Parafibromin, a Component of the Human PAF Complex, Regulates Growth Factors and Is Required for Embryonic Development and Survival in Adult Mice^{∇†}

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Parafibromin, a transcription factor associated with the PAF complex, is encoded by the *HRPT2* gene, mutations of which cause the hyperparathyroidism-jaw tumor syndrome (OMIM145001). To elucidate the function of parafibromin, we generated conventional and conditional *Hrpt2* knockout mice and found that *Hrpt2*^{−/−} mice were embryonic lethal by embryonic day 6.5 (E6.5). Controlled deletion of *Hrpt2* after E8.5 resulted in apoptosis and growth retardation. Deletion of *Hrpt2* in adult mice led to severe cachexia and death within 20 days. To explore the mechanism underlying the embryonic lethality and death of adult mice, mouse embryonic fibroblasts (MEFs) were cultured and *Hrpt2* was deleted in vitro. *Hrpt2*^{−/−} MEFs underwent apoptosis, while *Hrpt2*^{+/+} and *Hrpt2*^{+/-} MEFs grew normally. To study the mechanism of this apoptosis, *Hrpt2*^{+/+} and *Hrpt2*^{+/-} MEFs were used in cDNA microarray, semiquantitative reverse transcription-PCR, and chromatin immunoprecipitation assays to identify genes regulated by parafibromin. These revealed that *Hrpt2* expression and the parafibromin/PAF complex directly regulate genes involved in cell growth and survival, including *H19*, *Igf1*, *Igf2*, *Igfbp4*, *Hmga1*, *Hmga2*, and *Hmgcs2*. Thus, our results show that expression of *Hrpt2* and parafibromin is pivotal in mammalian development and survival in adults and that these functions are likely mediated by the transcriptional regulation of growth factors.

Loss-of-function *HRPT2* mutations are associated with the autosomal dominantly inherited hyperparathyroidism-jaw tumor (HPT-JT) syndrome. Patients with HPT-JT develop parathyroid tumors, ossifying fibromas of the mandible and maxilla, uterine tumors, and renal abnormalities that may include cysts, mixed epithelial and stromal tumors, and Wilms' tumors (3). The *HRPT2* gene is located on chromosome 1q31.2, and the observed loss of heterozygosity in HPT-JT-associated tumors and the identification of combined somatic and germ line *HRPT2* mutations in some other HPT-JT tumors indicate that *HRPT2* is a tumor suppressor gene (2, 7). In addition, *HRPT2* mutations are frequently found in sporadic parathyroid carcinomas (18, 43), and expression of parafibromin, which is the 531-amino-acid protein encoded by *HRPT2* (7), in parathyroid

carcinomas has been reported to be lower than in normal parathyroid tissues (49). Sporadic human renal tumors, from two patients, have also been reported to have frequent allelic imbalances and to harbor *HRPT2* mutations (55). These findings are consistent with a tumor suppressor role for *HRPT2* and its encoded protein, parafibromin.

Parafibromin shares 32% sequence similarity with the *Saccharomyces cerevisiae* protein Cdc73, which is a component of the yeast polymerase II-associated factor (PAF) complex, and it also has homologs in *Caenorhabditis elegans*, *Drosophila melanogaster*, and mice (7). The yeast PAF complex, which is associated with RNA polymerase II and is composed of Paf1, Ctr9, Leo1, Rtf1, and Cdc73, is involved in RNA polymerase II-mediated transcription initiation and elongation (36), mRNA processing and maturation through maintaining proper poly(A) tail length (28, 34, 44), Rad6-mediated histone H2B-K123 monoubiquitination (30, 52), Set1-mediated histone H3-K4 methylation (21, 31), and Dot1-mediated histone H3-K79 methylation (20). Thus, the PAF complex is involved in gene transcription through the following pathway: PAF complex → histone H2B ubiquitination → histone H3 methylation → chromatin remodeling (33).

Previous studies have shown that the PAF complex is associated with RNA polymerase II on transcriptionally active yeast genes (28, 46). However, only a small subset of genes, related to the cell cycle, protein synthesis, lipid metabolism, and nucleic acid turnover, are reported to be directly regulated by the PAF complex (1, 37, 45). Consistent with the findings in

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yeast, three separate studies have shown that parafibromin is a member of a human PAF complex that also includes hCtr9, hLeo1, and hPaf1 (40, 53, 57). Moreover, the human PAF complex is necessary for trimethylation of histone H3-K4 and dimethylation of H3-K79, which suggests that the complex may affect gene expression through a mechanism similar to that of the yeast PAF complex (57).

Although Cdc73 is not essential for the growth of *S. cerevisiae*, a recent study demonstrated that the *D. melanogaster* homolog of parafibromin, termed Hyrax, is necessary for early development (27). Importantly, human parafibromin, but not yeast Cdc73, can rescue the phenotypic defect caused by a loss of Hyrax, indicating that the biological function of parafibromin may be more complicated than that of Cdc73. In order to further elucidate the function of parafibromin and its target genes, we generated knockout mouse models of *Hrpt2*. Our study reveals that *Hrpt2* heterozygous mice are viable but that *Hrpt2* homozygous mice are embryonically lethal. Moreover, deletion of *Hrpt2* at various stages of embryonic development demonstrated that *Hrpt2* expression and its protein, parafibromin, are necessary for development and that their loss promotes apoptosis. In addition, loss of *Hrpt2* expression was found to alter the expression of several target genes that are associated with cell growth and cell death. Thus, our study sheds new light on the functions of *Hrpt2* and parafibromin.

MATERIALS AND METHODS

Generation of *Hrpt2* conventional knockout mice. The *Hrpt2* conventional knockout mouse was established using the embryonic stem (ES) cell line RRE190, from the BayGenomics Genetrap resource (48). The linearized Genetrap vector had inserted 2,584 bp downstream of the *Hrpt2* exon 6, in intron 6, and this was shown to lead to a loss of exons 7 to 17, which would result in a parafibromin/beta-galactosidase fusion protein that lacked the C-terminal 361 amino acids of parafibromin. The RRE190 ES cells were used to generate chimeric mice, which were mated to generate heterozygous (*Hrpt2*^{+/-}) mice. *Hrpt2*^{+/-} mice were viable and were intercrossed to obtain *Hrpt2*^{-/-} mice. Mice were kept in accordance with United Kingdom welfare guidelines and approved licensed restrictions and genotyped using PCR primers F1 (5'-GTCAACAAAC CAAAGCCTCTGGAACG-3') and R (5'-GTTACAAGGTCATGGATATTTT CACC-3') to yield a wild-type band of 281 bp and F2 (5'-CTGCAAGGCGAT TAAGTTGGGTAACG-3') and R (5'-GTTACAAGGTCATGGATATTTTCCA CC-3') to yield a mutant band of 289 bp (Fig. 1A).

Generation of *Hrpt2* conditional knockout mice. The *Hrpt2* targeting vector and *Hrpt2*^{2L/+neo}^{F/+} mice were generated, and germ line deletion of the *neo* gene was achieved by crossing a *Hrpt2*^{2L/+neo}^{F/+} mouse with a FLP^{er} mouse (13) to establish *Hrpt2*^{2L/+} mice (51). *Hrpt2* heterozygotes were generated by mating the *Hrpt2*^{2L/+} mouse with a cytomegalovirus (CMV)-Cre transgenic mouse (42) (Fig. 1B). All animals were treated and maintained in accordance with the Institutional Animal Care and Use Committee of Van Andel Research Institute.

Hrpt2^{2L/L} mice and Cre-ER transgenic mice (17) were mated to generate three genotypes: Cre-ER/*Hrpt2*^{2L/L}, Cre-ER/*Hrpt2*^{2L/+}, and Cre-ER/*Hrpt2*^{+/+}. At age 4 to 6 weeks, these animals were injected intraperitoneally (i.p.) daily with 4-OH-tamoxifen (4-OH-TM; 3 mg/40 g of body weight) dissolved in corn oil, for five consecutive days. Timed-pregnant mice were injected i.p. with tamoxifen (4 mg/40 g of body weight) dissolved in corn oil, as previously described (17). Mice were euthanized 2 days later after exposure to tamoxifen and the embryos dissected. For genotyping, primers F (5'-GTATACAGTGGGGTGGAGATG-3') and R (5'-ATTCCAAGTGGCTTCCAAGCAG-3') that detected the LoxP site were used to yield a wild-type band of 179 bp and a mutant band of 242 bp; to detect the wild-type and mutant alleles of the *Hrpt2* gene, three primers, F1 (5'-ATCTGTGAAGTGCGTCCCTA-3'), F2 (5'-CACCATATCTCACTGTAT GT-3'), and R (5'-ATCTGGGGAACCATCTTATT-3'), were used to yield bands of 253 bp and 546 bp, respectively (Fig. 1C).

Murine embryonic fibroblast cultures. Murine embryonic fibroblasts (MEFs) were isolated from embryonic day 12.5 (E12.5) or E13.5 embryos and maintained

in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin. *Hrpt2*^{+/+}, *Hrpt2*^{2L/+}, and *Hrpt2*^{2L/L} MEFs, as well as Cre-ER/*Hrpt2*^{+/+} and Cre-ER/*Hrpt2*^{2L/L} MEFs, were harvested and frozen for later experiments. *Hrpt2*^{+/+}, *Hrpt2*^{2L/+}, and *Hrpt2*^{2L/L} MEFs were infected with a multiplicity of infection of 25 Ad5CMV-Cre or Ad5CMV-GFP (University of Iowa) for 2 h. In the Cre-ER system, Cre-ER/*Hrpt2*^{2L/L} and Cre-ER/*Hrpt2*^{+/+} MEFs were treated with 1 µM 4-OH-TM for 48 h (Sigma).

Embryo culture. E3.5 blastocysts from natural matings were isolated and placed singly into 96-well plates. Acidic Trypse solution (Sigma) was used to remove the zona pellucida of blastocysts before plating. Embryos were cultured in Dulbecco's modified Eagle's medium and 15% fetal bovine serum for a total of 2 to 5 days and examined by phase-contrast microscopy prior to genotyping by PCR (12).

Histological analysis and immunoblot analysis. Tissues were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Sections were used for hematoxylin and eosin (H&E) staining and immunostaining using an antiparafibromin antibody as previously reported (49).

Whole-cell lysates were prepared in radioimmunoprecipitation buffer (1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 10 mM Tris pH 8.0, 0.14 M NaCl) with protease inhibitor complex (Roche). Western blot analysis was performed using the following antibodies: monoclonal anti-parafibromin at 1:5,000 (49), caspase 3 at 1:1,000 (Cell Signaling), cleaved caspase 3 at 1:1,000 (Cell Signaling), LC3 at 1:1,000 (MBL), and HMG1/HMG2 at 1:1,000 (Stressgen).

Cell death and proliferation assays. Apoptosis terminal deoxynucleotidyl-transferase-mediated biotin-dUTP nick end labeling (TUNEL) staining was conducted according to the manufacturer's protocol with the in situ cell death detection kit with POD and the in situ cell death detection kit with fluorescein (Roche). The staining patterns with annexin V and propidium iodide (BD Bioscience) were analyzed by flow cytometry according to the manufacturer's protocol. The bromodeoxyuridine (BrdU; Roche) incorporation assay was carried out following the manufacturer's protocol.

Gene expression profiling and semiquantitative RT-PCR. *Hrpt2*^{+/+} and *Hrpt2*^{2L/L} MEFs were infected with a multiplicity of infection of 25 Ad5CMV-Cre for 2 h, and 4 days later RNA was extracted using TRIzol (Invitrogen), purified, and used for murine cDNA microarray analysis. Experiments were performed on a 15,360-feature cDNA microarray containing full-length cDNA clones that were PCR amplified from the NIA 15k clone set (Bethesda, MD). Arrays were spotted in-house using a custom 204-slide microarrayer (Beta Integrated Concepts, MI) with 32 Point Technologies PT-3000 print pins. All batches of microarrays passed the quality control and quality assurance criteria. These experiments were also performed using RNA extracted from Cre-ER/*Hrpt2*^{+/+} and Cre-ER/*Hrpt2*^{2L/L} MEFs that had been treated with 1 µM 4OH-TM for 4 days. All microarray hybridizations were performed four times, and the signal for each gene was expressed as the signal ratio of the log₂(test signal/control signal), where the test signal represented that obtained from the *Hrpt2*^{2L/L} or Cre-ER/*Hrpt2*^{2L/L} MEFs and the control signal represented that obtained from the *Hrpt2*^{+/+} or Cre-ER/*Hrpt2*^{+/+} MEFs. A gene was designated as being up-regulated if all four of its log₂ signal ratios were >0 (i.e., positive) and as being down-regulated if all four of its log₂ signal ratios were <0 (i.e., negative). Genes that did not fulfill these criteria were not included in any further analyses. The means ± standard deviations of the log₂ signal ratios were calculated for each of the genes that fulfilled the criteria of up-regulation or down-regulation and the anti-log₂ value yielded the mean change ± standard deviation (*n* = 4) in altered expression for each gene, which was designated as being up-regulated if the mean showed a ≥2-fold increase and down-regulated if the mean showed a ≥2-fold decrease. Semiquantitative reverse transcription-PCR (RT-PCR) of selected genes was applied on the same RNA extracted from MEFs.

ChIP assay. Chromatin immunoprecipitation (ChIP) was undertaken by using the ChIP assay kit (Upstate Biotechnology). Polyclonal antiparafibromin and anti-Paf1 antibodies (Bethyl Laboratories) were used for IP.

RESULTS

Germ line disruption of *Hrpt2* leads to embryonic lethality. *Hrpt2*^{+/+} mice from the conventional knockout were intercrossed to obtain homozygous (*Hrpt2*^{-/-}) mice; however, no viable *Hrpt2*^{-/-} neonates were identified, indicating that *Hrpt2*^{-/-} mice are embryonically lethal. The time and cause of this embryonic lethality was investigated by obtaining embryos

TABLE 1. *Hrpt2* genotypes of mice and embryos obtained from *Hrpt2*^{+/-} intercrosses

Stage	No. of mice with indicated <i>Hrpt2</i> genotype		
	+/+	+/-	-/- ^a
Neonate	14	16	0**
E14.5	2	5	0*
E13.5	1	4	0*
E11.5–E12.5	11	15	0**
E8.5–E9.5	11	15	0**
E6.5–E7.5	13	21	0**
E5.5	3	14	2
E3.5	3	10	3
Total	58	100	5***

^a Significantly different from expected: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

at different stages of gestation. A total of 133 embryos ages E3.5 to E14.5 were obtained. The *Hrpt2*^{+/+}, *Hrpt2*^{+/-}, and *Hrpt2*^{-/-} genotypes of these 133 embryos were identified at a ratio of 1.16:2:0.1, which deviated significantly (*P* < 0.001) from the expected 1:2:1 Mendelian ratio. Moreover, this genotype ratio (*Hrpt2*^{+/+}/*Hrpt2*^{+/-}/*Hrpt2*^{-/-}) also deviated significantly (*P* < 0.01) from the expected Mendelian ratio at all the gestational stages above E6.5 to E7.5 (Table 1); the expected Mendelian ratio with *Hrpt2*^{-/-} embryos was only obtained at gestational stages E3.5 and E5.5. *Hrpt2*^{-/-} embryos at E3.5 appeared normal and indistinguishable from the *Hrpt2*^{+/-} and *Hrpt2*^{+/+} embryos (Fig. 1A). These results reveal that *Hrpt2* null mice are embryonically lethal before E6.5 and indicate that the lethality is likely to occur either at hatching (E4.5) or implantation (E4.5 to 5.5). In order to investigate further these possibilities and the cause of embryonic lethality, we pursued studies in the conditional knockout as it allowed us to temporally control the timing of *Hrpt2* deletion during development. For the conditional knockout mouse model, *Hrpt2* heterozygous mice were obtained by mating the *Hrpt2*^{L/+} mouse with a CMV-Cre transgenic mouse. In order to obtain *Hrpt2*-null mice, *Hrpt2* heterozygous mice were intercrossed. Live *Hrpt2*-null mice were not obtained, consistent with embryonic lethality. Thus, the conditional and conventional knockout mice yielded similar results.

To gain insight into the etiology of this embryonic lethality, blastocysts at E3.5 were collected and cultured in vitro. Normally, blastocysts are composed of two distinct cell lineages; approximately two-thirds of cells make up the trophectoderm (TE), while the remainder forms the inner cell mass (ICM). When blastocysts are cultured in vitro, these two types of cells have been shown to differentiate normally and to continue growing (12). Under these conditions, the wild-type and heterozygous *Hrpt2* blastocysts were observed to attach to the bottom of the plate at E4.5, develop into the ICM and TE at about E5.5, and continue growing. However, the *Hrpt2*-null blastocysts did not attach to the bottom of the plate, failed to proliferate into the ICM and TE cells, and consequently died within 3 days (Fig. 2A).

To identify whether *Hrpt2* expression is essential at other points in development, controlled deletion of *Hrpt2* was undertaken by mating *Hrpt2*^{L/L} mice to *Cre-ER* transgenic mice. *Cre-ER* is widely expressed upon administration of tamoxifen

FIG. 2. A) Culture of E3.5 blastocysts in vitro. The wild-type (*Hrpt2*^{+/+}) and heterozygous (*Hrpt2*^{+/-}) blastocysts differentiated into an ICM and TE at about E5.5 and continued to grow at E6.5 and E7.5. *Hrpt2*-null (*Hrpt2*^{-/-}) blastocysts failed to differentiate into ICM and TE and died. Experiments were repeated three times. B) Temporally controlled deletion of *Hrpt2* in conditional knockout mouse embryos. Pregnant mice were treated with tamoxifen at 8.5 dpc, 10.5 dpc, and 12.5 dpc, and embryos were collected 2 days later. Embryos were viewed by microscopy (magnification is indicated in parentheses): E8.5 to 10.5 (4 $\times$), E10.5 to 12.5 (2 $\times$), and E12.5 to 14.5 (1.6 $\times$). *Cre-ER/Hrpt2*^{L/L} embryos exposed to tamoxifen at E8.5 to 10.5 died and had a developmental defect of the brain (arrow). C) The body sizes of *Cre-ER/Hrpt2*^{L/L} embryos were smaller than those of control embryos (E8.5 to 10.5, *P* < 0.01; E10.5 to 12.5, *P* < 0.01; E12.5 to 14.5, *P* < 0.05).

in tissues of the Cre-ER transgenic mouse at embryonic and adult stages. For example, in E8.5 embryos, the Cre enzyme recombination can be detected at 6 h and the efficiency of recombination is up to 95% after exposure to tamoxifen for 48 h but declines to about 50 to 60% in E11.5 and E14.5 embryos (17). Tamoxifen was therefore injected i.p. into pregnant mice at 8.5, 10.5, 12.5, and 14.5 days postcoitum (dpc), and the embryos were harvested 2 days later. Exposure of Cre-ER/*Hrpt2*^{L/L} embryos to tamoxifen at E8.5 to E10.5 resulted in a deletion of the *Hrpt2* gene as assessed by PCR, and this was associated with a >50% reduction in parafibromin as assessed by Western blot analysis (data not shown). Cre-ER/*Hrpt2*^{L/L} embryos at E8.5 to 10.5 were significantly smaller, had a significant delay in the development of the central nervous system, and died rapidly (Fig. 2B and C). The deletion of *Hrpt2* in Cre-ER/*Hrpt2*^{L/L} embryos at later stages, i.e., E10.5 to 12.5 and E12.5 to 14.5, affected growth but not organogenesis or survival.

Widespread deletion of *Hrpt2* in adult mice causes damage to multiple tissues and death. In order to investigate the effects of a loss of *Hrpt2* expression in adult tissues, the *Hrpt2* gene was deleted in somatic cells using the Cre-ER transgenic mouse. Eleven Cre-ER/*Hrpt2*^{L/L} mice were generated, and a PCR-based genotype analysis using DNA samples from 15 different organs confirmed deletion of *Hrpt2*. As expected, low levels of the wild-type band were detected because of incomplete penetration of the Cre enzyme (Fig. 3A). All 13 of the control mice and 7 of the Cre-ER/*Hrpt2*^{L/L} mice that were injected with corn oil alone were viable and remained healthy until euthanization 3 months later. Null Cre-ER/*Hrpt2*^{L/L} mice died within 20 days after being exposed to tamoxifen. Prior to this, all of these mice had low activity, labored breathing, slow reaction, weight loss (Fig. 3B), and a reduction in abdominal and subcutaneous adipose tissue (Fig. 3C). In addition, the liver, kidneys, heart, spleen, lungs, stomach, testes, seminal vesicles, and salivary glands were smaller in size than those in the controls (Fig. 3D). Some of the Cre-ER/*Hrpt2*^{L/L} mice also developed dilation of the gastrointestinal tract and/or developed ascites. The expression of *Hrpt2* was detected, by RT-PCR, in multiple adult normal mouse tissues (Fig. 4A), and the presence of parafibromin in these multiple tissues was confirmed by Western blot analysis (Fig. 4B). In addition, the expression and nuclear localization of parafibromin in E12.5 and E16.5 embryos (Fig. 4C) and normal adult tissues (Fig. 4D) were demonstrated by immunohistochemistry. However, in adult Cre-ER/*Hrpt2*^{L/L} mice parafibromin expression was reduced in the kidney, liver, stomach, and salivary glands (Fig. 5). Moreover, in those adult Cre-ER/*Hrpt2*^{L/L} mice with a widespread deletion of *Hrpt2* there was a rapid onset of cachexia, which suggested the occurrence of multiorgan failure. Indeed, this was consistent with the finding of necrotic cells in the liver and kidneys of the most morbid Cre-ER/*Hrpt2*^{L/L} mice (Fig. 5). In addition, the morbid Cre-ER/*Hrpt2*^{L/L} mice had a reduced secretion by the submandibular salivary glands, a decreased number of tubular glands, and diminished secretion in the seminal vesicles. Furthermore, the use of TUNEL staining revealed significant apoptosis in the salivary glands, tongue, stomach, intestine, liver, and kidneys (Fig. 5). The parathyroid glands of the morbid Cre-ER/*Hrpt2*^{L/L} mice were not found to contain abnormalities (data not shown).

FIG. 3. Adult conditional *Hrpt2* knockout mice of ages 4 to 6 weeks were treated with tamoxifen for 5 days. A) Deletion of the *Hrpt2* gene as detected by PCR analysis in DNA obtained from different organs. The asterisk indicates the band following deletion of *Hrpt2*, and the black arrow indicates the control, wild-type band of *Hrpt2*. B) Tamoxifen-treated Cre-ER/*Hrpt2*^{L/L} mice ($n = 11$) lost 2.28 ± 0.56 g of body weight, while similarly treated control, i.e., Cre-ER/*Hrpt2*^{+/+} and Cre-ER/*Hrpt2*^{L/+} mice ($n = 13$) gained 3.50 ± 0.50 g at the time of euthanization ($P < 0.01$). C) All Cre-ER/*Hrpt2*^{L/L} mice died within 20 days and had less adipose in the abdominal cavity (upper panel), a smaller body size, less subcutaneous fat, and loss of muscle (lower panel). D) The organs (from top to bottom, heart, lung, spleen, kidney, testis, seminal vesicle, stomach, liver, and salivary gland) of the tamoxifen-treated Cre-ER/*Hrpt2*^{L/L} mice were also smaller than those of similarly treated Cre-ER/*Hrpt2*^{+/+} mice.

Loss of *Hrpt2* promotes apoptosis in vitro and in vivo. The mechanism of cell death caused by disruption of *Hrpt2* was further investigated using *Hrpt2*-null MEFs. MEFs were obtained from E12.5 embryos of the following genotypes: *Hrpt2*^{+/+}, *Hrpt2*^{L/+}, *Hrpt2*^{L/L}, Cre-ER/*Hrpt2*^{+/+}, and Cre-ER/

FIG. 4. Expression of *Hrpt2* and parafibromin. A) Expression of *Hrpt2* in different tissues of normal adult mice was detected by RT-PCR. Lanes: 1, heart; 2, liver; 3, spleen; 4, lung; 5, kidney; 6, brain; 7, eye; 8, tongue; 9, salivary gland; 10, esophagus; 11, bronchus; 12, stomach; 13, duodenum; 14, pancreas; 15, intestine; 16, skin; 17, muscle; 18, bladder; 19, uterus; 20, testis; 21, placenta; 22, pcDNA3.1-HRPT2 (positive control) (54). S is the size marker (1-kb ladder). B) Expression of parafibromin in normal mouse tissues as detected by Western blot analysis. Lanes: a, kidney; b, liver; c, spleen; d, intestine; e, stomach; f, heart; g, lung; h, brain; i, tongue; j, salivary gland; k, muscle; l, eye; m, testis; n, seminal vesicle; o, duodenum; p, ovary; q, uterus. C) Expression of parafibromin in embryos (E12.5 and E16.5) as detected by immunohistochemistry. The inset represents a magnified view of the area denoted by the red square, to show nuclear localization of parafibromin. D) Expression and nuclear localization of parafibromin in adult normal mouse tissues. (I) Brain; (II) liver; (III) kidney; (IV) testis.

Hrpt2^{L/L}. The conditional nature of *Hrpt2*^L allowed for the generation of a null allele of *Hrpt2* via the use of either an adenoviral Cre system or exposure of the cultures to tamoxifen in the case of Cre-ER/*Hrpt2*^{L/L} cells. Loss of *Hrpt2* (*Hrpt2*-

deficient MEFs), induced by either method, resulted in reduced cell proliferation (Fig. 6A) and increased cell death (Fig. 6B), and this was associated with decreased amounts of parafibromin (Fig. 6C). This is consistent with the in vivo observa-

FIG. 5. Pathological changes, detected by H&E, TUNEL, and parafibromin staining, in tamoxifen-treated Cre-ER/*Hrpt2*^{L/L} mice prior to death. All the tissues from the tamoxifen-treated Cre-ER/*Hrpt2*^{L/L} mice had more TUNEL staining but less expression of parafibromin compared with control mice. In the tamoxifen-treated Cre-ER/*Hrpt2*^{L/L} mice, there was reduced glandular tissue in the salivary glands (H&E) but significantly increased cell death in the liver and kidneys (H&E).

FIG. 6. Studies with *Hrpt2*^{+/+}, *Hrpt2*^{L/L}, and *Hrpt2*^{L/L} MEFs infected with Ad5CMV-Cre and *Hrpt2*^{L/L} MEFs infected with Ad5CMV-GFP for 2 days. A) Growth curves; 2 days after infection with adenovirus, all the *Hrpt2*-containing MEFs (Ad-Cre/*Hrpt2*^{+/+}, Ad-Cre/*Hrpt2*^{L/L}, and Ad-GFP/*Hrpt2*^{L/L}) grew in a similar pattern, while the *Hrpt2*-deficient MEFs (Ad-Cre/*Hrpt2*^{L/L}) failed to proliferate. B) Significantly increased cell death was found to occur in the *Hrpt2*-deficient MEFs. C) Two days after Ad5CMV-Cre infection, parafibromin expression, as detected by Western blot analysis, was found to be decreased in the *Hrpt2*-deficient MEFs (Ad-Cre/*Hrpt2*^{L/L} MEFs) but was unchanged in the *Hrpt2*-containing MEFs (Ad-Cre/*Hrpt2*^{+/+} MEFs). All experiments were repeated three times.

tions detailed above. Infecting the *Hrpt2*^{L/L} MEF cells with a control adenovirus did not reveal differences in cellular proliferation or survival relative to *Hrpt2*-containing cells.

Hrpt2-deficient MEFs also showed increased apoptosis as revealed by the following: increased positive TUNEL staining at day 3 adenovirus infection, relative to the controls (Fig. 7A); significantly increased annexin V staining relative to control cells at 48 h following deletion of *Hrpt2* (Fig. 7B); significantly increased levels of cleaved caspase 3 (Fig. 7C). Electron microscopy and investigation for the expression of LC3-II (8, 26) did not reveal any double membrane autophagosomes (data not shown) or the conversion of LC3-I into LC3-II (Fig. 7C), thereby indicating that the *Hrpt2*-deficient cells were not predisposed to higher levels of autophagic cell death. Moreover, expression of HMGB1/B2 (11) in these *Hrpt2*-deficient cells was not altered compared to controls (Fig. 7C), thereby indicating that cell death was not associated with an increased level

of necrosis. These in vitro findings indicate that there is increased apoptosis following *Hrpt2* disruption, and this was further investigated in vivo utilizing *Hrpt2*-deficient embryos. *Hrpt2*-deficient embryos at E10.5 had significantly increased TUNEL-positive cells relative to control embryos (Fig. 7D). In addition, cell proliferation, which was assessed in BrdU incorporation assays, was notably decreased in Cre-ER/*Hrpt2*^{L/L} embryos (Fig. 7D). Thus, these results demonstrate that the loss of *Hrpt2* expression is associated with increased apoptosis and reduced cell proliferation.

***Hrpt2* and its encoded protein, parafibromin, regulate genes involved in genomic imprinting and cell growth.** Our findings indicate that *Hrpt2* and its encoded protein, parafibromin, are involved in regulating mammalian embryonic development and survival in adulthood. In order to identify genes that are regulated in these pathways, differences in gene expression were sought by means of cDNA microarrays between *Hrpt2*^{L/L} and *Hrpt2*^{+/+} MEFs 96 h after exposure to Ad5CMV-Cre and Cre-ER/*Hrpt2*^{+/+} and Cre-ER/*Hrpt2*^{L/L} MEFs 96 h after exposure to 4-OH-TM. This revealed that the expression of 111 genes was altered by the decreased expression of *Hrpt2* (threshold, twofold change; see Tables S1, S2, and S3 in the supplemental material). Forty-three of these genes showed up-regulation (see Tables S1 and S3 in the supplemental material) and 68 showed down-regulation (see Tables S1 and S3 in the supplemental material). One group of down-regulated genes was related to cell growth and energy metabolism, and this included H19 fetal liver mRNA (*H19*), insulin-like growth factor 2 (*Igf2*), insulin-like growth factor 1 (*Igf1*), insulin-like growth factor binding protein 4 (*Igfbp4*), 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 (*Hmgcs2*), carbonyl reductase 2 (*Crb*), bone marrow stromal cell antigen 2 (*Bst2*), high-mobility group AT-hook 1 (*Hmga1*), high-mobility group AT-hook 2 (*Hmga2*), and signal transducer and activator of transcription 2 (*Stat2*) (Fig. 8A). These changes were confirmed for several representative dysregulated genes by the use of semiquantitative RT-PCR on total RNA isolated from the MEFs (Fig. 8A). These findings are consistent with the previous report that the yeast PAF complex is related to protein synthesis and to lipid and nucleic acid metabolism (1).

To further identify the direct target genes whose transcription is controlled by parafibromin or the PAF complex, ChIP assays were used to investigate some growth factors and imprinting genes. This revealed that parafibromin and Paf1 bound to the promoter and/or coding regions of *H19*, *Igf1*, *Igf2*, *Igfbp4*, *Hmgcs2*, *Hmga2*, and *Hmga1* (Fig. 8B and C). Thus, these results demonstrate that *H19*, *Igf1*, *Igf2*, *Igfbp4*, *Hmga2*, *Hmgcs2*, and *Hmga1* are transcriptional target genes of the murine parafibromin/PAF complex and that these genes are normally regulated by murine parafibromin.

DISCUSSION

Our results have demonstrated that *Hrpt2* expression is critical for embryonic development (Table 1 and Fig. 2) and survival in adult mice (Fig. 3) and that disruption of the *Hrpt2* gene results in apoptosis (Fig. 5 and 7) by decreasing the expression of several target genes, including *H19*/*Igf2*, *Igf1*, *Igfbp4*, *Hmgcs2*, *Hmga1*, and *Hmga2* (Fig. 8). *IGF1* and *IGF2* are known to provide trophic support for multiple cell types

and organ cultures derived from various species, to delay the onset of programmed cell death through the mitochondrial pathway, and to antagonize activation of cytotoxic cytokine signaling (22). *Hmga1* and *Hmga2*, which belong to the HMG family that also includes *HMGA1b* (6, 39), bind DNA to build a nuclear scaffold that is critical for the assembly of a stereospecific transcriptional complex (41, 50). This complex has roles in promoting DNA repair and in either inducing cellular apoptosis (39) or inhibiting apoptotic activity as revealed by the interaction between HMGA1 and p53 (35). In addition, mitochondrial *Hmgcs2* is known to encode a key enzyme in the synthesis of ketones, which have a role in energy metabolism (29). A disruption of these mechanisms may therefore contribute to the observed apoptosis in the *Hrpt2*-deficient MEFs (Fig. 6 and 7).

These in vitro observations are supported by our in vivo results, which showed that *Hrpt2* expression is required for growth of the formed fetus and survival of the adult mouse (Fig. 2 and 3). Moreover, significant apoptosis and developmental defects were observed in the E10.5 fetus with a deletion of the *Hrpt2* gene for 48 h (Fig. 2). On the contrary, although deletion of *Hrpt2* for 48 h retarded fetal growth, gross developmental defects were not detected at E12.5 or E14.5. Explanations for these differences include the possibility that Cre recombination efficiency may not be as high in the E10.5 to E14.5 embryos as in the E8.5 to E10.5 embryos and that cells at the later embryonic stages may not be as sensitive to loss of *Hrpt2* expression as at the earlier stages. Nevertheless, *Hrpt2* expression clearly has essential roles in growth and development and in the survival of adult mice, although the organs and cells which are most sensitive to its loss remain to be defined. However, it is important to note that the target genes for *Hrpt2* expression and its encoded protein, parafibromin, include *Igf1*, *Igf2*, *Hmga2*, and *Hmga1* (Fig. 8), and these are involved in growth of embryos and adult mice. Indeed, *Igf1* and *Igf2* knockout mice have been reported to have late embryonic lethality and surviving offspring have a reduced body size that is 60% of their wild-type littermates (9, 10, 23, 24, 38). Furthermore, the surviving offspring from *Igf1* and *Igf2* double knockouts are

FIG. 7. *Hrpt2* loss promotes apoptosis in vitro and in vivo. A) Equal numbers of control MEFs and *Hrpt2*-deficient MEFs were plated and stained (left panel) using TUNEL and DAPI on day 3 after deletion of the *Hrpt2* gene. There were more cells with TUNEL staining among the *Hrpt2*-deficient MEFs (right panel) than among control MEFs ($P < 0.05$). B) *Hrpt2*-deficient MEFs had more annexin V-positive cells than did control MEFs at days 2 (D2) and 3 (D3) after deletion of the *Hrpt2* gene. C) Whole-cell lysates were isolated from control MEFs and *Hrpt2*-deficient MEFs at days 2, 3, 4, and 5 after deletion of the *Hrpt2* gene. Compared to control MEFs, there was no change of total caspase 3, but the cleaved caspase 3 was increased in *Hrpt2*-deficient MEFs. There was no conversion of LC-3 I to LC-3 II, but the expression of LC-3 I was decreased in *Hrpt2*-deficient MEFs. There was no increase of HMGB1/B2 in the supernatant obtained from the *Hrpt2*-deficient MEFs compared to the control MEFs. All experiments were repeated three times. D) BrdU incorporation and TUNEL and H&E staining of E10.5 Cre-ER/*Hrpt2*^{+/+} and Cre-ER/*Hrpt2*^{L/L} embryos, obtained from tamoxifen-treated pregnant mice at 8.5 dpc. Cre-ER/*Hrpt2*^{L/L} embryos had increased cell death (H&E), less BrdU incorporation, and significantly more cells with positive TUNEL staining than the control Cre-ER/*Hrpt2*^{+/+} embryos.

FIG. 8. Confirmation of altered gene expression by semiquantitative RT-PCR and CHIP assays. A) Total RNA was extracted from control MEFs and *Hrpt2*-deficient MEFs at day 4 after deletion of the *Hrpt2* gene and semiquantitative RT-PCR was performed. *CD24*, *CK*, *CXCL7*, *HSP1*, and *MnK2* were up-regulated, whereas *H19*, *Igf1*, *Igf2*, *Igfbp4*, *Hmgs2*, *Stat2*, *Hmga2*, *Hmga1*, *Crb2*, *Clu*, *Bst2*, *Clca1*, and *Hrpt2* were down-regulated in *Hrpt2*-deficient MEFs compared to control MEFs. GAPDH was used as the loading control. Experiments were repeated three times. B) The CHIP assay was performed using normal MEFs and anti-Paf1 and antiparafibromin polyclonal antibodies. Rabbit immunoglobulin G (IgG) was used as the negative control. Parafibromin and Paf1p bound to the promoter and/or open reading frame (ORF) and/or the 3' untranslated region (3'UTR) of several genes, including *Igf2*, *H19*, *Hmgs2*, *Igf1*, *Hmga1*, *Hmga2*, and *Igfbp4*. C) The CHIP assay was also performed using Ad-GFP/*Hrpt2*^{L/L} MEFs and Ad-Cre/*Hrpt2*^{L/L} MEFs and the antiparafibromin polyclonal antibody. Real-time PCR was performed to assess for quantitative differences in the expression levels of *H19*, *Igf2*, and *Igf1* in Ad-GFP/*Hrpt2*^{L/L} MEFs and Ad-Cre/*Hrpt2*^{L/L} MEFs.

even smaller, with 30% of the body size of the wild-type littermates (15). *Hmga2* knockout mice also have a smaller body size that is associated with a reduction in adipose synthesis (56), while *Hmga1* knockout mice develop insulin resistance and diabetes mellitus (14). Thus, it is likely that the observed rapid death of adult *Hrpt2*-deficient mice (Fig. 3) may be related to a decreased expression of *Igf1*, *Igf2*, *Hmga2*, and *Hmga1*.

The balance of proapoptotic and antiapoptotic signals is likely to have a major role in contributing to the observed embryonic lethality of *Hrpt2*^{-/-} embryos before E6.5. This lethality is likely to occur at hatching (E4.5) or implantation (E4.5 to E5.5). *Hrpt2*^{-/-} blastocysts were normal at E3.5 (Fig. 1A), although they could not proliferate into ICM and TE cells in vitro (Fig. 2A) and died 5.5 to 6.5 dpc. Cell death normally occurs in both the ICM and TE lineages at the blastocyst stage (16), and the cell death index has been reported to be approximately 15% of total cells (19). The balance between anti- and proapoptotic factors is crucial for preimplantation development, and a death-by-default mechanism may force cells into committing suicide upon the withdrawal or nonsynthesis of critical survival factors (32). Indeed, the supplementation of culture medium with growth factors has been shown to increase rates of blastocyst formation that are accompanied by elevated numbers of cells, which likely result from a suppression of cell death (4, 5), whereas the addition of IGF-1 to culture medium results in a significant decrease in the number of apoptotic cells (25, 47). Hence, our results, which have demonstrated that loss of *Hrpt2* expression and parafibromin is associated with an induction of apoptosis, provide a likely mechanistic insight into the cause of *Hrpt2*^{-/-} embryonic lethality at the hatching or implantation stage. Thus, our findings demonstrate an important role of *Hrpt2* and its encoded protein, parafibromin, in the transcriptional regulation of growth factors and in mammalian development and survival in adults.

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