Loss of Dis3l2 partially phenocopies Perlman syndrome in mice and results in upregulation of Igf2 in nephron progenitor cells

Ryan W. Hunter  
*University of Texas Southwestern Medical Center at Dallas*

Yangjian Liu  
*Washington University School of Medicine in St. Louis*

Hema Manjunath  
*University of Texas Southwestern Medical Center at Dallas*

Asha Acharya  
*University of Texas Southwestern Medical Center at Dallas*

Benjamin T. Jones  
*University of Texas Southwestern Medical Center at Dallas*

*See next page for additional authors*

Follow this and additional works at:  [https://digitalcommons.wustl.edu/open_access_pubs](https://digitalcommons.wustl.edu/open_access_pubs)

Please let us know how this document benefits you.

**Recommended Citation**

[https://digitalcommons.wustl.edu/open_access_pubs/6993](https://digitalcommons.wustl.edu/open_access_pubs/6993)
Loss of Dis3l2 partially phenocopies Perlman syndrome in mice and results in up-regulation of Igf2 in nephron progenitor cells

Ryan W. Hunter,1,2 Yangjian Liu,1,13 Hema Manjunath,1 Asha Acharya,1 Benjamin T. Jones,1 He Zhang,3,4 Beibei Chen,3,4 Harini Ramalingam,1 Robert E. Hammer,5 Yang Xie3,4,6 James A. Richardson,1,7 Dinesh Rakheja,6,7,8,9 Thomas J. Carroll,1,6,10,11 and Joshua T. Mendell1,6,11,12

1Department of Molecular Biology, 2Medical Scientist Training Program, 3Department of Clinical Sciences, 4Quantitative Biomedical Research Center, 5Department of Biochemistry, 6Harold C. Simmons Comprehensive Cancer Center, 7Department of Pathology, 8Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, Texas 75390, USA; 9Division of Pathology and Laboratory Medicine, Children’s Health, Dallas, Texas 75235, USA; 10Department of Internal Medicine, 11Hamon Center for Regenerative Science and Medicine, 12Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, Texas 75390, USA

Loss of function of the DIS3L2 exoribonuclease is associated with Wilms tumor and the Perlman congenital overgrowth syndrome. LIN28, a Wilms tumor oncoprotein, triggers the DIS3L2-mediated degradation of the precursor of let-7, a microRNA that inhibits Wilms tumor development. These observations have led to speculation that DIS3L2-mediated tumor suppression is attributable to let-7 regulation. Here we examine new DIS3L2-deficient cell lines and mouse models, demonstrating that DIS3L2 loss has no effect on mature let-7 levels. Rather, analysis of Dis3l2-null nephron progenitor cells, a potential cell of origin of Wilms tumors, reveals up-regulation of Igf2, a growth-promoting gene strongly associated with Wilms tumorigenesis. These findings nominate a new potential mechanism underlying the pathology associated with DIS3L2 deficiency.

Supplemental material is available for this article.

Received April 17, 2018; revised version accepted May 23, 2018.

Several lines of evidence strongly implicate the let-7 family of microRNAs [miRNAs] as important suppressors of Wilms tumor, the most common pediatric malignancy of the kidney (Ward et al. 2014). Hot spot mutations in the miRNA-processing enzymes DROSHA and DICER that impair production of a subset of miRNAs, including let-7, are common in Wilms tumor (Rakheja et al. 2014; Torrezan et al. 2014; Walz et al. 2015; Wegert et al. 2015). Additionally, the negative regulator of let-7 maturation LIN28B is frequently overexpressed in advanced-stage Wilms tumors (Viswanathan et al. 2009), and overexpression of LIN28 in developing mouse kidneys leads to Wilms tumor-like pathology, which is suppressed by enforced expression of let-7 (Urbach et al. 2014). LIN28 inhibits let-7 maturation by binding to precursor let-7 [pre-let-7] hairpins, recruiting a terminal uridylyl transferase to catalyze the addition of a series of uridines to the pre-let-7 3′ end [Supplemental Fig. S1A; Hagan et al. 2009; Heo et al. 2009]. Uridylation of pre-let-7 serves as a trigger for degradation by DIS3L2 (Chang et al. 2013), an exoribonuclease that, interestingly, is also recurrently mutated in Wilms tumors (Astuti et al. 2012; Torrezan et al. 2014; Wegert et al. 2015; Gadd et al. 2017).

DIS3L2 is a highly conserved 3′–5′ exoribonuclease that preferentially degrades uridylylated RNA substrates (Chang et al. 2013; Lubas et al. 2013; Malecki et al. 2013; Ustianenko et al. 2013). In addition to pre-let-7, it has also been reported to target histone mRNAs and many small noncoding RNAs (Labno et al. 2016; Pirouz et al. 2016; Reimao-Pinto et al. 2016; Ustianenko et al. 2016). While somatic mutations in DIS3L2 occur in sporadic Wilms tumors, germline mutations in DIS3L2 result in Perlman syndrome, an overgrowth syndrome characterized by neonatal death, genitourinary [GU] anomalies, hypotonia, neurodevelopmental delay, and frequent Wilms tumors (Astuti et al. 2012; Higashimoto et al. 2013; Soma et al. 2017). DIS3L2 mutations in both Perlman-associated and sporadic cases of Wilms tumor suggest the importance of this gene as a tumor suppressor, yet the mechanisms through which it functions as such remain unclear.

The importance of let-7 in Wilms tumorigenesis and the established role of DIS3L2 in LIN28-dependent let-7 repression have led many to postulate that DIS3L2 acts as a tumor suppressor by influencing let-7 expression (Chang et al. 2013; Urbach et al. 2014; Hohenstein et al. 2015; Wegert et al. 2015). However, our current understanding of the role of DIS3L2 in the LIN28 pathway is not congruent with this hypothesis [Supplemental Fig. S1B]. For example, Dis3l2 knockdown in embryonic stem [ES] cells results in accumulation of uridylylated pre-let-7, but this species is not a substrate for DICER processing, and mature let-7 levels are accordingly unaffected by DIS3L2 inhibition in this cell type [Heo et al. 2008; Chang et al. 2013]. Furthermore, since DIS3L2 is believed to be the terminal nuclease in the LIN28 pathway, the expected consequence of loss of this protein would be a potential increase in let-7 expression, an effect that would be expected to suppress, rather than enhance, Wilms tumor development. Nevertheless, knockdown of DIS3L2 in HeLa cells was reported to result in reduced mature let-7 levels,
leading to speculation that this protein may act in a cell type-specific manner to regulate let-7 expression (Ustianenko et al. 2013).

Given the potentially paradoxical role of DIS3L2 in the LIN28–let-7 pathway and its role as a tumor suppressor in Wilms tumor, we sought to more thoroughly investigate the influence of DIS3L2 on let-7 expression in additional cell types, particularly those most relevant to Wilms tumor development. We observed that genetic inactivation of DIS3L2 does not affect mature let-7 family members in a broad panel of mammalian cell lines. To address whether this holds true in relevant cell types in vivo, we generated two distinct conditional Dis3l2 mutant mouse lines harboring a null allele that removes the DIS3L2 catalytic site or an allele modeled after the most commonly reported mutation in Perlman syndrome patients (Astrupi et al. 2012, Higashimoto et al. 2013). Resultant phenotypes in both mouse models not only resemble some key Perlman syndrome features but also are indistinguishable from each other, providing evidence that disease-causing DIS3L2 mutations observed in Perlman syndrome patients are phenotypically equivalent to null alleles. Moreover, examination of Dis3l2-null primary nephron progenitor cells (NPCs), a cell type proposed to be a Wilms tumor cell of origin (Huang et al. 2016), demonstrates that loss of this protein has no effect on mature let-7 expression in this context but rather results in upregulation of Igf2, a principal Wilms tumor oncogene (Hu et al. 2011).

Results and Discussion

**Loss of DIS3L2 does not affect mature let-7 levels in cell lines**

The effect of DIS3L2 loss on mature let-7 expression has thus far been examined in three cell types—mouse ES (mES) cells, HeLa cells, and P19 teratocarcinoma cells—exclusively using knockdown approaches (Chang et al. 2013; Ustianenko et al. 2013; Nowak et al. 2017). We first set out to examine whether DIS3L2 influences mature let-7 levels in a wider range of cell lines by using a genome-editing approach to completely eliminate DIS3L2 activity. Cell lines with undetectable LIN28A or LIN28B were selected (HeLa and HCT116), as were lines with varying levels of either LIN28 paralog (E14tg2a [a mES cell line]) and Igvr1 with LIN28A expression or HEK293T and Huh7 with LIN28B expression) [Fig. 1A]. For each cell line, we used either transcription activator-like effector nuclease [TALEN] pairs or clustered regularly interspaced short palindromic repeats [CRISPR]/Cas9 to generate frameshift insertion–deletion [indel] mutations in an early coding exon [Fig. 1B]. In all examined DIS3L2 knockout cell lines, confirmed by both DNA sequence analysis (Supplemental Fig. S2) and resultant loss of protein [Fig. 1C], mature let-7 levels remained comparable with parental cells [Fig. 1D,E]. Notably, this included HeLa cells, where DIS3L2 knockdown was reported previously to result in reduced let-7 abundance (Ustianenko et al. 2013). Consistent with prior reports, we did detect an increase in uridylated pre-let-7 in DIS3L2-deficient mES cells [Supplemental Fig. S3]. These data indicate that, irrespective of LIN28 expression, DIS3L2 is not a major regulator of mature let-7 abundance in commonly used mammalian cell lines.

**Dis3l2 loss of function partially recapitulates Perlman syndrome in mice**

Although DIS3L2 deficiency did not influence mature let-7 levels in a broad panel of cell lines, it remained possible that loss of DIS3L2 results in altered let-7 expression in Wilms tumor-relevant cell populations within the context of the developing kidney. To investigate this possibility, we used CRISPR/Cas9 to generate mice with either conditional or germline-inheritable Dis3l2 mutations. Given that Perlman syndrome-associated Dis3l2 mutations are presumed to result in loss of function (Astrupi et al. 2012), we initially generated a Dis3l2-null allele by targeting mouse exon 11 [orthologous to exon 10 of the human gene], which encodes highly conserved residues essential for catalytic activity [Fig. 2A; Chang et al. 2013] and whose deletion results in a frameshift and premature termination codon. Single-guide RNAs (sgRNAs) targeting sequences flanking exon 11 together with Cas9 mRNA and oligonucleotides containing loxP sites were coinfected into fertilized oocytes, resulting in the generation of both exon 11-deleted [Dis3l21/11] and exon 11-floxed [Dis3l21/10] alleles.

Analysis of offspring derived from Dis3l21/11 intercrosses revealed fully penetrant perinatal lethality of Dis3l21/11 mice, with no homozygous animals surviving the first postnatal day [Supplemental Table S1]. Consequently, litters were delivered by caesarean section at embryonic day 18.5 [E18.5], revealing the presence of all genotypes at the expected Mendelian frequencies and grossly normal development of Dis3l21/11 animals.
order to examine the consequences of DIS3L2 loss on later stages of kidney development. As expected, Wt1Cre/+; Dis3l2^11Δ/11Δ mice exhibit efficient elimination of exon 11-containing Dis3l2 mRNA in the kidneys [Supplemental Fig. S6A]. Nevertheless, these animals survive into adulthood and display normal kidney histology without evidence of malignancy up to at least 6 mo of age [Supplemental Fig. S6B], a time point by which other Wilms tumor mouse models develop malignancies at high penetrance [Hu et al. 2011; Urbach et al. 2014]. These findings demonstrate that loss of function of DIS3L2 in mice recapitulates some key features of Perlman syndrome, such as GU abnormalities and neonatal death, but is not sufficient to impair kidney development or initiate Wilms tumor formation.

Modeling the most common Perlman syndrome mutation in mice

Although it is widely assumed that Perlman syndrome results from complete loss of function of DIS3L2, the failure of Dis3l2^11Δ/11Δ mice to recapitulate all aspects of this disorder led us to consider the possibility that disease-causing alleles may function as hypomorphs or neomorphs. Interestingly, among the reported Perlman syndrome mutations, the majority is in-frame deletions [Supplemental Table S1]. These data indicate that Perlman syndrome patients who developed Wilms tumor carry at least one mutation of this type (Fig. 3A). To directly assess the phenotypic consequences of a disease-associated mutation, we again used CRISPR/Cas9 to produce deleted and floxed alleles of mouse Dis3l2 exon 10 (Dis3l2^10Δ and Dis3l2^10fl, respectively) (Fig. 3B), which mimic deletion of human DIS3L2 exon 9, the most common mutation observed in Perlman syndrome patients. Importantly, this deletion, which is associated with Wilms tumor development, preserves the DIS3L2 reading frame and removes only 1% of the residues in the catalytic RNB domain. Nevertheless, DIS3L2 protein levels were dramatically reduced in the kidneys of E18.5 Dis3l2^10Δ/10Δ mice, suggesting that this mutation destabilizes the protein [Supplemental Fig. S5]. Accordingly, the phenotype of Dis3l2^10Δ/10Δ mice was indistinguishable from Dis3l2^11Δ/11Δ mice, with fully penetrant perinatal lethality associated with lung hypoinflation, bradykinesia, and spinal curvature indicative of neuromotor defects (Supplemental Fig. S6A). Nevertheless, these animals survive into adulthood and display normal kidney histology without evidence of malignancy up to at least 6 mo of age (Supplemental Fig. S6B), a time point by which other Wilms tumor mouse models develop malignancies at high penetrance [Hu et al. 2011; Urbach et al. 2014]. These findings demonstrate that loss of function of DIS3L2 in mice recapitulates some key features of Perlman syndrome, such as GU anomalies and neonatal death, but is not sufficient to impair kidney development or initiate Wilms tumor formation.
DIS3L2 deficiency leads to Igf2 overexpression in NPCs

To identify potential let-7-independent tumor suppressor functions of DIS3L2, we further examined the RNA-seq data from Dis3l2Δ/Δ11 NPCs. As reported previously in other cell types (Labno et al. 2016; Pirouz et al. 2016; Reimao-Pinto et al. 2016; Ustianenko et al. 2016), we detected up-regulation of many histone mRNAs and RNA polymerase III [Pol III] transcribed small noncoding RNAs, including Vaultu5c, Rny1, Rny3, and Rpph1 [Fig. 4C; Supplemental Table S3]. Strikingly, among the most significantly up-regulated protein-coding genes was Igf2, along with the neighboring genes H19 and Igf2os. This finding was noteworthy, as Igf2 is recognized to be one of the most important Wilms tumor oncogenes. The Igf2 locus is imprinted in nearly all tissues such that Igf2 is transcribed exclusively from the paternal allele, while the neighboring H19 gene is transcribed from the maternal allele. Loss of imprinting or loss of heterozygosity of this locus, resulting in Igf2 overexpression, is observed in a majority of Wilms tumors [Wegert et al. 2015]. Moreover, biallelic expression of Igf2 underlies Beckwith-Wiedemann syndrome, an overgrowth syndrome with similarities to Perlman syndrome, including Wilms tumor susceptibility [Lapunzina 2005]. Therefore, overexpression of Igf2 could potentially contribute to both overgrowth and Wilms tumor development in Perlman syndrome. Quantitative RT–PCR [qRT–PCR] confirmed overexpression of both Igf2 and H19 in independently derived Dis3l2Δ/Δ11 NPCs [Fig. 4D].

Given that Igf2 and H19 share a common set of enhancers [Nordin et al. 2014], their coordinated up-regulation in DIS3L2-deficient NPCs is most consistent with increased transcription of these genes. Indeed, we observed up-regulation of unspliced Igf2 and H19 transcripts in Dis3l2Δ/Δ11 NPCs [Fig. 4E], strongly supporting this premise. Moreover, transcripts derived from all four annotated mouse Igf2 promoters exhibited a proportional increase in expression, consistent with a general increase in transcription across the Igf2/H19 locus [Supplemental Fig. S8]. Since loss of imprinting is a common mechanism of increased Igf2 transcription in Wilms tumor, we assessed the imprinting status of this locus in Dis3l2 mutant NPCs. Intercrosses of C57BL/6 and BALB/cJ mice carrying the Dis3l2Δ/Δ11 allele allowed generation of NPCs with defined single-nucleotide polymorphisms (SNPs) in exons of Igf2 and H19 that were used to track allele-specific expression of each gene [Supplemental Fig. S9]. Monoallelic expression of both Igf2 and H19 was observed in Dis3l2Δ/Δ11 NPCs, demonstrating that loss of DIS3L2 does not impact imprinting of this locus. We conclude that DIS3L2 loss of function results in transcriptional activation of the Igf2/H19 locus in NPCs, most likely by leading to the activation of shared cis-regulatory elements that control these genes.

IGF2: a new candidate driver of overgrowth and Wilms tumor in DIS3L2-deficient settings

The strong predisposition to Wilms tumor in Perlman syndrome as well as the presence of recurrent somatic mutations in DIS3L2 in sporadic Wilms tumor provide compelling genetic evidence that this gene functions as a tumor suppressor in this malignancy. Given the role of DIS3L2 as the terminal nuclease in the LIN28–let-7 pathway and the evidence linking the LIN28–let-7 pathway to...
The lack of tumor formation in Dis3l2-null mice is consistent with the previous demonstration that isolated Igf2 overexpression is not sufficient to trigger Wilms tumor development in mouse models [Sun et al. 1997; Hu et al. 2011]. Wilms tumors have been successfully generated in mice by combining multiple genetic lesions that co-occur in patients, such as Igf2 loss of imprinting and Wt1 deletion [Hu et al. 2011]. Similar strategies using the new conditional Dis3l2 mutant alleles reported here in combination with co-occurring Wilms tumor mutations in genes such as Dgcr8, Wtx, and others (Torrezan et al. 2014; Wegert et al. 2015; Gadd et al. 2017) are likely to produce new Wilms tumor models representing additional genetic subtypes of this malignancy. It is therefore anticipated that the new Dis3l2-deficient cell lines and mouse models generated in this study will provide a valuable resource for further study of the pathogenesis and treatment of Perlman syndrome and Wilms tumor.

Materials and methods

Mouse strains

Dis3l2 mutant alleles were generated using CRISPR/Cas9 as described previously [Yang et al. 2013]. In brief, Cas9 mRNA (Sigma), in vitro transcribed sgRNA, and loxP-containing Ultramer oligos (Integrated DNA Technologies) were injected directly into C57BL/6J oocytes. Relevant oligonucleotide sequences are in Supplemental Table S4. Successfully generated alleles were maintained by backcrossing to C57BL/6J. Wt1Cre [Zhou et al. 2008] and BALB/c mice were obtained from The Jackson Laboratory. All mouse experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center.

Data access

RNA-seq data have been deposited in Gene Expression Omnibus under accession number GSE114673.

Acknowledgments

We thank Richard Gregory, Keith Jong, and Feng Zhang for plasmids and cell lines, and Vanessa Schmid in the McDermott Center Next-Generation Sequencing Core, John Shelton in the University of Texas Southwestern Histopathology Core, and Kathryn O’Donnell and Kenneth Chen for helpful discussions. This work was supported by grants from the Cancer Prevention and Research Institute of Texas [RP160249 to J.T.M., D.R., and T.J.C., and RP150936 to the University of Texas Southwestern Bioinformatics Core Facility], the National Institutes of Health [R35CA197311 to J.T.M.; R24DK080004, R01DK095057, R01DK106743, and P30DK079328 to T.J.C.; and PO5CA196516 to Y.X., D.R., T.J.C., and J.T.M.], and the Welch Foundation [I-1961-20180324 to J.T.M.]. H.R. was supported by a University of Texas Southwestern Center for Regenerative Science and Medicine fellowship. J.T.M. is an investigator of the Howard Hughes Medical Institute.


References


Loss of Dis3l2 partially phenocopies Perlman syndrome in mice and results in up-regulation of Igf2 in nephron progenitor cells

Ryan W. Hunter, Yangjian Liu, Hema Manjunath, et al.

Genes Dev. 2018, 32: originally published online June 27, 2018
Access the most recent version at doi:10.1101/gad.315804.118

Supplemental Material
http://genesdev.cshlp.org/content/suppl/2018/06/27/gad.315804.118.DC1

Related Content
Overgrowth syndromes and pediatric cancers: how many roads lead to IGF2?
Ruthrothaselvi Bharathavikru and Nicholas D. Hastie
Genes Dev. August, 2018 32: 993-995

References
This article cites 40 articles, 10 of which can be accessed free at:
http://genesdev.cshlp.org/content/32/13-14/903.full.html#related-urls

Articles cited in:
http://genesdev.cshlp.org/content/32/13-14/903.full.html#ref-list-1

Creative Commons License
This article is distributed exclusively by Cold Spring Harbor Laboratory Press for the first six months after the full-issue publication date (see http://genesdev.cshlp.org/site/misc/terms.xhtml). After six months, it is available under a Creative Commons License (Attribution-NonCommercial 4.0 International), as described at http://creativecommons.org/licenses/by-nc/4.0/.

Email Alerting Service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.