Mutations causing Greenberg dysplasia but not Pelger anomaly uncouple enzymatic from structural functions of a nuclear membrane protein

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The lamin B receptor (LBR) is an inner nuclear membrane protein with a structural function interacting with chromatin and lamins, and an enzymatic function as a sterol reductase. Heterozygous *LBR* mutations cause nuclear hyposegmentation in neutrophils (Pelger anomaly), while homozygous mutations cause prenatal death with skeletal defects and abnormal sterol metabolism (Greenberg dysplasia). It has remained unclear whether the lethality in Greenberg dysplasia is due to cholesterol defects or altered nuclear morphology.

To answer this question we characterized two LBR missense mutations and showed that they cause Greenberg dysplasia. Both mutations affect residues that are evolutionary conserved among sterol reductases. In contrast to wildtype LBR, both mutations failed to rescue C14 sterol reductase deficient yeast, indicating an enzymatic defect. We found no Pelger anomaly in the carrier parent excluding marked effects on nuclear structure. We studied Lbr in mouse embryos and demonstrate expression in skin and the developing skeletal system consistent with sites of histological changes in Greenberg dysplasia. Unexpectedly we found in disease-relevant cell types not only nuclear but also cytoplasmatic LBR localization. The cytoplasmatic LBR staining co-localized with ER-markers and is thus consistent with the sites of endogeneous sterol synthesis.

We conclude that LBR missense mutations can abolish sterol reductase activity, causing lethal Greenberg dysplasia but not Pelger anomaly. The findings separate the metabolic from the structural function and indicate that the sterol reductase activity is essential for human intrauterine development.

Introduction

The lamin B receptor (LBR) is a multifunctional inner nuclear membrane protein with structural impact on nuclear shape and chromatin organization.[1] The nucleoplasmatic part directly and indirectly interacts with chromatin, lamins, heterochromatin proteins HP1α and γ, histones H3 and H4 and other nuclear components.[2,3] The transmembrane part belongs to the sterol reductase family and exhibits sterol reductase activity in vivo.[4-7]

LBR and lamins contribute to chromosome positioning, gene expression and distribution of nuclear pore complexes. Also, the lamin network has been shown to be involved in other essential cellular processes such as mitosis, meiosis and apoptosis. Laminopathies are diseases associated with lamin network proteins. Alterations of nuclear envelope components such as emerin or lamin A/C change nuclear shape and cause a variety of human diseases. Manifestations range from developmental to degenerative phenotypes, including cardiomyopathy, restrictive dermatopathy, lipodystrophy, mandibuloacral dysplasia, muscular dystrophy, peripheral neuropathy and premature ageing syndromes.[8-14] This demonstrates the essential cellular and clinical impact of the lamin network.

Lamin B receptor mutations cause human Pelger anomaly (Pelger-Huët anomaly, PHA [MIM 169400]), human lethal Greenberg dysplasia (HEM [MIM 215140]), and recessive ichthyosis in mice (ic/ic). We showed earlier that *LBR* mutations cause dose-dependent hyposegmentation of granulocyte nuclei in individuals with heterozygous or homozygous Pelger anomaly.[15] Heterozygous *LBR* mutations alter neutrophil morphology without causing disease, while homozygous *LBR* mutations cause a spectrum of systemic malformations ranging from cardiac defects, brachydactyly and mental retardation (as occurs in homozygous Pelger anomaly), to severe skin disease (modeled by ichthyotic ic/ic mice) and prenatal death, as found in Greenberg dysplasia.[15-18]

Greenberg dysplasia is also known as HEM skeletal dysplasia, an abbreviation derived from the characteristic features hydrops, ectopic calcification and moth-eaten skeletal dysplasia.[18-25] The disease is associated with an abnormal sterol metabolite, cholesta-8,14-dien-3β-ol. Defects in other sterol synthesis enzymes present with skeletal malformations, mental retardation, failure to thrive or even death, and are viewed as metabolic malformation syndromes.[26] Whereas some groups believe that in Greenberg dysplasia the sterol defect is causative,[18,26] others assume the primary problem in altered nuclear structure.[27]

So far, truncating mutations, namely nonsense, splice site and frameshift mutations, have been reported for Pelger anomaly.[15-17] For Greenberg dysplasia, one homozygous nonsense mutation and one homozygous missense mutation in the lamin B receptor gene were described.[18,28] In the family with Greenberg dysplasia due to homozygous missense mutation p.N547D, no sterol measurements or blood smears were available.[28] It thus remained unclear whether or not the lethal effect is due to altered sterol metabolism or due to altered nuclear structure.[28,29]

Results

We studied three fetuses that all fulfilled the clinical criteria of Greenberg dysplasia, namely intrauterine growth retardation, massive generalized edema (hydrops), extreme shortening of long bones (tetrabrachymelia) with a moth-eaten appearance of tubular bones, ectopic calcification centers and a narrow thorax (**Fig. 1A, Suppl. Table 1**). Detailed clinical examination was obtained from fetus A; fetus B has been described previously.[25] Sterol analyses were performed in muscle tissue of fetus B and revealed the abnormal sterol metabolite 5α-cholest-8,14-dien-3β-ol,[25] that was previously shown to be associated with Greenberg dysplasia.[18] Sterol analysis was not available for the other two fetuses.

Sequence analysis revealed frameshift and missense mutations in the LBR gene. We sequenced *LBR* and identified mutations in all three families (**Fig. 1B**, sequence traces and segregation in **Suppl. Fig. 1A**). Fetus A showed a homozygous frameshift mutation c.1492delT that is predicted to change residues 468 to 474 and to create a premature stop in codon 475 (p.Y468TfsX475). Fetus B revealed two different mutations, c.32delTGGT and c.1748G>A. The first is a deletion of 4 base pairs causing a frame shift with subsequent premature stop in codon 24 (p.V11EfsX24). The second is a missense mutation replacing arginine by glutamine at residue 583 (R583Q). Both parents of fetus C were carriers of the missense mutation p.N547D. Even though no material was available from fetus C to show homozygosity for mutation p.N547D, consanguinity of the parents and the presence of the same mutation in another fetus with Greenberg dysplasia[28] indicate that this mutation was causative. We proved that the nucleotide changes were not present in 150 controls, thereby making a polymorphism unlikely. For the missense mutations, we tested another 150 controls, to further reduce the possibility that they were rare variants.

The missense mutations reside in the sterol reductase domain and affect evolutionary conserved residues (**Fig. 1B**). We tested the potential relevance of the identified missense mutations by an interspecies comparison. The p.N547D and p.R583Q mutations both change residues that are evolutionary extremely conserved among LBR and other sterol reductases, indicating their functional relevance (**Suppl. Fig. 1B**). Sequencing of *DHCR7* and *TM7SF2* did not reveal alterations in any of the families, excluding a second hit in another gene of the C14 sterol reductase family.

LBR missense mutation did not alter nuclear shape in neutrophils. Since both missense mutations reside in the sterol reductase domain of the lamin B receptor, disruption of the sterol reductase function seemed likely. However, the position of these mutations made effects on the second function of the lamin B receptor, namely nuclear structure, less likely. Based on the assumption that the amino acid substitution does not affect essential regions for modification, transport, or lamin B receptor anchoring, we expected nuclear morphology to remain unchanged. To test this hypothesis, we obtained blood from the parents of fetus B. The blood smear of the father indeed showed apparently normal neutrophils with multisegmented nuclei, whereas the mother had an obvious heterozygous Pelger anomaly
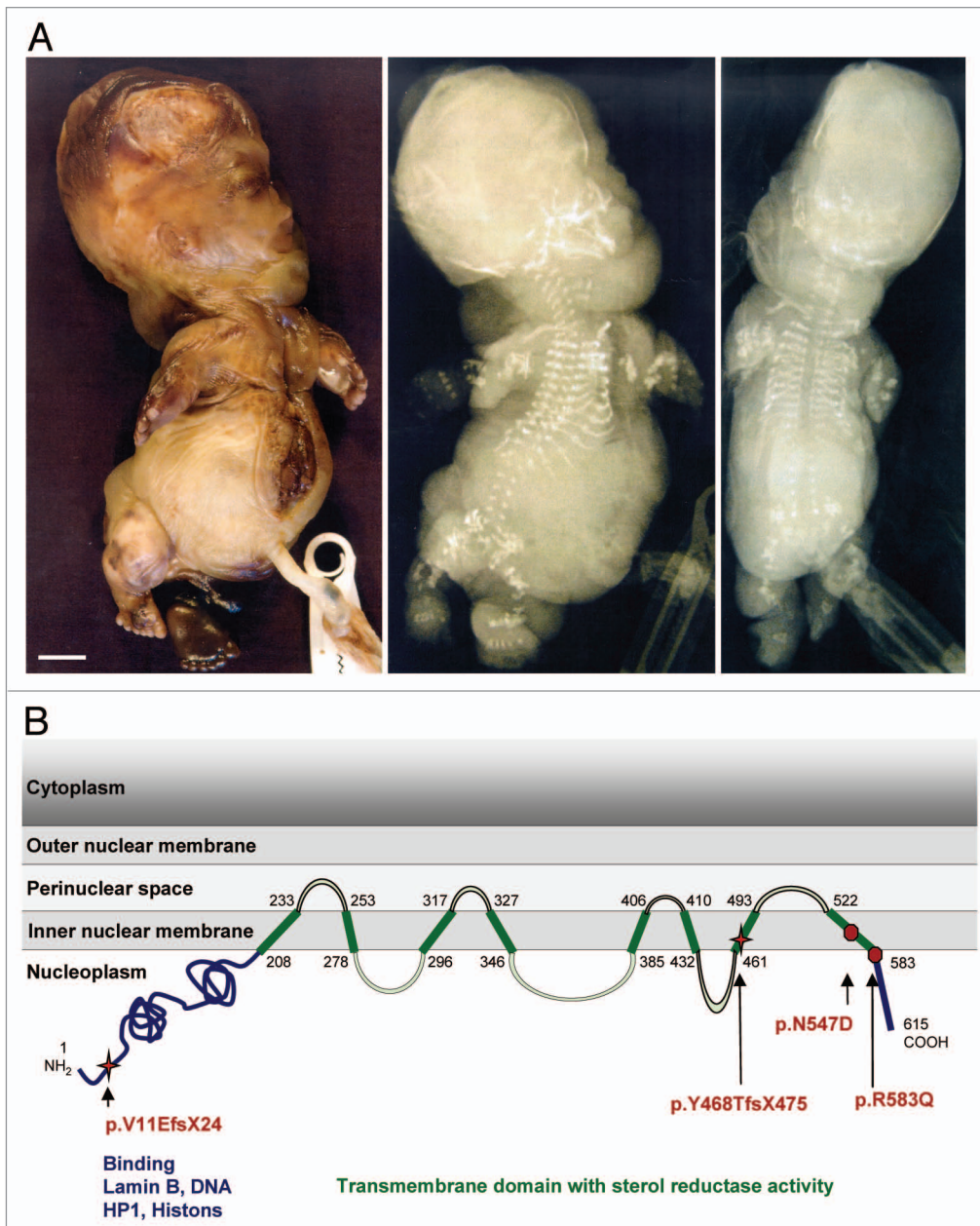
Figure 1. Phenotype and identified mutations. (A) Post mortem appearance of fetus A at 16 + 3 weeks pregnancy. Note the edema, extreme micromelia of all four limbs and roentgenographic moth-eaten appearance of tubular bones. The thorax is deformed and narrow. Note the large head with hygroma and the hexadactyly on hands and feet. Scale bar 1 cm. (B) Schematic view of the lamin B receptor as a protein of the inner nuclear membrane and identified mutations. The nucleoplasmatic part interacts directly and indirectly with lamins, chromatin and other nuclear proteins. The transmembrane domain belongs to the C14 sterol reductase family and displays sterol reductase activity. The missense mutations p.N547D and p.R583Q reside in the transmembrane domain (sterol reductase domain). The two frameshift mutations are predicted to create a premature stop codon and thus the RNA is likely to undergo nonsense mediated decay, abolishing both the structural and metabolic function. Missense mutation p.N547D was previously described.[28]

with nuclear hyposegmentation (**Fig. 2**). This state of affairs was confirmed by sequence analyses. The mother with the Pelger anomaly was the carrier of the nonsense mutation p.V11EfsX24. The father, with the normal neutrophils on blood smears, carried the heterozygous missense mutation p.R583Q, indicating that this mutation affects sterol metabolism but not nuclear shape. No blood smears from the parents of fetus A and C were available.

Both missense mutations failed to compensate for C14 sterol reductase deficiency in yeast. The human wildtype lamin B receptor can complement for the sterol reductase function in yeast.[5,30] LBR belongs to the C14 sterol reductase family as does yeast ERG24. The ERG24 deficient yeast has an altered sterol metabolism where the normal end product, ergosterol, is missing and the abnormal metabolite ignosterol is produced instead.
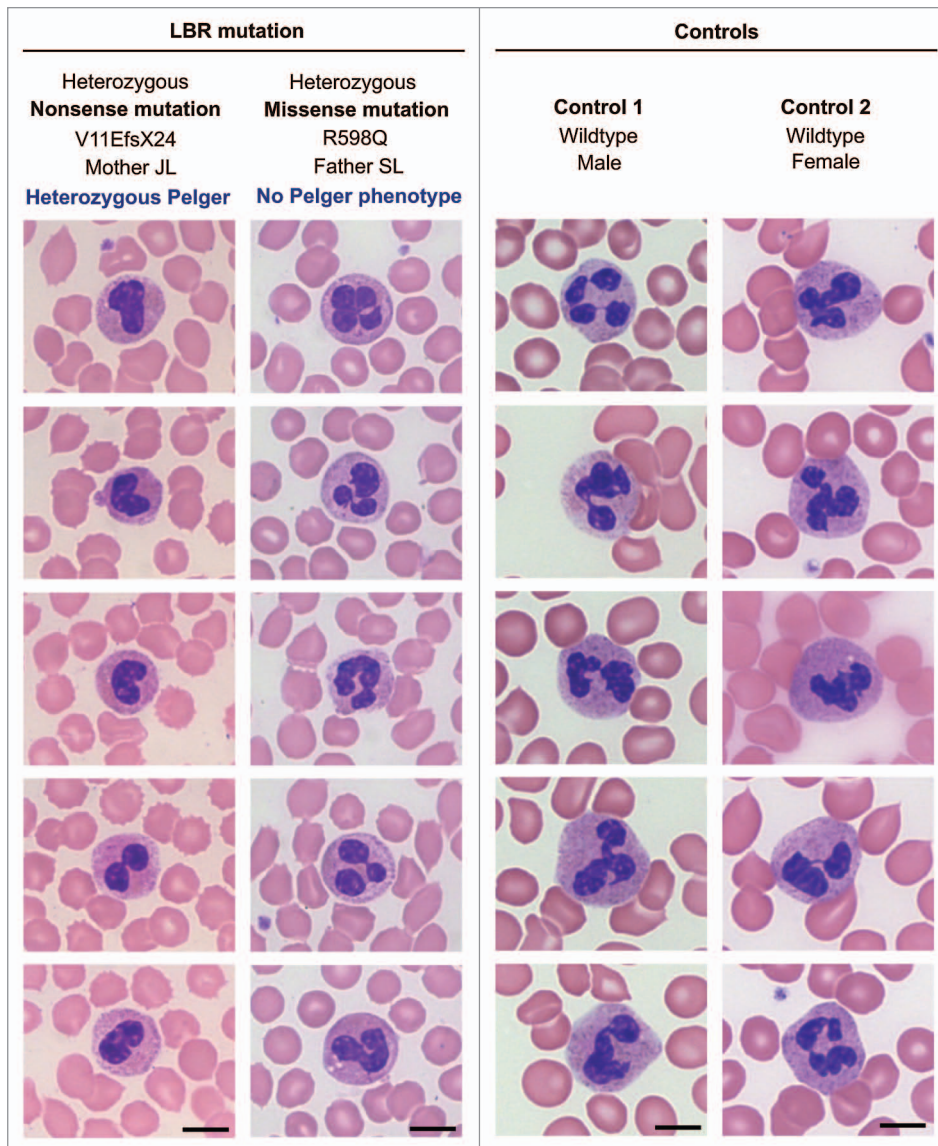
Figure 2. Neutrophils show normal nuclei with multiple segments in the father with heterozygous missense mutation p.R583Q and in controls, indicating that this mutation does not affect nuclear shape. In contrast, the mother is a heterozygous carrier of a nonsense mutation and shows Pelger anomaly with hypolobulated nuclei in blood smears. Five representative neutrophils per individual are shown. Scale bar 10 μm.

in an amount similar to the wildtype rescue, indicating a partial compensation. However, p.N547D caused a significant accumulation of the abnormal metabolites 4-methylzymosterol and ignosterol. Human mutation p.R583Q failed to produce the normal end product ergosterol in significant amounts. Instead, we observed a huge accumulation of the abnormal end product ignosterol. Both mutations increased the total amount of pathway metabolites, probably to provide at least trace amounts of ergosterol that is necessary for a number of essential cellular functions in yeast. Analyses of growth pattern confirmed these results with p.N547D partially restoring growth and p.R583Q failing to do so (**Fig. 3C**).

In fibroblasts, LBR is not only located at the nuclear rim but also shows significant non-nuclear localization. The lamin B receptor is a protein of the inner nuclear membrane. Accordingly predominant localization was so far only reported in the nucleus. To test the hypothesis that malformations observed in Greenberg dysplasia result from effects other than nuclear sterol synthesis, we studied the cellular distribution of the protein. The abnormal sterol metabolite in Greenberg dysplasia was initially identified by growing Greenberg fetal fibroblasts in lipid-depleted serum.[18] We therefore reasoned that fibroblasts are a reasonable cell type to test this hypothesis and indeed found extensive localization of the lamin B receptor outside the nucleus (**Fig. 4A and B**). De-novo endogeneous sterol synthesis takes place in the endoplasmatic reticulum

Since ergosterol is essential, ERG24 mutants show impaired growth (**Fig. 3A, Suppl. Fig. 2**).

We first demonstrated that wildtype human LBR and wildtype yeast ERG24 rescued the ERG24 deficient yeast phenotype in our system (data not shown). We then introduced the missense mutations p.N547D and p.R583Q, respectively, and transformed yeast with these mutant variants. We found that the mutants failed to rescue the yeast ergosterol phenotype (**Fig. 3B**). As expected, wildtype yeast showed metabolites of the normal pathway, lanosterol, fecosterol and ergosterol. The same applied for ERG24 deficient yeast rescued by transformation with the yeast ERG24 gene or by the human LBR wildtype gene. The human LBR mutant p.N547D also produced fecosterol and ergosterol

(ER). Accordingly, we found a co-localization of the non-nuclear LBR with calnexin as an ER membrane component (representative localization in **Fig. 4A**; more cells are shown in **Suppl. Fig. 3**). Further, we tested this in HeLa cells that have a higher growth rate and a higher expression level of LBR compared to fibroblasts. We found extensive cytoplasmic localization of LBR co-localizing with calnexin in immunostaining also in this cell type. Further, the western blot of fractionated HeLa cells revealed LBR in both the nuclear and cytoplasmic fraction whereas the nuclear marker lamin B was only present in the nuclear fraction (**Suppl. Fig. 4**).

LBR is expressed in human osteoclasts and osteoblast-like cells. The Greenberg phenotype manifests as hydrops and severe skeletal dysplasia with shortening of long bones and altered

Figure 3. Design and results of the yeast rescue experiment. (A) Systematic view of the rescue experiment. (B) Analysis of human LBR constructs by GC/MS. hLBRwt is able to complement ERG24 deficiency in yeast. hLBR_N547D is partially able to complement ERG24 deficiency, visible in the production of the end product of the sterol pathway, ergosterol. However, providing this essential component happens on the cost of also producing large amounts of the abnormal metabolites 4-methylzymosterol and ignosterol. hLBR_R583Q is not able to complement ERG24 deficiency, seen in the insufficient production of the normal endproduct ergosterol and massive accumulation of the irregular product ignosterol. (C) Growth pattern. Mutation p.R583Q failed to normalize the growth of Erg24 deficient yeast. Mutation p.N547D partially compensated for Erg24 deficiency with respect to growth.

growth plates in early fetal development. Therefore, we analyzed RNA levels in potentially disease-relevant human cell lines. We found expression of *LBR* in fibroblasts, lymphoblastoid cells, and to a very high degree in human in vitro differentiated osteoclasts (OC) and human osteosarcoma cells (HOS) which are osteoblast-like (**Fig. 5A**).

LBR is strongly expressed in liver, skin, brain as well as in specific regions of the developing cartilage and bone in mouse embryos. To analyze Lbr expression in vivo we studied wildtype mouse embryos at developmental stages consistent with the earliest manifestations in human Greenberg fetuses which have been reported as early as in gw 13 by ultrasound. In situ hybridization in wildtype mouse embryos at embryonic day E12.5 (corresponding to human gw 8 + 2) and qPCR of mouse tissues at postnatal day P4 showed strong expression of *Lbr*-RNA in the liver, lung, midgut, skin, brain, as well as in developing cartilage (**Fig. 5B and C**). To further analyze Lbr expression in growth plate cartilage we performed immunohistochemistry on E15.5 mouse forelimb sections in comparison to the chondrogenic marker Sox9 (**Fig. 6A**). Lbr is expressed throughout growth plate cartilage, with weaker expression in hypertrophic chondrocytes. At the sites of trabecular bone formation Lbr expression was also seen in osteoblasts. In addition to the cartilage/bone expression, a signal for Lbr protein was also observed in muscle and in connective tissue fibroblasts. Consistent with the findings in human fibroblast cultures, we found, in addition to localization at the nuclear rim, a non-nuclear staining in connective tissue fibroblasts and also in Lbr-expressing cells of the developing cartilage and bone (**Fig. 6A and** magnification in **B**).
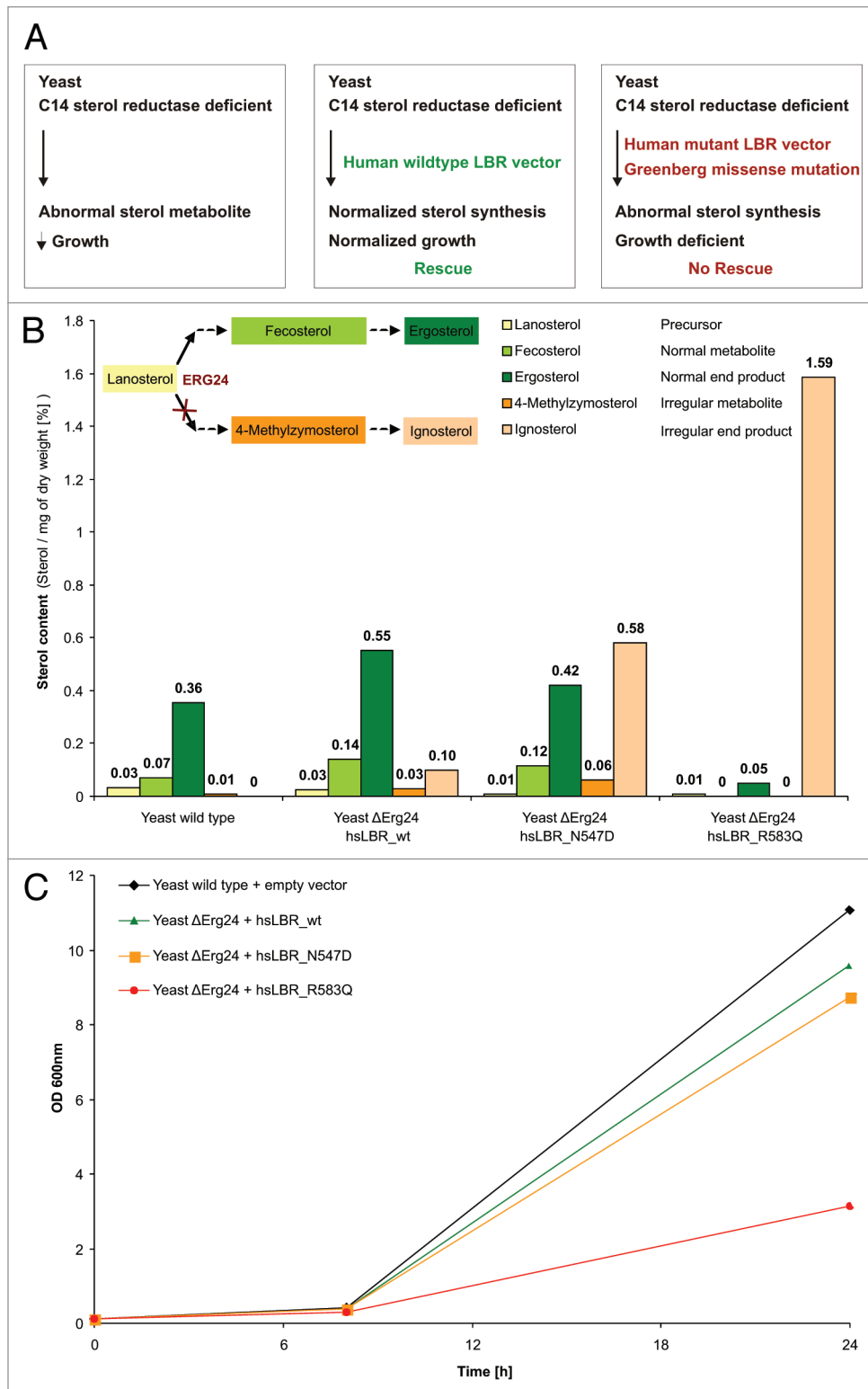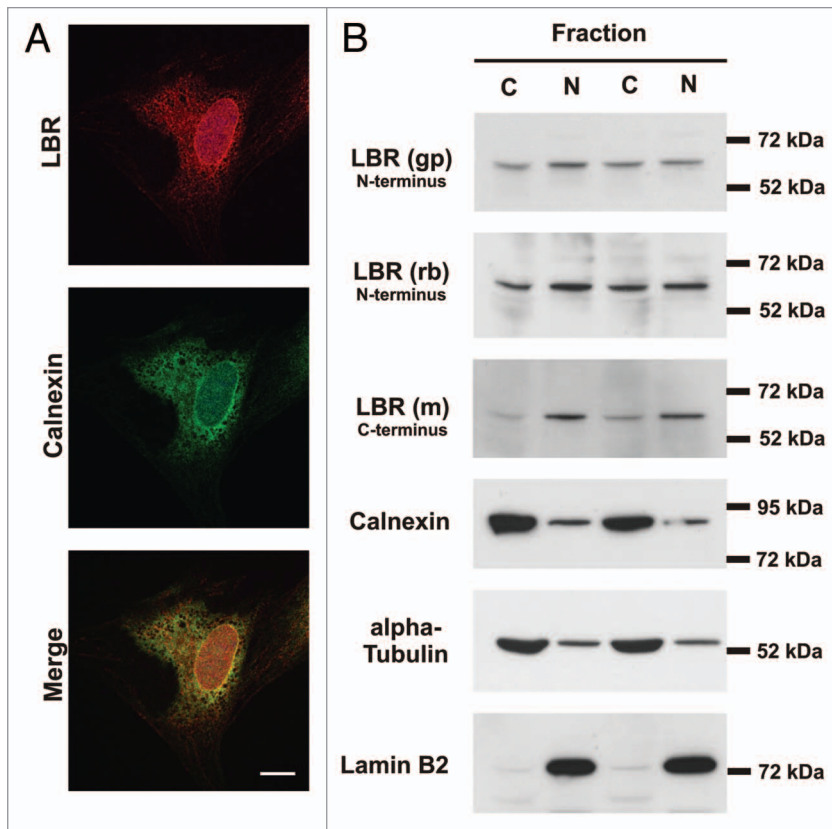
Figure 4. Localization of the lamin B receptor in human control fibroblasts. (A) Lamin B receptor staining (gp-anti-LBR_N-term) of the nuclear rim and in addition in the cytoplasm in control fibroblasts. The non-nuclear LBR showed a partial co-localization with the ER membrane protein calnexin (green), indicating a localization of LBR in this compartment. Scale bar 10 μm. (B) Nuclear (N) and cytoplasmic (C) extracts from human control skin fibroblasts showed a strong LBR expression in both the nuclear and the cytoplasmatic fraction by three different LBR antibodies. The nuclear protein lamin B2 and the cytoplasmatic alpha tubulin confirmed separation of the nuclear and cytoplasmatic fractions.

Discussion

We studied three cases of Greenberg dysplasia in which *LBR* missense and nonsense mutations were identified. There was no phenotypic difference between fetuses with nonsense and missense mutations. Both missense mutations changed evolutionary conserved residues of the sterol reductase domain and failed to rescue sterol reductase deficient yeast. We showed that carrying a heterozygous missense mutation affecting the sterol reductase domain did not alter nuclear shape in neutrophils. We demonstrated LBR expression in cytoplasmatic compartments (ER) and in embryonic structures essential for bone development. Our findings uncouple the metabolic from the structural function of LBR and indicate that the developmentally essential enzymatic function may be exerted in the ER.

LBR is a hybrid protein, is likely to mediate separate functions and thus could also contribute to separate distinct diseases. LBR is the only sterol reductase that gained an additional 200 amino acids at the N-terminus which added new functions such as the localization to the nucleus and interaction with chromatin and other nuclear components.[1-7] The combination of an

enzymopathy and a structural trait by mutations in the same gene product is, as to our knowledge, quite unique. We draw attention to the dominant structural effect in Pelger anomaly and the recessive enzymopathy in Greenberg dysplasia.

The N-terminal part mediates the interaction of the lamin B receptor with lamins, chromatin and heterochromatin proteins.[2,4,31] Nonsense mutations with subsequent loss of the encoded protein impair that structural function and result in hyposegmentation of neutrophil nuclei and altered chromatin structure, as seen in the Pelger blood phenotype in the mother with the heterozygous nonsense mutation p.V11EfsX24. In contrast, the nuclear structure of neutrophils was unaffected by missense mutation p.R583Q which affects a residue of the sterol reductase domain only. The father of fetus B was heterozygous for missense mutation p.R583Q and did not show any evidence for Pelger anomaly. We assume that the nuclear structure is also not markedly altered in other cell types since the nuclei of neutrophils are especially sensitive to loss of LBR. In addition, the position of this mutation within the membrane makes a marked structural effect on nuclear shape unlikely. The same applies for missense mutation p.N547D where no blood smears from the parents were available.

In contrast, both missense mutations failed to compensate for C14 sterol reductase deficiency in yeast, indicating severe enzymatic defects in sterol metabolism. We showed that both missense mutations affect evolutionary conserved residues of sterol reductases and experimentally proved that they both failed to compensate for sterol reductase deficiency in yeast. While p.R583Q completely failed to compensate, p.N547D could partially restore C14 sterol reduction, but produced a huge amount of abnormal metabolites. The relevant functional effect is underscored by the fact that mutations in homologous positions in another human sterol reductase, 7-dehydrocholesterol reductase [DHCR7 (MIM 602858)], cause Smith-Lemli-Opitz syndrome [SLOS (MIM 270400)].[6,32] Smith-Lemli-Opitz syndrome is a recessive disease with multiple malformations including skeletal and developmental defects, and mental retardation.[33,34] Notably, an abnormal sterol metabolite is also found in the same pathway where LBR is expected to function; whereas LBR acts in an earlier step in cholesterol synthesis, DHCR7 catalyzes the final conversion from 7-dehydrocholesterol to cholesterol (overview in **Fig.** 7, reviewed in ref. 26). The lamin B receptor mutation LBR. N547D corresponds to a mutation found in Smith-Lemli-Opitz syndrome affecting residue DHCR7.N407Y.[32] Mutation LBR. R583Q has a homolog DHCR7 mutation with even an identical amino acid exchange from arginine to glutamine (DHCR7. R443Q).[6] We excluded mutations in *DHCR7* and *TM7SF2* in all three of our families. Thus, both LBR missense mutations
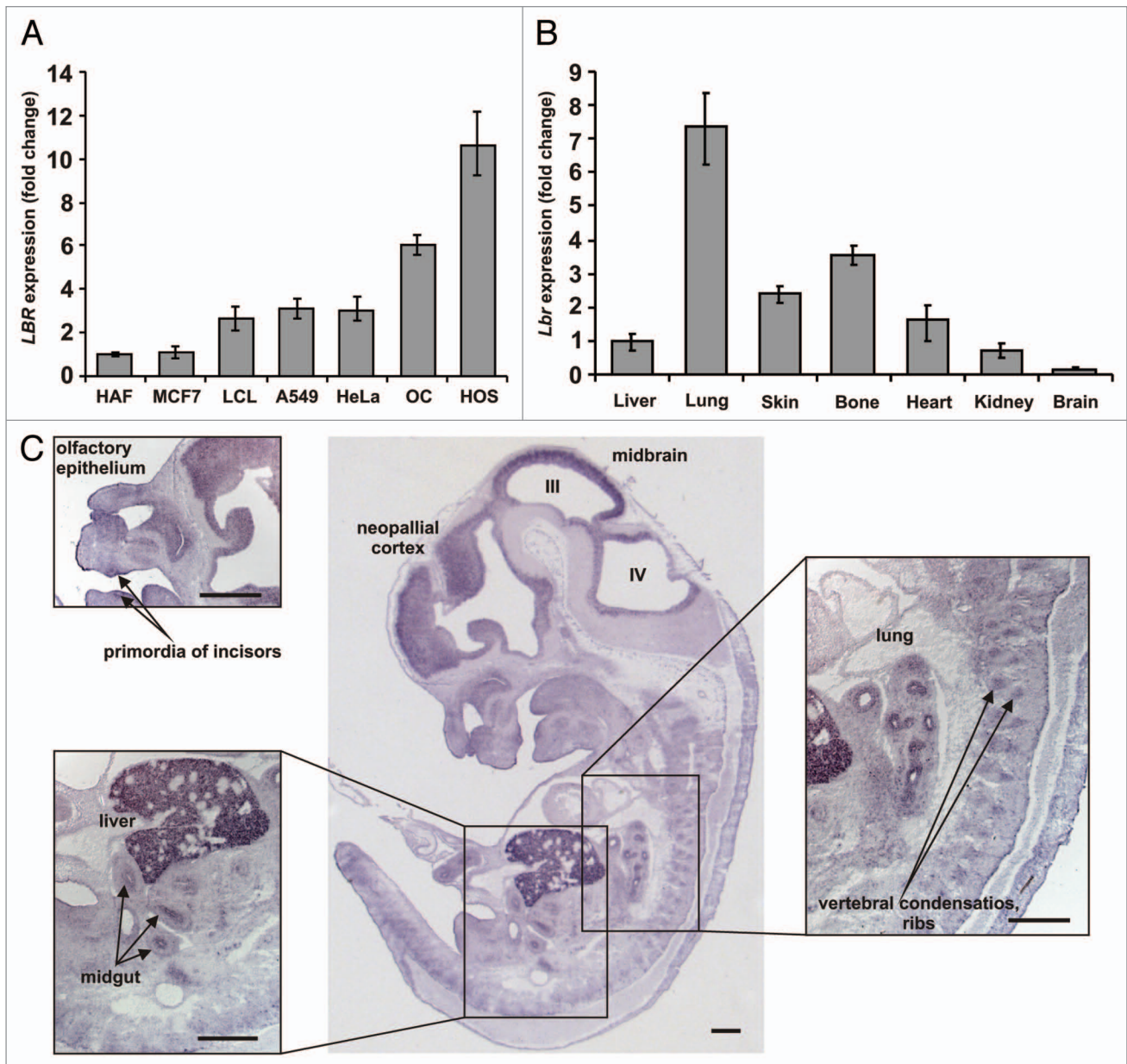
Figure 5. Expression of the lamin B receptor in different cell types and in development. (A) Relative LBR mRNA expression in different human cell lines, calibrated to fibroblasts. The highest expression is found in cell types associated with skeletal development, consistent with the most obvious manifestations in Greenberg dysplasia. Analyzed cell types: Skin fibroblasts (HAF), breast cancer cells (MCF-7), lymphoblastoid cells (LCL), lung cancer cells (A549), cervical cancer cells (HeLa), in-vitro differentiated osteoclasts (OC) and osteosarcoma cells (HOS). (B) Lbr expression in mouse tissues from developmental stage P4, calibrated to liver. Lung, skin and bone show the highest Lbr expression which is consistent with the main manifestation sites in patients with Greenberg dysplasia. (C) In-situ hybridization of Lbr in day 12.5 mouse embryos. Pronounced expression is found in liver, in several regions of the brain, in skin, developing incisors, in midgut epithelium, lung epithelium and in vertebral condensations. Mouse embryonic day E12.5 corresponds to human gestational week 8 + 2 (post menstruation). Scale bar 500 μm.

appear to result in functional null effects with respect to sterol metabolism.

The relevance of the metabolic function of LBR for Greenberg dysplasia is underscored by a phenotypic and pathogenic overlap with other diseases associated with cholesterol synthesis in humans. The congenital hemidysplasia with ichthyosiform erythroderma and limb defects [CHILD syndrome (MIM 308050)], chondrodysplasia punctata X2 [CDPX2

(MIM 302960)], lathosterolosis [MIM 607330], desmosterolosis [MIM 602398] and Smith-Lemli-Opitz syndrome all have defects in enzymes of the cholesterol synthesis pathway downstream of LBR (**Fig. 7**). Skeletal defects, complete or increased prenatal or perinatal lethality and dysmorphic facies are present in all these conditions (reviewed in ref. 26). The results presented here fit with the view that the enzymopathy in LBR deficiency is causative for Greenberg dysplasia and thus indeed belongs to
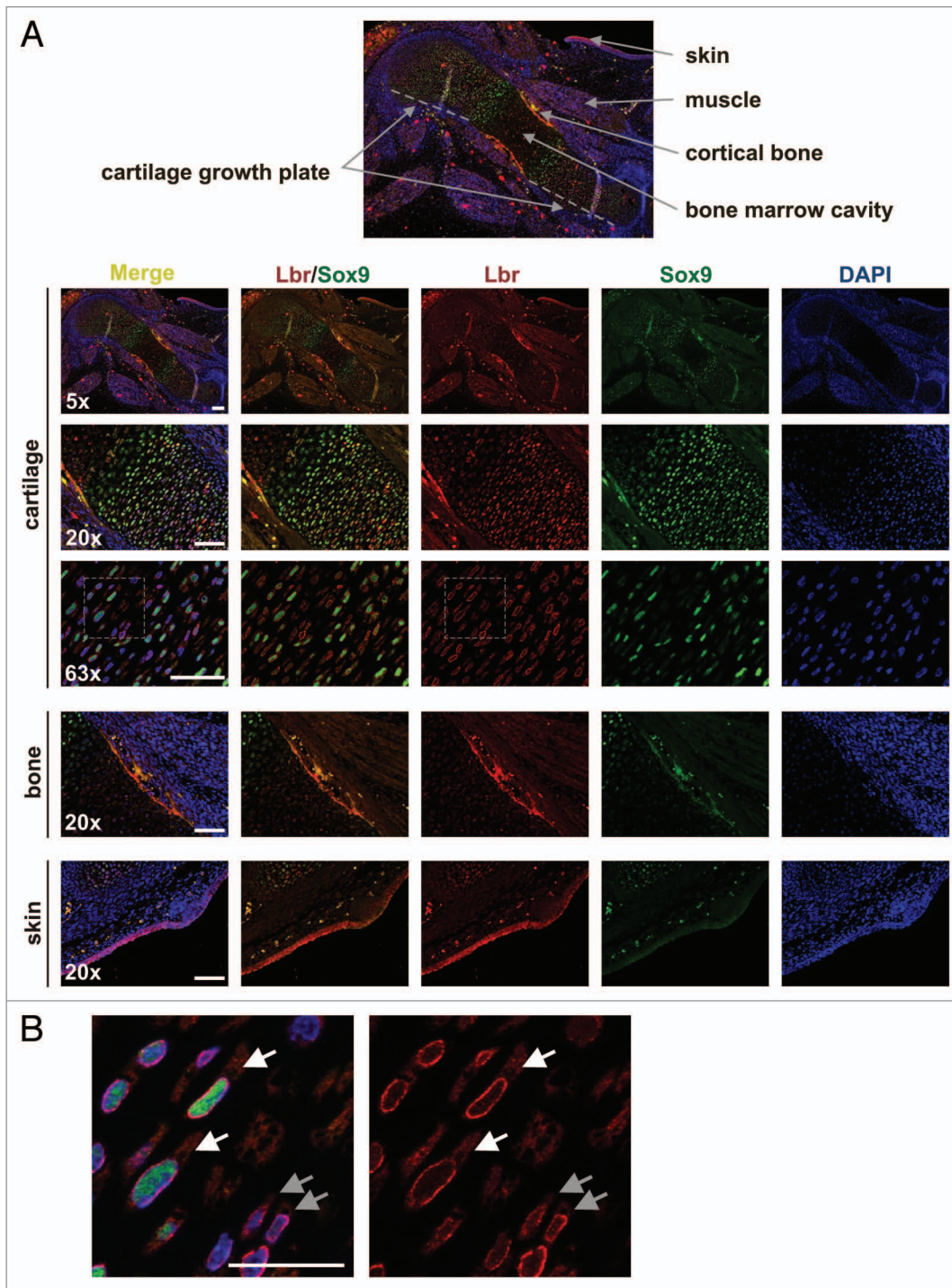
Figure 6. Expression analysis of Lbr protein in wildtype mouse fore limb on embryonic day E15.5. (A) Lbr (red) is strongly expressed in skin and in growth plate cartilage with exception of the hypertrophic zone. Immunostaining for the cartilage marker Sox9 (green) is shown for comparison. Positive staining is also detected in skin, muscle cells and connective tissue fibroblasts. In the region of the cortical bone, osteoblasts show a specific Lbr staining in higher magnification. DNA was stained by DAPI (blue). (Note that the bright Lbr and Sox9 staining at the diaphysic bone collar is an artifact due to unspecific antibody binding to the bone matrix). Scale bars: 5x and 20x 100 μm, 63x 50 μm. (B) Cytoplasmatic staining in chondrocytes and connective tissue fibroblasts. White arrowheads point to cytoplasmatic LBR staining in chondrocytes, grey in connective tissue fibroblasts. Magnification from (A) 63x cartilage. Scale bar 25 μm.

the metabolic malformation syndromes.[26] Detailed mechanistic information is not yet available how the loss of sterol reductase activity of the lamin B receptor causes Greenberg dysplasia. The abnormal metabolite, cholesta-8,14-dien-3β-ol, may mediate toxic effects. Alternatively, upstream or downstream intermediates in cholesterol metabolism that are altered in quantity or
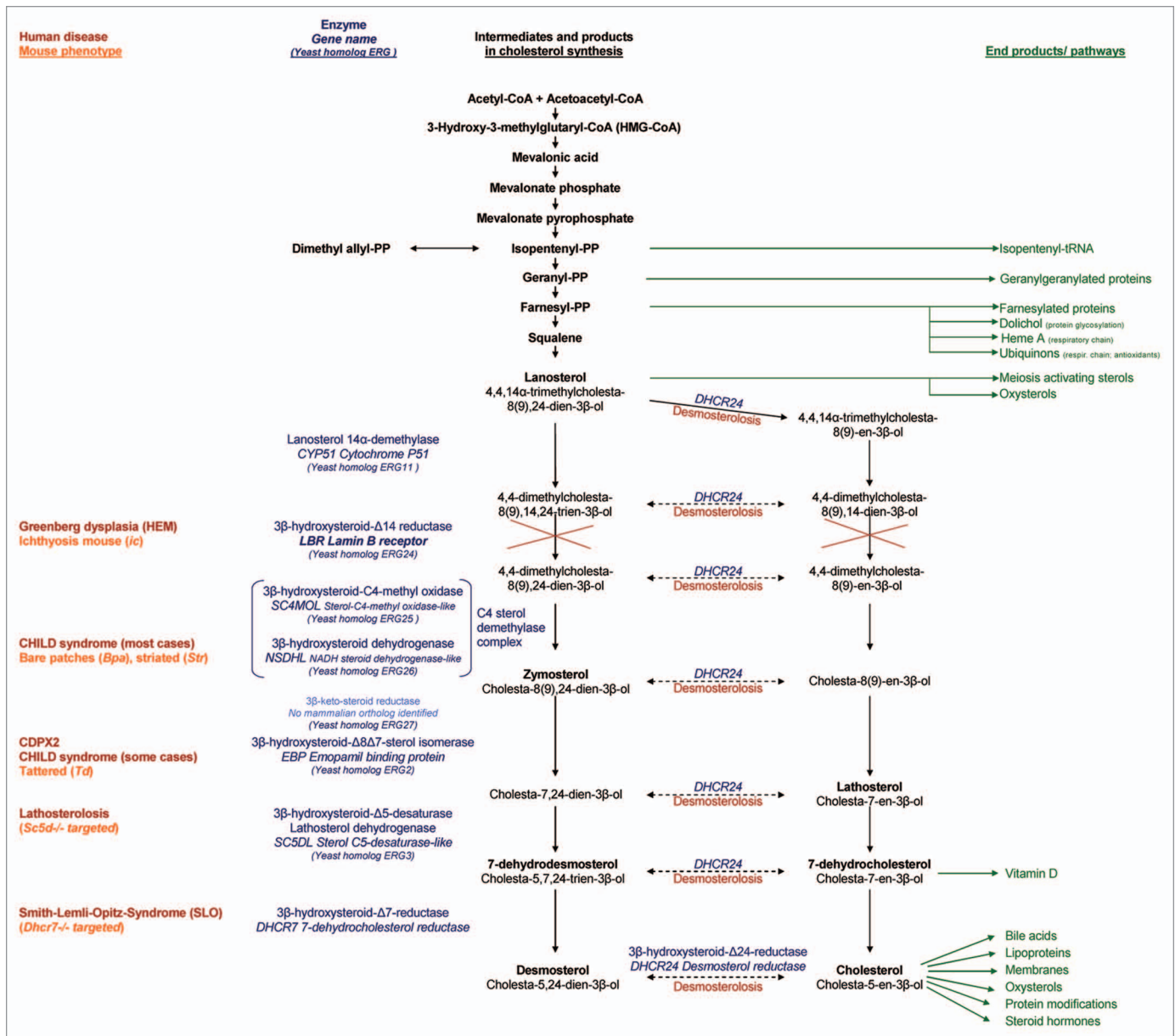
Figure 7. Cholesterol synthesis pathway, associated diseases and putative pathogenic mechanisms (reviewed in refs. 26, 49 and 50). Blockade of the C14 sterol step might lead to an accumulation of precursors with subsequent up or downregulation. This accumulation could affect important pathways such as farnesylation, heme and ubiquinone synthesis, or even the first hydroxyl methyl glutaryl CoA reduction step. Equally or more likely might be a deficiency in downstream products. LBR catalyzes an early step in post-squalene cholesterol synthesis. Failure in this step might subsequently result in different amounts or composition of derivates from intermediate steps, such as meiosis activating sterols, oxysterols, vitamin D, and finally of the end-product cholesterol and its derivates bile acids and steroid hormones.[26] Cholesterol is a major component of membranes and lipid rafts and is produced in significant amounts by the fetus itself.[51] Insufficient amounts and altered membrane composition could impair fetal development. Greenberg dysplasia could even feature a modified hedgehog pathway, as a result of cholesterol modification. Hedgehog proteins are modified by cholesterol.[52] Altered hedgehog signaling was shown in other diseases of the post-squalene pathway[35] and mutations in genes of the hedgehog pathway cause a number of skeletal defects. These defects include brachydactyly and polydactyly that are also seen in Greenberg dysplasia. Impaired vitamin D metabolism might be another potential effector in Greenberg dysplasia since LBR affects a step upstream of the vitamin D precursor 7-dehydrocholesterol. Vitamin D is essential for bone development. Vitamin D is produced in significant amounts in the placenta and the fetus itself. Though to our knowledge, whether or not there is de novo synthesis of vitamin D in the fetus is not entirely clear; however, at least locally such synthesis might be possible. There are overlapping pathophysiologic and histologic findings in Greenberg dysplasia, rickets in children, and osteomalacia in adults. Skeletal mineralization depends on the presence of sufficient amounts of calcium and phosphate at the sites of mineralization. Furthermore, chondrocytes, osteoblasts and collagen matrix must position and function properly. Mineralization occurs in chondrocytes. If osteoblasts produce more matrix than the chondrocytes can mineralize, rickets or osteomalacia can develop. Similar mechanisms could be operative in a very early stage of skeletal development in Greenberg dysplasia. The imbalance could either be due to absence or mal-position of calcium by vitamin D deficiency or by abnormal chondrocyte function. In addition, rickets show inadequate mineralization of the chondrocyte matrix in the growth plates. In both rickets and Greenberg dysplasia there is a disorganization or complete failure of chondrocytes to form chondrocyte columns. Epiphyses are stippled, growth of long bones is impaired in both conditions. Osteomalacia is also seen in neurofibromatosis and as a complication of anticonvulsive therapy.[53]

quality could affect farnesylation, oxysterols, steroid hormones, bile acids, vitamin D, hedgehog signaling, modification of other nuclear components as well as cytoplasmatic and nuclear lipid signaling.[26,35-37] These effects and alteration of the structural and metabolic function of cholesterol itself are all possible participants in the disease process.

The other sterol reductases of the postsqualene synthesis reside and act in the cytoplasm. We showed that the lamin B receptor is not only present in the nucleus but also exhibits an extensive presence in cytoplasmatic structures. The lamin B receptor has been so far viewed as a protein of the inner nuclear membrane. In contrast, fibroblasts showed in addition to the nuclear localization an extensive lamin B receptor staining in cytoplasmatic compartments (shown for three different LBR antibodies). This could either be due to a functional demand of cholesterol synthesis in certain cell types. Alternatively, cells with higher LBR expression levels might exceed the nuclear binding capacity for the lamin B receptor.[38] However, the abnormal sterol metabolite in Greenberg dysplasia was initially identified in fibroblasts that were grown in lipid depletion, forcing endogenous cholesterol synthesis.[18] Since fibroblasts show both this feature of sterol synthesis and this exceptional extranuclear localization of LBR, the lamin B receptor is likely to participate in cytoplasmatic cholesterol synthesis. Further, we demonstrated partial co-localization with the endoplasmatic reticulum membrane-protein calnexin. These findings are in agreement with the cytoplasmatic localization of other sterol reductases and the endogeneous cholesterol synthesis in the ER.[5] LBR localized to cytoplasmatic structures in significant amounts especially in certain cell types such as skin fibroblasts and bone-related cells, indicating the potential and probably the in vivo need for a de-novo endogeneous sterol synthesis.

We further showed that LBR is expressed in critical times and tissues of prenatal development in mice, corresponding to the predominant phenotypic defects skeletal dysplasia and edema seen in Greenberg fetuses. At mouse embryonic day 12.5 and 15.5 Lbr is present at RNA or protein level, respectively, in skin, epithelia of lung and midgut, liver as the organ of major fetal hematopoiesis but also in brain and distinct regions of skeletal development. Lbr expression in cartilage is pronounced in all growth plate chondrocytes and also present in osteoblasts forming the primary cortex. This is consistent with the histologic changes in bones from Greenberg fetuses such as disorganization or lack of chondrocyte columns, lack of growth plates, abrupt transition from cartilage to bone and premature excessive ossification of the diaphysic bone collar. These histologic changes were found, to our knowledge, in all histologically analyzed fetuses with Greenberg dysplasia,[18,19,21-25,28,39] and were also found in our fetuses. The expression pattern we document for the embryonic and postnatal mouse in combination with our finding that human osteoclasts and osteoblast-like cells (HOS) also strongly express *LBR*, indicate that the lamin B receptor is involved in cartilage and bone development in both human and mouse.

Complete loss or functional loss of LBR as a sterol reductase is developmentally lethal in humans whereas trace amounts

might enable survival. We described a patient earlier with a homozygous *LBR* mutation, IVS12-5-10del, where the intronic deletion almost completely abolished normal splicing with subsequent skipping of exon 13, frameshift, and a premature stop codon.[15] However, the splicing defect was not complete. Trace amounts of normally spliced mRNA and normal protein could be shown. This patient clearly had homozygous Pelger manifestations with round neutrophil nuclei, indicating that there was an almost 100% penetrance of this mutation with respect to the structural function. However, with respect to sterol metabolism, even these tiny amounts of normal LBR seemed to have been sufficient to permit survival. This patient had mild mental retardation, brachydactyly due to shortened metacarpals, and a cardiac defect[15] all far less severe phenotypes than observed in Greenberg dysplasia. The findings in this Pelger patient and the presented Greenberg cases suggest that minimal amounts of functional LBR are essential for human fetal development and survival.

Complete loss of LBR is lethal in humans but not necessarily in mice. Lbr deficiency in mice (ic/ic) presents with severe skin alterations (alopecia and ichthyosis), a Pelger blood phenotype, growth deficit, increased perinatal death, variable syndactyly, and hydrocephalus.[7,17,40] Wassif et al. analyzed sterol metabolism in Lbr deficient mice bred on different backgrounds and identified a sterol defect only on specific backgrounds and at a defined point of development in the brain.[27] They concluded that the phenotype in Greenberg dysplasia and ichthyosis is more likely to be caused by altered nuclear structure than by sterol metabolism. Based on our finding that *LBR* missense mutations affected the sterol metabolism but not the nuclear morphology and that no additional mutation in *DHCR7* or *TM7SF2* were found, we conclude that in humans the sterol defect of the lamin B receptor is pathogenic for Greenberg dysplasia.

Manifestations in response to homozygous failure of the lamin B receptor differ between species. Complete lamin B receptor deficiency seems to be not compatible with life in humans, shows a severe phenotype in mice but is tolerated by Drosophila.[41] Drosophila Lbr is evolutionary not highly conserved and lacks sterol reductase activity,[41] probably because cholesterol is an essential nutrient in flies not requesting an endogenous cholesterol synthesis. There is no explanation yet for the different phenotypes of LBR deficiency in man and mice; hypotheses include variations in cholesterol synthesis, transport or placental transfer. Phenotypic differences between human and mice are also seen in defects of other components of cholesterol synthesis. Thus, 3β-hydroxysteroid dehydrogenase defects cause limb defects in humans (CHILD syndrome) but not in Bpa mice.[26]

In humans, the fetal deaths in Greenberg dysplasia and severe skeletal defects in other cholesterol synthesis disorders underscore the importance of normal sterol synthesis for intrauterine development. This is of relevance for considering potential teratogenic side effects of lipid lowering drugs but also of antimicrobial agents that often target sterol synthesis.[42-45]

We conclude that LBR missense mutations in the transmembrane domain can abolish sterol reductase activity, thereby causing lethal Greenberg dysplasia but not Pelger anomaly. This finding separates the metabolic from the structural function of

LBR and indicates that its sterol reductase function is essential for intrauterine development in humans.

Material and Methods

Patients. We studied three fetuses with the clinical diagnosis of Greenberg dysplasia. The Charité University Medicine ethics committee approved the study. Written, informed consent was obtained from all participants or their legal representatives. Sterol analyses for cholesta-8,14-dien-3β-ol was performed from fetal material (liver or muscle) as published in Offiah et al.[25]

Sequence analyses. We sequenced all exons including the flanking intron regions of the *LBR* gene (NM_002296.2) as described previously[15] in either the fetus or the parents as obligate heterozygous carriers. All mutations were tested for correct segregation in the patient's families (where available). We further analyzed 300 control chromosomes to exclude a previously undescribed polymorphism. To explore the functional effect of missense mutations, we proved the evolutionary conservation of the affected residues by BLAST alignment and interspecies comparison. We sequenced additionally all coding exons of the genes encoding the two other members of the C14 sterol reductases family, *DHCR7* (NM_001360.2) and *TM7SF2* (NM_003273.2), respectively. Primer sequences are available on request.

C14 deficient yeast complementation assay. C14 sterol reductase deficient yeast has a defect of ERG24, resulting in abnormal sterol metabolism and consequent failure to grow (**Suppl. Fig. 2**). ERG24 belongs to the same C14 sterol reductase family as the lamin B receptor. Human wildtype LBR rescues the ERG24 deficiency in yeast.[5,30] We therefore tested whether or not human LBR carrying the missense mutations rescued the yeast phenotype.

Vectors p1023 (hsLBR_wt) and p1032 (empty) were kindly donated by Gerard Loison. We introduced the missense mutations p.N547D and p.R583Q by mutagenesis following the manufacturer's instructions (QuikChange[R] XL Site-directed Mutagenesis Kit, Stratagene). ERG24, wild type LBR, LBR_N547D and LBR_R583Q were each cloned in the yeast expression vector pEMR1032. We performed all analyses under the control of two different promoters (*PGK1* and *TP1*) to ensure that the rescue deficit of mutants is not due to chance variations in vector insertion or promoter activity.

ERG24 deficient yeast *Saccharomyces cerevisiae* BY4742*erg24* (Y11164) and the corresponding reference strain *S. cerevisiae* BY4742 (Y10000) were obtained from Euroscarf (Frankfurt, Germany). Transformation, expression and sterol analysis in yeast were performed as previously described.[30,46] We replicated all measurements in at least two independent experiments and in 2 different colonies per transformation.

To confirm expression of LBR in the yeast transformants we prepared 1 ml from log phase cultures. We pelleted ~3 x 10^{8} cells, resuspended in 100 µl distilled water, added 100 µl 0.2 M NaOH and vortexed for 5 min at room temperature. We pelleted the solution at 14,000 rpm for 2 min and resuspended in 50 µl 2x LDS buffer with 10% β-mercaptoethanol. After boiling for 3 min and pelleting, the samples were processed as previously

described.[15] Human wildtype LBR and both mutants showed the same expression pattern in western blot indicating that transfection efficiency was comparable (data not shown).

Used LBR antibodies. The most widely used LBR antibody, the guinea pig polyclonal anti-LBR antibody (gp-anti-LBR_N-term) is directed to the first 210 aminoacids of LBR and was kindly provided by Harald Herrmann-Lerdon.[15] The specificity of this antibody was shown in Shultz and co-workers: Immunostaining and immunoblot of Lbr deficient cells showed no background staining by the LBR antibody.[17] The rabbit monoclonal anti-LBR antibody (rb-anti-LBR_N-term) is also directed against the N-terminus of human LBR (Epitomics, Burlingame, CA, catalogue number 1398-1). The mouse monoclonal anti-LBR antibody (mouse-anti-LBR_C-term) is directed against the C-terminal domain of LBR (klh-19, kindly provided by Harald Herrmann-Lerdon).

Immunostaining in human fibroblasts. Human dermal fibroblasts were cultivated in Dulbecco's Modified Eagle Medium (Lonza, Basel, Switzerland) with 10% fetal calf serum (FCS) and 2 mM L-glutamine. Cells were grown on coverslips, fixed in 4% paraformaldehyde in 1x phosphate buffered saline (PBS) for 10 min at 4°C, then blocked for 30 min with 10% bovine serum albumine (BSA) in PBS, incubated for 1 h with polyclonal gp-anti-LBR_N-term (1:100 dilution) followed by an 1 h incubation with the secondary antibody (Alexa 555-conjugated goat anti-guinea pig antibody, Molecular Probes, 1:1,000) and DAPI (Sigma, 1:1,000). Cells were mounted with Fluoromount G (SouthernBiotech) and imaged using an LSM 510 meta microscope (Carl Zeiss, Göttingen, Germany) with a x63 Plan Apochromat oil immersion objective.

Nuclear extraction and immunoblotting. Nuclear and cytosolic fractions of whole cell lysates of skin fibroblasts were extracted by the Nuclear-Extraction Kit (Cayman Chemicals) and resolved by electrophoresis in SDS poly-acrylamide gels. The following antibodies were used for immunoblot analysis on PVDF membranes: as described above the polyclonal gp-anti-LBR_N-term, monoclonal rb-anti-LBR_N-term, monoclonal mouse-anti-LBR_C-term, further a rabbit polyclonal calnexin antibody (catalogue number GTX13504, Acris Antibodies, Herford, Germany), a mouse monoclonal alpha-tubulin antibody (ab7291, abcam, Cambridge, USA), and a mouse monoclonal anti-lamin B2 antibody (clone X223, catalogue number 65147C, Progen, Heidelberg, Germany). All immune reactions were carried out in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20 (TBST) with 5% dried milk at RT with washing steps in TBST.

Quantitative PCR (qPCR). We studied *LBR* mRNA levels in different human cell lines and mouse tissues from postnatal day four. Following lysis with Trizol® and standard phenol/chloroform RNA extraction, total cDNA was transcribed by RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas). For qPCR on ABI Prism 7500 (Applied Biosystems Foster City US) we mixed cDNA, CyberGreen (Invitrogen), and primers. We analyzed the data using the ABI Prism SDS Software package (ΔΔCt method, normalisation against GAPDH). Primer sequences are available on request.

In-situ hybridization. We generated probes for *Lbr* by RT-PCR from mouse E14.5 whole cDNA. Primer sequences are available on request. Antisense riboprobes were transcribed with SP6 or T7 polymerase using the Roche Dig-RNA labeling kit according to the manufacturer's instructions. Protocols for whole-mount in-situ hybridizations and in-situ hybridizations on paraffin sections have been previously described.[47,48]

Immunostaining in mouse embryos (paraffin sections). For immunostaining, paraffin sections were deparaffinized, rehydrated and boiled for 10 minutes in 0.01 M Sodium citrate pH 6.0. Sections were blocked in 10% goat serum for 1 h, primary antibodies (1:100 polyclonal guinea pig anti-LBR_N-term, generated by Monika Zwerger, initially described in;[40] 1:100 rabbit anti-Sox9, Santa Cruz) were applied in 5% goat serum at 4°C over night. Secondary antibodies (goat anti-guinea pig-Alexa Fluor 546, goat anti-rabbit Alexa Fluor 488, Molecular Probes, 1:1,000, together with DAPI, 1:2,000) were applied for 1 h at room temperature. Sections were analyzed using an Axiovert 200 (Zeiss) equipped with ApoTome optical section device and AxioVision software.

Web Resources

1. Basic Local Alignment Search Tool (BLAST), http://blast.ncbi.nlm.nih.gov/Blast.cgi

2. Ensembl Genome Browser, www.ensembl.org/

3. GenBank, www.ncbi.nlm.nih.gov/Genbank/

4. Online Mendelian Inheritance in Man (OMIM), www.ncbi.nlm.nih.gov/Omim/

5. National Center for Biotechnology Information (NCBI), www.ncbi.nlm.nih.gov

Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/ClaytonNUC1-4-Sup.pdf

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