Gene Dosage Effects of the Imprinted Delta-Like Homologue 1 (*Dlk1/Pref1*) in Development: Implications for the Evolution of Imprinting

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Abstract

Genomic imprinting is a normal process that causes genes to be expressed according to parental origin. The selective advantage conferred by imprinting is not understood but is hypothesised to act on dosage-critical genes. Here, we report a unique model in which the consequences of a single, double, and triple dosage of the imprinted *Dlk1/Pref1*, normally repressed on the maternally inherited chromosome, can be assessed in the growing embryo. BAC-transgenic mice were generated that over-express *Dlk1* from endogenous regulators at all sites of embryonic activity. Triple dosage causes lethality associated with major organ abnormalities. Embryos expressing a double dose of *Dlk1*, recapitulating loss of imprinting, are growth enhanced but fail to thrive in early life, despite the early growth advantage. Thus, any benefit conferred by increased embryonic size is offset by postnatal lethality. We propose a negative correlation between gene dosage and survival that fixes an upper limit on growth promotion by *Dlk1*, and we hypothesize that trade-off between growth and lethality might have driven imprinting at this locus.

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Introduction

Genomic imprinting, the process causing genes to be differentially expressed according to their parental origin acts on a subset of developmentally regulated genes [reviewed in 1]. Since imprinting results in functional haploidy of target genes, the evolution of such a mechanism must have conferred a significant advantage to its recipients to offset the cost. In addition, the maintenance of this method of gene dosage regulation must confer a continuing selective benefit to the individual. By creating genetic models that mimic the loss of imprinting of genes regulated in this way, we can study phenotype and consider what selective pressures act to maintain gene dosage, and begin to examine what may have driven the acquisition of mono-allelic expression.

*Dlk1*, Delta-like homologue 1 also known as Predipocyte factor 1 (*Pref1*), on mouse chromosome 12 is part of an imprinted gene cluster [2,3]. It encodes a transmembrane glycoprotein that possesses six epidermal growth factor-like motifs in the extracellular domain similar to those present in the Delta/Serrate/Lin-12 (DSL) family of signalling molecules. In contrast to other NOTCH ligands, DLK1 does not have the Delta/Serrate/Lin-12 (DSL) domain believed to mediate the interaction and activation of the NOTCH receptor [4]. Nevertheless, DLK1 can interact with NOTCH through specific EGF-like repeats and can act as a Notch antagonist, both in culture and in vivo, binding to the receptor without activating it [5,6,7]. *In vivo*, *Dlk1* maintains precursor cell populations and inhibits differentiation [4,8,9]. *Dlk1* is expressed at high levels in a wide range of embryonic tissues [10,11] however its developmental functions in *vivo* are largely unknown.

In more ancestral vertebrates such as the fish *Oryzias latipes* and *Fugu rubripes*, *Dlk1*, and its neighbour *Dio3* which encodes a negative regulator of thyroid hormone metabolism, are only 10–15 Kb apart. In eutherian mammals, the genes have become separated by the insertion of sequences including a retrotransposon-like gene (*Rtl1*) and several non-coding RNAs, including a large microRNA cluster, and have acquired imprinting [12]. *Dlk1*, *Rtl1* and *Dio3* are expressed from the paternally inherited chromosome and the non-coding RNAs from the maternally-inherited chromosome [13].

Imprinting on chromosome 12 is controlled by an intergenic differentially methylated region (IG-DMR) that is methylated on the paternally inherited chromosome [14,15]. The unmethylated maternal IG-DMR is necessary to repress protein-coding transcription and to activate the non-coding RNAs [14]. In terms of gene expression, the paternal chromosome 12 most likely resembles the ancestral (pre-imprinted) state as *Dlk1* and *Dio3* are expressed and the non-coding RNAs are silenced [12]. Furthermore, paternal deletion of the methylated IG-DMR has no effect on transcription [14].

Alteration of imprinting at chromosome 12 has considerable consequences for embryonic fitness. Uniparental disomy mice with two paternal copies and no maternal copy (*PatDp*(12)/PatDp
Author Summary

Genomic imprinting, the process that causes genes to be expressed from one of the two chromosome homologues according to parental origin, is likely to act on genes whose dosage is important for their correct function. To test this, we compared the phenotype of transgenic mice expressing a double and triple dose of the imprinted gene Dlk1/Pref1 with animals expressing the normal single dose expressed from the paternally inherited chromosome. Our results showed that a triple dose causes severe developmental abnormalities and death before or at birth. Embryos expressing a double dose, recapitulating absence of imprinting, are bigger at birth but then around one-third of them died within the first three days of life. Those that survived had poor early growth performance in the first week of life becoming small and remaining small, thus offsetting any benefit conferred by being born bigger. Therefore, imprinted levels of Dlk1/Pref1 represent the optimal balance of growth versus lethality. These findings lead to speculation about the evolutionary pressures acting to establish and maintain imprinting at this locus.

(dist12) or mice with two maternal copies and no paternal copy (MatDi(12)/MatDp(dist12)) of chromosome 12, are lethal and show distinct phenotypes. These include growth abnormalities and developmental defects in muscle, cartilage/bone and placenta [16,17,18]. Animals die prenatally commencing at E16. The embryonic uniparental disomic phenotypes can be ascribed to the Dlk1-Dio3 imprinted cluster because embryos with maternal deletion of the IG-DMR (ΔIG-DMR\textsuperscript{MAT}) recapitulate the transcriptional profile and embryonic mutant phenotypes of the PatDi(12)/PatDp(dist12) conceptuses [14,19].

Since loss of imprinting at chromosome 12 is lethal, there are clear advantages in maintaining the imprintated state. However since in PatDi(12)/PatDp(dist12) and ΔIG-DMR\textsuperscript{MAT} conceptuses multiple paternally expressed coding genes are up-regulated, and multiple maternally expressed non-coding genes are repressed, we are unable to determine if selection is acting to maintain the mono-allelic expression of one or all protein coding genes or to maintain expression of the non-coding RNAs. As Dlk1 is an ancestral gene at this imprinted locus and it is expressed at high levels in tissues where uniparental disomy conceptuses have the ancestral gene at this imprinted locus and it is expressed at high levels in PatDi(12)/PatDp(dist12) or mice with two maternal copies and no paternal copy (MatDi(12)/MatDp(dist12)) of chromosome 12, are lethal and show distinct phenotypes. These include growth abnormalities and developmental defects in muscle, cartilage/bone and placenta [16,17,18]. Animals die prenatally commencing at E16. The embryonic uniparental disomic phenotypes can be ascribed to the Dlk1-Dio3 imprinted cluster because embryos with maternal deletion of the IG-DMR (ΔIG-DMR\textsuperscript{MAT}) recapitulate the transcriptional profile and embryonic mutant phenotypes of the PatDi(12)/PatDp(dist12) conceptuses [14,19].

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Manipulating imprinted gene dosage in vivo provides a powerful tool to study imprinted gene function and evolution. Here we describe a Dlk1 over-expression model in which the gene is driven by its own endogenous regulatory sequences. We generate mouse lines harbouring bacterial artificial chromosome (BAC) transgenes that encompass the entire unmanipulated Dlk1 gene with different lengths of flanking sequence. Of these, transgenic Dlk1 expression at sites where the endogenous gene is expressed, was achieved from a 70 kb BAC transgene in three independent lines and the outcome was the same for all three lines. This allowed us to compare the phenotypes of mice expressing a double (transgene hemizygotes) or triple (transgene homozygotes) dose of Dlk1 with normal animals expressing a single imprinted dose. This transgenic system of regulated Dlk1 over-dose allowed us to explore the developmental and physiological functions of this gene in comparison with other models, and infer evolutionary scenarios for Dlk1 imprinting.

Results

Construction of a Transgenic System for the Alteration of Dlk1/Pref1 Dosage

BAC transgenes were generated that encompass the entire Dlk1 gene and endogenous flanking sequences but without other genes in the cluster. Transgenic mice were created by pronuclear injection of two BAC transgenes differing in the amounts of flanking regulatory sequences (Figure 1A): Tg\textsuperscript{Dlk1-70} starts 8 kb upstream of the Dlk1 gene and ends approximately 18 kb downstream of the Dlk1 transcriptional start site. The Tg\textsuperscript{Dlk1-31} transgene shares its 3’ end with the Tg\textsuperscript{Dlk1-70} transgene but contains 49.4 kb of sequence upstream of Dlk1 (Figure 1A). The four independent lines containing the Tg\textsuperscript{Dlk1-31} transgene failed to express Dlk1 and no Dlk1-associated phenotypes were observed (data not shown) regardless of transgene copy number (Figure S1A). These mice were not analysed further. In contrast, the Tg\textsuperscript{Dlk1-70} transgenic animals successfully expressed Dlk1 (Figure 1B and C and Table S1), and were subjected to further analysis.

The four independent Tg\textsuperscript{Dlk1-70} lines of mice, hitherto referred to as 70A, 70B, 70C and 70D were maintained as hemizygotes (referred to as WT/TG) and bred to C57BL/6 for more than ten generations to ensure stability of copy number and phenotype. Copy number was stable from the third generation onwards and estimated to be 4–5 (70A), 5–6 (70B), 7 (70C) and 1 (70D) (Figure S1 and Table S1). Once stable lines were established, analysis of the overall level of Dlk1 expression was performed by Northern Blotting for the different lines.

In the single copy 70D line, no transgene-derived Dlk1 expression was observed and no phenotypic consequences were noted (data not shown). This line was not analysed further.

Importantly, for the 70A, 70B and 70C lines, WT/TG E16 embryos expressed approximately twice as much Dlk1 as their normal littersmates regardless of copy number. Representative quantitative Northern blot expression data is illustrated in Figure 1B and 1C for 70C and 70B families. Detailed expression data (both from Northern blots and RT-qPCR) are shown independently for the three lines in Figure S2B and STable S1). This expression analysis using two independent methods clearly shows that WT/TG E16 and E18 embryos for the three lines express approximately twice as much Dlk1.

Interestingly, expression of the transgene occurred independent of the parental-origin of the transgene in the three independent lines (Figure 1B; Table S1). Absence of transgene imprinting was confirmed using a single nucleotide polymorphism (SNP) located in the 3’UTR of Dlk1 (Lin et al., 2003) allowing the endogenous gene to be distinguished from the transgene (Figure S2A; Table S1).

As the transgene is not imprinted, homozygous transgenic mice (TG/TG) were generated to further increase the dosage of Dlk1 in the three transgene expressing lines. As illustrated in Figure 1C for 70B and shown also for 70A and 70C (Figure S2 and Table S1) levels of Dlk1 in the E16–E18 TG/TG embryos were approximately 3-fold higher than normal littersmates.

Dlk1 regulation is complex involving alternative splicing and extensive post-translational modifications, therefore we compared DLK1 protein isoforms between the genotypes (for the 70B family). DLK1 protein levels in the different genotypes are consistent with the Dlk1 mRNA expression and we saw no change in isoform preference (Figure 1D; Figure 2B). Furthermore, DLK1 protein levels in WT/TG embryos are comparable to that of...
Figure 1. Generation of Dlk1 transgenic mice. A. Two different BAC transgenes containing the Dlk1 gene. The left panel displays a schematic representation of the two BAC transgenes containing Dlk1 presented in this study; TgDlk1-31 and TgDlk1-70. TgDlk1-31 did not express the transgene and was not further analysed. Red box - maternally expressed gene Gtl2; blue box – paternally expressed gene Dlk1; white circle – unmethylated IG-DMR; black circle – methylated IG-DMR; brown line – vector sequence; Probes: violet line – Dlk1 upstream region probe, green line – Dlk1 exon 5 probe. The right panel contains representative Southern hybridizations of the restriction enzyme fragments of the BAC 153O05 clone from where TgDlk1-31 (arrowhead) and TgDlk1-70 (arrow) were obtained by restriction digestion; the two probes were used to confirm the presence of appropriate restriction fragments containing the Dlk1 gene and upstream sequences within the transgene. Lane 1: SgrAI; Lane 2: SgrAI+Sall; Lane 3: Sall; Lane 4: Sall+Sall; Lane 5: PvuI; Lane 6: PvuI+Sall; Lane 7: Sall. B. Expression of Dlk1 in transgene hemizygotes is independent of parental inheritance of the transgene. Quantitative Northern Blot showing that Dlk1 is over-expressed upon both maternal and paternal transmission of the TgDlk1-70 transgene in E16 70C fetuses. The graph represents mean normalized Dlk1 expression ± SEM (ratio: Dlk1/Gapdh) from WT/TG and WT/WT littermates upon maternal or paternal transmission of the transgene. Significant differences between the WT/TG and WT/WT littersmates are indicated by asterisks (p-value < 0.05, unpaired Student’s t test); Abbreviations: N – WT/WT fetus; T – WT/TG fetus; Mat – maternal transmission; Pat – paternal transmission. C. Double and triple dosage of Dlk1 in hemizygous and homozygous TgDlk1-70 transgenic mice. The graph on the left represents normalized Dlk1 expression from E16 70B WT/WT, WT/TG and TG/TG fetuses from heterozygous intercrosses obtained by Northern Blot analysis (as in B). Significant differences between the WT/TG and WT/WT and between TG/TG and WT/WT are indicated by asterisks (p-value < 0.01, unpaired Student’s t test; n=4 for all genotypes). D. Comparative DLK1 protein levels in transgenic and PatDi(12) E16 70B fetuses and control littermates were evaluated by Western Blot (right panel). Antibodies used were anti-DLK1 and anti α-TUBULIN (TUB) as a normalization control. Comparable protein isoforms are evident between WT/WT and the transgenic animals on longer exposure.
PatDi(12) embryos, and are further increased in TG/TG conceptuses.

Tg<sup>Dlk1</sup>-70 Transgene Recapitulates the Spatio-Temporal Expression of the Endogenous Locus in Embryonic Tissues

Overall levels of Dlk1 expression were assessed in WT/WT and WT/TG embryonic tissues at E16 (Table S1) and E18 by TaqMan RT-qPCR for 70B (Figure 2A) and 70A (Table S1). Results show that Dlk1 levels are over-expressed around 2–2.5 fold in embryonic tissues analysed in the WT/TG when compared to normal littermates (Figure 2A). The placenta was the only tissue with no significant over-expression (109% ± 6% compared to WT/WT) (Figure 2A and Table S1). This suggests that key placenta specific regulators lie outside the region delineated by the transgene.

Western Blot analysis of DLK1 protein expression was conducted in control, WT/TG and TG/TG tissues and compared with the RT-qPCR analysis. In all cases, using this semi-quantitative method, protein levels correlated with RNA levels for the tissues and stages analysed (Figure 2B). Curiously, a slight increase in DLK1 protein levels was observed in the TG/TG placenta, suggesting that there is minimal expression of the transgene in this tissue.

Tissue-specific expression of Dlk1 was compared between normal and transgenic conceptuses by in situ hybridisation and no ectopic Dlk1 activity was evident (Figure 3 and Table S4) at E16 or E18. This suggests that endogenous regulators are present on the transgene and are driving transgenic Dlk1 transcription. Tissue-specific expression of the endogenous neighbouring non-coding RNA gene, Gtl2 was unaffected in all genotypes (data not shown) allowing phenotypes of the transgenic mice to be attributed to Dlk1 over-expression. Phenotypic characterization was performed independently in all three lines and all the observed phenotypes were consistent so data was combined. Results generated from the individual lines are presented and also summarised in the Supplementary Data.

Dlk1 Over-Dose Leads to Embryonic Growth Enhancement Independent of the Placenta

Imprinted genes have long been known to function in controlling pre-natal growth and nutrient acquisition [20]. To address a growth function for Dlk1, we performed extensive measurements of wet and dry embryonic masses, placental wet masses and crown-rump lengths. WT/TG fetuses are expressing a double dose of Dlk1 and showed consistent overgrowth from E16 to the day of birth. Wet and dry embryonic masses and crown-rump (C–R) length values increased by 6–10% compared to WT/WT littermates (Figure 4A–B; Table S3). TG/TG fetuses show growth enhancement at E16 (wet mass and dry mass increases of 23–25%). In contrast, no differences are observed in E18 C–R length and dry mass compared to WT/WT littermates most likely due to the failure to thrive of these severely compromised fetuses at the later stages as has been observed in other models [16]. Differences in wet mass between TG/TG and WT/WT at late...
Figure 3. Transgenic expression in embryos recapitulates endogenous expression. A. WT/WT (left and middle) and TG/TG (right) e16.70B embryos were sectioned, then in-situ hybridisation was performed using sense control (left panel) and antisense probes to Dlk1 (exons 3 to 5, middle and right panels). No ectopic expression was observed. 2.5× magnification. B. High power images of WT/WT (left) and TG/TG (right) kidney (20×, top), neck (10×) and lung (40×). In the WT/WT kidney, Dlk1 is largely absent except in the capsule and some punctuate staining in the mesenchyme. This pattern is recapitulated in the TG/TG, with greater intensity. In the neck, Dlk1 is expressed in the ossifying cartilage of the spinal cord, in the muscle and in the brown adipose tissue deposits, in both the WT/WT and TG/TG. In WT/WT lung Dlk1 is expressed in the epithelium of branching alveoli and in the mesenchyme. This pattern is also clearly present in the TG/TG.

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gestation are due to oedema (Figure 6A–B). This late gestation data correlates with lethality of the TG/TG fetuses commencing around E16 (Table 1, Figure 4 and see below).

To determine the contribution of individual tissues to the increase in fetal mass, E18–19 brains, livers, lungs, forelimbs, hindlimbs and brown adipose tissue (BAT) were weighed and analysed histologically (Figure 4C, Figure 5, Figure 6). In the growth-enhanced WT/TG fetuses, lungs, forelimbs and hindlimbs were proportionally heavier. WT/TG brains, that significantly over-express Dlk1, were spared. E18 liver growth was unaffected, suggesting that the milder Dlk1 over-expression in this tissue (E18: 1.31 ± 0.21 of WT/WT Dlk1 liver expression and at E16: 1.67 ± 0.21) may not be sufficient to cause overgrowth. Overgrowth was observed in TG/TG livers evident from E16. Histological examination revealed no obvious defects and portal triads, and hepatocyte size appeared unaffected (Figure 4G, Figure 6 and data not shown). In general, this suggests that, with the exception of the brain, multi-organ overgrowth is associated with Dlk1 over-expression in an organ-autonomous manner.

In the placentas, no significant differences between the genotypes were observed at any stage (Figure 4A and Table S3). Histological analyses indicated no obvious phenotypic abnormalities in the placenta at E16 and E19 for the three genotypes (data not shown). This is consistent with the finding that Dlk1 is expressed at very low levels from the transgene in this tissue (Figure 2 and Table S1) and suggests that embryonic growth enhancement occurs independently of the placenta.

Dlk1 Dosage Modulates Skeletal Maturation

PatDi(12)/PatDp(dist12) and ΔIG-DMRMat exhibit costal cartilage defects and hypo-ossification of mesoderm-derived bones [16,18,19]. Since WT/TG embryos have increased crown-rump length, we examined the skeletal development of WT/TG and TG/TG mice. WT/TG skeletons show signs of growth enhance-
ment and minor ossification delays in the sternum and the closure of the sagittal suture (Figure 5). Further increasing the dosage of Dlk1 in TG/TG animals led to more severe skeletal defects. Overall the TG/TG skeleton was smaller and the delay in the closure of the sagittal suture was more pronounced. In addition, we observed a bell-shaped thorax with a hypo-ossified sternum and thinner ribs and vertebrae. We can therefore relate the severity of hypo-ossification with increasing levels of Dlk1.

Another major defect associated with PatDi(12/PatDi(dist12)) and ΔIG-DMR	extsuperscript{MAY} is skeletal muscle immaturity [16,18,19]. Interestingly, in contrast to the defects in the skeleton, no muscle maturation abnormalities were observed even when Dlk1 dosage was tripled. Detailed morphometric analysis assessing myofiber diameter and % of myofibers with centrally located nuclei was performed on the E18 diaphragm and on a subset of muscles in the forearm and no differences were observed (Figure S3). Dlk1 over-expression of all isoforms was confirmed at the protein level (Figure 2B). Therefore our data clearly show no prenatal muscle immaturity or hypertrophy induced by Dlk1 over-expression from endogenous regulators.

**Table 1.** Frequency and viability of Dlk1 transgenic mice from E16 to early postnatal life.

| TRANSMISSION          | GENOTYPE | E16 | E18–E19 | P1–P3 |
|-----------------------|----------|-----|---------|-------|
| Maternal              | WT/WT    | 37  | 29      | -     |
|                       | TG/WT    | 48  | 47 (1)  | -     |
| Paternal              | WT/WT    | 31  | 56      | 116 (12) |
|                       | WT/TG    | 45  | 41      | 133 (42)* |
| Heterozygous Intercross | WT/WT | 34  | 58      | 13 (1) |
|                       | WT/TG    | 87 (1) | 153    | 23 (4) |
|                       | TG/TG    | 37 (4) | 80 (7)  | 7 (7)  |

The values represent number of animals genotyped in each time-point from crosses involving Dlk1 transgenic animals; the numbers in brackets represent number of dead animals.

WT/TG neonatal lethality is statistically significantly increased compared to WT/WT (p-value<0.01; χ²-test).

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Increasing Dlk1 Expression Reduces Embryonic and Perinatal Fitness in a Dose-Dependent Manner

Hemizygous transgenic embryos are viable and fertile and observed at Mendelian frequencies at birth indicating that a double dose of Dlk1 expression is compatible with embryonic viability (Table 1 and Table S2). In contrast to WT/TG animals, TG/TG embryos have distinct morphological features from E16 including severe oedema, a small thoracic region and a protruding abdomen (Figure 6A and B). This is associated with lethality from E16 with none of the TG/TG animals surviving more than a few hours after birth (Table 1 and Table S2). We also observed that the weight of the TG/TG lungs was significantly reduced (Figure 4C) with a denser cellular arrangement at E18 (Figure 6C). Dlk1 is strongly expressed in the bronchioles of the lung (Figure 3 and ref [10]) and an intrinsic defect in lung development caused by over-expression of Dlk1 is likely to contribute to the lethality of these animals.

Despite the absence of major embryonic abnormalities caused by doubling Dlk1 dosage in vivo, we observed a significant increase in early postnatal lethality in WT/TG animals. 32% of WT/TG pups died during the first three days after birth compared to 10% of WT/WT littersmates, in crosses from wild type mothers (Table 1 and Table S2). In order to understand the cause of death, we studied processes essential to early postnatal survival in rodents such as temperature regulation, suckling ability and glucose homeostasis.

Temperature regulation is required for adapting to cold exposure after birth and relies on the process of non-shivering thermogenesis (NST) mediated by brown adipose tissue (BAT) metabolism. Dlk1 is believed to inhibit adipogenesis of both brown and white adipose tissue [21,22] thus is a likely candidate for involvement in the NST process. We measured BAT per body weight and analysed markers for both fat differentiation (such as Pparγ2) and thermogenesis (such as Usp1) before and after birth. At E19, WT/TG/BAT per body weight and levels of Pparγ2 and Usp1 are comparable to WT/WT (Figure S4). At birth, we observed that Usp1 and Pparγ2 expression was slightly elevated, perhaps to compensate for a slight decrease in BAT mass per body weight (Figure 7A and B). Failure to thrive, therefore, cannot be ascribed to compromised NST.

The sucking ability of Dlk1 WT/TG pups at birth was assessed by using stomach weight as a measure of milk content [23]. We observed that WT/WT animals were frequently found with stomach content in the range of 0.03–0.08 grams, whilst WT/TG animals never exceeded 0.03 grams (Figure 7A). In situ data shows that at late gestation (E18), highest expression of Dlk1 is in the tongue and the upper and lower lips, which is consistent with a role of Dlk1 in suckling (Table S4 and data not shown). Blood glucose levels were not different at birth between the two genotypes (Figure 7C).

We also decided to monitor the growth performance of WT/TG animals during the first three weeks of life, when juveniles are still dependent on the mother for nutrition. WT/TG animals are born bigger than their littermates (Figure 4A and Figure 7A), but they fail to gain weight at the same rate as WT/WT neonates during the first two weeks (Figure 7D). By 14 days, WT/TG animals are small and remain small thereafter (−10%) (Figure 7D and data not shown). The differences in growth performance are most pronounced within the first week after birth (Figure 7D), correlating with poor sucking and the increased lethality of these animals. In conclusion, despite an early growth advantage, animals expressing a double dose of Dlk1 fail to thrive in early life, and thus any benefit conferred by an increased embryonic size is offset by postnatal lethality.

**Discussion**

We have developed a unique model in which the consequences of a single, double and triple dosage of one imprinted gene, Dlk1, can be assessed in the growing embryo. The double dose is reminiscent of the situation where there is no imprinting of this gene. BAC and YAC transgenes have been widely used in mouse genetics and in imprinting studies as a molecular tool for the localisation of transcriptional or imprinting regulatory elements [24,25,26]. We generated transgenes differing in the extent of the regulatory sequences upstream of the gene, is expressed. We have therefore determined that the majority of embryonic tissue-specific enhancer sequences for Dlk1 expression are located in the 8 kb to 49 kb interval upstream of the gene. This is consistent with a previous report showing that enhancers for Dlk1 are absent from 3 kb upstream to 175 kb downstream of the gene [26]. Minimal transgene expression in the placenta suggests the absence of the specific regulatory sequences for this organ in the 70 kb transgene.
Figure 5. Skeletal defects in E19 WT/TG and TG/TG fetuses. A, Sagittal view of the whole skeleton. B, Frontal view of the thoracic cage. C, Dorsal view of the vertebrae in the dorsal zone and D, cranial view of the skull of E19 WT/WT, WT/TG and TG/TG skeletons stained with Alcian Blue/Alizarin Red. Arrow in B indicates the fifth ossification centre in the WT/WT sternum, absent in the WT/TG and TG/TG skeleton. Asterisk in D marks the closure of the sagittal suture. Scale bars: 0.5 mm (B and C) and 1 mm (D). All images shown A–D are derived from 70A animals; results are exactly the same for 70B and 70C.
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At E16, we detected growth enhancement in a Dlk1 dose-dependent manner. TG/TG were more than 20% larger than WT/WT littermates, whereas WT/TG were growth-enhanced by 10%. At later stages, progressive morbidity and mortality of TG/TG embryos precluded a meaningful comparative assessment of their growth rate. This was not the case for viable WT/TG embryos which clearly maintained an increased growth trajectory from E16 up to birth. This is reciprocal to the 20% reduction in weight reported for Dlk1-null E19 fetuses [21]. In general, tissue-specific overgrowth correlated with levels of Dlk1 over-expression, except in the brain. This suggests Dlk1 acts locally on organ growth, though we cannot rule out an endocrine role [27].

With the exception of studies on placental specific Igf2 [28,29], previous functional analyses of imprinted genes expressed in embryo and placenta have not been able to discern whether growth effects are intrinsic to the embryo or are secondary to placental function. This is because genetic manipulation of imprinted genes often results in altered gene dosage in both embryonic and extra-embryonic tissues [reviewed in 30]. In contrast, the WT/TG model reported here generates embryonic Dlk1 over-expression in the presence of a normal placenta with a normal Dlk1 dose, and the results indicate that Dlk1 can modulate embryonic growth independently of the placenta. To complement these studies, it will be important to determine the extent to which placental Dlk1 expressed in the fetal endothelium and some trophoblast cells of the labyrinthine zone [10] also contributes to embryonic growth.

Importantly, this study shows that Dlk1 has a dual role on growth. WT/TG neonates are born larger but fail to thrive during the first week of life. The reduced postnatal growth rate is concurrent with reduced food intake in the neonatal period. Maternally repressed imprinted genes are expected to favour growth performance and resource acquisition from embryonic stages to weaning according to the kinship theory which posits that imprinting arose as a consequence of a conflict between males and females over the allocation of maternal resources to the offspring [31,32]. The embryonic overgrowth generated by Dlk1 double dosage follows the directionality predicted by this theory however the postnatal failure to thrive phenotype is contrary to it. Our model implicates Dlk1 in reduced postnatal nutrient acquisition. Indeed the results suggest that maternal repression of Dlk1 may have evolved to increase postnatal nutrient acquisition. Furthermore it has been hypothesized that maternally repressed imprinted genes, such as Dlk1, would minimize heating contribution within huddles [33,34]. NST is not impaired in BAT over-expressing Dlk1. In fact, slight over-expression of Ppara and Ucp1 may suggest the opposite scenario, whereby a maternally repressed gene may contribute more to the communal heating. Our results are therefore inconsistent with the conflict hypothesis. It is possible that imprinting may have evolved due to more than one type of selective pressure, perhaps different for different domains.

In TG/TG embryos over-growth occurs at E16 coincident with an increased frequency of embryonic mortality. Embryos expressing this triple dose did not survive past birth. A double dose of Dlk1 was compatible with embryonic viability but resulted in significant neonatal lethality. We propose a negative correlation between gene dosage and survival that may fix an upper limit on growth promotion by Dlk1.

One hypothesis for the lethality could be that Dlk1 dosage regulates the balance between proliferation and differentiation resulting in a trade-off between pre-natal size and developmental maturity. Increasing Dlk1 dosage incrementally shifts the embryo towards increased growth, perhaps at the expense of organ maturation as was suggested for PatDi(12) embryos [16]. The major abnormalities found in the liver, lungs and skeleton of the TG/TG fetuses may be signs of developmental immaturity of these organs. The inability of the lungs to support TG/TG animals surviving to term, as well as pre-natal oedema, is consistent with this. The Notch signalling pathway is required for branching morphogenesis and maturation of the lungs, and disruption of this pathway can lead to peri-natal lethality due to lung hypotrophy [reviewed in 35, 36, 37]. In contrast, except for subtle ossification delays in the WT/TG fetuses, no other clear signs of organ immaturity were identified, so we cannot conclude whether this contributes to the neonatal lethality of the hemizygous genotype.

In order to explore the cause of lethality in the WT/TG neonates more closely, we measured several parameters related to rodent well-being. WT/TG animals were significantly less likely to have milk-filled stomachs on the day of birth. This may be a result of defects in suckling behaviour engendered by many causes, such as appetite or olfactory regulation, or motor function. Starvation may therefore be the cause of death of WT/TG neonates, and reduced feeding in the first week is also a likely cause of the reduced growth rate of surviving transgenic animals during this period. Further analysis of the physiological consequences of this interesting growth trajectory of prenatal growth enhancement followed by postnatal compromised growth is in progress.
Figure 7. Poor early post-natal fitness in animals with Dlk1 double dose. A. Neonatal total mass (left panel) and organ weights (right panels) at birth (P1). Graphs represent weight of individual animals/organs. Significant differences from wild-type are indicated by asterisks on top of the WT/TG (p-value < 0.05, unpaired Student’s t test). For stomach weights, the non-parametric Mann-Whitney U-test was performed (p-value < 0.05). B. BAT expression analysis by TaqMan RT-qPCR. Graphic representation of relative levels of expression of Dlk1, Pparc2 and Ucp1 normalized against 18S (loading control) at P1 (mean ± SEM, n=4). Significant differences between WT/TG and WT/WT for each gene are indicated by asterisks on top of the WT/TG bar (p-value < 0.05; unpaired Student’s t test). C. Free-fed glycemia at P1; Graph represents individual levels of glycemia (n=15). D. The left panel represents the pre-weaning growth curve of WT/WT and WT/TG male juveniles. Graph represents mean values ± SEM per genotype (n=7). Significant reduced weight from WT/WT is indicated by # and significant increased weight from WT/WT is indicated by * (p-value < 0.05, unpaired Student’s t test). The right panel shows the growth rate of WT/WT and WT/TG animals in the first 5 weeks of age (calculated using the following formula: for week n: [weight at week n - weight at week (n-1)]/weight at week n. All data shown in A–D are the combined results for the three transgenic lines.

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Imprinting of the Dlk1-Dio3 cluster is important. Disruption of imprint expression in several models results in lethality therefore there is a clear selective pressure to maintain imprinting at this cluster [13,38]. The IG-DMR, like other imprinting control regions, controls the dosage of many linked genes. It has been postulated that in imprinting clusters, some genes are the target of dosage regulation while others are merely bystanders whose altered dosage do not have phenotypic consequences and therefore are not targets for selection [39]. We show that Dlk1 is a dose-dependent regulator of embryonic growth, as well as modulating processes necessary for postnatal survival. WT/TG animals model dependent regulator of embryonic growth, as well as modulating modulation. It therefore could have been the target of selection for to be such a dosage-critical gene through its significant consequences on animal fitness upon dosage modulation. It therefore could have been the target of selection for dosage control by imprinting at this locus. Current levels of Dlk1 represent an optimal balance of maximized growth with minimal neonatal lethality.

Materials and Methods

Generation and Breeding of Transgenic Animals

Tg(Dlk1-35) and Tg(Dlk1-70) transgenes were obtained by restriction endonuclease mapping of the BAC clone 163O05, screened from a BAC library of the mouse strain 129/Sv. The two transgenes were microinjected individually into the male pronuclei of (C57BL/6 × CBA) F1 zygotes and then implanted into foster (C57BL/6 × CBA) F1 mothers. The first generation of animals was obtained by crossing the founder with a (C57BL/6 × CBA) F1 animal. After the first generation, all transgenic animals were mated on a C57BL/6 background to maintain the lines. Animals were housed four per cage (maximum) in a temperature-controlled room (24°C) with a 12-hr light/dark cycle. Food and water were available ad libitum. All experiments involving mice were carried out in accordance with UK Government Home Office licensing procedures. For the embryonic studies, the day of vaginal plug was considered day E1.

Expression Studies

RNA was extracted from whole embryos using TRI Reagent (Ambion), following the manufacturer’s guidelines. mRNA was then isolated from 120 µg of total RNA using Dynabeads Oligo (dT)25 kit (Dynal) following the supplied protocol. We carried out northern-blot hybridization and used probes as described previously for Dlk1 and Gt12 and Gapdh [3]. For RT-qPCR, total RNA (10 µg) was DNase-treated with RQ1 RNase-free DNase (Promega) following the manufacturer’s guidelines. All cDNA was synthesized using random hexamers and Superscript III RNase H reverse transcriptase (Invitrogen), following standard procedures.

Taunquantitative real-time PCR (qRT-PCR) was used to measure expression levels of Dlk1 and Gt12 normalized to β-2-microglobulin (β2m) in different E16 and E18 embryonic tissues. All RT-qPCR reactions were performed in a 25 µl final volume using standard Taqman qPCR conditions (Applied Biosystems protocols) and amplified on a DNA engine Opticon 2 thermocycler (MJ Research). All reactions were conducted in triplicate. Dlk1 expression levels were measured using the TaqMan gene expression assay ID - Mm00494477_m1 (Applied Biosystems). Gt12 gene expression was measured using the forward primer 5'-GGGGGCGGCCAGAAGAAA-, the reverse primer 5'-GGTTGTAGCCCATGTATGCTA-3' and the TaqMan MGB FAM probe FAM-5'-CTTTACTGCGCTTCT-3'-NFQ, spanning the Gt12 exon 1-exon 2 boundary. Finally, for the β2m gene, the forward primer B2M-32 5'-CACCCTCCTGAGACT-GATA-3', the reverse primer B2M-38 5'-TGGGCTCGCCCATACTG-3' and the TaqMan MGB VIC-labelled fluorogenic probe VIC-5'-CCTGCGAGATTAAGC-5'-NFQ were used. Relative expression was calculated by normalisation to 100% using fetal E18 WT/WT tongue for the expression profile of the embryonic tissues and quantified using the comparative method (2^−ΔΔCT) [40]. Phary and Upi1 analysis was performed as previously described [41]. The fluorescent signal emitted during PCR was detected using the DNA engine Opticon 2 sequence detection system (MJ Research) and post-PCR data analysis was performed using the Opticon Monitor analysis software version 2.02 (MJ Research).

In situ hybridisation was conducted on paraformaldehyde fixed, wax embedded embryo and placenta sections at E16 and E18 of gestation according to the procedures previously described [42].

DLK1 Protein Analysis

Tissue lysates from whole embryos, whole placentas and multiple organs were used for SDS/PAGE analysis with a 10% polyacrylamide gel. Resolved proteins were transferred to a polyvinylidene difluoride (PVDF) Western blotting membranes Immobilon-P (Millipore) which were incubated with anti-DLK1 H-118 rabbit polyclonal antibody (1:500) (Santa Cruz Biotechnology) or anti-DLK1 (1:500) for placenta (Proteintech) and anti-α-TUBULIN mouse monoclonal antibody (Sigma) (1:5000) overnight at 4°C, followed by the secondary HRP-conjugated antibodies: polyclonal goat anti-rabbit (1:5000) or polyclonal goat anti-mouse (1:7500) (DakoCytomation), respectively, on the next day. Any signal was detected using ECL plus Western Detection Technology or anti-DLK1 (1:500) for placenta (Proteintech) and anti-DLK1 for placenta (Proteintech) and anti-α-TUBULIN mouse monoclonal antibody (Sigma) (1:5000) overnight at 4°C, followed by the secondary HRP-conjugated antibodies: polyclonal goat anti-rabbit (1:5000) or polyclonal goat anti-mouse (1:7500) (DakoCytomation), respectively, on the next day. Any signal was detected using ECL plus Western Detection System (Amersham Biosciences). The intensity of the signal was measured by scanning densitometry using Image J software (NIH).

Phenotypic Characterisation of Dlk1 Transgenic Fetuses and Neonates

All embryonic dissections were recorded in terms of number and genotype of embryos/pups, dead embryos/pups, necrotic embryos and reabsorptions for all Dlk1 transgenic families. Wet masses were recorded for all fetuses/newborns/juveniles and placentas dissected at different developmental stages. Wet organ weights were also determined for E18, E19 and P1. For determination of the dry weight, pre-weighed embryos were dried by incubation at 60°C for 48 h plus 100°C for a further 24 h and, then, weighed. Crown-Rump length was accurately measured using callipers. Glycemia levels were measured using a One Touch Ultra glucometer (LifeScan).

For histology, freshly harvested embryos were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated and embedded in paraffin wax using standard protocols. Sections of 7–10 µm were cut and 1 section in 10 was stained with Haematoxylin and Eosin.
(H&E). For skeletal preparation, the skeleton of E19 embryos was stained with Alcian Blue and Alizarin Red as described previously [16]. Muscle was analysed after immunocytochemistry with MY32 antibody (M4276 Sigma), a monoclonal antibody specific for skeletal muscle myosin heavy chain, performed according to the procedure described previously [16]. Comparative morphometrics were carried out on MY32-stained histological sections through the largest cross-sectional area of the forelimbs and diaphragm. Comparable sections were carefully selected for the TG/TG, WT/TG and WT/WT littermates and 3 sections (7 μm thick) with 20 section intervals were selected for each embryo. Measurements were performed by randomly selecting the fields across the whole section. Details were as described previously [19].

Ethics statement
All animal work in this study was conducted under a licence (30/2042) from the UK Government Home Office.

Supporting Information
Figure S1 Dlk1 transgene copy number and expression. A. Schematic representation of the endogenous and transgenic Dlk1 locus; Restriction digestion with EcoRI (arrows) results in a 4.5 kb band for the endogenous gene and a 4.0 kb band for the transgenic gene, when detected by the probe represented in green. B. Determination of copy number in transgenic and normal (N) animals from different families (upper panel). Genotyping of the WT/TG and TG/TG animals of the 70C and 70A families (lower panels). Found at: doi:10.1371/journal.pgen.1000392.s001 (2.47 MB TIF)

Figure S2 TG<sup>Dlk1-70</sup> is expressed at double dose in all three lines and is not imprinted. A. Sequence analysis of RT-PCR products from E16 WT/WT, TG/WT (maternal transmission) and WT/TG (paternal transmission) embryos showing expression of the transgene regardless of parental-origin; the endogenous locus is indicated by the T allele (DBA/2) in the nucleotide marked by the arrow head, while the transgene is indicated by the C allele (129/Sv). Allele-specific sequence analysis was conducted for 70B and 70C families. B. Quantitative Northern blotting and histograms showing that Dlk1 is expressed at twice the normal dose in WT/TG animals in all three transgenic lines analysed. Found at: doi:10.1371/journal.pgen.1000392.s002 (3.57 MB TIF)

Figure S3 Transgenic mice do not have skeletal muscle defects. A. Comparable sections through the diaphragm of E18 PatDi(12), WT/WT, WT/TG and TG/TG fetuses stained with the myofibrill specific antibody MY32. Scale bar: 50 μm. B. Morphometric analysis of the skeletal muscle of E18 WT/WT, WT/TG and TG/TG fetuses (a) Standard section through the forearm stained with MY32 antibody; Forel I (extensor carpi radialis longus+brachioradialis), Forel II (extensor digitorum+extensor carpi ulnaris) were used for morphometric measurements; Scale bar: 200 μm; Abbreviations: r - radius; u -ulna. (b) Morphometric analysis of the myofiber diameter of the skeletal muscles (diaphragm, forel I and forel II) of E18 WT/WT, WT/TG and TG/TG. Graphs show mean values±SEM (n=4). (c) Morphometric analysis of the percentage of myofibers with centrally-located nuclei from the same material used to analyse myofiber diameter; Graphs show mean values±SEM (n=4). Found at: doi:10.1371/journal.pgen.1000392.s003 (2.32 MB TIF)

Figure S4 BAT expression analysis by TaqMan RT-qPCR at E19. Graphic representation of relative levels of expression of Ppar<sup>y2</sup> and Ucp1 normalized against 18S (loading control) at E19 (mean±SEM, n=6) (70B). Found at: doi:10.1371/journal.pgen.1000392.s004 (0.43 MB TIF)

Table S1 Summary of the copy number and Dlk1 expression levels in E16 WT/TG fetuses relative to wild type for the 70 kb Dlk1 transgenic lines. Significant differences in expression for the different families are indicated by asterisks (p-value<0.05; unpaired student’s t-test); Abbreviations: F3 - Generation 3. Found at: doi:10.1371/journal.pgen.1000392.s005 (0.05 MB DOC)

Table S2 Frequency and viability of WT/WT, WT/TG and TG/TG animals from E16 to early postnatal life for the three over-expressing 70 kb transgenic lines (70A, 70B and 70C). The values represent number of animals genotyped in each time-point from crosses involving Dlk1 transgenic animals; the numbers in brackets represent number of dead animals. Found at: doi:10.1371/journal.pgen.1000392.s006 (0.06 MB DOC)

Table S3 Overall embryonic and placental growth of Dlk1 transgenic fetuses from the three over-expressing 70 kb transgenic lines (70B, 70A and 70C). Values represent mean±SEM; Means were obtained from fetuses originated from at least three independent heterozygous intercross litters (n=5). Percentage values represent the ratio between WT/TG or TG/TG weights to WT/WT counterparts. Significant differences between the WT/TG and WT/WT and between TG/TG and WT/WT are indicated by asterisks on the percentage value (unpaired Student’s t test). Found at: doi:10.1371/journal.pgen.1000392.s007 (0.07 MB DOC)

Table S4 Comparison of expression of Dlk1 between WT/WT and WT/TG or TG/TG embryos. In situ hybridisation was conducted on E16 and E18 conceptuses using antisense and sense (control) probes for Dlk1. Sections were scored for expression of Dlk1. Expression of Dlk1 was evident in all endogenous sites and ectopic expression was not observed in transgenic embryos or placenta. Found at: doi:10.1371/journal.pgen.1000392.s008 (0.04 MB DOC)

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Author Contributions
Conceived and designed the experiments: MC SPL ACFS. Performed the experiments: STdR MC SPL IG YI DG WD. Analyzed the data: STdR M. Author Contributions

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