The most centromeric imprinted gene is an isoform of *Begain 1b* ([Deguchi et al. 1998](#)), and the most telomeric is *Dio3* – encoding the type 3 deiodinase ([Hernandez et al. 1998](#)). In the mouse, both of these genes are expressed predominantly from the paternally inherited chromosome ([Hernandez et al. 2002](#), [Tsai et al. 2002](#), [Tierling](#)

Figure 1 The imprinted cluster on human chromosome 14/mouse chromosome 12. (A) Genes normally expressed from the maternally inherited chromosome are shown in red, and those expressed from the paternally inherited chromosome in blue. Grey boxes represent silenced genes. Regions with a high-density CpG dinucleotides are shown as lollipops, white when unmethylated and black when methylated. The IG-DMR is shown as a white (unmethylated) or black (methylated) circle between *Dlk1* and *Meg3*, adapted from Charalambous *et al.* (2012). (B) Developmental regulation of methylation at the IG-DMR, *Meg3* promoter DMR and the *Rian* DMR throughout development. Rows of the table represent the maternally- (MAT) or paternally- (PAT) inherited chromosomes. Methylation is indicated by the box filled in black, and unmethylated by the box filled in pale grey. Grey fill indicates an intermediate level of methylation.

et al. 2009), but their imprinting status in humans has not been well established. The imprinting status of the intervening genes is well described: *Dlk1* and *Rtl1* are expressed predominantly from the paternally inherited chromosome, and *Meg3* (*Gtl2*); *anti-Rtl1* (encoding 7 miRNAs that target *Rtl1* and suppress its expression *in trans*), *Rian* (encoding C/D Sno-RNAs), and *Mirg* (a large microRNA cluster) are all expressed from the maternally inherited chromosome (Kobayashi *et al.* 2000, Schmidt *et al.* 2000, Takada *et al.* 2000, Wylie *et al.* 2000, Cavaille *et al.* 2002, Seitz *et al.* 2003, Seitz *et al.* 2004). These maternally expressed genes comprise a long multicistronic non-coding RNA (ncRNA) transcript that initiates at the *Meg3* promoter (Tierling *et al.* 2006).

Regulatory regions

The promoter CpG-islands of *Begain 1b*, *Dlk1* and *Dio3* remain unmethylated in most cell types examined (Takada *et al.* 2000, Tsai *et al.* 2002, Tierling *et al.* 2009). Four additional cytosine-rich DNA regions have been mapped, which are differentially methylated between parental chromosomes (differentially methylated regions or DMRs, Fig. 1): an intragenic DMR within

Dlk1 whose regulatory function is unknown; an intergenic DMR (IG-DMR) that is located ~15 kb upstream of the *Meg3* promoter. The *Meg3* promoter is differentially methylated, and this correlates with the expression of the ncRNAs. Each of these three DMRs is methylated on the paternally inherited chromosome and unmethylated on the maternal chromosome (Schmidt *et al.* 2000, Takada *et al.* 2000, Takada *et al.* 2002). Finally, a maternally methylated DMR within the *Rian*-C/*D* SnoRNA cluster has recently been described in both humans and mice (Court *et al.* 2014, Zeng *et al.* 2014).

The IG-DMR bears all of the hallmarks of the ICR, because it acquires differential methylation in the germline (methylated in sperm, unmethylated in oocytes), and retains its differential methylation throughout preimplantation development. In contrast, the *Meg3* promoter, the *Dlk1* DMR and the *Rian* DMR are secondary DMRs because they only acquire their differential methylation post-implantation (Schmidt *et al.* 2000, Takada *et al.* 2000, Nowak *et al.* 2011; summarised in Fig. 1B).

The definitive proof that the IG-DMR is the ICR at the *Dlk1*-*Dio3* cluster comes from experiments using targeted deletions in the mouse. Lin *et al.* (2003) created a deletion of ~4 kb including the IG-DMR and generated crosses where the modified chromosome could be inherited from either parent. When the deletion was inherited through the male germline, gene expression *in cis* was not affected, indicating that a methylated DMR is dispensable for the expression of the protein-encoding genes and for the silencing of the ncRNAs. This also suggested that the structure of the paternally inherited chromosome was the evolutionarily ancestral state – a hypothesis supported by the finding that in non-imprinting species such as birds and fish, *DLK1* and *DIO3* are biallelically expressed and the ncRNAs are not present (Edwards *et al.* 2008). When the IG-DMR deletion was inherited through the female germline, the chromosome was modified such that an epigenotype switch occurred – the ncRNAs were silenced and the protein-encoding genes reactivated. In addition, the *Meg3* promoter DMR was no longer methylated (Lin *et al.* 2003), summarised in Fig. 2A and B.

The mechanism of imprinting control has been elucidated further by additional genetic modifications of the *Dlk1*-*Dio3* domain (Fig. 2C, D and E). Several lines of evidence suggest that the IG-DMR directs the methylation status of the *Meg3* promoter DMR, which then controls gene expression across the entire cluster. Firstly, insertion of a transgene array between the IG-DMR and *Meg3* promoter, when paternally inherited, prevents promoter methylation, leading to reactivation of the ncRNAs *in cis* and concomitant reduced expression of the protein-encoding genes. Insertion on the maternally inherited chromosome causes transcriptional interference with the *Meg3* promoter and reciprocal gene mis-expression (*Gtl2*-*LacZ* mice

A WT locus

DNA methylation		Gene expression									
IG-DMR	Meg3-DMR	<i>Begain 1b</i>	<i>Dlk1</i>	<i>Meg3</i>	<i>Anti-Rtl1</i>	<i>Rtl1</i>	<i>Rian</i>	<i>Mirg</i>	<i>Dio3</i>		
Methylated	Methylated	High	High	None	None	High	None	None	High	PAT	
Unmethylated	Unmethylated	None	None	High	High	None	High	High	None	MAT	

B IG-DMR deletion

	IG-DMR	Meg3-DMR	<i>Begain 1b</i>	<i>Dlk1</i>	<i>Meg3</i>	<i>Anti-Rtl1</i>	<i>Rtl1</i>	<i>Rian</i>	<i>Mirg</i>	<i>Dio3</i>		
Paternal inheritance	Deleted	Methylated	High	High	None	None	High	None	None	High	PAT	
	Unmethylated	Unmethylated	None	None	High	High	None	High	High	None	MAT	
Maternal inheritance	Methylated	Methylated	High	High	None	None	High	None	None	High	PAT	
	Deleted	Methylated	High	High	None	None	High	None	None	High	MAT	

C *Gtl2-LacZ* insertion

	IG-DMR	Meg3-DMR	<i>Begain 1b</i>	<i>Dlk1</i>	<i>Meg3</i>	<i>Anti-Rtl1</i>	<i>Rtl1</i>	<i>Rian</i>	<i>Mirg</i>	<i>Dio3</i>		
Paternal inheritance	Methylated	Unmethylated	?	Low	Low	Low	Low	Low	Low	Low	PAT	
	Unmethylated	Unmethylated	?	None	High	High	None	High	High	None	MAT	
Maternal inheritance	Methylated	Methylated	?	High	None	None	High	None	None	High	PAT	
	Unmethylated	Unmethylated	?	Low	Low	Low	Low	Low	Low	Low	MAT	

D *Meg3* deletion (includes *Meg3*-DMR)

	IG-DMR	Meg3-DMR	<i>Begain 1b</i>	<i>Dlk1</i>	<i>Meg3</i>	<i>Anti-Rtl1</i>	<i>Rtl1</i>	<i>Rian</i>	<i>Mirg</i>	<i>Dio3</i>		
Paternal inheritance	Methylated	Deleted	?	Low	Deleted	Low	Low	Low	Low	Low	PAT	
	Unmethylated	Unmethylated	?	None	High	High	None	High	High	None	MAT	
Maternal inheritance	Methylated	Methylated	?	High	None	None	High	None	None	High	PAT	
	Unmethylated	Deleted	?	Low	Deleted	Low	Low	Low	Low	Low	MAT	

E *Meg3* deletion (includes half of *Meg3*-DMR)

	IG-DMR	Meg3-DMR	<i>Begain 1b</i>	<i>Dlk1</i>	<i>Meg3</i>	<i>Anti-Rtl1</i>	<i>Rtl1</i>	<i>Rian</i>	<i>Mirg</i>	<i>Dio3</i>		
Paternal inheritance	Methylated	Methylated/Del	?	High	Deleted	None	High	None	None	High	PAT	
	Unmethylated	Unmethylated	?	None	High	High	None	High	High	None	MAT	
Maternal inheritance	Methylated	Methylated	?	High	None	None	High	None	None	High	PAT	
	Methylated	Methylated/Del	?	High	Deleted	None	High	None	None	High	MAT	

F Paternal UPD

	IG-DMR	Meg3-DMR	<i>Begain 1b</i>	<i>Dlk1</i>	<i>Meg3</i>	<i>Anti-Rtl1</i>	<i>Rtl1</i>	<i>Rian</i>	<i>Mirg</i>	<i>Dio3</i>		
	Methylated	Methylated	?	High	None	None	High	None	None	High	PAT	
	Methylated	Methylated	?	High	None	None	High	None	None	High	MAT	

G Maternal UPD

	IG-DMR	Meg3-DMR	<i>Begain 1b</i>	<i>Dlk1</i>	<i>Meg3</i>	<i>Anti-Rtl1</i>	<i>Rtl1</i>	<i>Rian</i>	<i>Mirg</i>	<i>Dio3</i>		
	Unmethylated	Unmethylated	?	None	High	High	None	High	High	None	MAT	
	Unmethylated	Unmethylated	?	None	High	High	None	High	High	None	MAT	

Figure 2 Summary of genetic manipulations of the *Dlk1-Dio3* region in the mouse. (A) Summary of gene expression and methylation status of the unmodified, WT imprinted locus on mouse chromosome 12. The paternally-inherited chromosome (Pat) on the top row and maternally inherited chromosome (Mat) on the bottom row. Gene expression status arising from deletion of the IG-DMR (B); the *Gtl2-LacZ* insertion (C); deletions of *Meg3* including the promoter (D) and (E); and uniparental disomy of Chromosome 12 (F) and (G). The methylation status of the DMRs is stated in the left box and gene expression in the right box. Genes normally paternally expressed are indicated by blue text and those normally maternally expressed by red text, black text indicates that the gene is silenced. ? indicates that the expression status has not been empirically tested. Yellow areas highlight the deleted regions, yellow triangles indicate the *Gtl2-LacZ* transgene insertion site.

(Schuster-Gossler *et al.* 1996, Schuster-Gossler *et al.* 1998, Sekita *et al.* 2006, Steshina *et al.* 2006), Fig. 2C). Deletion of the *Meg3* promoter DMR and the first few exons of *Meg3* also interferes with ncRNA transcription with reciprocal effects on protein-encoding gene expression (Takahashi *et al.* 2009; Fig. 2D). Both of the manipulations above affect methylation of the *Meg3* promoter without changes in the methylation status of the IG-DMR, supporting a mechanism whereby the ICR directs *Meg3* promoter methylation in a hierarchical fashion. This hierarchical mechanism is further supported by work in patients with small deletions at the *DLK1-DIO3* cluster. Maternal transmission of a micro-deletion at the IG-DMR resulted in a gain of methylation at the *MEG3* promoter DMR. This was accompanied by protein-encoding gene expression *in cis* and silencing of the ncRNAs (Kagami *et al.* 2010), and overall was strikingly similar to the effects of IG-DMR deletion in the mouse (Lin *et al.* 2003). Similarly, maternal transmission of micro-deletions encompassing the *MEG3* promoter DMR does not result in changes in methylation status of the IG-DMR (Kagami *et al.* 2010, Beygo *et al.* 2015). However, in the mice, one deletion of the *Meg3* promoter and associated sequences caused ectopic methylation of the IG-DMR on the maternally inherited chromosome, with associated gene expression changes (Zhou *et al.* 2010), Fig. 2E, indicating that communication from the *Meg3* promoter to the IG-DMR may also occur.

There is still much to discover about how the IG-DMR acts at a distance to control the transcriptional status of genes in the cluster, and such mechanisms likely involve three-dimensional interactions between the components of the nuclear architecture and local regulatory regions (reviewed in da Rocha *et al.* (2008)).

Acquisition and maintenance of IG-DMR methylation

The IG-DMR is one of three ICRs in the mouse/human genome that are methylated in sperm and unmethylated in oocytes. The acquisition of differential methylation by this region in gametes and the maintenance of this epigenetic mark throughout the reprogramming events in the early embryo are the subject of intense research, but are outside the scope of this review. Some of the current data and ideas on this topic have been summarised recently (Hanna & Kelsey 2014). However, recent work in an *in vitro* model has suggested that the IG-DMR might function as an enhancer to drive transcription of small ncRNAs on the maternally inherited chromosome that protect the region from methylation in the early embryo, because the region demonstrates the hallmarks of enhancer sequences including characteristic histone modifications and hypersensitivity to nuclease digestion (Kota *et al.* 2014).

Phenotypes associated with the *Dlk1-Dio3* cluster

Human *upd(14)* syndromes

Paternal uniparental disomy of chromosome 14 was first described in 1991 (Wang *et al.* 1991), and is now known as Kagami–Ogata syndrome. Since then, at least 30 cases have been described with the following symptoms: placentomegaly associated with hyperproliferation of the chorionic villi; skeletal abnormalities, specifically ‘coat hanger ribs’ that without intervention cause perinatal death by pulmonary obstruction; limb shortening; polyhydramnios which often leads to premature delivery; abdominal wall defects; craniofacial abnormalities; feeding difficulties with developmental delay; and moderate mental retardation (Kagami *et al.* 2010). Kagami–Ogata syndrome can be caused by paternal uniparental disomy or other chromosomal abnormalities/epimutations that lead to elevated expression of the protein-encoding genes (*BEGAIN 1B*, *DLK1*, *RTL1*, *DIO3*) and/or loss of expression of the ncRNAs (Kagami *et al.* 2012a; Fig. 2F). Based on careful analysis of patients with small deletions at the *DLK1-DIO3* cluster, it is likely that gain of *RTL1* expression dosage underlies placentomegaly, whereas more complex combinations of loss and gain of function are responsible for skeletal abnormalities, failure to thrive and other *upd(14)*pat-like phenotypes (Kagami *et al.* 2008).

The 51 cases of TS published to date have been summarised in a recent clinical review (Ioannides *et al.* 2014). This syndrome is characterised by intrauterine growth restriction (placental insufficiency has been reported), commonly followed by hypotonia and perinatal failure to thrive. TS newborns have reduced skeletal length often with relative macrocephaly. Adults with TS have reduced final height, but frequently display high BMI with onset in late childhood. Elevated BMI can be accompanied by metabolic diseases such as type 2 diabetes and/or dyslipidaemias. The majority of TS cases of both sexes experience early puberty. There are additionally some dysmorphic characteristics associated with TS, specifically a characteristic face with a broad nose and high forehead, and small hands and feet. The most common molecular cause of TS reported to date is maternal uniparental disomy of chromosome 14, but loss of methylation of IG-DMR and paternal deletions have also been reported (summarised in Ioannides *et al.* (2014)). All of these perturbations would be predicted to reduce the expression dosage of the protein-encoding genes and increase that of the ncRNAs (Fig. 2G).

Mouse chromosome 12 uniparental disomy

The critical region for *upd(14)* syndromes has been mapped to 14q32, and the syntenic region in the mouse lies on the distal portion of chromosome 12qF1. Robertsonian translocations have been used to generate uniparental disomic mice for this region (Cattanach *et al.*

1993), and the resultant phenotypes have much in common with upd(14) syndromes (Georgiades *et al.* 2000,2001).

pUPD12 conceptuses exhibit placentomegaly with impairments to the labyrinthine zone of the placenta – the analogous structure to the chorionic villi. The bones of the rib cage develop the classical ‘bell shape’ and there is delayed ossification of the long bones and bones of the face and head. The misshapen rib cage causes compression of the visceral organs and breaches of the body wall. Skeletal muscle development is also delayed. The animals die in late gestation, probably due to placental insufficiency (Georgiades *et al.* 2000,2001).

mUPD12 conceptuses are extremely growth retarded from the latter third of gestation, and this is accompanied with reduced placental mass. The skeleton is proportionally small, and there are minor malformations of the facial bones. The skeletal muscle mass is disproportionately reduced – muscles have fewer and smaller myofibres, probably due to premature differentiation. The mUPD12 conceptuses die within the first few days of life, usually following respiratory failure (Georgiades *et al.* 2000,2001).

The pre- and perinatal lethality of UPD12 precludes the comparison with the human upd(14) syndromes for postnatal phenotypes. However, live animals are recovered from mice models of partial disruption of chromosome 12 imprinting caused by a transgene insertion into the cluster (the *Gtl2–LacZ* model described above and in Fig. 2C). Inheritance of a disrupted chromosome from the father mimics TS – with intrauterine growth restriction, relative macrocephaly and small placenta, failure to thrive in the early postnatal period with subsequent growth retardation into adulthood (Schuster-Gossler *et al.* 1998, Charalambous *et al.* 2014a). Maternal inheritance of the transgene insertion partially reproduces the gene expression pattern associated with Kagami–Ogata syndrome and causes placentomegaly, perinatal lethality and failed metabolic adaptations to postnatal life (Charalambous *et al.* 2012). Taken together, the phenotypes of mice with disruptions to chromosome 12 imprinting phenocopy many of the clinical characteristics of human chromosome 14 syndromes.

Expression and functions of the genes in the *Dlk1–Dio3* domain

In both humans and mice, the failure to regulate dosage of *DLK1–DIO3* cluster imprinted genes is detrimental to survival in early life. Moreover, the impairments associated with mis-regulation appear to converge on a small number of physiological processes, namely those concerned with ensuring adequate growth and nutrition before birth and in early postnatal life. In addition, syndromes associated with reciprocal inheritance

patterns appear to have opposite effects on these processes, i.e. mutations causing upd(14)pat-like gene expression profiles cause skeleto-muscular immaturity, delayed placental formation (slow migration of glycogen cells) and failure to survive the transition to birth. On the other hand, upd(14)mat-like mutations appear to advance development, particularly long bone growth and the onset of puberty. ‘Cluster-wide’ murine models are extremely useful in understanding the actions of co-ordinated gene regulation on physiological processes, but cannot be used to understand how altered expression dosage of individual genes act on developmental pathways to effect physiological change. Over the last ~10 years, single gene deletions and overexpression models have been developed and described for many of the genes in the *Dlk1–Dio3* cluster in the mouse. These are summarised in the sidebar.

Begain

Very little work has been done to date on the BEGAIN protein. It is expressed in the developing and postnatal brain, and co-localises at postsynaptic junctions of neurons with other important regulators of postsynaptic transmission (Deguchi *et al.* 1998). To date no murine model of *Begain* gain or loss of function has been described.

Dlk1

Since its independent discovery by three groups in 1993 (Jensen *et al.* 1993, Laborda *et al.* 1993, Smas & Sul 1993), more than 200 papers have been published with *Dlk1* as their main subject. *Dlk1* encodes a protein that is widely expressed during embryogenesis in mesoderm/neuroectoderm and in the placenta, particularly the labyrinthine zone (Yevtodiyenko & Schmidt 2006, da Rocha *et al.* 2007). *DLK1* is a vertebrate-specific member of the Delta family of Notch ligands, although it lacks the canonical interacting DSL domain (Laborda *et al.* 1993). It is a transmembrane protein that, due to alternative splicing, generates both membrane-bound and serum-soluble isoforms (Smas *et al.* 1997). The signalling pathway(s) through which *DLK1* acts has not been fully elucidated. Several deletion and overexpression lines for *Dlk1* have been generated (Moon *et al.* 2002, Lee *et al.* 2003, Raghunandan *et al.* 2008, da Rocha *et al.* 2009, Abdallah *et al.* 2011, Appelbe *et al.* 2013), and their phenotypes are described in ‘gene function–phenotype interactions’ section.

Meg3

Meg3 is expressed abundantly and widely in the embryo and placenta, in an overlapping but not identical pattern to *Dlk1* (Schuster-Gossler *et al.* 1998, da Rocha

et al. 2007). It has particularly strong expression in the CNS where expression persists after birth (Schmidt *et al.* 2000). The function of *Meg3* has not been established, but loss of function has been associated with neoplastic transformation in a large number of studies comparing tumours and healthy tissues (reviewed in Benetatos *et al.* (2011)). Several mice models have deleted *Meg3* (see Fig. 2D and E) but all also removed the promoter region and retained a neomycin insertion and thus cause disruptions to gene expression across the *Dlk1–Dio3* cluster. For this reason, the phenotypes arising from these manipulations are not discussed further.

Rtl1* and *Rtl1as

The retrotransposon-like 1 gene is a member of the Ty3/gypsy class of retroelements (Youngson *et al.* 2005). Its expression is widespread in the embryo and placenta, and similarly to *Meg3*, *Rtl1* expression persists in the postnatal brain (Brandt *et al.* 2005). Sekita *et al.* (2008) generated a mice model that deletes *Rtl1* when paternally inherited. When maternally inherited the same manipulation deletes the antisense transcript-encoding miRNAs that negatively regulate *Rtl1* *in trans*. Thus, both loss of function (paternal deletion) and gain of function (maternal deletion of the antisense transcript) of *Rtl1* can be assessed (Sekita *et al.* 2008). However, phenotypes arising from maternal transmission of the *Rtl1*-region deletion must be interpreted cautiously, because the miRNAs may regulate targets in addition to *Rtl1*.

***Rian*- C/D SnoRNAs**

The *Rian* transcript encodes a repeat array of two types of C/D-box small nucleolar RNAs (Cavaille *et al.* 2002). This catalytic RNA family has been shown to apply post-transcriptional modification to RNAs, in this case 2 hydroxymethylation which is thought to stabilise the molecule. Other members of the family stabilise the rRNA molecules, but the targets for the *Dlk1–Dio3* cluster SnoRNAs have not been discovered (reviewed in Girardot *et al.* (2012)). To date, phenotypes arising from the deletion of this gene have not been reported.

Mirg

Mirg encodes one of the largest microRNA clusters in the mammalian genome, with over 40 individual miRNAs (Seitz *et al.* 2004). Its expression follows *Meg3*, and is very high during embryogenesis and the first few days of life, but declines thereafter in most tissues except the brain (Labialle *et al.* 2014). Indeed many of the individual miRNAs and the cluster as a whole have been reported to be co-regulated by events in neuronal development and maturation (Fiore *et al.* 2009).

Intriguingly, the cluster is highly expressed in human pancreatic islets and this expression is reduced in Islets harvested from type 2 diabetic patients (Kameswaran & Kaestner 2014). *Mirg* has been recently deleted in mice (Labialle *et al.* 2014) and phenotypes arising from this are discussed in 'gene function–phenotype interactions' section.

Dio3* and *Dio3os

Dio3 encodes the type 3 iodothyronine deiodinase (D3) enzyme that acts in the thyroid hormone (TH) pathway. The main circulating TH is thyroxine (T₄) which is converted within tissues to bioactive tri-iodothyronine (T₃) by D1 and D2. D3 acts as a negative regulator of TH by converting T₄ and T₃ to biologically inactive rT₃ and T₂ respectively (Bianco 2011). Both global (Hernandez *et al.* 2002) and conditional (Medina *et al.* 2011) loss of function alleles of *Dio3* have been generated. An additional transcript overlaps the *Dio3* transcript on the opposite DNA stand, *Dio3os* (Hernandez *et al.* 2002). This transcript encodes a microRNA, *miR1247* (Martinez-Sanchez & Murphy 2013). *Dio3os* is not imprinted in the mouse foetus, and it is not known if its expression is regulated by the IG-DMR (Tierling *et al.* 2006).

Gene function–phenotype interactions

Placentation

Some of the earliest defects associated with upd(14) syndromes are failures of placentation. The mature placenta is the primary organ of nutrient exchange and foetal hormone production, and its adequate growth and differentiation are essential for successful development. Kagami *et al.* (2012b) reported abnormal placental physiology in Kagami–Ogata syndrome term placentae; all of the observed abnormalities affected the chorionic villi, with hyperproliferation and thickened endothelium of intermediate but not terminal villi, swelling of vascular endothelial cells and hypertrophy of the pericytes in the terminal villi. This was associated with a narrowed capillary lumen. In the mouse, pUPD12 is associated with several placental defects in late gestation; i) defects in the cell-cell contacts between endothelial and trophoblast layers of the labyrinth, ii) abnormal behaviour of glycogen cells, causing delayed and/or shallow invasion of the maternal decidual layer (Georgiades *et al.* 2001). Only a few cases of TS have reported placental size (Mitter *et al.* 2006), but in these cases where there was foetal growth retardation the placenta was also small, suggesting that IUGR may have been secondary in part to placental growth restriction.

Dlk1 is expressed in the labyrinth compartment of the mature placenta, predominantly in the endothelial cells that enclose the foetal circulation (Floridon *et al.* 2000, Yevtodiyenko & Schmidt 2006, da Rocha *et al.* 2007).

Deletion of *Dlk1* causes a reduction in foetal and placental size from late gestation (Moon *et al.* 2002, Appelbe *et al.* 2013), with birth weight at about 90% that of WT littermates. In global *Dlk1*-null mice, the cell composition of the placenta is altered; the labyrinthine compartment is reduced in volume and the junctional layer is increased. In addition, the *Dlk1*-null placental labyrinth shows increased cellularity and decreased vascularity in comparison with WT, and the junctional zone has a decreased number of glycogen cells with an increase in pre-glycogen cells (Appelbe *et al.* 2013).

The ncRNAs *Meg3*, *Rian* and *Mirg* are expressed at high levels in the late gestation placenta (da Rocha *et al.* 2007, Charalambous *et al.* 2012). The *Meg3* promoter is active in the foetal endothelial and trophoblast compartments (da Rocha *et al.* 2007), but the function of *Meg3* or other ncRNAs in the placenta has not been elucidated. However, deletion of *Mirg* does not affect the overall size of the placenta, and does not cause an embryonic growth phenotype (Labialle *et al.* 2014).

Rtl1 is expressed in the foetal endothelial cells of the late gestation placenta (Sekita *et al.* 2008). Mice with loss of *Rtl1* expression have pre- and post-natal growth retardation (~80% of WT) with small placentas (72% of WT). The *Rtl1*-deleted placenta has reduced passive diffusion of inulin, indicating that the maternal-foetal barrier is abnormal (Sekita *et al.* 2008). Gain of *Rtl1* expression dosage by deletion of six of the seven miRNAs in the *Rtl1*-AS transcript causes placentomegaly (150% of WT) with abnormal labyrinthine trophoblast morphology. Mice are born at normal weight but fail to thrive and die perinatally (Sekita *et al.* 2008).

Dio3 is known to be expressed at high levels in the placenta where its product D3 protects the foetus from high maternal circulating TH (Hernandez *et al.* 2006, Charalambous *et al.* 2012). To date there have been no reports of placental phenotypes arising from the deletion of *Dio3*.

Taken together, these data suggest that the gain of *Rtl1* expression dosage alone may cause the enlarged placenta and defects of the chorionic villi of upd(14) pat/labyrinth of pUPD12. Impaired *Dlk1* dosage may additionally play a role in the glycogen cell migration defect, but without a placental overexpression model for *Dlk1* this is not proven. Loss of expression of *Dlk1* and *Rtl1* is likely to act in combination to reduce placental size in mUPD12 conceptuses. However, the molecular pathways by which these genes act and potentially interact to modulate placental development are still unclear.

Birth transition and failure to thrive

Both upd(14) syndromes have reduced viability in the immediate postnatal period. Kagami-Ogata syndrome neonates experience feeding difficulties and fail to

thrive. Two genetic manipulations in mice with upd(14)pat-like expression profiles reproduce this phenotype: increased expression dosage of *Dlk1* (da Rocha *et al.* 2009) and loss of *Mirg* expression (Labialle *et al.* 2014), both cause lethality in the perinatal period. Doubling the expression dosage of *Dlk1* impairs survival by more than one mechanism. Firstly, lung development is incomplete at birth causing respiratory failure, and secondly the animals fail to feed from the mother (da Rocha *et al.* 2009). Mice with a *Mirg* deletion also die within a day or two of delivery, with evidence of a feeding failure as well as a profound defect in the ability of the animal to face the metabolic challenges of postnatal life. Mice lacking *Mirg* are hypoglycaemic due to an inability to mobilise their hepatic glycogen stores, and in addition fail to supplement low glucose with the production of ketones (Labialle *et al.* 2014). Combined, these data implicate the *Dlk1*-*Dio3* cluster in mechanistic events that synchronise essential adaptations to life outside the womb. Intriguingly, maternal inheritance of the *Gtl2*-*LacZ* mutation (Fig. 2C), which has a gene expression profile of a 'mild' upd(14)pat (elevated protein-encoding gene expression and reduction in ncRNA expression dosage), fails to adapt to a second early life transition, from sucking to weaning, due to impairments in fuel metabolism, adipogenesis and thermogenesis (Charalambous *et al.* 2012).

Poor perinatal survival in TS may be caused by loss of expression of any one of the protein-encoding genes, because in mice deletion of *Dlk1* (Moon *et al.* 2002), *Rtl1* (Sekita *et al.* 2008) and *Dio3* (Hernandez *et al.* 2006) each causes lethality post-delivery, largely by unknown mechanisms. However, *Dio3* deletion causes perinatal thyrotoxicosis (Hernandez *et al.* 2006) which to date has not been observed in TS.

Muscle

Problems with skeletal muscle development probably underlie some of the failure to thrive phenotypes that we observe in the upd(14) syndromes, such as breathing (independently of the rib defect) and feeding difficulties. The mouse UPD12 studies demonstrated that increased paternal gene dosage and/or reduced ncRNA expression causes defects in late gestation muscle development – the fibres are large and contain centrally located nuclei – characteristic of immature muscle. Conversely, reduced expression of the protein-encoding genes and upregulation of the ncRNAs are associated with reduced muscle mass – both smaller and fewer fibres (Georgiades *et al.* 2000).

Most of the chromosome 12 genes are expressed in the developing muscle – from the beginning of somitogenesis to terminal differentiation (Tsai *et al.* 2002, Seitz *et al.* 2003, Brandt *et al.* 2005, da Rocha *et al.* 2007). Overexpression of *Dlk1* does not cause the muscle defects reported for pUPD12 (da Rocha *et al.*

2009). However, loss of *Dlk1* expression from the myogenic lineage causes a reduction in muscle mass by reducing myofibre number (Waddell *et al.* 2010). Roles for *Rtl1*, *Dio3* or the ncRNAs in muscle development have not been established, and thus the contribution of *Dlk1*–*Dio3* cluster genes to control of myogenic development has yet to be elucidated.

Skeletal growth

All of the long bones, the vertebrae and some of the facial bones are formed by endochondral ossification, a process through which a cartilage blueprint condenses from the mesenchyme, are in turn invaded by osteoclasts, and then are mineralised to form bone. This process starts *in utero* and is completed by the fusion of the growth plates at the end of the pubertal growth spurt. Skeletal development is under tight control both by factors within the developing skeletal tissue, and external endocrine signals (reviewed in Mackie *et al.* (2011)). Genes within the *Dlk1*–*Meg3* domain have been linked to a number of chondrogenic factors and processes. These include modulating the expression of key transcriptional regulators as well as roles within chondrogenic differentiation, bone mineralisation and hormonal control of bone growth.

Intrinsic control of skeletal development

Mesenchymal condensation forms the template for the future skeleton and in the mouse is near completion by E10.5. The mesenchymal cells then transition from secreting a matrix rich in collagen type 1 to the one rich in collagen type 2 and aggrecan by becoming chondrocytes, and forming the cartilaginous anlagen (reviewed in Mackie *et al.* (2011)), summarised in Fig. 3A. One of the first markers of chondrocyte condensation is *Sox9*, a transcription factor that is required for the expression of a number of key cartilaginous extracellular matrix proteins. The chondrocytes then passage through a specific sequence of phenotypes: the cells undergo proliferation, arrange themselves in columns and then enter a hypertrophic phase. This is followed by cell death and entry of blood vessels, along with osteoclasts and osteoblasts, which alongside other factors mineralise the tissue (reviewed in Mackie *et al.* (2011)).

The long bones extend by maintaining cartilage proliferation at the growth plate, and ossifying from the central region out. The maturation zone of the growth plate is where cells transition from proliferation to hypertrophy and is an important area for the control of endochondral ossification. Parathyroid-related peptide (PTHrP) and Indian Hedgehog (IHH) are key regulators of this process. IHH is produced by the cells at the junction of the hypertrophic zone. IHH then induces PTHrP expression in the perichondrium. However, the

Figure 3 Linking chromosome 12/14 genes with the major pathways of cartilage and bone formation. (A) During early development, mesenchymal cells (dark grey) condense to form a cartilaginous anlagen (light grey). *Dlk1* blocks this condensation but also drives *Sox9* expression, which promotes the expression of extracellular matrix component genes. *Mirg* also affects these extracellular matrix genes by blocking their inhibition by *Twist1*. *Dio3* blocks *T3* activation and drives the process of condensation, while *miR-1247* blocks *Sox9* expression and is in turn inhibited by *Sox9*. (B) Following the formation of the cartilaginous anlagen, the cells arrange themselves into a growth plate and are influenced by various genes and feedback loops. Within this process, *Dlk1* drives *Sox9* and *miR-1247* blocks it just as in (A). Known pathways are illustrated with black arrows (positive effect) and blunted arrows (negative effect). Chromosome 12/14 genes are shown in orange, and their effects are illustrated with green arrows (positive effect) and red blunt arrows (negative effects).

receptor of PTHrP is mainly found in the prehypertrophic cells and lower proliferating zone cells. Once it has diffused to these cells, the PTHrP maintains them in a prehypertrophic phenotype by preventing hypertrophic differentiation (and expression of *RUNX2*, a transcriptional master regulator of hypertrophic cartilage), thereby decreasing the number of *IHH* expressing cells. This negative feedback loop-ensured control is exerted on the rate of hypertrophic differentiation (reviewed in Mackie *et al.* (2011) and summarised in Fig. 3B).

Dlk1 is expressed extensively in the cartilaginous anlagen at E12.5, but this expression is reduced as the cells undergo ossification (Yevtodiynenko & Schmidt 2006). In micromass cultures, *DLK1* can drive upregulation of *Sox9*, resulting in increased chondrogenesis but decreased chondrocyte maturation and ossification. Consistently, *Dlk1* knock-out mice have elevated *Ihh* and reduced collagen type 2 expression, followed by an upregulation in collagen type X. This suggests an accelerated transition of cells into the hypertrophic phenotype, and indeed at birth the hypertrophic zone is increased in size by 40%. The converse transgenic mouse downregulated *Ihh* and upregulated collagen type 2, suggesting that cells are held in the proliferative phenotype. The conclusion from this work was that *Dlk1* promotes the expression of *Sox9* and in so doing maintains chondrocytes in an immature state (Wang & Sul 2009), summarised in Fig. 3. An additional pathway by which *Dlk1* influences chondrogenesis has been elucidated. Insulin induces chondrogenesis in ATDC5

cells (a well-established chondrogenic cell line) through the AKT pathway. Activation of AKT was found to be completely abolished in *Dlk1* overexpressing cells, and chondrogenic differentiation was impaired (Chen *et al.* 2011). *Dlk1* has also been implicated in the process of bone remodelling. An osteoblast-specific *Dlk1*-overexpressing mouse showed growth retardation while body weight and bone mineral density were both reduced (Abdallah *et al.* 2011).

Recent research in isolated human chondrocytes has linked *miR-1247* to *Sox9*. Although *miR-1247* modulation did not alter *Sox9* mRNA levels, overexpression showed a decrease in SOX9 protein. Interestingly, SOX9 was found in turn to repress *miR-1247*, creating a negative feedback loop (Martinez-Sanchez & Murphy 2013).

Several of the miRNAs contained in *Mirg* have been shown to block the activity of *Twist1* in an *in-vitro* model of epithelial-to-mesenchymal transition (EMT). *Twist1*, a target of Wnt signalling (Reinhold *et al.* 2006), is known to be an important inhibitor of *Runx2* expression and consequently chondrocyte maturation (Karsenty *et al.* 2009). During EMT, the expression of these miRNAs is progressively reduced while the expression of *Twist1* and associated genes is upregulated (Haga & Phinney 2012). In this way *Mirg* may promote chondrogenesis by blocking *Twist1*.

T_3 promotes chondrocyte differentiation in ATDC5 cells (Miura *et al.* 2002) as well as mesenchymal stem cells (Karl *et al.* 2014) and in cultured epiphyseal chondrocytes (Wang *et al.* 2007). *Dio3* is expressed in developing cartilage (Tsai *et al.* 2002) and the direct action of D3 in cartilage may thus result in a reduction in chondrogenesis. Consistent with this, *Dio3* knockout mice are growth retarded in terms of both weight and length, and this could be due to a premature maturation of chondrocytes resulting in a poorly functioning growth plate. However, since *Dio3* knock-out mice are hypothyroid following perinatal thyrotoxicosis, reduced skeletal growth could also be due to reduced circulating TH (Hernandez *et al.* 2006).

In summary, the expression of *Sox9* is modulated by *Dlk1* and *miR-1247*, and *Dlk1* also regulates insulin signalling via AKT in chondrogenic differentiation. *Dlk1* is additionally implicated in the process of bone mineralisation. *Dio3* interferes with TH regulation of chondrogenesis, and *Mirg* blocks a key chondrogenic pathway. Combined, this evidence indicates a convergent role for these imprinted genes in skeletal development that is consistent with the growth restriction observed in TS and skeletal abnormalities in upd(14)pat, summarised in Fig. 3.

Endocrine control of skeletal development

Both growth hormone (GH) and insulin-like growth factor 1 (IGF1) have important roles in the modulation of

long bone growth, as well as other developmental processes. GH is known to stimulate local *Igf1* expression in the growth plate in addition to elevating circulating IGF1 levels by promoting hepatic production (reviewed in Mackie *et al.* (2011)). The 'dual effector hypothesis' posits that GH recruits chondrocytes to the proliferative zone while also stimulating local and systemic IGF1, which further promotes this proliferation (Green *et al.* 1985). In this way, GH and IGF1 ensure that chondrocytes are in the proliferative zone where they contribute to long bone growth rather than transitioning to hypertrophic chondrocytes.

Several studies link the *Dlk1*–*Dio3* cluster genes to the GH–IGF1 axis (summarised in Fig. 4). Paternal inheritance of the *Gtl2*–*LacZ* mutation (TG-PAT, Fig. 2C) causes pre- and postnatal growth retardation due to derangements in the IGF1 signalling pathway at multiple levels. Firstly, late gestation TG-PAT embryos are small with altered expression of the IGF1 binding proteins, IGFBP1 and IGFBP4. Secondly, pre-pubertal TG-PAT mice have reduced circulating IGF1 levels and a reduced growth rate (Charalambous *et al.* 2014a). TG-PAT mice have reduced protein-encoding and increased ncRNA

Figure 4 Action of *Dlk1*–*Dio3* cluster genes in the GH–IGF axis control of skeletal development. GH and TH release from the anterior pituitary gland is primarily controlled by the hypothalamus. GH acts on the liver to cause the release of IGF1, as well as acting directly at the growth plate. Circulating and locally produced IGF1 act at the growth plate, as does TH. *Dlk1* increases GH production and release by the pituitary gland, *Dio3* blocks TH signalling locally and by central axis modulation. In the liver, *Mirg* blocks the action of *Igf1r* and therefore blocks the effects of IGF1 on the growth plate. Known pathways are illustrated with black arrows (positive effect) and blunted arrows (negative effect). Chromosome 12/14 genes are shown in orange, and their effects are illustrated with green arrows (positive effect) and red blunt arrows (negative effects).

expression dosage, suggesting that loss of *Dlk1*, *Rtl1* or *Dio3* function, or gain of ncRNA expression, might modulate the IGF1 pathway.

Consistent with this, *Dlk1*-null mice have reduced pituitary GH content (Cheung *et al.* 2013) while *Dlk1* overexpressing mice have excess pituitary *Gh* mRNA and circulating levels (Charalambous *et al.* 2014b). As discussed above, *Dio3* affects the levels of active TH (Hernandez *et al.* 2006). TH and GH are linked in a number of key growth processes including long bone growth, adipose tissue generation and bone mineralisation (Xing *et al.* 2012).

Mirg may regulate the expression of the *Igf1* receptor (IGF1R). Overexpression of two miRNAs (*mir-376a* and *mir-376c*) *in vitro* reduced the proliferation of a melanoma cell line. Bioinformatic analysis of these miRNAs predicted binding sites in the 3'-UTR of IGF1R. In the cell lines overexpressing *mir-376a* and *c*, IGF1R mRNA and protein levels were decreased (Zehavi *et al.* 2012). There is evidence of reduced IGF1 signalling in *Mirg* knock-out mice, because they have reduced hepatic *Igf1* mRNA, and have shown transient growth restriction around the time of weaning (Labialle *et al.* 2014). Combined, these results show that *Mirg* may modulate the IGF1 pathway by more than one mechanism.

The GH-IGF1 axis has many important roles in development and *Dlk1-Dio3* cluster genes affect this axis in a number of different ways. *Dlk1* promotes GH expression, while *Dio3* modulates TH levels and local sensitivity, affecting the GH/IGF1 pathway indirectly. Lastly, *Mirg* acts directly on IGF1 signalling, by modulation of both ligand and receptor expression.

Other phenotypes

To date, much less is known about how mis-regulated imprinting in the *Dlk1-Dio3* cluster might contribute to the other clinical manifestations of upd(14) syndromes, such as precocious puberty, metabolic phenotypes and mental retardation. Recently, a large parental origin-specific genome-wide association study has highlighted paternal inheritance of variants at the *Dlk1-Meg3* region to be associated with delayed age at menarche (Perry *et al.* 2014). In the mouse, alterations in pubertal timing have not been reported for any deletion or overexpression model. Mice with loss of *Dlk1* function are more prone to weight gain when fed an obesogenic diet (Moon *et al.* 2002), but this weight gain is cumulative in adult life and does not mirror the prepubertal truncal obesity observed in TS. All of the *Dlk1-Dio3* cluster genes are expressed in the embryonic brain, and could potentially contribute to brain development and cognitive function. However, we must await behavioural testing experiments on the various animal models to elucidate the actions of imprinted gene function on this aspect of the upd(14) syndrome phenotypes.

Conclusions

Imprinted genes are commonly clustered, sharing both tissue and temporal-specific regulatory elements as well as the ICRs that direct their parental origin-specific expression (Ferguson-Smith 2011). The *Dlk1-Dio3* region is no exception to this, and the linkage between *Dlk1* and *Dio3* is conserved across vertebrates, in both imprinting and non-imprinting species (Edwards *et al.* 2008). This suggests that synchronised expression of genes in the cluster is functionally important. *Dlk1-Dio3* region genes, like most other genes, are controlled by enhancers and repressors that act in specific tissues to drive their correct temporal and spatial expression pattern. The imprinting mechanism overlies an additional level of transcriptional control, by modulating gene dosage. There is strong evidence from genetic studies in the mouse that regulating/limiting expression dosage of at least two genes in the cluster – *Dlk1* and *Rtl1* – is crucial for the completion of development (Sekita *et al.* 2008, da Rocha *et al.* 2009).

The clinical phenotypes associated with mis-expression of genes in the *Dlk1-Dio3* cluster converge on several key processes concerned with early life: they control the energy supply to the developing foetus by modulating placental size and permeability, their appropriate dosage is critical for physiological adaptations that promote survival in the perinatal period and for musculoskeletal growth. Remarkably, experiments in mice that cause loss or gain of expression of single genes in the cluster do not delineate clear one-gene one-phenotype relationships. Rather, it appears that multiple genes in the cluster act in a concerted fashion to modulate common processes. We speculate that by controlling the expression dosage of multiple genes acting on overlapping physiological processes, the imprinting mechanism may be acting to fine-tune the adaptations necessary for survival in early life. Selection acting on the ICR can modulate expression dosage across the cluster for optimum outcome, by co-ordinately controlling intrauterine growth and relative maturity at birth, the rate of musculoskeletal development, and metabolic adaptations necessary for life outside the uterus.

Although rare, Kagami-Ogata and TSs have significant consequences for the survival and quality of life for their sufferers. Studies of the regulation and action of *Dlk1-Dio3* cluster genes have already vastly improved the clinical diagnosis of these conditions. Furthermore, understanding the actions of *Dlk1-Dio3* cluster genes in, for example, the GH-IGF1 pathway could considerably inform treatment options for patients with short stature and incipient metabolic disease. Beyond the upd(14) syndromes, growth restriction and failure to thrive affects between 5 and 10% of pregnancies in the developed world, with largely unknown aetiology (Figueras & Gardosi 2011). Understanding the functions

of *Dlk1–Dio3* cluster genes further will increase our understanding of the basic biology of growth, placenta-tion and early-life metabolic adaptation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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