See related Commentary on page xii

Homozygous and Compound Heterozygous Mutations in *ZMPSTE24* Cause the Laminopathy Restrictive Dermopathy

Casey L. Moulson,* Gloriosa Go,* Jennifer M. Gardner,† Allard C. van der Wal,‡ J. Henk Sillevis Smitt,§ Johanna M. van Hagen,¶ and Jeffrey H. Miner*#

*Department of Internal Medicine, Renal Division and †Department of Genetics, Washington University School of Medicine, St Louis, Missouri, USA; ‡Department of Cardiovascular Pathology and §Department of Dermatology, Academic Medical Center, Amsterdam, The Netherlands; ¶Department of Clinical Genetics and Human Genetics, VU University Medical Center, Amsterdam, The Netherlands; #Department of Cell Biology and Physiology, Washington University School of Medicine, St Louis, Missouri, USA

Restrictive dermopathy (RD) is a lethal human genetic disorder characterized by very tight, thin, easily eroded skin, rocker bottom feet, and joint contractures. This disease was recently reported to be associated with a single heterozygous mutation in *ZMPSTE24* and hypothesized to be a digenic disorder (Navarro *et al*, Lamin A and ZMPSTE24 (FACE-1) defects cause nuclear disorganization and identify restrictive dermopathy as a lethal neonatal laminopathy. Hum Mol Genet 13:2493–2503, 2004). *ZMPSTE24* encodes an enzyme necessary for the correct processing and maturation of lamin A, an intermediate filament component of the nuclear envelope. Here we present four unrelated patients with homozygous mutations in *ZMPSTE24* and a fifth patient with compound heterozygous mutations in *ZMPSTE24*. Two of the three different mutations we found are novel, and all are single base insertions that result in messenger RNA frameshifts. As a consequence of the presumed lack of ZMPSTE24 activity, prelamin A, the unprocessed toxic form of lamin A, was detected in the nuclei of both cultured cells and tissue from RD patients, but not in control nuclei. Abnormally aggregated lamin A/C was also observed. These results indicate that RD is an autosomal recessive laminopathy caused by inactivating *ZMPSTE24* mutations that result in defective processing and nuclear accumulation of prelamin A.

Key words: FATP4 protein/lamin A/nuclear envelope/STE24 protein
J Invest Dermatol 125:913–919, 2005

Restrictive dermopathy (RD), also known as lethal tight skin contracture syndrome (MIM 275210), is a rare genetic disorder that results in death, usually within several hours or days of birth. Babies with RD have tight, translucent, partially eroded skin, joint contractures, rocker bottom feet, and distinctive craniofacial abnormalities that typically include micrognathia, a facial expression with the mouth fixed in an "O" position, a small pinched nose, and low-set ears (Welsh *et al*, 1992; Mau *et al*, 1997; Sillevis Smitt *et al*, 1998). In most patients, death results from respiratory distress as a result of severely restricted movements.

Of the few RD cases reported in the literature, many arose in children of consanguineous parents, and the disease recurs within families; its mode of inheritance had therefore been presumed to be autosomal recessive (Welsh *et al*, 1992; Sillevis Smitt *et al*, 1998). Recently, however, Navarro *et al* (2004) used a candidate gene approach to identify a single inactivating heterozygous mutation in *ZMPSTE24* (c.1085dupT) associated with RD in six unrelated, nonconsanguineous families of French and Dutch heritage.

Abbreviations: DHPLC, denaturing high-performance liquid chromatography; FACE, farnesyiated protein-converting enzyme 1; mRNA, messenger RNA; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RD, restrictive dermopathy; SNP, single nucleotide polymorphism

ZMPSTE24 encodes a zinc metalloproteinase required for lamin A maturation (Bergo *et al*, 2002; Pendas *et al*, 2002), suggesting that RD is a laminopathy that affects the structure of the nucleus. Because only one allele was found to be mutated in each affected individual, and the parents carrying that allele were normal, the authors hypothesized a digenic mode of inheritance for RD (Navarro *et al*, 2004). Contrary to this, we have identified four patients with three different homozygous mutations and one patient with compound heterozygous mutations in *ZMPSTE24* that are associated with RD, providing strong support for a simple autosomal recessive mode of inheritance. We also show that in two patients with different homozygous mutations, there are nuclear abnormalities associated with accumulation of unprocessed prelamin A, thereby providing novel evidence that RD is a laminopathy.

Results

Identification of *ZMPSTE24* mutations associated with RD We became interested in RD upon discovering a spontaneous, autosomal recessive mouse mutation that we named *wrinkle free* (*wrfr*). *wrfr* −/− mice have very tight, thick skin, and their mouths are fixed in an open position (Moulson *et al*, 2003). They die within 24 h of birth from an inability to breathe properly or to suckle, because of

severely restricted movements. The overall phenotype is thus reminiscent of RD. Positional cloning of *wrfr* revealed a retrotransposon insertion in *Slc27a4*, which encodes fatty acid transport protein 4 (Moulson *et al*, 2003).

In an effort to determine whether *SLC27A4* plays a role in human RD, we obtained blood and/or tissues from members of six RD kindreds, three of which were known or suspected to involve consanguinity. DNA was prepared from individuals with RD, all of whom died within hours or days of birth and exhibited the classic features described by Witt (Witt *et al*, 1986). DNA was also prepared from parents and any unaffected full siblings when available. We sequenced *SLC27A4* exons and intron–exon junctions in three unrelated patients but found no mutations. To formally rule out *SLC27A4* as a candidate, and to identify potential candidate regions, we performed genome-wide single nucleotide polymorphism (SNP) genotyping using Affymetrix GeneChip Mapping arrays (Matsuzaki *et al*, 2004) on a small but potentially informative subset of these DNA (data not shown). Using DNA-Chip (dCHIP) software (Lin *et al*, 2004) to display the results, and assuming an autosomal recessive mode of inheritance, we identified a 15 Mb locus on chromosome 1p32–35 as a major RD candidate region, whereas the *SLC27A4* locus at 9q34 was ruled out. Consistent with the report of Navarro *et al*, 1p32–35 contains *ZMPSTE24*. Therefore, we sequenced *ZMPSTE24* exons and flanking intronic sequences amplified by polymerase chain reaction (PCR) from RD family members' DNA to search for mutations and to determine their association with RD.

A consanguineous Dutch family had one child affected with RD and five unaffected children. We identified in this family the same c.1085dupT insertion reported by Navarro *et al* (2004) (Fig 1*A* and *B*). In contrast to the findings of Navarro *et al*, however, here the mutation was homozygous in the affected patient, whereas both parents were heterozygous (Fig 1*A* and *B*). DNA was also available from four of the five unaffected siblings; three were heterozygous for the insertion, and one did not carry it. We found this same mutation in a nonconsanguineous American family of German ancestry (Fig 1*C*). The mutation was homozygous in one affected child, and both parents and one brother were heterozygous; the other brother did not carry the mutation. No DNA from the second affected child was available. In this family, the apparently identical mutations were linked to different genotypes at a novel SNP in *ZMPSTE24* intron 4 (data not shown), suggesting that the mutations arose independently.

Analysis of *ZMPSTE24* in a consanguineous Guatemalan family with two affected children revealed a novel single base duplication in exon 5 (c.591dupT) (Fig 1*D* and *E*). This mutation was homozygous in one affected child and heterozygous in both parents (Fig 1*D* and *E*). DNA from the second child was not available.

A novel single base duplication was present in *ZMPSTE24* exon 1 (c.54dupT) in a Mennonite kindred with five affected children out of six born to two families (Fig 1*F* and *G*). DNA was only available from one affected child; the mutation was homozygous in her and heterozygous in both her parents. The other parents and their unaffected son were heterozygous for the same mutation. RD has been reported in a Mennonite kindred before (Lowry *et al*, 1985), suggesting that this unique mutation is segregating in the Mennonite population.

We also assayed DNA extracted from archived paraffin-embedded tissues taken from a Dutch RD patient. Two different heterozygous mutations were detected: the exon 5 mutation found in the Guatemalan family (c.591dupT) and the previously identified exon 9 insertion (c.1085dupT). We conclude that this patient is compound heterozygous, although we cannot formally prove it because parental DNA are not available.

Might these mutations actually be common polymorphisms? The c.1085dupT mutation in exon 9 has been previously reported, and it was not found to be present on 300 control chromosomes (Agarwal *et al*, 2003; Navarro *et al*, 2004). Denaturing high-performance liquid chromatography (DHPLC) analysis of exons 1 and 5 amplified by PCR from 50 CEPH grandparent DNA showed no evidence of the c.54dupT and c.591dupT mutations, respectively (data not shown). Searches of the Celera Discovery System (http://myscience.appliedbiosystems.com/) and public SNP (http://www.ncbi.nlm.nih.gov/SNP/) databases did not reveal any insertions in the *ZMPSTE24* coding region, although >70 SNP have been identified in the gene. Together with the fact that all three sequence variants shift the messenger RNA (mRNA) reading frame, these findings suggest that they are genuine pathogenic mutations.

Consequences of the mutations on expression of *ZMPSTE24* All three mutations are single base duplications in *ZMPSTE24* coding exons. They are therefore frameshift mutations that would be expected to lead to production of truncated versions of ZMPSTE24, which normally consists of 475 amino acids. c.54dupT would encode p.Ile19-TyrfsX28; c.591dupT would encode p.Ile198TyrfsX20; and c.1085dupT would encode p.Leu362PhefsX19, according to standard nomenclature (den Dunnen and Antonarakis, 2000; see also http://www.genomic.unimelb.edu.au/mdi/mutnomen/recs.html). These truncated proteins would be expected to have little if any normal function, as ZMPSTE24 is conserved in terms of both sequence and enzymatic activity from yeast to humans. Indeed, human ZMPSTE24 can complement the yeast *ste24* mutation (Schmidt *et al*, 2000), but p.Leu362PhefsX19, which is encoded by the c.1085dupT mutation, is noncomplementing (Agarwal *et al*, 2003).

Aside from encoding truncated proteins, the mutant transcripts might also be subject to nonsense-mediated mRNA decay (NMD) (Hentze and Kulozik, 1999), which could preclude synthesis of any truncated ZMPSTE24 proteins. To assay for NMD, we analyzed RNA isolated from fibroblasts derived from the American patient carrying the c.1085dupT mutation and from a control. Northern blotting did not reveal a significant decrease in ZMPSTE24 mRNA levels in the RD fibroblasts (data not shown), suggesting that NMD was not a major factor. One possibility to explain this is that exon 9 containing the frameshift might be skipped, leading to an in-frame splice from exon 8 to exon 10 and elimination of the premature stop codon. But reverse transcriptase-polymerase chain reaction (RT-PCR) analysis did not reveal any evidence for skipping of exon 9 (Fig 2*A*).

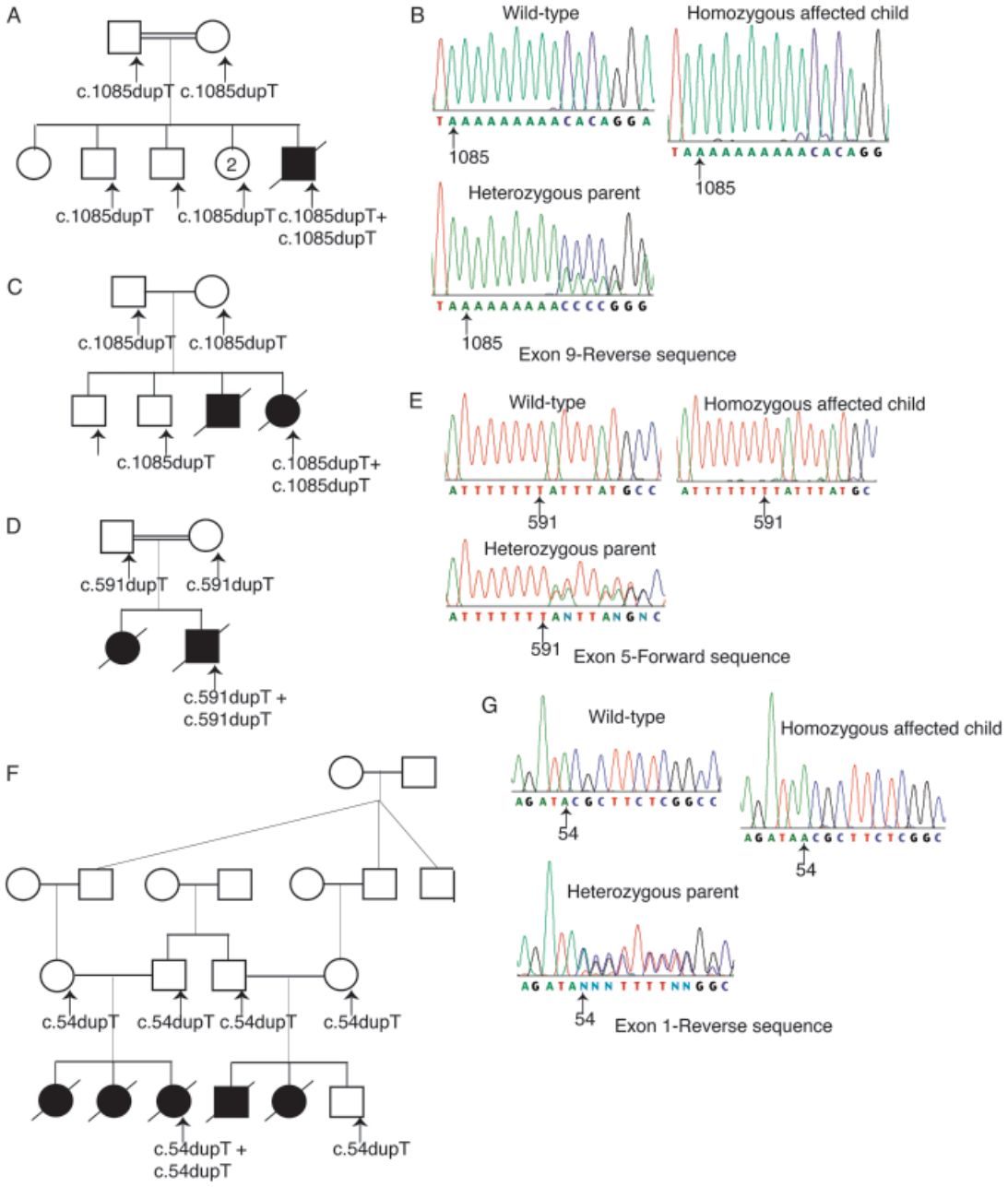

Figure 1
Family pedigrees, *ZMPSTE24* genotypes, and sequence chromatograms. Arrows indicate individuals whose DNA was tested. *ZMPSTE24* mutations are shown below the pedigree symbols; all are hypothesized to cause loss of activity. (*A*) Pedigree of a consanguineous Dutch family; the father's maternal grandmother and the mother's paternal grandmother were sisters. The affected child had a homozygous thymidine duplication in *ZMPSTE24* exon 9. (*B*) Sequence chromatograms of exon 9 (reverse direction) show a duplicated thymidine in a stretch of nine. Overlap of the normal and mutated strand sequences occurred after the insertion in this and in all the other heterozygous samples described. (*C*) Pedigree of a nonconsanguineous American family carrying the same mutation as the Dutch family. (*D*) Pedigree from a consanguineous Guatemalan family; the father's paternal grandfather was a paternal uncle of the mother. (*E*) Sequence chromatograms of exon 5 (forward direction) show a duplicated thymidine in a stretch of seven. (*F*) Five affected children were born to two pairs of related parents from a Mennonite kindred; the fathers were brothers of each other, and the mothers were first cousins of each other. (*G*) Sequence chromatograms of *ZMPSTE24* exon 1 (reverse direction) show an extra thymidine homozygous in the affected child.

RNA was also prepared from the liver of the Guatemalan patient, but it was highly degraded and not suitable for northern blotting. Nevertheless, RT-PCR analysis revealed that there was no in-frame skipping of the mutated exon 5 (Fig 2*A*), which could have led to production of a protein. Consistent with these RT-PCR data, western blotting with a ZMPSTE24 COOH-terminal antibody detected little if any ZMPSTE24 in patient fibroblasts or liver, but an appropriate ~50 kDa band was observed in controls (Fig 2*B*).

Consequences of the mutations on localization of nuclear envelope proteins The only known substrate for ZMPSTE24 is lamin A, encoded by the *LMNA* gene. This gene also encodes lamin C, a shorter form that is translated

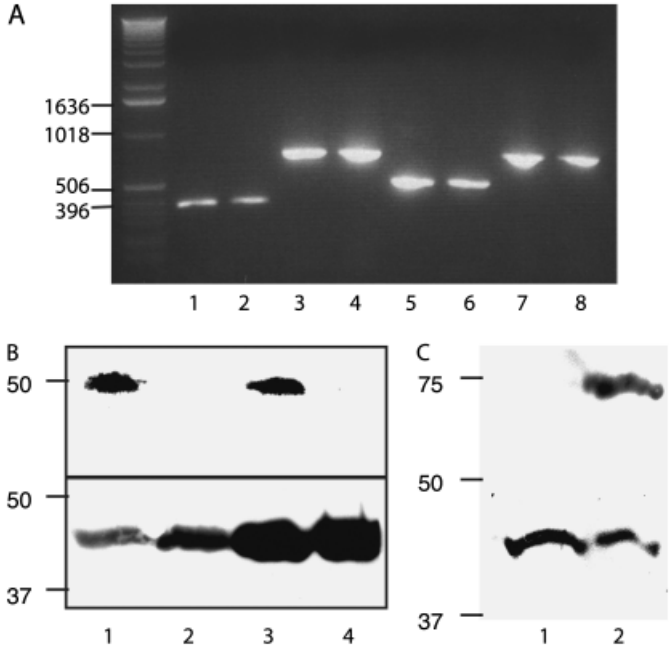

Figure 2
Analysis of ZMPSTE24 messenger RNA (mRNA) and protein in control and restrictive dermopathy (RD) samples. (*A*) Reverse transcriptase polymerase chain reaction analysis of total RNA from liver and fibroblasts reveals no evidence of splicing around the exons containing the c.591dupT (exon 5; *lanes 1* and *2*) and c.1085dupT (exon 9; *lanes 5* and *6*) mutations. Glyceraldehyde-3-phosphate dehydrogenase primers were used as a control in *lanes 3, 4, 7*, and *8*. Control fetal liver, *lanes 1* and *3*; c.591dupT liver, *lanes 2* and *4*; control fibroblasts, *lanes 5* and *7*; c.1085dupT fibroblasts, *lanes 6* and *8*. (*B*) Western blot analysis of ZMPSTE24 (*top*) and β-actin (*bottom*). ZMPSTE24 was detected in control but not RD extracts; the same blot reprobed for β-actin shows protein in all lanes. *Lane 1*, control fetal liver; *Lane 2*, RD liver (Guatemalan patient); *Lane 3*, control fibroblasts; *Lane 4*, RD fibroblasts (American patient). (*C*) Western blot analysis of prelamin A and β-actin. Prelamin A (~ 70 kDa) was detected in liver from the RD patient (*lane 2*) but not from the control (*lane 1*). The blot was reprobed for β-actin to show that protein was present in both lanes.

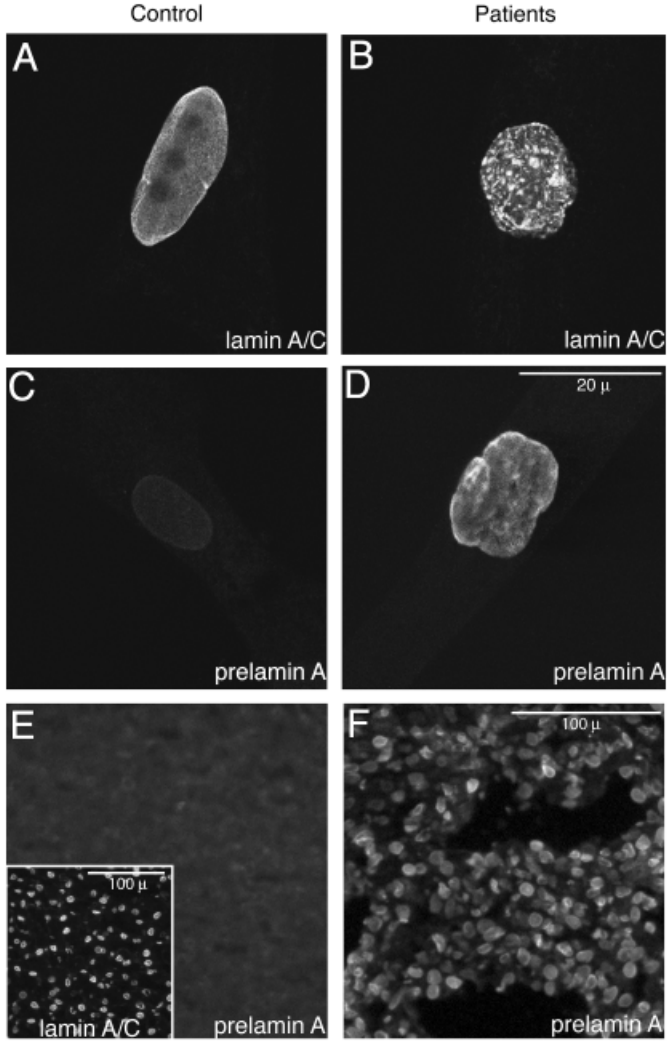

Figure 3
Immunofluorescent localization of lamin A/C and prelamin A in control and restrictive dermopathy (RD) samples. The organization of lamins in fibroblast nuclear envelopes is disrupted by accumulation of prelamin A in the absence of ZMPSTE24. Confocal micrographs show localization of lamins A and C in nuclei of a control fibroblast (*A*) and a fibroblast from the American RD patient (*B*). Prelamin A was undetectable in control fibroblasts (*C*), but there was nuclear accumulation in RD patient fibroblasts (*D*). In liver sections, prelamin A was undetectable in the control (*E*) but clearly present in RD patient nuclei (*F*). The control section did contain nuclei, which are stained for lamin A/C (inset in *E*).

from an alternatively spliced mRNA. Lamin A maturation requires ZMPSTE24 activity, but lamin C maturation does not. Lamin A/C distribution is abnormal in RD patients reported to have a heterozygous c.1085dupT mutation in *ZMPSTE24* (Navarro *et al*, 2004). Because the c.1085dupT mutation in the patient from whom we obtained fibroblasts was homozygous, we examined whether there was aberrant distribution of lamin A/C using an antibody that recognizes both (Fig 3*A*, *B*). Lamin A/C appeared to be distributed in clusters in the nucleus, as opposed to the homogeneous, smooth, uniform distribution in nuclei from normal fibroblast cultures.

Accumulation of unprocessed prelamin A, which is toxic (Fong *et al*, 2004), has been reported in *Zmpste24* knockout mice (Bergo *et al*, 2002; Pendas *et al*, 2002). To examine whether there was accumulation of prelamin A in RD patients as well, cultured fibroblasts were stained with a prelamin A-specific antibody. Strong prelamin A staining was observed in RD fibroblast nuclei (Fig 3*D*), whereas no prelamin A was detected in control fibroblasts (Fig 3*C*). Nuclear prelamin A was also observed in frozen liver sections from the Guatemalan patient (Fig 3*F*) but not in liver from a control, although lamin A/C was clearly present (Fig 3*E*). The presence of prelamin A in patient but not control liver was confirmed by western blotting (Fig 2*C*). We conclude that RD is associated with a specific defect in prelamin A processing caused by the absence of functional ZMPSTE24.

Discussion

In this study, we identified two novel mutations and a third previously described mutation in *ZMPSTE24* that are associated with RD. Importantly, we also show that RD is a simple autosomal recessive disorder. Though *ZMPSTE24* had already been implicated in RD, only one heterozygous mutation was identified, and it was also present in unaffected family members, leading to the conclusion that RD

was a digenic disorder (Navarro *et al*, 2004). Rather than there being a digenic mode of inheritance, we favor the possibility that there exist *ZMPSTE24* mutations that are difficult to detect by conventional PCR-based methods. This is perhaps the most straightforward explanation for how there could be an absence of mature lamin A even with one apparently wild-type copy of *ZMPSTE24*. Such mutations could lie in the gene's promoter, or could involve segmental deletions that are complemented—only as far as PCR is concerned—by normal regions of the other allele. Indeed, we could not find any mutations in a pair of affected fraternal twins from Finland, though SNP genotyping showed that the twins share genotypes on the segment of chromosome 1 containing the *ZMPSTE24* locus (data not shown).

All mutations identified thus far to cause RD are single base duplications that result in mRNA frameshifts, which likely prevent production of a functional protein. One missense *ZMPSTE24* mutation (c.1018T>C; p.Trp340Arg) has been found, however, in a Belgian patient with severe mandibuloacral dysplasia with type B lipodystrophy (MADB—MIM 608612) (Agarwal *et al*, 2003). This patient was actually compound heterozygous; the other allele bore the same c.1085dupT-inactivating mutation that Navarro *et al* (2004) and we have subsequently found to be associated with RD. The mutant protein carrying the p.Trp340Arg substitution is partially active in yeast complementation assays, whereas p.Leu362PhefsX19, which is encoded by c.1085dupT, is totally inactive (Agarwal *et al*, 2003). Thus, the limited information available regarding *ZMPSTE24* mutations suggests that inactivating mutations cause RD, whereas partial loss of function mutations are associated with less severe disease, as is MADB. Identification of mutations in additional patients will help determine whether a definitive genotype/phenotype correlation exists.

Accumulation of prelamin A has been reported in *Zmpste24* knockout mice (Bergo *et al*, 2002; Pendas *et al*, 2002) and in cultured cells from human patients with Hutchinson–Gilford progeria syndrome (HGPS) (MIM 176670) (Goldman *et al*, 2004); its presence has also been hypothesized but not proved in other human laminopathies (Agarwal *et al*, 2003; Navarro *et al*, 2004). Our study demonstrates accumulation of prelamin A in affected human tissue *in situ* (Fig 3*F*), suggesting that it is not merely a consequence of cell culture. Accumulation of prelamin A has recently been shown to be toxic; disease phenotypes in *Zmpste24* $-/-$ mice are "rescued" by only a 50% reduction in the amount of prelamin A, via knockout of one *Lmna* allele (Fong *et al*, 2004). The presence of large amounts of prelamin A may also account for the increased severity of RD as compared with laminopathies with either lamin A mutations or a missense mutation in *ZMPSTE24*. Given the lack of detectable nuclear prelamin A in normal cells, it is possible that a simple immunofluorescence assay for prelamin A in amniocyte and/or chorionic villi cell nuclei, in conjunction with DNA analysis, might serve as a definitive prenatal test for RD and perhaps for other laminopathies. Preimplantation genetic diagnosis (Sermon *et al*, 2004) should also be possible. Further studies will be necessary to determine the feasibility of these approaches.

We found lamins A and C clustered and distributed in aggregates in the nuclei of RD patient fibroblasts, whereas normal fibroblast nuclei showed a homogeneous and smooth, even distribution, concentrated at the nuclear periphery. Abnormal distribution of lamins A and C in foci, aggregations, and honeycombs has been reported in a number of human patients carrying lamin A/C gene mutations (Novelli *et al*, 2002; Capanni *et al*, 2003; Caux *et al*, 2003; Muchir *et al*, 2004) as well as in cells transfected with lamin A/C mutants (Ostlund *et al*, 2001; Holt *et al*, 2003).

The mechanism whereby mutations in *LMNA* and *ZMPSTE24* that result in aberrant lamin A processing cause disease has not been determined. Several lines of evidence suggest that it is likely to be a combination of impaired nuclear stability and the resulting nuclear deformations (Lammerding *et al*, 2004) causing secondary alterations in heterochromatin localization and significant changes in gene expression (Nikolova *et al*, 2004). (For a review of these hypotheses, see reference Worman and Courvalin (2004).) In addition, lamin A binds directly or indirectly to a number of nuclear proteins, including emerin (Lee *et al*, 2001; Sakaki *et al*, 2001) and nesprin (Mislow *et al*, 2002), and attach them to the nuclear envelope. Lamin A also binds to transcription factors such as MOK2 (Dreuillet *et al*, 2002), retinoblastoma (Mancini *et al*, 1994), and SREBP1 (Lloyd *et al*, 2002). (See (Zastrow *et al*, 2004) for a review of lamin-binding proteins.) Mislocalization of lamin A, as we and others have observed, is likely indicative that some of its binding partners are also mislocalized and not bound to the nuclear envelope, which may have profound secondary effects on cells.

Interestingly, the neonatally lethal wrinkle-free phenotype of *Slc27a4* $-/-$ mice that we and others have reported (Herrmann *et al*, 2003; Moulson *et al*, 2003) appears to be much more similar to the human RD phenotype than that exhibited by *Zmpste24* $-/-$ mice, which survive for several months. This may be coincidence, but the possibility exists that there is some special mechanistic relationship in humans between nuclear architecture and expression of genes involved in fatty acid homeostasis. Indeed, one of the features of the diverse laminopathies that are less severe than RD is lipodystrophy.

Genetic and allelic heterogeneity appear to explain in part the phenotypic differences among the related laminopathies HGPS, MAD, and most recently, RD. Navarro *et al* included in their study two patients several months old with a skin disease less severe than typical RD, and they found one novel and one previously reported mutation in the *LMNA* gene. These are both likely to be sporadic, heterozygous, dominant-negative mutations. The latter mutation had been found previously in several patients with HGPS. There is clearly a marked difference in disease severity between *ZMPSTE24*-based neonatally lethal RD and *LMNA*-based progeria. To avoid confusion, we propose that the descriptor "restrictive dermopathy" be reserved for individuals with the severe, neonatally lethal disease described by Witt (Witt *et al*, 1986). Children with some RD-like features who survive well past the neonatal period are more likely to manifest features of progeria or MAD and should be described as such. The ability to screen for *ZMPSTE24* and *LMNA* mutations may make these distinctions possible at the molecular level.

Materials and Methods

Amplification of genomic DNA, DNA sequencing, and DHPLC The Washington University School of Medicine Human Studies Committee approved this study, which was conducted according to the Declaration of Helsinki principles. Informed parental consent was obtained in all cases, except for one that involved archived tissues; for this a waiver of consent was approved. Genomic DNA was extracted from the blood of six affected individuals from five different kindreds and from their parents and available unaffected siblings using QiaAMP DNA Blood Kit (Qiagen Inc., Valencia, California). DNA was extracted from paraffin-embedded tissues from a seventh affected individual as described (Coombs *et al*, 1999). Exons and intron–exon junctions from genomic DNA samples were amplified using KlentaqLA (BD Biosciences Clontech, Palo Alto, California). Each 20 μL reaction contained 1 × KLA (KlentaqLA) buffer, 125 μM dNTP (deoxribonucleotide triphosphates) 2.5 mM $MgCl_2$, 10 pmoles of each primer, 20 ng genomic DNA, and 1 U KlentaqLA. Cycling conditions were a 3 min initial denaturation at 95°C, followed by 35 cycles of 30 s at 94°C, 1 min at 57°C, and 3 min at 70°C. PCR products were gel purified and recovered using the Wizard SV Gel Clean-up System (Promega, Madison, Wisconsin). The Washington University Protein and Nucleic Acid Chemistry Lab performed automated sequencing reactions using internal primers with Big-Dye Terminator 3.1 and an Applied Biosystems 3730 DNA sequencer (Applied Biosystems, Foster City, California). The PCR and sequencing primer sequences used are shown in Table SI.

For DHPLC, exons 1 and 5 were amplified from 50 CEPH individuals and from heterozygous parents as described above. Following denaturation and slow reannealing, amplicons were subjected to DHPLC analysis on a Transgenomic WAVE System containing a DNASepHT column (Transgenomic, Omaha, Nebraska) in a linear acetonitrile gradient.

RNA purification and RT-PCR Total RNA was isolated from liver homogenized in Tri-Reagent (Molecular Research Center, Cincinnati, Ohio) and from fibroblasts scraped into Tri-Reagent according to the manufacturer's instructions. 0.5 μg of total RNA was reverse transcribed using Superscript III (Invitrogen, Carlsbad, California) with oligo$(dT)_{12–18}$ primers in a 20 μL reaction. Touchdown PCR was performed in a 20 μL reaction using 1 μL of complementary DNA. PCR reactions contained manufacturer-supplied 1 × reaction buffer, 2.5 mM magnesium chloride, 0.125 mM of each dNTP, 10 pmoles of each primer, and 0.5 U Biolase DNA polymerase (MidSci, St Louis, Missouri). Primers are shown in Table SII.

Western blotting Proteins were extracted from liver by sonication in 8 M urea, 10 mM tris pH 8.0, 1 mM ethylenediaminetetraacetic acid, and Complete Protease Inhibitors (Roche Diagnostics, Indianapolis, Indiana). Fibroblasts were scraped into cold phosphate-buffered saline (PBS) containing protease inhibitors, pelleted, resuspended in the urea buffer, and sonicated. Proteins were separated on 8% sodium dodecyl sulfate-polyacrylamide gels and blotted on to nitrocellulose. Blots were incubated in 5% nonfat dry milk in TBST (Tris-buffered saline/0.02% Tween-20) for 1 h prior to incubation with primary antibodies overnight at 4°C. Primary antibodies were goat anti-human prelamin A (sc-6214; Santa Cruz Biotechnology, Santa Cruz, California), rabbit anti-human ZMPSTE24 (AP2415b; Abgent, San Diego, California), and rabbit anti-human β-actin (4967; Cell Signaling Technology, Beverly, Massachusetts). The secondary antibody for β-actin was conjugated to horseradish peroxidase, whereas secondary antibodies for prelamin A and ZMPSTE24 were detected with avidin–biotin and horseradish peroxidase. Visualization was by enhanced chemiluminescence (Amersham, Arlington Heighst, Illinois).

Cell culture and immunofluorescence Skin fibroblasts from the American RD patient and normal skin fibroblasts (Detroit 551 from the American Type Culture Collection, Manassas, Virginia) were cultured in Earle's Minimal Essential medium modified to contain 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g per liter sodium bicarbonate, and 10% fetal bovine serum. For immunostaining, cells were cultured for several days on glass Falcon BioCoat CultureSlides (BD Biosciences Discovery Labware, Bedford, Massachusetts). Cells were fixed for 10 min at 4° C in 2% fresh paraformaldehyde in PBS, washed in PBS, permeabilized for 10 min in cold methanol, and air-dried. Liver tissue from the Guatemalan RD patient and from a control were frozen in OCT, sectioned on a cryostat, air-dried for 30 min, and fixed and permeabilized as described for cultured cells.

For immunofluorescence, samples were blocked in either 4% bovine serum albumin (BSA) in PBS or 1% heat-inactivated normal goat serum in PBS. Antibodies were as follows: anti-lamins A and C (MAB3538) and fluorescein isothiocyanate-conjugated anti-goat immunoglobulin G (IgG) from Chemicon (Temecula, California); goat anti-prelamin A; and Alexa 488-conjugated anti-mouse IgG1 from Molecular Probes (Eugene, Oregon). All antibody dilutions were in PBS containing 1% BSA, and washes were in PBS. Following primary and secondary antibody incubations at room temperature for 1 h each and washing, slides were mounted in 1 mg per mL *p*-phenylenediamine/0.1 × PBS/90% glycerol.

Confocal microscope images were obtained using a Zeiss compound microscope (Carl Zeiss International, Jena, Germany) with a BioRad MRC 1024 confocal adaptor (BioRad Laboratories, Inc., Hercules, California). All other micrographs were obtained with a Spot 2 cooled color digital camera (Diagnostic Instruments, Sterling Heights, Michigan) attached to a Nikon Eclipse E800 compound microscope (Nikon USA, Melville, New York). Images were imported into Adobe Photoshop 5 and Adobe Illustrator 9 for processing and layout.

We are extremely grateful to the families who participated in this study and to the referring physicians, scientists, and genetic counselors for their invaluable contributions. We thank The Laboratory of Developmental Biology at the Univerisity of Washington in Seattle, supported by National Institutes of Health grant R24HD000836, for supplying human fetal liver tissue, and Edward Fox and the Dana-Farber Cancer Institute Microarray Core for performing SNP genotyping; the Washington University School of Medicine Division of Human Genetics Genotyping Core for facilitating DHPLC analysis; Martin Pollak and Alan Beggs for advice; Phillip Stahl and Didier Hodzic for use of the confocal microscope; and Paul Goodfellow for helpful suggestions and comments on the manuscript. This work was funded by National Institutes of Health grant R01AR049269 to JHM.

Supplementary Material

The following material is available online for this article.
Table S1 Primers used to amplify and sequence *ZMPSTE24* exons and flanking itronic sequences.
Table S2 Primers used for RT-PCR.

DOI: 10.1111/j.0022-202X.2005.23846.x

Manuscript received February 4, 2005; revised April 11, 2005; accepted for publication May 3, 2005

After this paper was accepted, we found a novel *ZMPSTE24* nonsense mutation, c.691G>T, predicted to encode p.Glu231X, in both parents of an affected child from southern India. DNA from the child was not available, but we consider this to be a pathogenic mutation.
Navarro et al. recently published a paper (Loss of ZMPSTE24 (FACE-1) causes autosomal recessive restrictive dermopathy and accumulation of Lamin A precursors. Hum Mol. Genet 14:1503–1513, 2005) showing that both alleles of ZMPSTE24 are in fact mutated in their patients with restrictive dermopathy; these patients were previously reported to carry only heterozygous mutations. Based on these new findings, they have withdrawn their hypothesis of digenic inheritance.

Address correspondence to: Dr Jeffrey H. Miner, Renal Division, Box 8126, Washington University School of Medicine, 660 S. Euclid Ave., St Louis, Missouri 63110, USA. Email: minerj@wustl.edu

References

Agarwal AK, Fryns JP, Auchus RJ, Garg A: Zinc metalloproteinase, ZMPSTE24, is mutated in mandibuloacral dysplasia. Hum Mol Genet 12:1995–2001, 2003

Bergo MO, Gavino B, Ross J, *et al*: Zmpste24 deficiency in mice causes spontaneous bone fractures, muscle weakness, and a prelamin A processing defect. Proc Natl Acad Sci USA 99:13049–13054, 2002

Capanni C, Cenni V, Mattioli E, *et al*: Failure of lamin A/C to functionally assemble in R482L mutated familial partial lipodystrophy fibroblasts: Altered intermolecular interaction with emerin and implications for gene transcription. Exp Cell Res 291:122–134, 2003

Caux F, Dubosclard E, Lascols O, *et al*: A new clinical condition linked to a novel mutation in lamins A and C with generalized lipoatrophy, insulin-resistant diabetes, disseminated leukomelanodermic papules, liver steatosis, and cardiomyopathy. J Clin Endocrinol Metab 88:1006–1013, 2003

Coombs NJ, Gough AC, Primrose JN: Optimisation of DNA and RNA extraction from archival formalin-fixed tissue. Nucleic Acids Res 27:e12, 1999

den Dunnen JT, Antonarakis SE: Mutation nomenclature extensions and suggestions to describe complex mutations: A discussion. Hum Mutat 15:7–12, 2000

Dreuillet C, Tillit J, Kress M, Ernoult-Lange M: *In vivo* and *in vitro* interaction between human transcription factor MOK2 and nuclear lamin A/C. Nucleic Acids Res 30:4634–4642, 2002

Fong LG, Ng JK, Meta M, *et al*: Heterozygosity for Lmna deficiency eliminates the progeria-like phenotypes in Zmpste24-deficient mice. Proc Natl Acad Sci USA 101:18111–18116, 2004

Goldman RD, Shumaker DK, Erdos MR, *et al*: Accumulation of mutant lamin A causes progressive changes in nuclear architecture in Hutchinson–Gilford progeria syndrome. Proc Natl Acad Sci USA 101:8963–8968, 2004

Hentze MW, Kulozik AE: A perfect message: RNA surveillance and nonsense-mediated decay. Cell 96:307–310, 1999

Herrmann T, van der Hoeven F, Grone HJ, *et al*: Mice with targeted disruption of the fatty acid transport protein 4 (Fatp 4, Slc27a4) gene show features of lethal restrictive dermopathy. J Cell Biol 161:1105–1115, 2003

Holt I, Ostlund C, Stewart CL, Man N, Worman HJ, Morris GE: Effect of pathogenic mis-sense mutations in lamin A on its interaction with emerin *in vivo*. J Cell Sci 116:3027–3035, 2003

Lammerding J, Schulze PC, Takahashi T, *et al*: Lamin A/C deficiency causes defective nuclear mechanics and mechanotransduction. J Clin Invest 113: 370–378, 2004

Lee KK, Haraguchi T, Lee RS, Koujin T, Hiraoka Y, Wilson KL: Distinct functional domains in emerin bind lamin A and DNA-bridging protein BAF. J Cell Sci 114:4567–4573, 2001

Lin M, Wei LJ, Sellers WR, Lieberfarb M, Wong WH, Li C: dChipSNP: Significance curve and clustering of SNP-array-based loss-of-heterozygosity data. Bioinformatics 20:1233–1240, 2004

Lloyd DJ, Trembath RC, Shackleton S: A novel interaction between lamin A and SREBP1: Implications for partial lipodystrophy and other laminopathies. Hum Mol Genet 11:769–777, 2002

Lowry RB, Machin GA, Morgan K, Mayock D, Marx L: Congenital contractures, edema, hyperkeratosis, and intrauterine growth retardation: A fatal syndrome in Hutterite and Mennonite kindreds. Am J Med Genet 22: 531–543, 1985

Mancini MA, Shan B, Nickerson JA, Penman S, Lee WH: The retinoblastoma gene product is a cell cycle-dependent, nuclear matrix-associated protein. Proc Natl Acad Sci USA 91:418–422, 1994

Matsuzaki H, Loi H, Dong S, *et al*: Parallel genotyping of over 10,000 SNPs using a one-primer assay on a high-density oligonucleotide array. Genome Res 14:414–425, 2004

Mau U, Kendziorra H, Kaiser P, Enders H: Restrictive dermopathy: Report and review. Am J Med Genet 71:179–185, 1997

Mislow JM, Holaska JM, Kim MS, Lee KK, Segura-Totten M, Wilson KL, McNally EM: Nesprin-1alpha self-associates and binds directly to emerin and lamin A *in vitro*. FEBS Lett 525:135–140, 2002

Moulson CL, Martin DR, Lugus JJ, Schaffer JE, Lind AC, Miner JH: Cloning of wrinkle-free, a previously uncharacterized mouse mutation, reveals crucial roles for fatty acid transport protein 4 in skin and hair development. Proc Natl Acad Sci USA 100:5274–5279, 2003

Muchir A, Medioni J, Laluc M, *et al*: Nuclear envelope alterations in fibroblasts from patients with muscular dystrophy, cardiomyopathy, and partial lipodystrophy carrying lamin A/C gene mutations. Muscle Nerve 30:444–450, 2004

Navarro CL, De Sandre-Giovannoli A, Bernard R, *et al*: Lamin A and ZMPSTE24 (FACE-1) defects cause nuclear disorganization and identify restrictive dermopathy as a lethal neonatal laminopathy. Hum Mol Genet 13:2493–2503, 2004

Nikolova V, Leimena C, McMahon AC, *et al*: Defects in nuclear structure and function promote dilated cardiomyopathy in lamin A/C-deficient mice. J Clin Invest 113:357–369, 2004

Novelli G, Muchir A, Sangiuolo F, *et al*: Mandibuloacral dysplasia is caused by a mutation in LMNA-encoding lamin A/C. Am J Hum Genet 71:426–431, 2002

Ostlund C, Bonne G, Schwartz K, Worman HJ: Properties of lamin A mutants found in Emery–Dreifuss muscular dystrophy, cardiomyopathy and Dunnigan-type partial lipodystrophy. J Cell Sci 114:4435–4445, 2001

Pendas AM, Zhou Z, Cadinanos J, *et al*: Defective prelamin A processing and muscular and adipocyte alterations in Zmpste24 metalloproteinase-deficient mice. Nat Genet 31:94–99, 2002

Sakaki M, Koike H, Takahashi N, Sasagawa N, Tomioka S, Arahata K, Ishiura S: Interaction between emerin and nuclear lamins. J Biochem (Tokyo) 129: 321–327, 2001

Schmidt WK, Tam A, Michaelis S: Reconstitution of the Ste24p-dependent N-terminal proteolytic step in yeast a-factor biogenesis. J Biol Chem 275: 6227–6233, 2000

Sermon K, Van Steirteghem A, Liebaers I: Preimplantation genetic diagnosis. Lancet 363:1633–1641, 2004

Sillevis Smitt JH, van Asperen CJ, Niessen CM, *et al*: Restrictive dermopathy. Report of 12 cases. Dutch Task Force on Genodermatology. Arch Dermatol 134:577–579, 1998

Welsh KM, Smoller BR, Holbrook KA, Johnston K: Restrictive dermopathy. Report of two affected siblings and a review of the literature. Arch Dermatol 128:228–231, 1992

Witt DR, Hayden MR, Holbrook KA, Dale BA, Baldwin VJ, Taylor GP: Restrictive dermopathy: A newly recognized autosomal recessive skin dysplasia. Am J Med Genet 24:631–648, 1986

Worman HJ, Courvalin JC: How do mutations in lamins A and C cause disease? J Clin Invest 113:349–351, 2004

Zastrow MS, Vlcek S, Wilson KL: Proteins that bind A-type lamins: Integrating isolated clues. J Cell Sci 117:979–987, 2004