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Dissertation

Nonsense-mediated mRNA decay in collagen X

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Chapter One

Introduction

1.1 CHONDRODYSPLASIAS

Chondrodysplasias are a heterogenous group of inherited disorders of skeletal development and linear bone growth. They are caused by disturbances to the structure or expression of cartilage components in the growing skeleton. By definition, chondrodysplasia mutations disrupt the function of the skeletal growth plate, which is where postembryonic bone growth occurs [1, 2]. The growth plate is a dynamic structure that resides near the ends of immature bones. Growth occurs when the cartilage template produced at the leading edge of the structure is converted into bone at its trailing edge. This highly complex process involves an interplay of differentiating chondrocytes and other cell types such as osteoblasts, osteo(chondro)clasts, perichondrial, vascular and perivascular cells [3, 4]. Key cellular events such as chondrocyte proliferation and terminal differentiation and vascular invasion of the cartilage template are regulated by feedback control systems. These involve locally synthesized growth factors and their receptors, including fibroblast growth factors (FGFs), parathyroid hormone-related protein (PTHrP), bone morphogenetic proteins (BMPs), and Indian hedgehog (Ihh). Furthermore, matrix proteins influence these cellular events through direct interactions with membrane receptors and growth factors (a detailed description can be found in [4]).

Abnormal cartilage growth and function can result from mutations in genes that are involved in cartilage structure or the complex regulation of chondrocyte differentiation, patterning, maturation and hypertrophy[4]. Mutations involving cartilage matrix proteins like collagens may alter the mechanical properties of cartilage, and protein misfolding mutations may also result in downstream effects on cellular gene expression[5] as well as exerting dominant negative effects on extracellular matrix assembly[6]. Matrix protein mutations disturb the biological properties of cartilage, such as by modification of growth factor availability, alteration of signals initiated by matrix-protein-receptor interactions, or by mechanisms such as activation of transmembrane receptors (e.g. FGFR3 or the PTHR), that result in abnormal chondrocyte growth and maturation (a detailed description can be found in the references[2, 7, 8].

Although the chondrodysplasias separately are very rare diseases, as a group they have a prevalence of 1:4000 births. The hallmark of chondrodysplasias is disproportionate short stature, with a disproportion between limbs and trunk (Figure 1.1 shows how chondrodysplasias are classified, based on the site of the primary bone

defect). Their severity ranges from lethal disorders to disorders that are so mild that they prove difficult to detect. Although much is known about the molecular genetics of chondrodysplasias, the clinical diagnosis remains essential, including clinical history, physical examination, skeletal radiographs, family history, and laboratory testing.

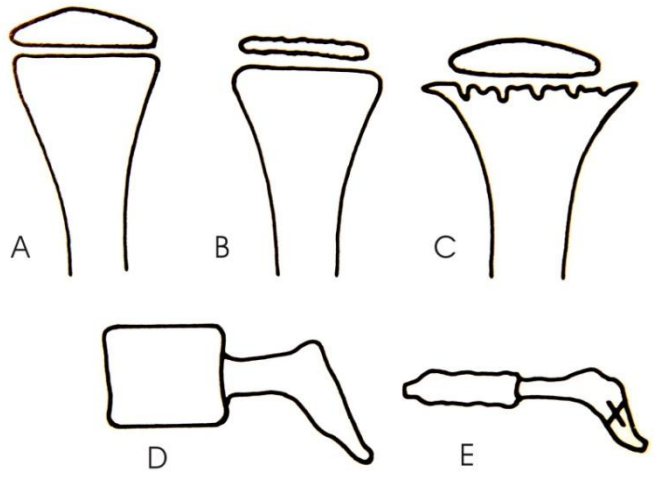


Figure 1.1 Classification of chondrodysplasias based on radiographic involvement of **A** to **C** long bones and **D**, **E** vertebrae. Combinations of **A**-**E** display different clinical pictures. **A** and **D** (both physiological) normal. **B** and **D** epiphyseal dysplasia. **C** and **D** metaphyseal dysplasia. **B** and **E** spondyloepiphyseal dysplasia. **B**, **C** and **E** spondyloepimetaphyseal dysplasia. Modified from Emery et al., 1990[9].

1.1.1 Schmid Metaphyseal Chondrodysplasia

Schmid Metaphyseal Chondrodysplasia (OMIM #156500) was first encountered in 1949 by Schmid in a 6 1/12 year old boy. This boy was treated for rickets from three years of age, when his mother became aware of his waddling gait and a reduced growth in length. The radiographic changes of metaphysis and epiphysis found in this boy led to the original name of SMCD, dysostosis enchondralis metaphysaria[10]. Later the name was changed to Schmid metaphyseal chondrodysplasia. This is a much more accurate description since the disease is not a defect in bone formation (dysostosis) but rather of the growth plate cartilage. Therefore, SMCD is now classified as a metaphyseal chondrodysplasia [1]. Metaphyseal chondrodysplasias (MCD) are clinically and aetiologically heterogenous disorders grouped together [1] because they display predominantly metaphyseal abnormalities comprising flaring, sclerosis and irregularity on radiographs. SMCD has been found to be the most common of all MCDs.

1.1.1.1 Clinical and radiographic features of Schmid Metaphyseal Chondrodysplasia

In 1988, Lachman et al. published a clinical and radiographic delineation of SMCD, to distinguish it from other genetic disorders that had been commonly confused with SMCD[11]. SMCD is an autosomal dominant disorder. It is not detectable clinically or radiographically at birth, but usually presents after the age of 2–3 years. The children show mild short stature (usually lower than the 5th percentile), S-like bowing of the lower extremities, coxa vara and a waddling gait. Hip pain may be present, enlargement of the wrists, inability to extend completely the fingers, and flaring of the lower rib cage are associated findings. The degree of short stature relative to norms increases with age. Facial appearance and craniofacial skeleton are normal, and there are no consistent extraskeletal manifestations in SMCD, an important consideration when assessing possible differential diagnoses. Basic metabolic tests (serum calcium, alkaline phosphatase, phosphate, vitamin D) are normal and allow the differentiation from rickets. Furthermore, it is of note that intelligence and lifespan are normal in affected individuals and there is considerable inter- and intrafamilial variability in the severity of the condition.

The radiographic findings are essential for the differential diagnosis of SMCD (refer to Figure 1.2). The hallmark is metaphyseal abnormality, comprising widening, sclerosis and irregularity. The changes are typically observed from age two years onwards. Distal femoral and proximal tibial metaphyses are the most consistently and severely affected. Proximal femoral metaphyses and anterior rib ends (metaphyseal equivalents) are also consistently abnormal. Coxa vara (reduction of the normal angle between shaft and neck of the femur), often in association with mild femoral bowing, is observed in the majority of cases and is an important radiographic sign in differentiating SMCD from other forms of metaphyseal chondrodysplasia [1]. Skull, craniofacial skeleton and pelvis are not affected in SMCD. Over time, secondary arthritic changes may occur in affected joints, which further complicate diagnosis of SMCD in adults. Recently, hand and spinal involvement have been reported ([12], [13], respectively). The studies also suggested that these features of SMCD may regress with age, and thus account for their omission in all of the previous literature regarding this condition.

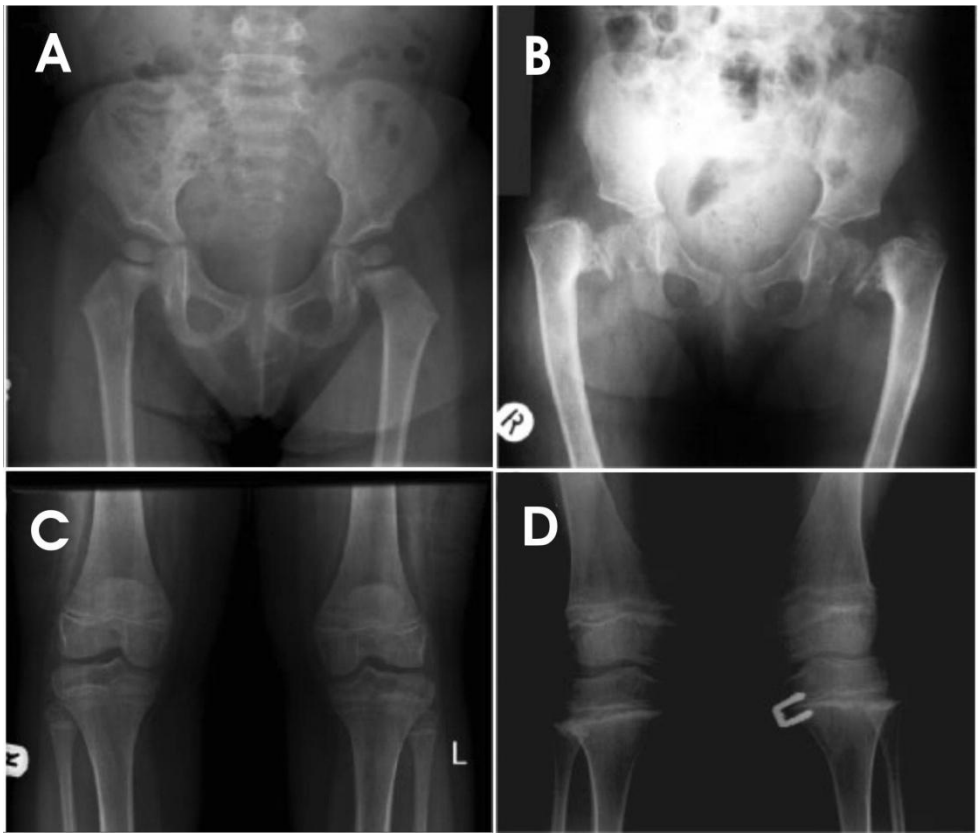


Figure 1.2 Radiographic findings in Schmid MCD patients. Anterior-posterior radiographs of a normal individual and an SMCD patient. **A** Normal pelvis and upper femur of a 6 year old child. **B** Pelvis of a 5 year old SMCD patient showing coxa vara and femoral bowing. **C** Radiograph of the knees of a normal 9 year old individual and **D** of the knees of an 9.5 year old SMCD patient, showing genu valga, metaphyseal widening and sclerosis. Source: modified from Bateman et al, 2005[14]

1.1.2 Mutations in collagen X cause Schmid Metaphyseal Chondrodysplasia

The clinical and radiographic findings in SMCD patients highlighting underlying growth-plate abnormalities suggested that SMCD was likely to be caused by a mutation in a gene expressed in this region of the developing bone. In 1991, an SMCD mutation was found in the gene for collagen X, a matrix protein expressed in the growth plate during a short phase of endochondral ossification [15].

Collagen X is a member of the family of the hexagonal network forming collagens, which are non-fibrillar short-chain collagens. It shows a very restricted expression pattern, confined to the developmental stage of endochondral ossification, and is present in hypertrophic chondrocytes of the foetal and juvenile growth plate [16, 17], fracture repair sites [18], and as a consequence of joint degeneration in osteoarthritis [19]. Collagen X appears in the cartilage matrix of the growth plate just before its mineralization and replacement by bone, when the chondrocytes undergo dramatic

changes in their gene expression, which include changing from collagen type II to type X as the major collagen type produced [20, 21].

In contrast to most other collagen genes, which have the characteristic multiexon-structure coding for the collagenous domains [22], the collagen X gene *COL10A1* consists of only three exons. Collagen X is a homotrimeric collagen containing three identical α1(X)-chains. Each α1(X)-chain has 680 amino acids, consisting of a helical domain (COL1, 463 amino acids) that is flanked by a large carboxyl terminal domain (NC1, 161 amino acids) and a short amino-terminal domain (NC2, 56 amino acids). It is of note that most of the collagen X protein sequence is coded for by exon 3 (about 3kb in length), including the 3' untranslated region (UTR), the NC1 domain, the COL1 domain, and part of the NC2 domain. Exon 2 (169bp) encodes the remaining NC2 sequence, the short signal peptide sequence (SP) and 15 bases of the 97bp-5' UTR. Exon 1 codes for the remaining 82bp of the 5'UTR .

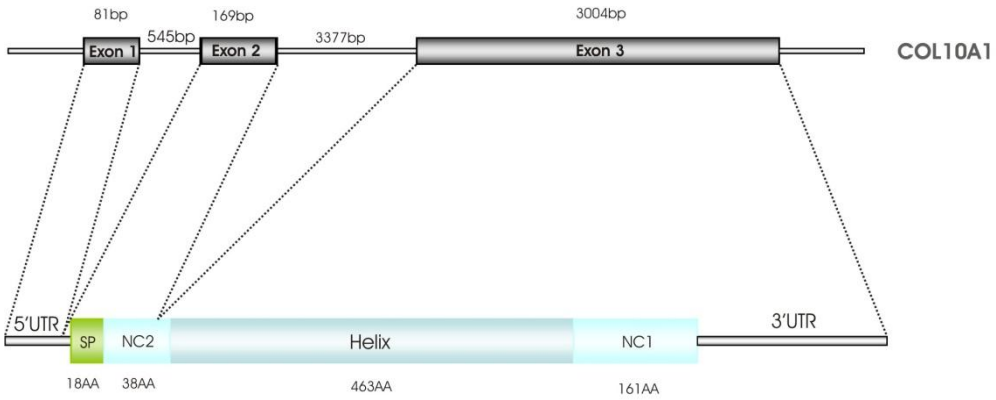


Figure 1.3 Gene, mRNA and protein structure of collagen X. Diagram of the intron-exon structure of COL10A1 gene, the mRNA and protein organization of the alpha1(X)-chain. In the upper part of the figure, exons are represented by black boxes, introns by horizontal lines. Intron and exon sizes are illustrated above the gene. The mRNA and protein structure is shown below the gene. The signal peptide (SP) is marked by the green box, 5' and 3' untranslated regions are grey horizontal lines. The blue box shows the collagenous (helix) and the turquoise boxes the noncollagenous (NC1 and NC2) domains of the protein. The sizes of the individual protein domains are indicated below. Different to other collagens, exon 3 of the COL10A1 gene codes for the majority of the polypeptide chain, including the triple helical domain. Source: modified from Bateman et al. (2005) [14]

Apart from the cleavage of the signal peptide in the rough endoplasmic reticulum, there appears to be no post-translational proteolytic processing of collagen X [23, 24]. The NC1 and NC2 domains are retained in mature collagen X molecules extracellularly. This is significantly different to the fibrillar collagens, which are synthesized as procollagens, containing large C- and N-terminal propeptides which are proteolytically removed after secretion [4]. In the helical domain, the Gly-X-Y triplet repeat sequence which is the

common feature of all collagens, contains eight helix sequence imperfections. Two of these coincide with collagenase cleavage sites.

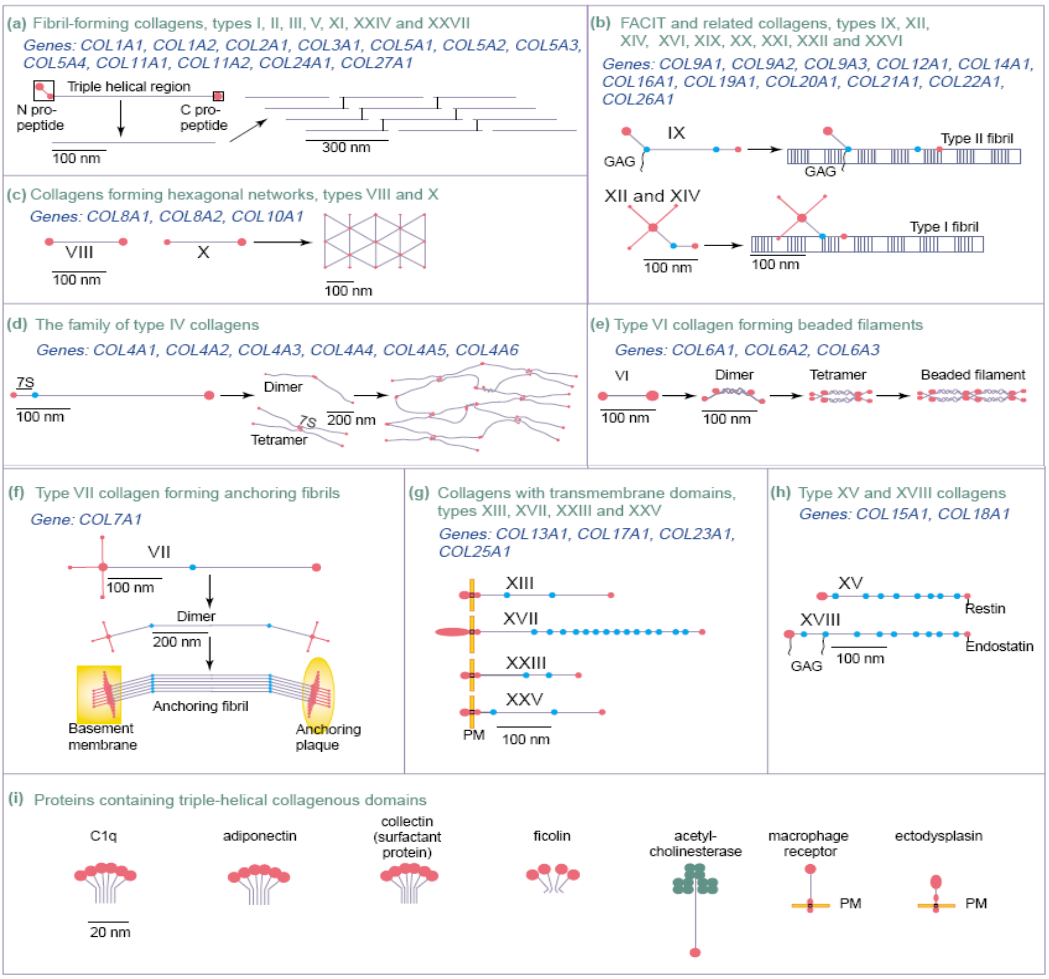


Figure 1.4 The collagen superfamily. The collagen superfamily consists of nine families, grouped together according to their supramolecular assembly: (a) fibril-forming collagens; (b)fibril-associated collagens with interrupted triple helices (FACITs) located on the surface of fibrils, and structurally related collagens; (c) collagens forming hexagonal networks; (d) the family of type IV collagens located in basement membranes; (e) type VI collagen, which forms beaded filaments; (f) type VII collagen, which forms anchoring fibrils for basement membranes; (g) collagens with transmembrane domains; and (h) the family of type XV and XVIII collagens. (i) is a group of highly heterogenous proteins that have not been identified as collagens but nevertheless contain collagenous domains. Supramolecular assemblies of (g) and (h) are not known and therefore not shown in this figure. 42 genes (blue) code for a total of 27 polypeptide chains, with each collagen molecule consisting of three different or identical polypeptide chains. Collagenous domains are shown in purple, and C-terminal non-collagenous domains are in dark pink, and the non-collagenous domains interrupting the triple helix in light blue, short interruptions of a few amino acids are not shown. Source: Myllyharju, 2004 [25] Abbreviation: PM, plasma membrane.

1.1.3 The NC1 domain is responsible for correct assembly of the three α1-(X)chains

Rotary shadowing visualization of type X collagen molecules using electron microscopy reveals the short triple helical domain of 132nm, flanked by two globular protuberances that correspond to the NC1 and NC2 domains. *In vitro*, collagen X molecules form hexagonal networks similar to those of type VIII collagen in the Descemet's membrane (a basement membrane of corneal endothelial cells). The supermolecular assembly of those hexagonal lattices seems to be driven, at least partly, by the NC1 domains [26, 27].

The importance of the NC1 domain is suggested by the high degree of conservation between collagen types VIII and X and between species. It plays a critical role in intracellular trimer formation [28], and also contributes to the stability of extracellular collagen X networks [29]. It contains a sequence of about 130 amino acids, the C1q module, which is also found in collagen VIII and other proteins including C1q itself. This module contains 13 tyrosine residues conserved throughout species. The C1q module is crucial in the initiation and maintenance of the ordered assembly of collagen type X and is involved in the trimerization process. The crucial role of the NC1 domains of the collagen X subunits in driving assembly has been shown by *in-vitro* translation and assembly studies[30, 31]. Protein structural studies [32]) confirmed that the NC1 trimer is highly stable due to the hydrophobic contacts formed between the subunits. These include a hydrophobic region at the base of the NC1 domain and the 12 amino acid long highly conserved 'aromatic zipper' on the surface of each NC1 subunit The trimers are more hydrophilic at the top where a cluster of four Ca2+ ions is present [32]).

The NC2 domain of α1(X) is structurally quite divergent from the NC2 domains of other collagens (even of the structurally very similar collagen type VIII). Although less conserved throughout the species than the NC1 domain, the NC2 domain is also thought to be involved in intermolecular interactions and the formation of extracellular supramolecular assemblies[26].

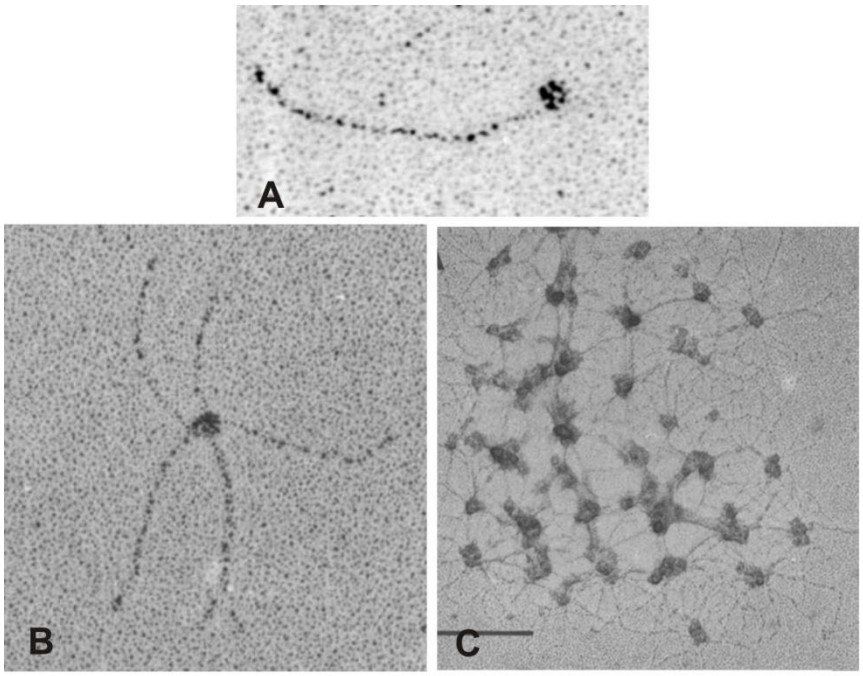


Figure 1.5 Collagen X rotary imaging. Molecular aggregates of type X collagen. (A) The electron micrograph of a single alpha1(X) chain shows the C-terminal non-collagenous (NC1) domain clearly visible in the intact monomer as a small globule. (B) Collagen X multimers associate at the NC1 domain (during in vitro incubating of purified chick type X collagen in PBS at 34°C). (C) The multimers assemble to form a hexagonal lattice. The network contains regularly spaced nodules of aggregated carboxy-terminal noncollagenous domains interconnected with filamentous structures formed via interactions between adjacent triple helices. The nodules in the lattice are arranged in a regular hexagonal arrangement in this rotary shadowing electron micrograph. Source: from Kwan et al., 2005[26]

1.1.4 The function of collagen X remains unclear

Despite recent data on the expression, structure and assembly of collagen X, very little is known about its precise function in the hypertrophic cartilage. Since the hypertrophic zone is structurally the weakest point in the growth plate, collagen type X may provide additional strength to the matrix. Due to its very restricted distribution it may well be involved in direct or indirect initiation of matrix mineralization[33], since it has been shown to bind Ca^{2+} and matrix vesicles, cell-derived microstructures found in calcifying cartilage that are thought to be essential for initiation of mineral deposition. Consistent with this postulated role, collagen X binds calcium directly at about 15 binding sites per chain[34]. Studies on mice where collagen X expression had been ablated showed a mild SMCD in homozygous null mice [35] with displacement of proteoglycans in the growth plate, altered mineral deposition, compression of the growth plate and hematopoietic changes [36].These data suggest that the collagen X network plays a role in extracellular matrix organization. Furthermore, two of the eight imperfections in the Gly-X-Y triple helical sequence are mammalian collagenase cleavage sites which may facilitate collagen X breakdown and supply the structural conditions that permit matrix removal, vascularization and bone development [37]

1.1.5 Mutations in COL10A1

In Schmid MCD patients, a total of 38 mutations in the collagen X gene *COL10A1* have been identified so far (mutations reviewed in [38] and [14], refer to Figure 1.6).

Most of these collagen X mutations are localized to the C-terminal NC1 domain of the protein. Despite the lack of phenotypic variation, in affected patients, the SMCD mutations identified to date range from amino acid substitutions to nonsense mutations and deletions resulting in premature termination codons. All of these mutations are about equally divided into missense mutations (47%) and mutations that cause premature termination codons (PTC, see below, frameshift and nonsense mutations, 53%).

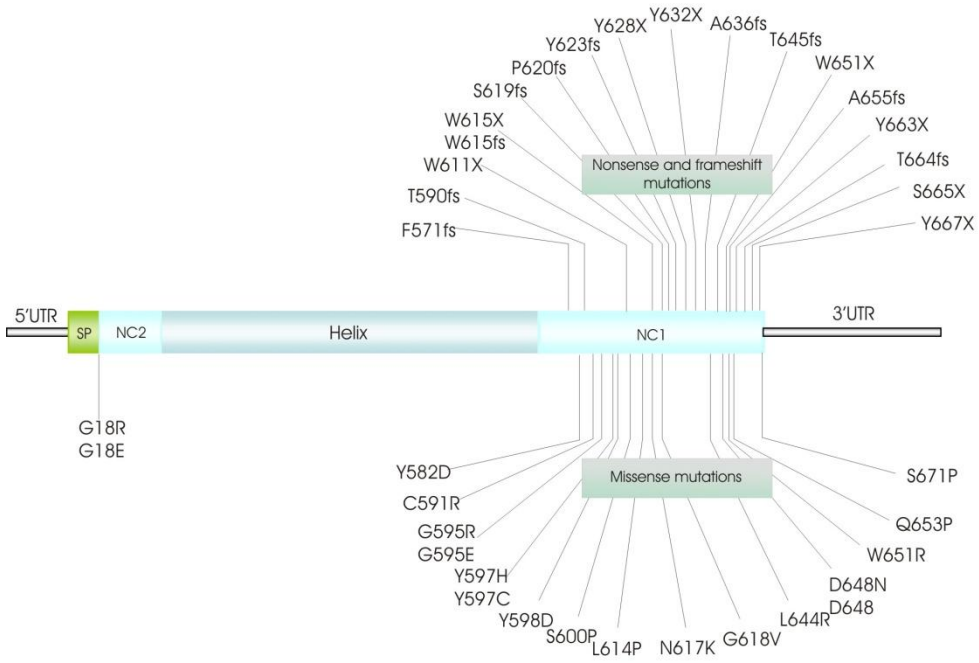


Figure 1.6 Collagen X mutations in SMCD. Schematic representation of the mutations that have been identified in SMCD patients. SMCD mutations that cause premature stop codons (PTCs, nonsense and frameshift mutations) are depicted above the protein structure and mutations causing single amino acid substitutions (missense mutations) are shown below the protein structure. The 5' and 3' untranslated regions (UTRs) are depicted by the black horizontal lines.The signal peptide (SP) is shown as a green box, the triple helical domain (helix) is indicated by a blue box, and the globular noncollagenous domains (NC1 and NC2) are shown as turquoise boxes.

1.1.5.1 Missense mutations

Of the 19 heterozygous missense mutations that have been found so far, 17 lie within the C-terminal NC1 domain of collagen X. The remaining two mutations occur at the N-terminal signal peptide cleavage site Gly^{18}-Val^{19} [39]. *In vitro* expression studies of

these two mutations c.52G>A*(p.G18R) and c.53G>A (p.G18E) demonstrated that the mutations inhibit cleavage of the signal peptide by its peptidase. Thus, the mutant chains remain anchored to the microsomal membranes and are degraded. Moreover, the chains bound to the membrane might assemble with normal collagen X chains and consequently cause further reduction of secreted functional collagen[41]. All other missense mutations have been found to cluster in the NC1 trimerization domain, between Tyr^{582} and Ser^{671} [14].

Missense mutations in the NC1 domain have been shown to prevent assembly in vitro [42, 43] and may thus result in a functional haploinsufficiency of α□ (X) by stopping trimer assembly and targeting of the mutant for breakdown. Recent data also shows that the misfolding of the collagen X caused by the missense mutations can result in a cellular stress response, the unfolded protein response [44], and this has been shown in other situations to result in changes in cellular gene expression leading ultimately to apoptosis [6]. This may be a major contributor to the pathophysiology of SMCD. In addition, small amounts of the mutant may also associate with the normal allele product and disturb assembly and function in a dominant-negative fashion.

1.1.5.2 Nonsense and frameshift mutations

Seven unique heterozygous nonsense and 12 unique heterozygous frameshift mutations have been identified in SMCD patients [38]. These mutations introduce a PTC at the site of the base change (nonsense mutation) or downstream (frameshift). All PTC causing mutations that have been found in SMCD patients occur within the C-terminal NC1 trimerization domain of collagen X.

When first reported, these PTC mutations were suggested to cause the production of truncated proteins lacking regions essential for trimer formation [43]. The molecular mechanism underlying SMCD in these cases was a matter of debate. Wallis et al. [45] argued for a dominant effect of nonsense mutations. However, studies on cartilage tissue from two patients with PTC mutations demonstrated that no mutant collagen X mRNA and protein was present, strongly arguing that the underlying mechanism to SMCD in these patients is haploinsufficiency [42, 46]. The complete lack of mutant collagen X mRNA is most likely the result of nonsense-mediated mRNA decay (NMD),

* Sequences are numbered from the translation initiation codon according to the nomenclature of den Dunnen[40] den Dunnen JT, Antonarakis SE. Nomenclature for the description of human sequence variations. Hum Genet 2001; 109:121-4.

an evolutionary conserved mRNA surveillance pathway that detects and eliminates mRNAs harboring PTCs. NMD thus protects the cell from the possibly deleterious effects of a truncated protein. This thesis focuses on the novel nature of the effects of PTC mutations in SMCD patients and their implications on the understanding of the process of NMD to date. Therefore the basic principles of NMD will be discussed in detail in following sections.

1.2 NONSENSE-MEDIATED DECAY – AN IMPORTANT FACTOR IN DISEASE AS WELL AS REGULATION OF NORMAL GENE EXPRESSION

While there are numerous types of mutational changes that can result in disease, nonsense mutations are very common, and reportedly cause one-third of all inherited genetic disorders [47]. Nonsense mutations, resulting from point mutations, insertions or deletions, generate a premature stop codon (PTC). This stop codon occurs upstream of the genuine stop codon that signals the termination of translation of the mRNA into the appropriate full length protein. The theoretical consequence of this PTC is the synthesis of a shorter truncated protein that could exert a dominant negative or gain of function effect on the cell that could be deleterious. To overcome this, cells have developed an elegant quality control surveillance mechanism called nonsense-mediated mRNA decay (NMD). This pathway allows the cell to identify a premature stop codon from a genuine stop codon and destroy the PTC-containing mRNA before it could result in a truncated protein. Recently, it has become evident that degradation of disease-associated transcripts is not the only purpose of the NMD pathway. The finding that NMD is a post-transcriptional process highly conserved throughout evolution indicated that it serves important roles in cellular RNA metabolism. For example, the advantages of alternative splicing of 55% of human pre-mRNAs[48] that lead to a huge proteome diversity come at the price of some inefficient or inaccurate intron removal during splicing. The NMD pathway allows PTCs caused by such an inaccuracy to be quickly and efficiently removed. Furthermore, the pathway is responsible for down-regulation of natural targets [49, 50], indicating that NMD also plays a role in regulation of gene expression.

Despite conservation of the NMD pathway, the nature of PTC definition and the decay pathway of targeted mRNAs vary across species. Two models are of particular

importance for understanding NMD in collagen X, NMD in mammalian cells and NMD in yeast. These two models are therefore explained in more detail below.

1.2.1 NMD in mammalian cells depends on exon-junction complexes

The widely accepted model of NMD interconnects pre-mRNA splicing, RNA transport from the nucleus and translation in the regulation of NMD[51-53].

During pre-mRNA splicing, removal of the introns leaves the exons adjoining each other, in so called exon-exon boundaries (see Figure 1.8, A). At each of these exon boundaries or junctions, a complex of proteins is deposited, called the Exon-Junction-Complex (EJC). EJCs are deposited approximately 20 nucleotides (nt) upstream (5') of each exon-exon junction[54]. Additionally, the mRNA is bound by a cap-binding protein CBP 80/20 heterodimer, and Poly-A-binding protein PABPN1[55].

EJCs comprise a range of proteins involved in pre-mRNA splicing, mRNA export, proteins responsible for NMD, as well as proteins whose functions are not yet fully understood (refer to Figure 1.7, reviewed in Maquat, 2005 [56]).

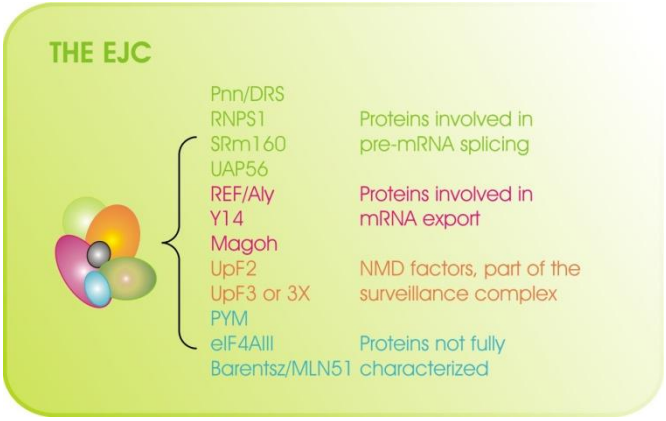


Figure 1.7 Components of the EJC. Modified from Maquat, 2005[56]

The resulting mRNP (messenger ribonucleoprotein, an mRNA decorated by a range of proteins) then undergoes an initial, or pioneer round of translation, during which the ribosomes scan along the mRNA. If the ribosomes do not encounter any PTCs, they displace the proteins of each EJC as they move along the mRNA[53].

However, when a stop codon is reached by a ribosome (Figure 1.8, B), a different RNA binding protein complex, a so called surveillance complex is recruited, including the termination factors eRF1 and eRF3 (eukaryotic release factors). The UPF (Up-Frameshift) proteins 1-3 are core components of the surveillance complex, the basic

function of which is conserved in eukaryotes. A deletion of one of these components results in mRNA stability despite the presence of a PTC[57].

If an EJC is found downstream of the stop codon (and if the distance between EJC and stop codon is greater than 50-55nt), it is identified as ‘premature’. In this situation, UpF1 as a part of the surveillance complex targets the mRNA containing the premature stop codon for NMD by interacting with the EJC-related UpF2[58]. The mRNA is then degraded.

On the other hand, if there is no EJC downstream of this surveillance protein complex, the stop codon is recognized as the correct stop and the initiation complex is ultimately remodelled to the steady-state initiation complex (refer to Figure 1.8, A). That is, eukaryotic translation initiation factor eIF4E replaces CBP 80/20, PABPC has replaced PABPN1 and all EJCs are removed. In mammals, the mRNA is now immune to NMD[55, 59, 60]. Multiple rounds of translation begin.

This rule also applies for nonsense codons located within the last exon (refer to Figure 1.8, C). Since the premature stop codon is not followed by an EJC, the surveillance machinery considers it as the correct stop codon. This way, PTCs within the last exon do not elicit NMD, resulting in a shorter, or truncated protein. This ‘gap’ in the NMD pathway could be useful because 3’ located nonsense codons will generate almost full length proteins that might be more likely to function than proteins generated by 5’ located nonsense codons. Also, the stop codon is located in the last exon in 93% of all examined genes[61], leaving the last exon as a comparatively small area for nonsense mutations that could possibly cause damage by not eliciting NMD.

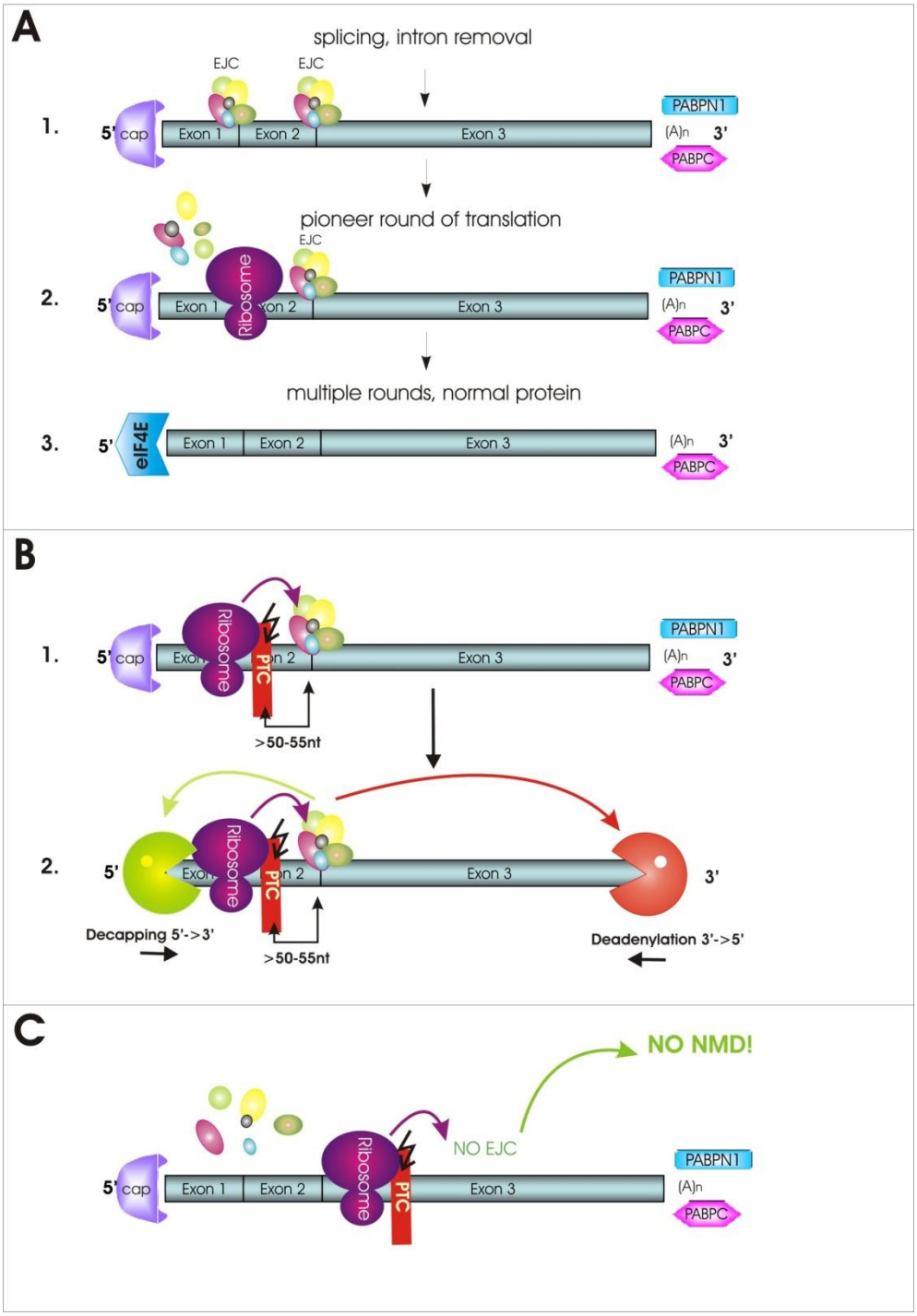


Figure 1.8 Nonsense-mediated decay in mammalian cells. A Normal translation. After pre-mRNA splicing, the site of intron removal, the exon-exon junction, is marked by an exon-junction complex (EJC) (1). The resulting mRNP undergoes a pioneer round of translation, during which the ribosome scans along the mRNA and displaces the EJC's (2). After this pioneer round, multiple rounds of translation start, and ultimately a full length protein will be produced. **B** If a ribosome encounters a stop codon during the pioneer round of translation, it is considered premature if the ribosome is able to detect an EJC downstream of this stop (1). The mRNA is rapidly degraded (2). **C** A premature stop codon in the last exon is not followed by an EJC. Therefore the ribosome is not able to identify the mutation as a PTC, and the mRNA will be translated.

1.2.1.2 NMD can be either nucleus- or cytoplasm associated

It has been shown by cell fractionation studies that most PTC-containing mRNAs are subject to nucleus-associated NMD, and therefore occurs before the newly synthesized mRNA is released into the cytoplasm (reviewed in ref. [62]). This form of NMD most likely takes place during or after transport across the nuclear pore complex, since some of the components of the EJC are involved in mRNA export out of the nucleus, allowing decay to take place during transport of the mRNA towards the cytoplasm[63, 64]. However, some mutant mRNA's are subject to cytoplasmic NMD and it is not yet known what renders mRNA to be subject to either form of NMD.

1.2.1.3 Recent findings in mammalian NMD reveal additional complexity

Recent data revealed surprising new aspects of nonsense-mediated decay. Studies on the hepatocyte nuclear factor 1β (HNF-1β) demonstrated that truncated mutant transcripts show a gradient of NMD between 5' and 3' ends, where mutations that are more 5'-located cause a higher susceptibility to nonsense-mediated decay than mutations that were more 3'-located [61].

Polarity of NMD had been seen before: Wang and colleagues [65] described 'boundary-independent polar NMD' in the T-cell receptor (TCR)- β gene, with a 3' to 5' polar susceptibility to NMD, where 5' mutations showed the lowest susceptibility to NMD. Interestingly, the polarity described by Harries et al[61] seems to be a 5' to 3' polarity, with the highest susceptibility rates close to the 5' end. Mutations in close proximity to the 3'end were resistant to NMD. It was hypothesised that this polarity could be caused by translation reinitiation downstream of the PTC, but that other factors such as the distance from the original initiation codon could also be involved in this unusual form of NMD [61].

Another interesting finding was recently reported on the NMD factors UpF1, UpF2, UpF3B, RNPS1, Y14 and MAGOH. Not only are they part of the surveillance complex in NMD, but they seem to also promote translation of normal mRNAs in mammalian cells[66, 67]. Previously it had been discovered that NMD factors appear to play a role in efficient translation termination[68, 69].

These findings implicate that NMD factors adopt multiple functions within a cell, and the NMD pathway is integrated into other cellular processes apart from translation surveillance.

1.2.2 PTC definition in yeast occurs independently of exon boundaries

A second model of NMD, different to mammalian NMD, is found in yeast. The recognition of premature termination codons in yeast follows rules different to the ones in mammals. There are two theories as to how a stop codon is assessed and determined premature in yeast.

The first theory on stop codon recognition in yeast is found in *S.cerevisiae.* Here, PTC definition occurs independently of exon-exon boundaries. This notion is consistent with the finding that PTC-containing mRNAs transcribed from naturally intronless genes are still subjected to NMD in *S.cerevisiae* [70]. In some yeast mRNAs, loosely defined downstream destabilizing elements (DSEs) have been found to function in the same way as EJCs in mammalian cells. Hrp1p (a heterogenous RNP-like protein) seems to bind to the DSE, thus marking it for NMD[71]. However, since there seems to be varying information about these yeast DSE's it is likely that multiple cis-acting sequence elements and trans-acting binding factors exist[47, 72].

The second theory on PTC recognition in yeast is the 3'UTR: Amrani et al. suggest that a generic feature of the mRNA, such as the poly(A)tail or a mark deposited during cleavage and polyadenylation, may hold the information necessary to discriminate a PTC from the natural stop[73] (refer to Figure 1.9). The 3'UTR is marked by a specific set of proteins (3'protein complex, 3'PC). Upon encountering a stop codon, a ribosome seeks to interact with these 3'UTR-bound proteins. This being possible, normal termination takes place (Figure 1.9, A).

However, the inability of the ribosome to establish these interactions with 3'UTR-bound proteins leads to rapid degradation of the mRNA (Figure 1.9, B). The recent finding that translation termination is aberrant at PTCs, and that prematurely terminating ribosomes fail to release efficiently strongly supports this model. Moreover it was found that this effect is abolished in mRNA lacking the NMD factor Upf1p or if the PTC is flanked with a normal 3'UTR (or the poly(A) binding protein Pab1p that mimics a normal 3'UTR) [73].

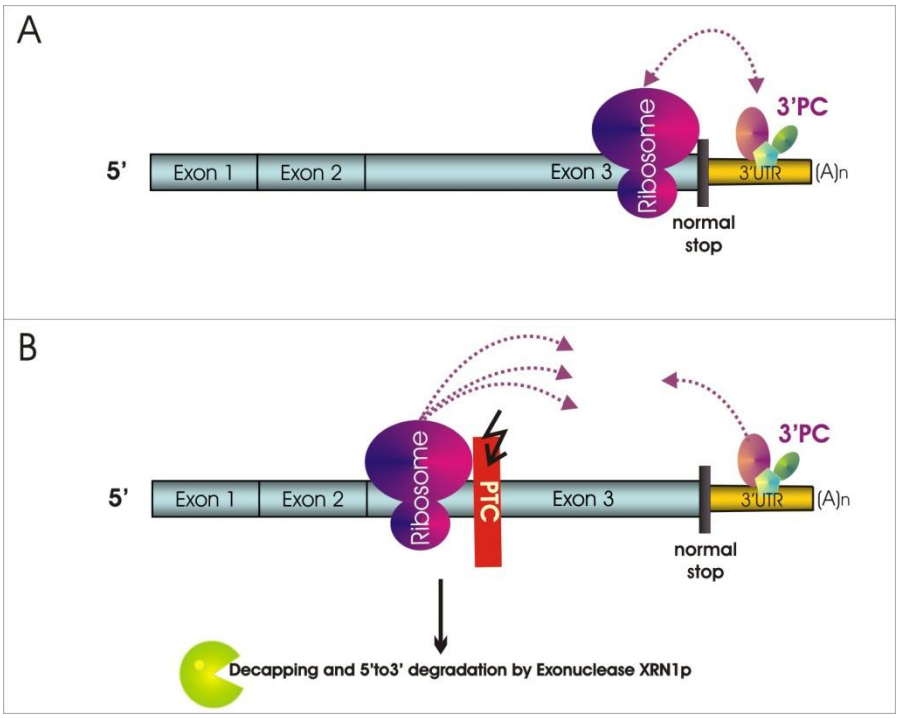


Figure 1.9 NMD in yeast. **A** Upon encountering the stop codon, a ribosome searches for a 3' protein complex (3'PC) that confirms the appropriate context of the stop codon. If a 3'PC is found, normal termination takes place. **B** If a premature termination signal impedes interaction between ribosome and 3'PC, the mRNA is degraded.

1.2.3 NMD in mammals and in yeast involves mRNA decay from both ends.

NMD in mammalian cells occurs both 5'-to-3' and 3'-to-5'. Data indicate that NMD initiates from the mRNA 5'end, where it involves decapping by decapping proteins Dcp1 and 2, followed by 5'-to-3'exonucleolytic decay by XRN1 or 2. From the mRNA 3'end, NMD involves deadenylation followed by 3'-to-5'exonucleolytic decay[62]. In mammals, it is not yet possible to determine the relative efficiencies of 5'-to-3' and 3'-to-5' decay during NMD (in yeast, 5'-to-3' decay predominates over 3'-to-5' decay[74]. Neither is it possible to determine if an mRNA can be degraded simultaneously from both ends.

1.3 NMD IN COL10A1 FALLS OUTSIDE THESE RULES AND OFFERS NEW INSIGHTS INTO THE COMPLEXITY OF NONSENSE-MEDIATED DECAY

Studies on nonsense mutations in the human collagen X gene, causing Schmid MCD, clearly illustrate that the current accepted model of NMD is incomplete. It was demonstrated that in cartilage biopsies from SMCD patients, two heterozygous premature termination mutations (W611X and Y632X, refer to Figure 1.6), 210bp and 147bp upstream of the normal stop codon lead to complete NMD of the mutant mRNA [42, 46].

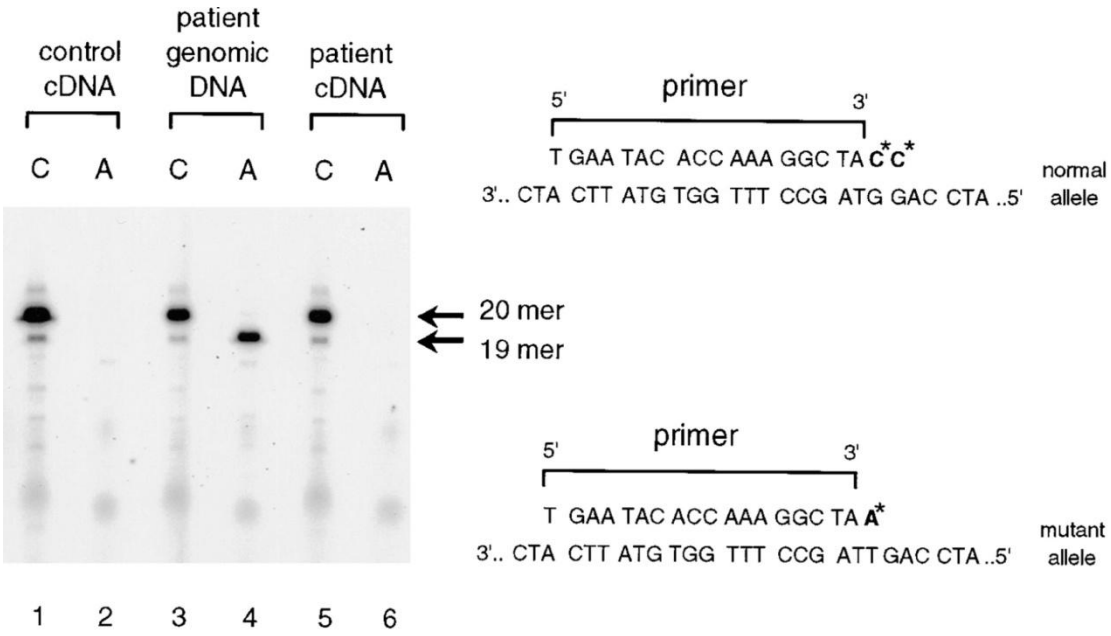


Figure 1.10 Complete NMD in SMCD patients cDNA. Single nucleotide primer extension (SNuPE) analysis of growth plate cartilage from a Schmid MCD patient and a control. cDNA and mRNA fragments of the mutated region were amplified using PCR and RT-PCR. The sequence of the 18mer extension primer, annealing 5' and one base short of the mutation, is shown to the right of the figure. Using (32P)cytosine (C*) or (32P)adenosine(A*), normal and mutant alleles were extended to a 20mer or 19mer, respectively. Lanes 1 and 2 show a control cDNA, extended by two C's showing that only wildtype cDNA is present. In the patients gDNA sample (lanes 3 and 4), as expected, normal (C) as well as mutant(A) alleles were found. Lanes 5 and 6 show the cDNA of the same patient. In contrast to the findings in the patient's gDNA, no amplified mutant allele could be detected indicating a complete loss of the mutant allele on mRNA level. Source: from Chan et al., 1998[46].

These results are surprising, considering the fact that all SMCD mutations including the two mutations mentioned above are located within the last exon of collagen X. Therefore, according to the currently accepted model of NMD, these mutations would not be expected to result in NMD. These data demonstrate a significant difference between NMD in collagen X and other genes that have been

studied. Only one other gene has been reported to also undergo last-exon NMD, the immunoglobulin light chain (κ)[75]. The κ gene, like collagen X, consists of a large last exon, that leaves no spliceable intron downstream of a potential premature termination mutation.

In genes with a short last exon this solution is sensible. Here, a premature termination codon would be in close proximity to the bona fide termination codon that usually resides within the last exon. The resulting protein would be only marginally shorter, and most likely functional. However, in a gene like collagen X that consists of only three exons, and with the last exon coding for the major part of the protein, such a large last exon is a great risk, leaving a huge area of the gene unprotected from the negative effects of a PTC. Relatively few genes have been studied for NMD, but extensive coding sequence within the last exon of transcripts is relatively common. Over 20% of multi-exon transcripts have coding sequences greater than 300bp within their last exon (NCBI Reference Sequences data human genome) and 10% have coding sequences greater than 600bp. It is likely that a number of these genes undergo last-exon NMD and therefore last-exon NMD will be involved in the pathology of a significant number of human diseases.

Further studies on the SMCD mutations mentioned above revealed another surprising finding: NMD of the mutant mRNA proved to be cell specific, since it only occurred in chondrocytes, where collagen is endogenously expressed, but did not do so in bone cells or lymphoblasts[42].

It is not yet clear whether last-exon NMD and/or cell specificity of NMD is an extension of the current model of NMD, or if this type of NMD is an independent mechanism.

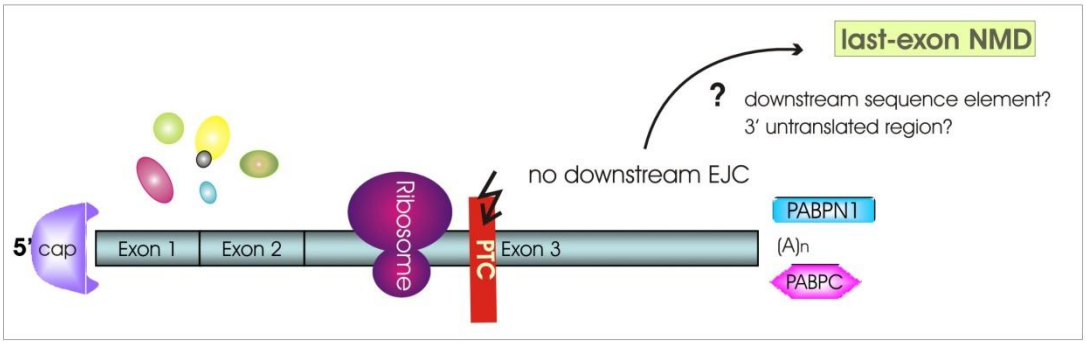


Figure 1.11 NMD in collagen X. A PTC within the last exon of collagen X elicits NMD in spite of the lack of a downstream exon-junction complex. The pathway by which this is accomplished is not yet known.

1.4 AIMS OF THIS STUDY

My studies aimed on dissecting the molecular mechanisms of collagen X last-exon NMD.

1. Effect of position of the PTC mutations in Col10a1 last-exon NMD in chondrocytes.

The localisation of all the PTC mutations in SMCD patients in a region of the NC1 trimerization domain gene sequence suggests that only a part of the Col10a1 may be competent for NMD. To address this experimentally, premature termination mutations will be introduced into a full-length genomic construct of the mouse collagen X gene[35]. This 13kb genomic construct contains the entire COL10A1 gene including 2kb of upstream promoter that drives expression levels comparable to wild type collagen X in chondrocytes. Sequential mutations will be made along the coding region for the NC1 domain and the helical domain. To validate the effects of mutations on the mRNA, mutations that cause NMD *in vivo* in SMCD patients will also be engineered in the mouse genomic construct to confirm comparability.

2. Role of specific conserved sequences in the 3'UTR in mediating last-exon NMD.

Since collagen X PTC mutations in the last exon cause NMD, the normal EJC-dependent NMD mechanisms do not apply and another uncharacterised NMD mechanism must be involved. One possible mechanism such as a downstream sequence element comparable to the DSE in yeast may be responsible for specifying this last-exon-NMD. A likely localization for such a DSE would be inside the sequence downstream of the normal termination codon, the 3'UTR.

Col10a1 has a long 3'UTR (993bp) and bioinformatic analysis revealed that it contains three regions of homology strongly conserved across species, suggesting that these may have an important biological role. Region I (23bp with 91.3% identity to UTR sequence alignments from human and mouse), region II (117bp, 87% homology) and region III (79 bp with 86% homology), refer to figure 2.1. Furthermore, region III contains a 13bp AU-rich element, which have been shown in other genes to have a role in regulating mRNA stability [76].

To determine the role of the 3'UTR on last-exon NMD, different deletions will be introduced into the col10a1 sequence. The whole 3'UTR will be deleted (+78 to +984bp, leaving the polyA addition site at +985bp intact), region I, region II and region III will be deleted separately. Furthermore, the AU-rich element (13bp) and a sequence containing the AU-rich element (32bp, region IIIb), will be deleted. The mutagenesis will be performed in a Col10a1 construct containing a mutation which normally undergoes NMD. Thus, when a region responsible for NMD is deleted, this mutation will no longer elicit mRNA NMD, but will show equally high levels of endogenous and mutant collagen X when treated with and without CHX (refer to chapter 2.3.2)

Chapter Two

Materials and Methods

2.1 COMPOSITION OF SOLUTIONS

All chemicals were of analytical grade, and were purchased from either BDH or Sigma-Aldrich.

Solution	**Composition**
Orange G loading buffer (6x)	0.25% (w/v) Orange G; 15% (w/v) Ficoll^{TM} 400; 100mM EDTA
Phosphate buffered saline (PBS)	136mM NaCl; 10mM Na_2HPO_4; 1.7mM KH_2PO_4; 2.68mM KCl, pH 7.4
10xTBE	890mM Tris-borate, pH 8.0; 20mM EDTA
10x Disodium EDTA	0.136M NaCl; 2mM KCl; 3mM Na_2HPO_4; 1mM KH_2PO_4; 10M NaOH; 5mM EDTA
Trypsin/EDTA	0.025% (v/v) trypsin; 0.53mM EDTA; 1xPBS, pH7.3
2YT Medium	16% tryptone; 10% yeast extract; 0.08M NaCl; pH7.0
LB Medium (Luria-Bertani Medium)	10% tryptone (w/v); 5% yeast extract (w/v); 0.17M NaCl; pH7.0
12% acrylamide solution	12% (v/v) 19:1 acrylamide:bisacrylamide; 42% (w/v) urea; 5% (v/v) 10xTBE
10x primer extension buffer	100mM Tris/HCl pH8.0; 500mM KCl; 15mM $MgCl_2$; 0.01% gelatin
STOP buffer	95% deionized formamide, 20mM EDTA, 0.02% (w/v) xylene cyanol, 0.05% (w/v) bromophenol blue
TE buffer pH 8.0	100mM Tris/HCl pH 8.0, 10mM EDTA

Table 2.1: Chemical Composition of Solutions.

2.2 SYNTHESIS OF NONSENSE MUTATIONS IN MOUSE COL10A1

2.2.1 Mutations and deletions introduced into col10a1

Two different classes of mutations were introduced into the mouse col10a1 gene: nonsense and missense mutations (figure 2.1).

PTC mutations were introduced into the coding region of col10a1, causing premature termination codons (PTCs) in the COL1 and NC1 domains. G174X, G308X, G407X, G502X and Q518X lay within the COL1 domain (helix), while Y582X, Y632X, W651X, Y663X and G674X are in the NC1 domain. PTCs Y632X, W651X and Y663X are mutations that were found in cartilage biopsies of SMCD patients[38]. Sequences are numbered from the translation initiation codon according to the nomenclature of den Dunnen[40].

Inside the 3'untranslated region (3'UTR), a range of deletions was made. Three regions of homology between species were deleted (Y632Xdel1, 23bp, Y632Xdel2, 117bp and Y632Xdel3, 79bp). Furthermore, smaller elements of region III were deleted separately: the 13bp AU-rich element (Y632XdelARE) and a 32bp element containing the 13bp ARE (Y632Xdel3b). For the deletions, a ColXFlag plasmid containing the Y632X mutation was used as a template in mutagenesis.

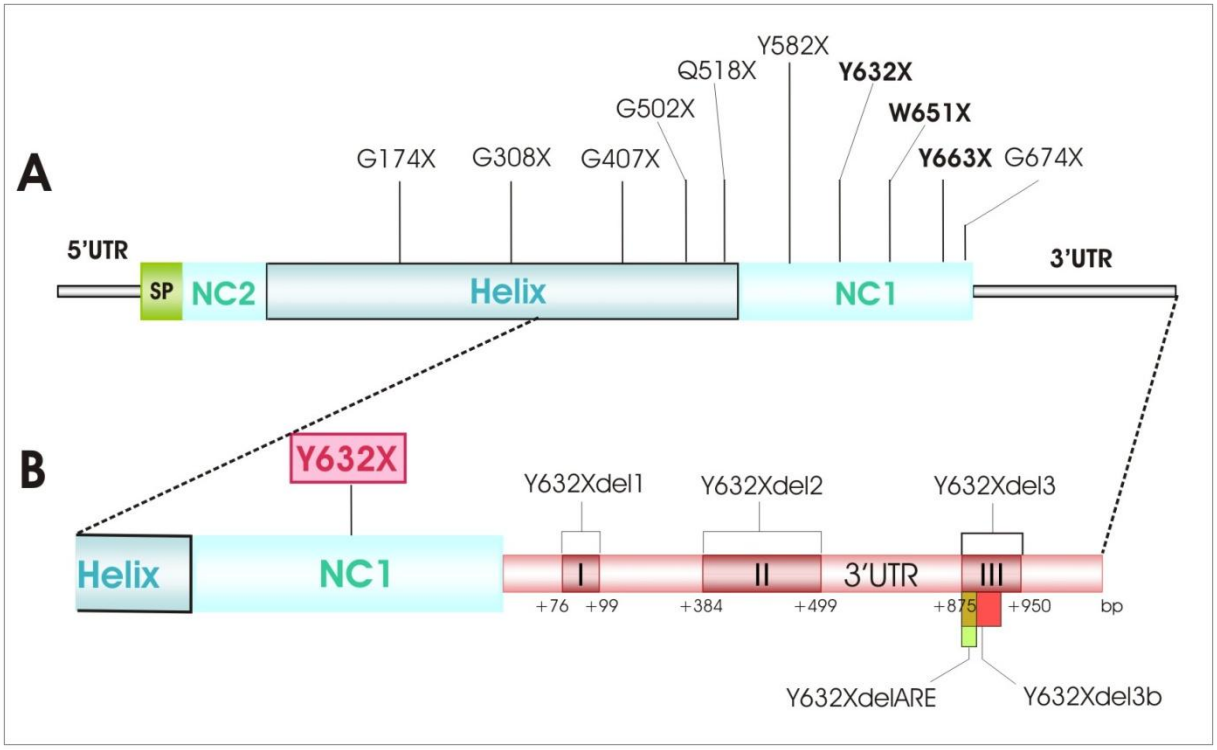


Figure 2.1 PTC mutations introduced into mouse col10a1. Diagram of the mRNA and protein structure of col10a1. The signal peptide (SP) is marked by the green box, 5' and 3' untranslated regions (UTR) are grey horizontal lines (A), the 3'UTR a red box (B). The blue box shows the collagenous (helix) and the turquoise boxes the noncollagenous (NC1 and NC2) domains of the protein. **A** PTC mutations introduced into col10a1. Locations are indicated by black lines. **Bold letters** indicate PTC mutations that were present in cartilage biopsies from SMCD patients. **B** Deletions of three regions of homology, marked by dark red boxes inside the 3'UTR (Y632Xdel1, Y632Xdel2 and Y632Xdel3) and of two smaller elements inside the third region of homology (Y632XdelARE and Y632Xdel3b). The deletions were made using a ColXFlag vector as a template that contained the Y632X mutation (red box).

2.2.2 Site directed mutagenesis using splicing-by-overlap extension (SOE)

The mutations were introduced into the coding sequence of *col10a1* using splicing-by-overlap-extension (SOE) PCR (refer to figure 2.3). Amplified products were initially subcloned into pRK23, a 6.8kb pBluescript genomic construct containing a 4kb *EcoRI* fragment of exon 3 of *col10a1*[77] in its multiple cloning site. Subsequently, the construct was subcloned into ColXFlag. ColXFlag is a 13kb mouse genomic *col10a1* construct in the N-terminal pFLAG-CMV vector containing 2kb promoter sequence and 2.2kb of 3'sequence including the entire 3'UTR and poly A signal.

In the overlap extension mutagenesis, two overlapping DNA fragments were amplified in two separate PCR's. The mutations were constructed in the region of overlap and present in both amplified fragments. Both PCR products were subsequently purified from agarose gels. Equal amounts of the overlapping fragments were mixed and, in a third PCR, were amplified into a full-length DNA using two primers that bound to the extremes of the two initial fragments (refer to tables 2.2 and 2.3)

All 50µl SOE-PCR reactions consisted of 0.25mM dNTPs, 0.25µl DeepVent proof-reading polymerase (New England Biolabs), 100ng forward and reverse primers, 20ng of template DNA and 1x reaction buffer. Hybaid PCR-Express thermocyclers or Applied Biosystems GeneAMP 2400 PCR machines were used for each PCR. Touchdown PCR conditions were 95°C for 5 mins, 14 cycles of 95°C for 20 seconds, annealing at 63-56°C (decreasing by 0.5°C each cycle) for 20 seconds, and 72°C for 45 seconds, then 16 cycles of 95°C for 20 seconds, 56°C for 20 seconds, and 72°C for 45 seconds, followed by a final extension at 72°C for 5 mins. All primers are listed in tables 2.2 and 2.3.

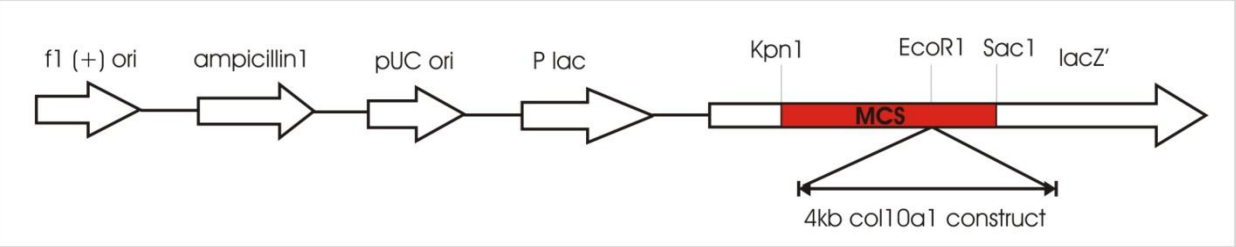


Figure 2.2 pRK23: a pBluescript genomic construct containing a 4kb col10a1 fragment[77]

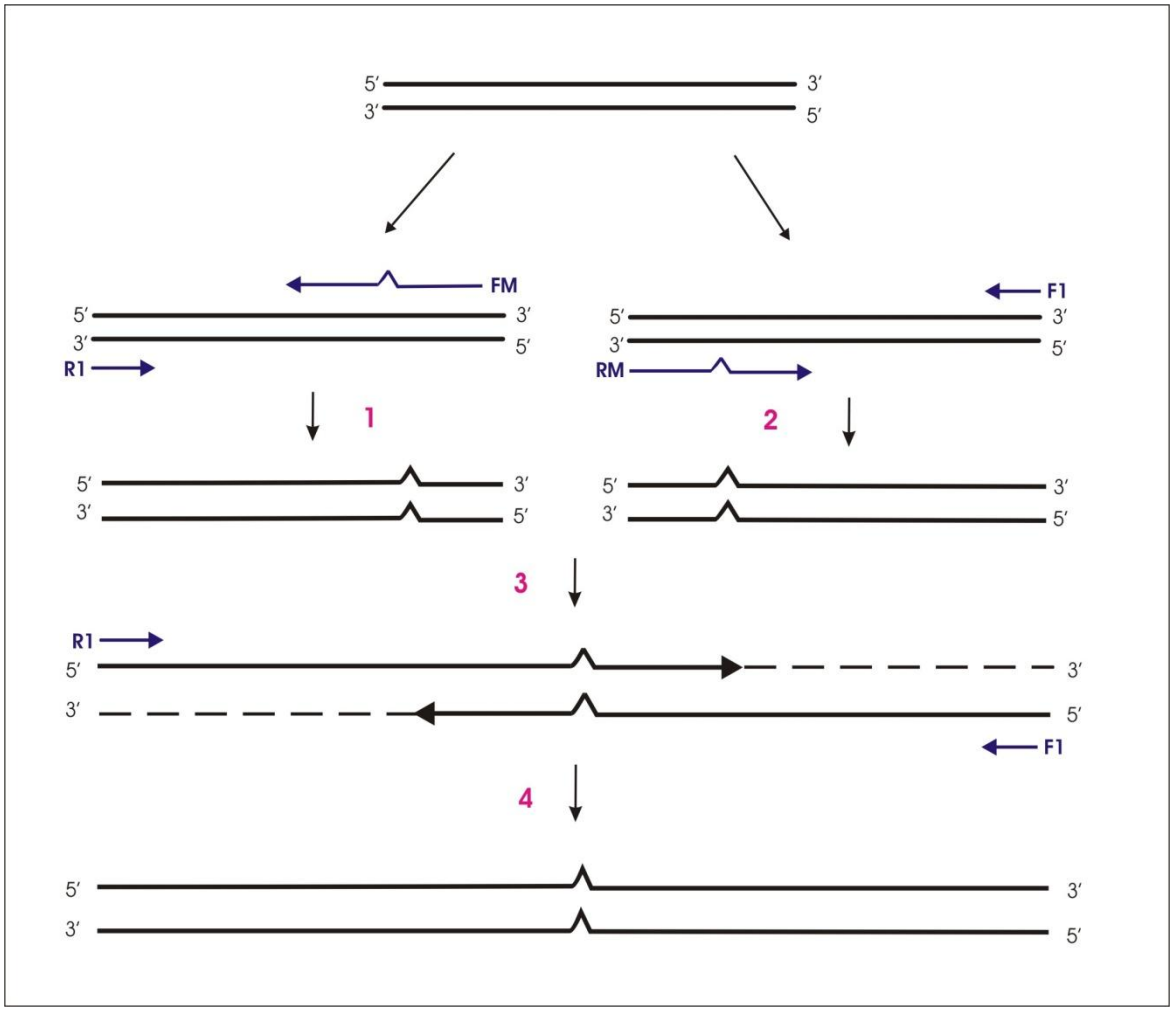


Figure 2.3 Splicing-by-overlap-extension (SOE)-PCR. In two separate amplifications, 1 and 2, two fragments of the target gene were amplified. PCR1 utilizes primers forward mutant (FM) and reverse1 (R1), whereas PCR2 utilizes primers forward1(F1) and reverse mutant (RM). The two amplicons are mixed together in PCR3 using primers R1 and F1, which bind to the extremes of the two initial fragments. The two fragments anneal at the region of overlap and are extended as shown by the dashed lines to form full length mutant DNA.

2.2.3 Directional cloning into plasmid vector pRK23

Directional cloning was performed using identical restriction enzymes for vector and fragment, which varied for each mutation. Refer to table 2.3 for restriction sites that were utilized. Restriction enzyme digests were performed for 16hrs at 37°C using appropriate buffers (New England Biolabs).

To suppress self-ligation and circularization of plasmid DNA, terminal 5'-phosphate groups were removed from the vector using bacterial alkaline phosphatase (BAP, Invitrogen™). Reactions contained 50µg plasmid DNA, 10µl 10xBAP buffer (provided by Invitrogen™) and 1µl BAP in a final volume of 100µl, and were incubated at 65°C for 60 mins.

Digested PCR fragments and plasmid DNA were separated on a 2% UltraPure™ Agarose-1000 (Invitrogen™)/0.5x TBE gel containing ethidium bromide and were visualized using FOTO/Prep®I UV Transilluminator (Fotodyne Inc.). The appropriately sized cDNA was excised from the gel and extracted from the agarose gel slice using the QIAquick® Gel Extraction Kit (QIAGEN) according to the manufacturers protocol.

Ligations were performed using T4 DNA Ligase (Invitrogen ™). To ensure a high yield of insert-containing plasmids, the molar ratio of insert to vector was 3:1. Each 20µl reaction contained a total of 100ng plasmid and insert, 4µl 5x ligation buffer and 1µl T4 DNA ligase. Reactions were incubated at room temperature for 15 mins, then on ice for 15 mins, and immediately used for transformation.

2.2.4 Transformation of competent E.coli

Transformation reactions consisted of 100ng recombinant plasmid DNA and 50µl Subcloning Efficiency™ DH5α™ Competent Cells (K-12 strands of *E.coli*, Invitrogen). Reactions were incubated on ice for 30 mins followed by a heat shock at 42°C for 45 seconds. Cells were immediately cooled on ice for 1-2 mins, and 300µl 2YT medium was added. For recovery, cells were incubated at 37°C, 225rpm for 45 mins. Transformations were applied to 90mm LB agar plates containing 100µg/ml ampicillin (Sigma-Aldrich) (ampicillin resistance gene in pBluescript) that were coated with 16µl X-gal solution (50mg/ml, 5-bromo-4-chloro-3-indolyl-B2;-D-galactopyranoside, Promega). Plates were incubated at 37°C for 16 hrs.

2.2.4.1 Blue/white colony screening: α-complementation

Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen) contain a *lacZΔM15* marker that provides α-complementation of the β-galactosidase gene, allowing blue/white screening of the colonies.

For each mutation, 6-8 white colonies were picked and used to inoculate 3ml of 2YT medium containing 100µg/ml ampicillin, and incubated at 37°C, 225rpm for 16 hrs. Plasmid DNA was extracted from the cells using the QIAPrep® Miniprep Kit (QIAGEN) according to the manufacturer's instructions. In brief, pelleted bacterial cells were resuspended in lysis buffer, neutralized and centrifuged DNA was bound to a QIAprep® spin column and washed using ethanol-containing wash buffer. Plasmid DNA was eluted in 30µl elution buffer. The plasmid obtained from each colony was examined for the correct insert either by sequencing or restriction endonuclease mapping.

2.2.4.2 Large-scale preparation of plasmid DNA

For large-scale preparation of the plasmid DNA, 250ml LB medium containing 100µg/ml ampicillin was inoculated with a DH5α colony expressing the mutant plasmid. The culture was incubated at 37°C for 16 hrs at 225rpm and plasmid DNA was isolated from the *E.coli* cells using the QIAfilter® Plasmid Maxi Kit (QIAGEN) according to the manufacturer's instructions. In brief, bacterial cells were harvested by centrifugation, resuspended in buffer containing RNase A, lysed and neutralized. The lysate was incubated at room temperature for 10 mins, filtered through a QIAfilter Cartridge and bound to an equilibrated QIAGEN-tip 500. It was washed twice, and DNA was eluted from the tip in buffer QF. DNA was precipitated at room temperature using isopropanol, centrifuged and washed with 70% ethanol, and the pellet resuspended in 300µl TE buffer, pH8.0

2.2.5 Subcloning of a 3.8KB **EcoRI fragment** *into COLXFLAG*

A 3.8kb fragment of *col10a1*, containing most of Exon 3, the 3'UTR and the poly A signal, was cut out of the ColXFlag construct using *EcoRI* restriction endonuclease, and replaced with a mutant-containing 3.8kb fragment digested from the pRK23 constructs.

Digestions of 100µl consisted of 25µg ColXFlag or 75µg pRK23 construct, 10µl 10x *EcoRI*-buffer and 1.5µl *EcoRI* endonuclease, and were carried out at 37°C for 16 hrs. Digested plasmid DNA was purified as described in section 2.2.3.
The ColXFlag construct was then transformed into DH5α cells, screened for the correct insert, and large-scale preparation of the mutant plasmids was performed (refer to section 2.2.4). Both pRK23 and ColXFlag plasmid preparations were stored at -20°C, and glycerol stocks of transformed DH5α cells were stored at -80°C.

2.3 EXPRESSION & QUANTITATION OF COL10A1 NONSENSE MUTATIONS

2.3.1 Transfection into chondrocyte cells

MCT cells (mouse chondrocytes, immortalized with a temperature-sensitive simian virus 40 large T antigen) [78] were used for transfections. MCT cells have been shown to grow continuously with a doubling time of approximately 2 days when incubated at 32°C, the temperature permissive for the activity of the large tumor antigen. At nonpermissive temperatures (37-39°C) the cells differentiate into hypertrophic chondrocytes and express higher levels of collagen X mRNA.
Furthermore, due to the reported tissue specificity of collagen X last-exon NMD[42], MCT cells were chosen because of their ability to produce endogenous collagen X. This ability depends on the state of differentiation in which the MCT cells are. At nonpermissive temperatures they express high levels of collagen X mRNA. This suggests that the expression of the collagen X gene is upregulated upon growth arrest, as is the case *in vivo* during chondrocyte hypertrophy. Therefore, endogenous collagen X served as an internal control when levels of endogenous collagen X were compared to those of mutant collagen X.

Cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Trace Biosciences) supplemented with 10% (v/v) foetal calf serum (CSL Ltd.), at 33°C in a humidified atmosphere of 5% CO_2/95% air. Medium was changed routinely every two to three days, and cells were passaged when they reached confluency. Passaging of the cells included a PBS wash to remove any serum, followed by a 5 min incubation in Trypsin/EDTA at 33°C. Cells were seeded at a 1:6 dilution.

For transfection, cells had to be approximately 50% confluent.

2.3.1.1 Preparation/Linearisation of Plasmids for Transfection

To enable stable integration into the genome, plasmids must be linearized before being transfected. Both the pGKhygro, a hygromycin resistance gene, and the ColXFlag constructs were linearized for cotransfection. pGKhygro was linearized utilizing *BamHI* restriction endonuclease. The 200µl reaction consisted of 20µg pGKhygro, 20µl 10x buffer #2 (provided by NEB), and 3ml *BamHI* endonuclease. ColXFlag was linearized utilizing *Sal I* endonuclease, a 200µl reaction containing 20µg ColXFlag construct, 2µl BSA, 20µl 10x NEB buffer #3. Both reactions were incubated at 37°C for 16 hrs.
Linearized DNA was then purified. Samples were run on a 1% UltraPure™ Agarose – 1000 (Invitrogen™) gel containing ethidium bromide, excised and extracted using the QIAquick® Gel Extraction Kit (QIAGEN) according to the manufacturer's protocol.

2.3.1.2 Cotransfection of MCT cells

FuGene 6 Transfection Reagent (Roche) was used for cotransfection of linearized pGKhygro and the ColXFlag construct, according to the manufacturer's instructions. Briefly, 40µl FuGene were added to 200µl serum-free DMEM, and incubated at room temperature for five mins. pGKhygro and ColXFlag were mixed with ColXFlag at a three times higher concentration than pGKhygro. FuGene was then added to the DNA and left at room temperature for an additional 15 mins. This solution was then added drop wise to the MCT cells.
Cells were incubated at 33°C for two days.
After two days, cells were passaged in 1:30, 1:50 and 1:70 dilutions and hygromycin was added at a final concentration of 150µg/ml (w/v). Cells were then incubated at 33°C for approximately 15 days.
Each colony was scraped from the dish using a sterile pipette tip, incubated in 0.5ml Trypsin/EDTA at 37°C for 5mins, and then expanded at 33°C in DMEM/Hygromycin until they reached confluency.
For long-term storage of transfected MCT cells, cells were resuspended in DMEM containing 10% (v/v) FCS and 10% (v/v) dimethyl sulfoxide, and stored in liquid nitrogen.

All tissue culture plastic ware was purchased from Nunc™.

2.3.2 Inhibition of Nonsense-Mediated Decay by Cycloheximide

Prior to RNA-extraction, confluent cells were incubated at 37°C for 7 days to allow MCT cells to differentiate into hypertrophic chondrocytes and thus express high levels of collagen X mRNA[78]. Cells were treated with 0.25mM ascorbic acid on the sixth day, and each clone was incubated in the absence or presence of 0.3mM cycloheximide (CHX) on the seventh day.

Cycloheximide is an antibiotic which acts as an inhibitor of protein synthesis in eukaryotes. It interferes with peptidyl transferase on the 60S ribosome, and thus blocks translational elongation. Although CHX is also known to be a potent inhibitor of NMD, the mechanism is not yet completely clear[79, 80].

Being a potent inhibitor of NMD, CHX was used to detect NMD in cells expressing mutant collagen X. For each mutation, cells were grown in the presence as well as in the absence of CHX. Collagen X mRNA levels were compared in the two groups.

If a mutation lead to NMD, collagen X mRNA decayed and thus mRNA levels were decreased. However, when the cells were treated with CHX, NMD was inhibited, and mRNA levels were normal. The ratio of mRNA levels (+CHX/-CHX) was >1.

If a mutation did not lead to NMD, mRNA levels were normal. The treatment of the clones with CHX did not alter the mRNA level. A ratio of mRNA levels of ≤1 followed.

MCT chondrocytes produce endogenous collagen X ([78], see above), which was used as internal control of collagen X mRNA levels.

To summarize the data collected for each mutation, the median of all analyzed clones was calculated. Since the median represents more accurately than the arithmetic mean and is also more robust to outliers, it was used as an indication of the central tendency of NMD caused by a particular mutation.

2.3.3 Total RNA Isolation

Total RNA was extracted 6-8 hrs after cycloheximide treatment of cells cultured in 6 well plates, using RNeasy™ columns (QIAGEN), according to the manufacturer's instructions. In brief, cells were lysed with buffer RLT (containing β-mercaptoethanol) and homogenized by passing through a sterile syringe fitted with a 23G needle. RNA was then ethanol-precipitated, bound to the RNeasy column by centrifugation at 10,000 rpm for 15 seconds, and washed with buffer RW1. An on-column DNase digest with RNase-free DNase was performed, incubating for 15 mins at room temperature. The RNA was then washed again with buffer RW1, and a third time, using buffer RPE. The RNA was eluted in 30µl diethylpyrocarbonate-treated water and stored at -80°C.
The concentration of each total RNA sample was determined by quantifying the absorbance (A) at 320nm, 280nm and 260nm on a DU®-64 Spectrophotometer (Beckman Instruments, Inc.). RNA concentration was calculated based on the A_{260} reading, where a value of 1 equates to 40µg RNA/ml.

2.3.4 Reverse Transcription and PCR

Each 20µl RT reaction contained 200ng total RNA, 50U MuLV Reverse Transcriptase, 20U RNase Inhibitor, 5mM $MgCl_2$, 1mM dNTPs, 2.5µM Oligo $d(T)_{16}$ and 1x PCR buffer II. All RT reagents were purchased from Applied Biosystems. Reverse Transcription was carried out at 42°C for 60 mins and followed by inactivation of the reverse transcriptase by heating for 5 min at 99°C. RT products were either stored at -20°C or used immediately as the template in PCRs.

Each 50µl PCR reaction contained 100ng cDNA, 0.8x PCR II buffer, 1mM $MgCl_2$, 100ng forward and reverse primers and 2.5U AmpliTaq® DNA Polymerase (Applied Biosystems). PCR conditions were denaturing at 94°C for 5 mins, then 30 cycles of 94°C for 30 seconds, annealing at variable primer-dependent temperatures for 30 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C for 7 mins (for primer sequences and annealing temperatures, please refer to Table 2.2 and 2.3).

2.3.5 Agarose Gel Electrophoresis

To separate the DNA fragments according to their size, the PCR reactions were run on 1% (w/v) agarose (Roche Diagnostics)/ 0.5x TBE gels containing ethidium bromide. DNA size and abundance were approximated using the 2log DNA ladder (0.1-10kb) (New England Biolabs). DNA bands were visualised using the Eagle Eye II™ UV transilluminator system (Stratagene).

2.3.6 cDNA Gel Purification

All PCR samples were electrophoretically separated on a 2% UltraPure™ Agarose – 1000 (Invitrogen™) gel containing ethidium bromide. cDNA bands were visualized using FOTO/Prep®I UV Transilluminator (Fotodyne Inc.), and the appropriately sized cDNA was excised from the gel.

cDNA was then extracted from the agarose gel slice using the QIAquick® Gel Extraction Kit (QIAGEN) according to the manufacturer's protocol, which includes a solubilization step using buffer QG, DNA precipitation by isopropanol, then binding of the DNA to a column, removal of oligo-dT-primers with wash-buffer PE and finally re-eluting the DNA in 30µl sterile water.

2.3.7 Primer Extension Assays

Protection of the mutated mRNA from NMD by CHX was assessed using the primer extension assay. The cDNA obtained from each clone (for each mutation) was used as a template in primer extension reactions. For each mutation, a primer was designed adjacent, but not complementary to the mutated nucleotide, binding one base short of the mutated base. This primer was therefore able to bind to either the endogenous or the mutant allele, both present in the cDNA template (refer to Tables 2.2 and 2.3). In two separate reactions, cDNA levels of either the endogenous or the mutant allele were assessed. This was accomplished using radioactively labelled nucleotides. The radioactive nucleotide complementary to the endogenous sequence was used in one reaction, resulting in radioactively labelled endogenous collagen X. In a separate reaction, the radioactive nucleotide complementary to the mutant sequence labelled only the mutant allele. The reactions were performed in the absence of nonradioactive

dNTPs. Thus, endogenous and mutant alleles could be analysed separately. PCR reactions were stopped after the first cycle to ensure that the radioactively labelled nucleotide bound solely at its specific location.

Each 10µl primer extension reaction consisted of 5ng cDNA, 1µM extension primer, 1µl 10x primer extension buffer (refer to Table 2.1), 0.5µl AmpliTaq® DNA Polymerase (Applied Biosystems) and 2µCi [α-^{32}P]dNTP (3,000 Ci/mmol; Perkin Elmer).

The conditions included one denaturing step of 95°C for 1 min, one annealing step of 2 mins (annealing temperatures were primer-dependent and identical to those in the PCR reactions), and one elongation step at 72°C for 1 min. The reaction was immediately stopped by adding 10µl STOP buffer (refer to table 2.1) and denatured at 99°C for 5 mins. The extended products were analysed on a 12% (w/v) denaturing polyacrylamide gel containing 7M urea. Radioactivity was quantified using the Typhoon^{TM}Scanner phosphor imager (Amersham Pharmacia Biotech). Radioactive samples excite the BaFBr:Eu^{2+} crystals inside a phosphor screen to $BaFBr^{-}$:Eu^{3+}. Information is released in form of blue light by the Typhoon^{TM}Scanner, by stimulation by red light at 633nm. Peak intensities of the emitted light pass through a band-pass filter and are detected and measured by the Typhoon^{TM}Scanner. Two of the advantages of storage phosphor autoradiography are the high sensitivity and the wide dynamic range and highly linear response[81]. Intensities are proportional to the radioactivity in the sample.

Collected data was analyzed using ImageQuant TL software (Amersham Biotech).

Table 2.2 Sequences of the primers used in SOE PCR, PCR and primer extension reactions.

primer	*sequence*	*primer*	*sequence*
AdelF	AAA CGA AAA GAC CCG GAG TAT TTA	**mSTOP582F**	ATA GGC AGC AGC ATT AAG ACC CAA GAT CTG
AdelR	AAA TAC TCC GGG TCT TTT CGT TT	**mSTOP582R**	CAG ATC TTG GGT CTT AAT GCT GCT GCC TAT
AREdelF	AAA CGA AAA GAC GCT CTG TAC TGT	**mSTOP651A**	GAC CAG GTA TGA CTC CAA TTG CCC
AREdelR	ACA GTA CAG AGC GTC TTT TCG TTT	**mSTOP651B**	GGG CAA TTG GAG TCA TAC CTG GTC
BdelF	TCC ATT TTT AAT AAC ACA CAT GGT T	**mSTOP663A**	AAC GGC CTC TAG TCC TCT GAG TAC
BdelR	AAC CAT GTG TGT ATT AAA AAT GGA	**mSTOP663B**	GTAC TCA GAG GAC TAG AGG CCG TT
BlueSpeF	GCG AAT TGG AGC TCC A	**mSTOP674A**	CGT CCT TCT CAT GAT TCC TAC TGG C
CdelF	CCC TCC CTG GAG GTA ATG TGA ACA	**mSTOP674B**	GCC ACT AGG AAT CAT GAG AAG GAC G
CdelR	TGT TCA CAT TAC CTC CAG GGA GGG	**MX1F**	GGC AGG TCC AAG AGG
DdelF	AAA CGA AAA GAC CAT TAT TGT TTA	**MX5R**	AAT GTC AGC CTT TAA GGG AG
DdelR	TAA ACA ATA ATG GTC TTT TCG TTT	**MX6F**	TAC CAC GTG CAT GTG AAA GG
M3'UTRDEL-A	*GCG CCC TCC CTG GAG* **AAT AAA ATG ACA TGA**	**MX7R**	AAT GTC AGC CTT TAA GGG TG
M3'UTRDEL-B	**TCA TGT CAT TTT ATT** *CTC CAG GGA GGG CGC*	**MXSN502R**	CTG GGA GAC CAG GTT CTC
mBgl2R	ACT CAG AGG AGT AGA GG	**MXSN632F**	TGA GTA CAG CAA AGG CTA
mNcoIF	GGA CCT AAA GGA ATC C	**MXSN674R**	TGG GAG CCA CTA GGA ATC
mNcoIR	ATC TCA CCT TTA GCG C	**PCR mX174F**	TAA GAG GAG AAC AAG GC
mSTOP174F	TAG GGG CTT TCC TTG AGA GAA GGG TGC ACA	**PCR mX308F**	TTC CAG GCC CTC AAG GTC CC
mSTOP174R	TGT GCA CCC TTC TCT CAA GGA AAG CCC CTA	**PCR mX407F**	AAG GTG AGA TAG GTC TAG TTG G
mSTOP308F	GCC AGG TTT GAG GTG ACA AAG GGG ACC TGC	**PCR mX518F**	TTG GCC CTG TAG GAG CTA AAG
mSTOP308R	GCA GGT CCC CTT TGT CAC CTC AAA CCT GGC	**PCR mX582R**	TAG AAG TGA GGA AAC TTG GTC
mSTOP407F	A CCC AGG GTT ACC ATG ACA AAA AGG TGA TCC	**SNUPE m174R**	TTG TGC ACC CTT CTC TC
mSTOP407R	GGA TCA CCT TTT TGT CAT GGT AAC CCT GGG T	**SNUPE m308R**	ACC AGC AGG TCC CCT TTG TC
mSTOP502A	GGC CAC AGT TGA GAA CCT GGT CTC	**SNUPE m407R**	ACT CCA GGA TCA CCT TTT TGT C
mSTOP502B	GAG ACC AGG TTC TCA ACT GTG GCC	**SNUPE m518R**	TGA CAT CAG GCA TGA CTG CTT
mSTOP518F	CAG GAC CCC CCG GCT AAG CAG TCA TGC CTG	**SNUPE m582F**	GTA CAA TAG GCA GCA GCA TTA
mSTOP518R	CAG GCA TGA CTG CTT AGC CGG GGG GTC CTG		

Table 2.3 Mutations introduced into the col10a1 gene with their associated primers and enzymes.

Mutation	*1°SOE primers*	*2°SOE primers*	*PCR primers*	*PCR annealing temperature*	*primer extension primer*	*restriction enzymes*
G174X	BlueSpeF & mSTOP174R mSTOP174F & mNcoIR	BlueSpeF & mNcoIR	PCR mX174F & SNUPE m174R	55	SNUPE m174R	SpeI & NcoI
G308X	BlueSpeF & mSTOP308R mSTOP308F & mNcoIR	BlueSpeF & mNcoIR	PCR mX308F & SNUPE m308R	60	SNUPE m308R	SpeI & NcoI
G407X	mSTOP407F & mBgl2R mNcoIF & mSTOP407R	mNcoIF & mBgl2R	PCR mX407F & SNUPE m407R	50	SNUPE m407R	NcoI & Bgl2
G502X	MXSN674R & mSTOP502A mSTOP502B & MX1F	MX1F & MXSN674R	MX1F & MXSN502R	60	MXSN502R	NcoI & Bgl2
Q518X	mSTOP518F & mBgl2R mNcoIF & mSTOP518R	mNcoIF & mBgl2R	PCR mX518F & SNUPE m518R	50	SNUPE m518R	NcoI & Bgl2
Y582X	mSTOP582F & mBgl2R mNcoIF & mSTOP582R	mNcoIF & mBgl2R	SNUPE m582F & PCR mX582	60	SNUPE m582F	NcoI & Bgl2
Y632X	MX1F & mSTOP632A mSTOP632B & MX5R	MX1F & MX5R	MX1F & MX5R	60	MXSN632F	Bgl2 & NsiI
W651X	MX1F & mSTOP651A mSTOP651B & MX5R	MX1F & MX5R	MX1F & MX5R	56	MXSN651F	Bgl2 & NsiI
Y663X	MX1F & mSTOP663A mSTOP663B & MX5R	MX1F & MX5R	MX1F & MX5R	60	MXSN663F	Bgl2 & NsiI
G674X	MX1F & mSTOP674A mSTOP674B & MX5R	MX1F & MX5R	MX1F & MX5R	60	MXSN674R	Bgl2 & NsiI
Y632Xdel3'UTR	M3'UTRDEL-B & MX7R MX6F & M3'UTRDEL-A	MX6F & MX7R	MXSN632F & MX5R	60	MXSN632F	EcoRI & SalI
Y632Xdel1	CdelF & MX7R MX1F & CdelR	MX1F & MX7R	MXSN632F & MX5R	60	MXSN632F	StuI
Y632Xdel2	BdelF & MX7R MX6F & BdelR	MX6F & MX7R	MXSN632F & MX5R	60	MXSN632F	StuI
Y632Xdel3	AdelF & MX7R MX6F & AdelR	MX6F & MX7R	MXSN632F & MX5R	60	MXSN632F	StuI
Y632Xdel3b	DdelF & MX7R MX6F & DdelR	MX6F & MX7R	MXSN632F & MX5R	60	MXSN632F	StuI
Y632XdelARE	AREdelF & MX7R MX6F & AREdelR	MX6F & MX7R	MXSN632F & MX5R	60	MXSN632F	StuI

Chapter Three

Results

3.1 EFFECT OF POSITION OF THE PREMATURE TERMINATION CODON MUTATIONS ON COL10A1 NONSENSE-MEDIATED MRNA DECAY IN CHONDROCYTES

3.1.1 Introduction

Schmid metaphyseal chondrodysplasia (SCMD) is a relatively mild cartilage disorder, usually manifesting in early childhood. The patients show a waddling gate, bowing of the lower extremities and coxa vara. It has been found that the disease is caused by mutations in the gene encoding collagen X, COL10A1 (refer to section 1.1.2). Unlike most collagens, COL10A1 contains only three exons, and the last exon encodes most of the collagen X protein sequence. Two different types of mutations were identified in SMCD patients, and the mutual effect of both missense and nonsense mutations inside the NC1 trimerization domain is functional haploinsufficiency[42, 43]. The mutations that have been found in SMCD patients so far are mostly clustered around the 3'region of exon 3 which encodes the NC1 trimerization domain (36/38 mutations). No mutations have been identified in the helix region (COL1). This restricted localization may have implications for how the position of stop codons influences mutant mRNA stability and thus be important for understanding disease mechanisms.

3.1.2 Premature termination mutations in the coding region of exon 3

To explore the detailed relationship between stop codon position and NMD within the coding region for the COL1 and NC1 domains, a range of premature termination codon mutations were engineered into a 13kb mouse genomic construct. These mutations were distributed along the coding region of exon 3. Therefore, mutant constructs were stably transfected into mouse MCT chondrocytes. NMD was assessed by incubation of the cells in the presence or absence of the NMD inhibitor cycloheximide (CHX), prior to RNA isolation (refer to chapter 2.3.2). cDNA was synthesised from mRNA using reverse transcription, and amplified by PCR. The clonal transfectants were analysed for mutant and endogenous collagen X mRNA levels using the allele-specific primer extension assay.

3.1.3 Results

The mutations assessed for NMD caused PTCs along the coding region for the NC1 and COL1 domains. G674X, Y663X, W651X, Y632X, Y582X, Q518X, G502X, G407X, G308X and G174X were engineered into exon 3 (refer to figure 2.1). Some of the mutations had been identified as stop mutations causing SMCD: Stop codons Y663X, W651X and Y632X. The Y632X mutation has been shown to elicit cartilage-specific NMD *in vivo* [42, 46] and thus serves as a positive control for NMD with mouse gene PTCs in the cell transfection system.

3.1.3.1 Stop codon G674X resides just 5' of the normal stop codon at amino acid 681.

PTC G674X was engineered into the col10a1 gene at -21bp from the normal stop codon, changing the Gly^{674} from GGA to a stop codon (TGA). Using the PCR primers MX1F and MX5R, a 954bp product was amplified and used as a template in the primer extension assay. Primer extension was performed in a one-cycle reaction with an annealing temperature of 60°C and utilising the 18-mer extension primer MXSN674R (refer to chapter 2.3.7). In the absence of nonradioactive dNTPs and presence of radioactively labelled [α-^{32}P] cytosine or [α-^{32}P] adenosine, endogenous and mutant templates were specifically elongated, respectively. Radioactivity was quantified using storage phosphor autoradiography.
The ratios of the eight clones varied from 0.18 to 1.01, with a median protection level of 0.95.

These protection levels indicate that even though the clones were each treated with and without the NMD inhibitor CHX, mRNA levels were equal in both the +CHX and the –CHX treated cells. This was only possible since no mRNA decay was elicited by the mutation. Thus, CHX treatment had no effect on mRNA levels.
Therefore the G674X mutation does not elicit last-exon NMD.

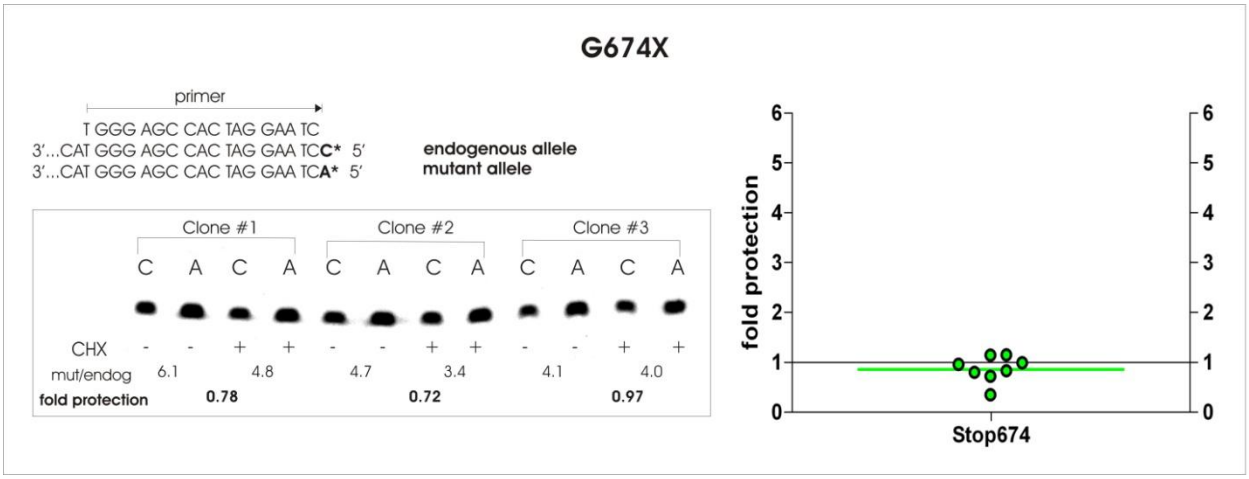


Figure 3.1 PTC mutation G674X. Primer extension assay of PCR-amplified cDNA fragments. The sequence of the 18-mer extension primer MXSN674R is shown above the figure. The corresponding wildtype and mutant allele sequences are depicted underneath the primer sequence. C and A represent radioactively labeled nucleotides: [α-^{32}P] cytosine (representing endogenous) and [α-^{32}P] adenosine (representing mutant collagen X), respectively. Primer extension samples were run on a 12% polyacrylamide gel, and measured using storage phosphor autoradiography. Emissions of mutant and endogenous collagen X were compared (mut/endog) and clones treated with and without CHX were compared to quantify NMD (fold protection). To the right a plot of all clones analyzed is depicted. The green line represents the median of 0.95, with ratios varying from 0.18 to 1.01. Therefore, G674X does not elicit NMD.

3.1.3.2 SMCD mutations Y663X, W651X and Y632X elicit NMD

Y663X, W651X and Y632X are premature termination mutations that were also found in the COL10A1 gene of SMCD patients[14]. We know that both Y632X and Y663X lead to NMD in patients. However, the effect of the W651X mutation is unknown. Recent data suggest a gain of function effect [77].

Y663X resides at -54bp from the normal stop codon. It mutates the Tyr^{663} from TAC to a stop codon (TAG). A 954bp product containing the Y663X PTC was amplified using the PCR primers MX1F and MX5R, and was then used as a template for the primer extension assay (refer to chapter 2.3.7). Primer extension was performed using the 18-mer MXSN663F as extension primer, and the radioactively labelled nucleotide [α-^{32}P]-dCTP was used to amplify the endogenous, and [α-^{32}P]-dGTP to amplify the mutant product. Both of the 19-mers resulting from this one-cycle extension reaction (annealing temperature 60°C) were run on a 12% polyacrylamide gel, and analysed using Storage Phosphor autoradiography using the ImageQuant TL quantification software (GE Healthcare). The calculated ratios of those clones treated with CHX to those grown in the absence of CHX were calculated (refer to chapter 2.3.2). Calculated ratios (+CHX/-CHX) ranged from 0.71 to 64.86, with a median of 3.56.
These data show that NMD was present in cells containing the Y663X mutation. When cells were not treated with the NMD inhibitor CHX, mRNA levels were about 3.5 times lower than in cells that were treated with CHX. Thus, cells treated with CHX showed normal levels of collagen X mRNA while those cells that were not treated with CHX showed levels of mRNA that were 3.5 times decreased by the effects of NMD.
Therefore, Y663X elicits NMD in chondrocytes.

The PTC mutation W651X was engineered into the col10a1 gene -90bp upstream of the normal stop at amino acid 681, changing the Trp^{651} from TGG to a stop codon (TGA). PCR was performed using the primers MX1F and MX5R, amplifying a 954bp product. Extension reactions were performed using the extension primer MXSN651F, amplifying a 21bp product elongated by either one [α-^{32}P]-dGTP for endogenous collagen X or one [α-^{32}P]-dATP for mutant collagen X.
Calculations of +CHX/-CHX showed ratios that varied from 1.25 to 5.36. A median protection of 2.13 was shown. In cells where NMD was prevented by CHX, collagen X

mRNA levels were 2.13 times higher than in those cells not treated with CHX. Therefore, W631X elicits NMD.

Y632X lies at -147bp of the normal stop codon, where it alters the Tyr^{632} from TAC to a stop codon (TAA). This mutation was also found in SMCD patients, where it elicits tissue specific last-exon NMD in chondrocytes from the SMCD patients growth plate cartilage biopsies [46].
Utilising MX1F and MX5R primers for PCR, a 954bp product containing the Y632X mutation was amplified and then used as template in the following primer extension. Utilizing the 18-mer extension primer MXSN632F, the products were elongated by either two [α-^{32}P]-dCTPs when the endogenous product was amplified or one [α-^{32}P]-dATP for the mutant product. The calculated ratios ranged from 0.78 to 23.22. The median was calculated to be 1.86. Thus, Y632X elicits NMD.

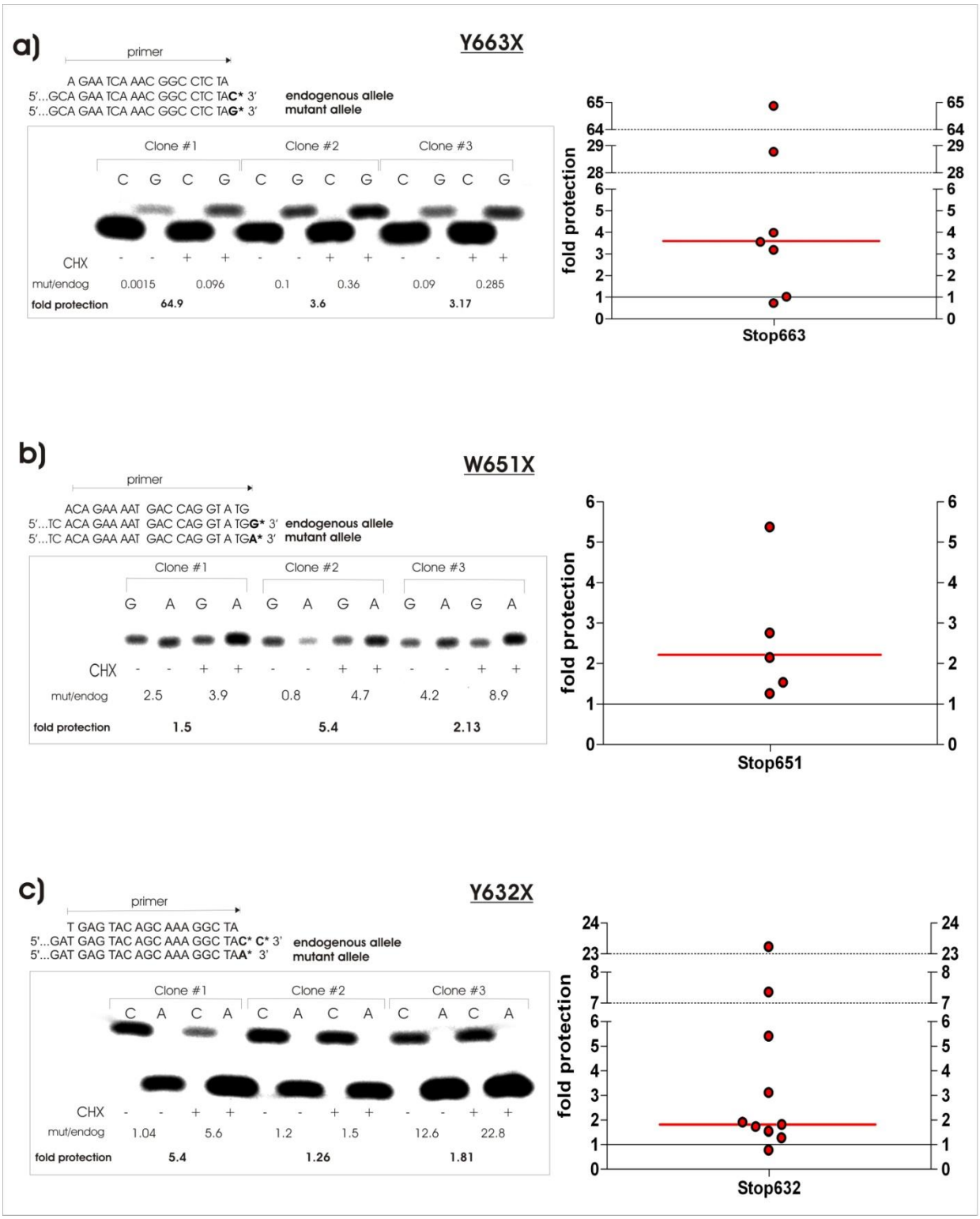

a)
Y663X
primer
A GAA TCA AAC GGC CTC TA
5'...GCA GAA TCA AAC GGC CTC TAC* 3' endogenous allele
5'...GCA GAA TCA AAC GGC CTC TAG* 3' mutant allele
Clone #1
Clone #2
Clone #3
C G C G C G C G C G C G
CHX - - + + - - + + - - + +
mut/endog 0.0015 0.096 0.1 0.36 0.09 0.285
fold protection 64.9 3.6 3.17
fold protection
Stop663
b)
W651X
primer
ACA GAA AAT GAC CAG GT A TG
5'...TC ACA GAA AAT GAC CAG GT A TGG* 3' endogenous allele
5'...TC ACA GAA AAT GAC CAG GT A TGA* 3' mutant allele
Clone #1
Clone #2
Clone #3
G A G A G A G A G A G A
CHX - - + + - - + + - - + +
mut/endog 2.5 3.9 0.8 4.7 4.2 8.9
fold protection 1.5 5.4 2.13
fold protection
Stop651
c)
Y632X
primer
T GAG TAC AGC AAA GGC TA
5'...GAT GAG TAC AGC AAA GGC TAC* C* 3' endogenous allele
5'...GAT GAG TAC AGC AAA GGC TAA* 3' mutant allele
Clone #1
Clone #2
Clone #3
C A C A C A C A C A C A
CHX - - + + - - + + - - + +
mut/endog 1.04 5.6 1.2 1.5 12.6 22.8
fold protection 5.4 1.26 1.81
fold protection
Stop632

Figure 3.2 Primer extension assay of PTC mutations Y663X (a), W651X (b) and Y632X (c). These three mutations were also found in SMCD patients. Y632X causes last-exon NMD *in vivo*, thus serving as a positive control. To determine the effect of each mutation, mutant collagen X mRNA levels of clones treated with the NMD-inhibitor CHX were compared to those of untreated clones (fold protection). Endogenous collagen X was used as internal control (mut/endog). Three representative clones for each mutation are shown on the left of each figure. On the right, mRNA levels of all analyzed clones are plotted, the red line representing the median. Extension primer sequences for each mutation are shown above the representative clones. **a)** Y663X. The 18-mer extension primer MXSN663F is elongated by one [α-^{32}P]-dCTP (C) for the endogenous allele or one [α-^{32}P]-dGTP (G) for the mutant allele. Ratios varied from 0.71 to 64.86 with a median of 3.56, showing that Y663X elicits NMD **b)** W651X. The 20-mer extension primer MXSN651F is elongated by one [α-^{32}P]-dGTP (G) for endogenous and one [α-^{32}P]-dATP (A) for mutant alleles. Ratios from 1.24 to 5.37, median 2.13, showing that W651X elicits NMD **c)** Y632X. The 18-mer extension primer is elongated by either two [α-^{32}P]-dCTP's (C) for the endogenous allele or one [α-^{32}P]-dATP (A) for the mutant allele. Calculated ratios ranged from 0.76 to 23.21, median 1.86. Y632X clearly elicits NMD in mouse chondrocytes.

3.1.3.3 Stop codons Y582X, Q518X and G502X elicit NMD

The PTC mutations Y582X, Q518X and G502X cluster around the part of exon 3 that codes for the transition zone between NC1 and COL1 domains. In SMCD patients so far, mutations were detected as far upstream of the normal stop codon as amino acid 571 (-330bp upstream of the normal stop).

Stop codon Y582X was engineered into the col10a1 gene -297bp upstream of the normal stop codon, in the area of exon 3 that encodes the 5'most region of the NC1 domain. The mutation changed Tyr^{582} from TAC to a stop codon (TAA). cDNA products containing the Y582X mutation were amplified using the SNUPEmX582F and PCRmX582R primers. The 476bp products were then used as template in the primer extension assay. The 21mer oligonucleotide SNUPEmX582F used for primer extension analysis annealed 5' and one base short of the mutation. Extension with radioactive dCTP detected primer extension of one cytosine from template sequence originating from the normal allele. By means of radioactive dATP a specific primer extension by one adenosine from the mutant allele template was detected. The ratios of mRNA levels of clones that were treated with CHX to those clones that were not treated with CHX were calculated. Ratios varied between 0.18 to 5.68. The mean of all clones analysed was a 1.38-fold protection from NMD in those cells treated with CHX. These data show that the Y582X mutation elicits last-exon NMD in mouse chondrocytes.

The PTC mutation Y518X was introduced into the col10a1 gene -489bp upstream of the normal stop codon. It changed the Tyr^{518} from CAA to the stop codon TAA. A 302bp cDNA product was amplified in a PCR reaction using PCRmX518F and SNUPEmX518R primers. In the following primer extension assay, the 21mer extension primer SNUPEmX518R annealed 3' and one base short of the mutation. Extension reactions with radioactive dGTP detected primer extension of two guanosines from the template sequence originating from the endogenous allele. This extension resulted in a radioactively labelled 23mer. When radioactive dATP was used, primer extension of one adenosine was detected from the mutant allele template, which resulted in a labelled 22mer. Radioactive emission was quantified and ratios were calculated of clones treated with CHX to those treated in the absence of the NMD-inhibitor. Ratios ranged

from 0.29 to 2.29. The median protection level was 1.16 fold, demonstrating that Y518X causes last-exon NMD in mouse chondrocytes.

Stop mutation G502X resides -537bp upstream of the normal stop codon. A 208bp product containing the G502X mutation was amplified by PCR utilising the primers MX1F and MXSN502R, with an annealing temperature of 60°C (refer to chapter 2.3.4). In the subsequent primer extension reaction the 18mer extension primer MXSN502R annealed 5' and one base short of the mutation. Thus, one radioactively labelled dCTP bound to the endogenous sequence resulting in a labelled 19mer, or two radioactive dATP'S bound to the mutant sequence adjacent to the 18mer extension primer, resulting in a labelled 20mer. Samples were run on a 12% polyacrylamide gel, then analyzed using storage phosphor autoradiography. Comparison of the clones treated with CHX to those not treated with the NMD inhibitor revealed a median protection from NMD by CHX of 1.17, ratios of the separate clones ranging from 0.4 to 4.78. These data show that the G502X collagen X mutation elicits last-exon NMD.

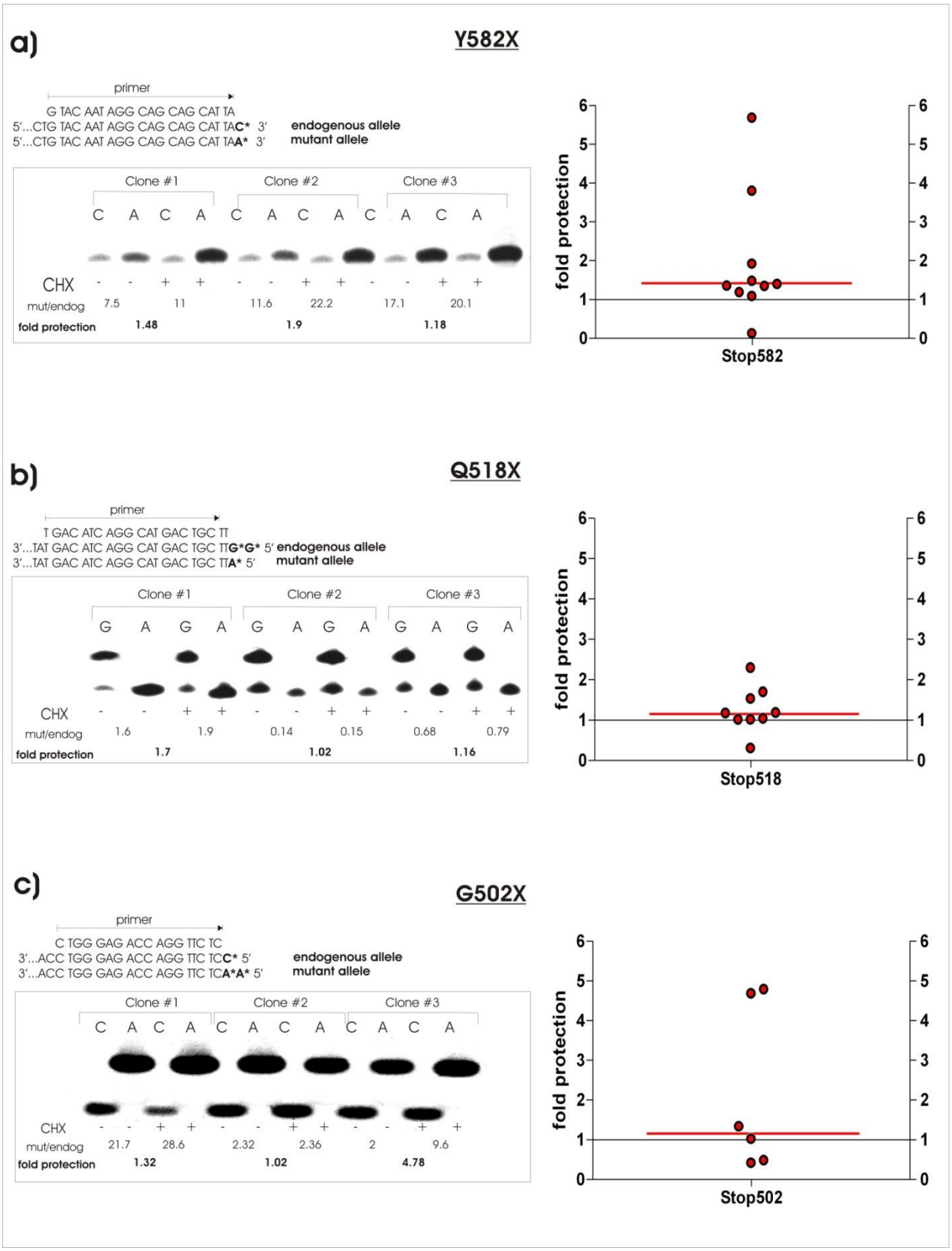

a)
Y582X
primer
G TAC AAT AGG CAG CAG CAT TA
5'...CTG TAC AAT AGG CAG CAG CAT TAC* 3'
5'...CTG TAC AAT AGG CAG CAG CAT TAA* 3'
endogenous allele
mutant allele
Clone #1
Clone #2
Clone #3
C A C A C A C A C A C A
CHX - - + + - - + + - - + +
mut/endog 7.5 11 11.6 22.2 17.1 20.1
fold protection 1.48 1.9 1.18
fold protection
Stop582
b)
Q518X
primer
T GAC ATC AGG CAT GAC TGC TT
3'...TAT GAC ATC AGG CAT GAC TGC TTG*G* 5'
3'...TAT GAC ATC AGG CAT GAC TGC TTA* 5'
endogenous allele
mutant allele
Clone #1
Clone #2
Clone #3
G A G A G A G A G A G A
CHX - - + + - - + + - - + +
mut/endog 1.6 1.9 0.14 0.15 0.68 0.79
fold protection 1.7 1.02 1.16
fold protection
Stop518
c)
G502X
primer
C TGG GAG ACC AGG TTC TC
3'...ACC TGG GAG ACC AGG TTC TCC* 5'
3'...ACC TGG GAG ACC AGG TTC TCA*A* 5'
endogenous allele
mutant allele
Clone #1
Clone #2
Clone #3
C A C A C A C A C A C A
CHX - - + + - - + + - - + +
mut/endog 21.7 28.6 2.32 2.36 2 9.6
fold protection 1.32 1.02 4.78
fold protection
Stop502

Figure 3.3 PTC mutations Y582X, Y518X and G502X. Primer extension assays performed on cDNA fragments spanning the respective mutant region. On the left, extension primers are shown above the col10a1 sequence adjacent to a mutation. Primers bound one base short of the mutations and were elongated by radioactively labelled nucleotides complementary to endogenous or mutant allele. Three representative clones for each mutation are shown below, and calculated ratios of clones treated with CHX to clones treated in the absence of the NMD-inhibitor are indicated (fold protection). On the right, the +CHX/-CHX ratios of all analysed clones are plotted, the red line representing the mean. **a)** Y582X. Ratios varied from 0.18 to 5.68 with a median protection level of 1.38, showing that Y582X elicits NMD **b)** Y518X. Ratios from 0.29 to 2.29, median protection level by CHX 1.16, demonstrating that Y518X elicits last-exon NMD **c)** G502X. The levels of protection ranged from 0.4 to 4.78 with a median 1.17-fold protection, showing that G502 elicits last-exon NMD in collagen X.

3.1.3.4 Stop codons G407X, G308X and G174X do not elicit NMD

The three PTC mutations were engineered into the region of exon 3 coding for the COL1 domain (helix) of collagen X (refer to figure 2.1). In this region, no mutations have been found *in vivo* in SMCD patients.

-822bp upstream of the normal stop codon, the PTC mutation G407X was designed. By PCR, a 159bp product enclosing the PTC region was amplified using PCR primers PCRmX407F and SNUPEmX407R with an annealing temperature of 50°C. In the following primer extension reaction, the 22mer primer SNUPEmX407R was utilized as extension primer, annealing 3' and one base short of the mutation. The radioactive nucleotides bound to their corresponding sequence, i.e. one [α-^{32}P]-dCTP bound to the endogenous template and one [α-^{32}P]-dATP bound to the template containing the mutant sequence.
The results showed no protection from NMD of those clones treated with CHX. Ratios varied from 0.3 to 1.03, the median protection level from NMD was 0.88. Therefore, the G407X mutation does not elicit last-exon NMD in mouse chondrocytes.

G308X was induced in the col10a1 sequence -1119bp upstream of the normal stop codon. PCR amplifictions of a 331bp product spanning the mutated sequence were carried out using the primers PCRmX308F and SNUPEmX308R, annealing temperature 60°C. Extension reactions were performed using the SNUPEmX308R 20mer extension primer which annealed 3' of the mutated sequence and was extended by three [α-^{32}P]-dCTPs in the endogenous sequence, resulting in a labelled 23mer or by one [α-^{32}P]-dATP in the mutant sequence, resulting in a radioactively labelled 21mer. Alike the G407X mutation, the G308X mutation did not show protection from NMD in the clones that had been treated with CHX. The protection levels ranged from 0.31 to 1.07, showing a median protection level of 0.92. These data show that the G308X mutation does not elicit last-exon NMD.

The PTC G174X was engineered into the col10a1 sequence coding for the COL1 domain (helix) at -1521bp upstream from the natural stop codon. It was the mutation farthest from the normal stop codon, only 351bp from COL1 and NC2 boundary. A 296bp PCR fragment was amplified in the PCR reaction, utilizing PCR primers

PCRmX174F and SNUPEmX174R with an annealing temperature of 55°C. The fragment was then used as template in the primer extension reaction. The extension primer annealed 3' and one base short of the mutation, elongated in the reaction by one [α-^{32}P]-dCTP for the endogenous sequence or three [α-^{32}P]-dATP's for the mutant sequence.

Ratios varied from 0.33 to 1.01, the median protection level being 0.95, showing that G174X does not elicit last-exon NMD.

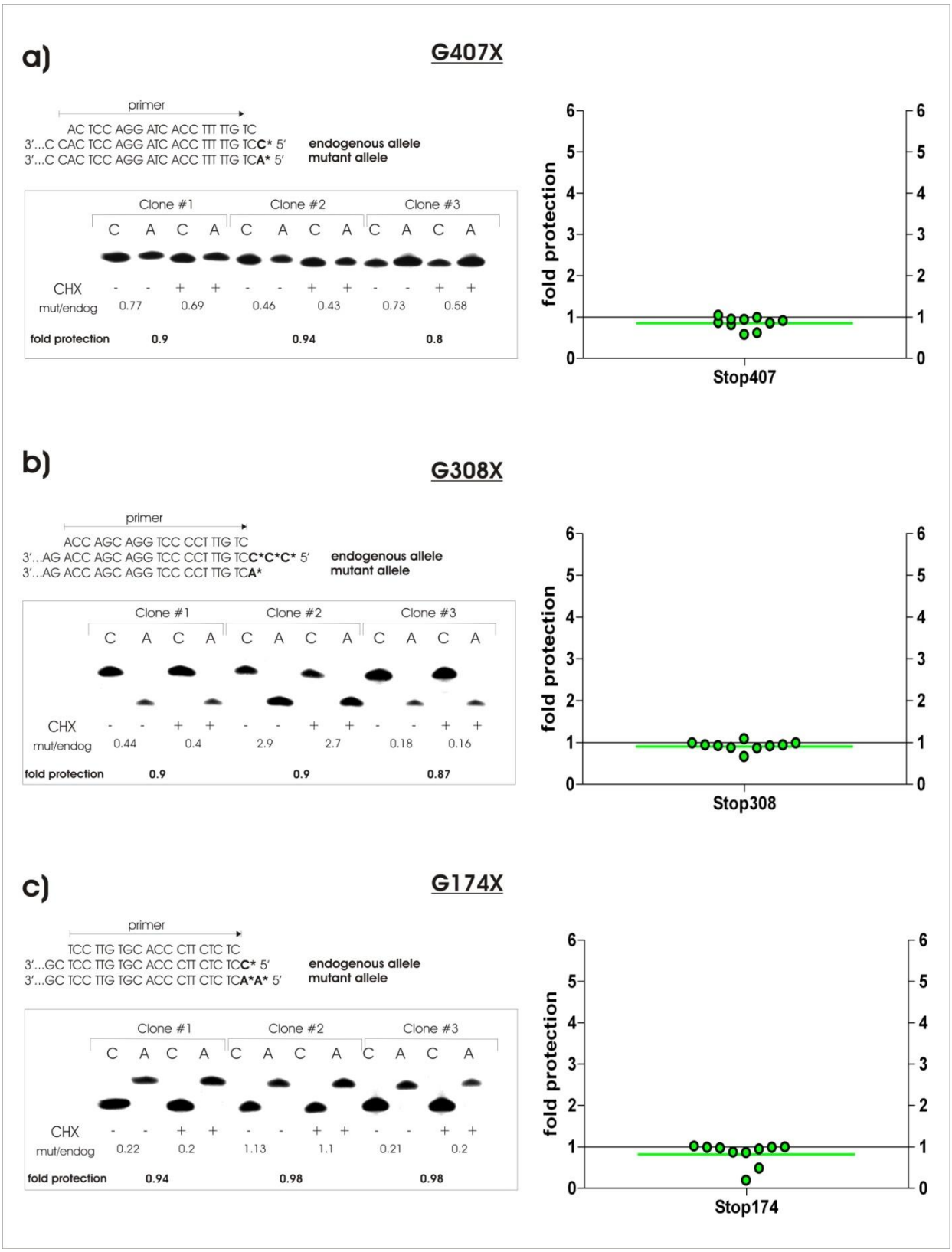

a)
G407X
primer
AC TCC AGG ATC ACC TTT TTG TC
3'...C CAC TCC AGG ATC ACC TTT TTG TCC* 5'
3'...C CAC TCC AGG ATC ACC TTT TTG TCA* 5'
endogenous allele
mutant allele
Clone #1
Clone #2
Clone #3
C A C A C A C A C A C A
CHX - - + + - - + + - - + +
mut/endog 0.77 0.69 0.46 0.43 0.73 0.58
fold protection 0.9 0.94 0.8
fold protection
Stop407
b)
G308X
primer
ACC AGC AGG TCC CCT TTG TC
3'...AG ACC AGC AGG TCC CCT TTG TCC*C*C* 5'
3'...AG ACC AGC AGG TCC CCT TTG TCA*
endogenous allele
mutant allele
Clone #1
Clone #2
Clone #3
C A C A C A C A C A C A
CHX - - + + - - + + - - + +
mut/endog 0.44 0.4 2.9 2.7 0.18 0.16
fold protection 0.9 0.9 0.87
fold protection
Stop308
c)
G174X
primer
TCC TTG TGC ACC CTT CTC TC
3'...GC TCC TTG TGC ACC CTT CTC TCC* 5'
3'...GC TCC TTG TGC ACC CTT CTC TCA*A* 5'
endogenous allele
mutant allele
Clone #1
Clone #2
Clone #3
C A C A C A C A C A C A
CHX - - + + - - + + - - + +
mut/endog 0.22 0.2 1.13 1.1 0.21 0.2
fold protection 0.94 0.98 0.98
fold protection
Stop174

Figure 3.4 Primer extension analysis of G407X, G308X and G174X. Premature termination mutations were engineered into the multiple cloning site of the ColXFlag vector that contained the col10a1 sequence. Clones were treated in the absence or presence of the NMD-inhibitor CHX. Primer extension reactions were performed, where in a one-cycle reaction the primer annealed 3' or 5' and one base short of the mutation. Radioactive labelled nucleotides were adjacent to either the endogenous or the mutant template sequence. Endogenous collagen X levels were used as internal control to the mutant levels (mutant/endogenous). Samples were run on a 12% polyacrylamide gel, and analyzed using storage phosphor autoradiography. To determine whether a mutation caused NMD, clones treated with CHX were compared to those not treated with the NMD inhibitor (+/-CHX). On the left, three representative clones are shown for each mutation, and their protection from NMD by CHX is shown underneath (fold protection). The sequence of the extension primer is shown above. On the right, a plot of all clones analyzed is depicted, the green line representing the median. **a)** G407X. Ratios ranged from 0.3 to 1.03, median 0.88, showing that G407X does not elicit NMD **b)** G308X. Ratios from 0.31 to 1.07, median 0.92. G308X therefore does not cause last-exon NMD **c)** G174X. Variation in protection levels from 0.18 to 1.01, median 0.95, showing that G174X does not elicit last-exon NMD.

3.1.4 Effect of Position of Premature Termination Mutations

The PTC mutation distributed along the collagen X gene showed varying effects on last-exon NMD. In close proximity to the 3'end of the coding sequence, mutations caused last-exon NMD. These were the PTC mutations Y663X, W651X, Y632X, Y582X, Q518X and G502X. Collagen X PTC mutations that were found in SMCD patients elicited NMD in mouse chondrocytes (PTC mutations Y663X, W651X and Y632X). Mutations further upstream from the bona fide stop codon failed to elicit last-exon NMD and showed equal levels of collagen X mRNA in all cells (PTC mutations G407X, G308X and G174X).

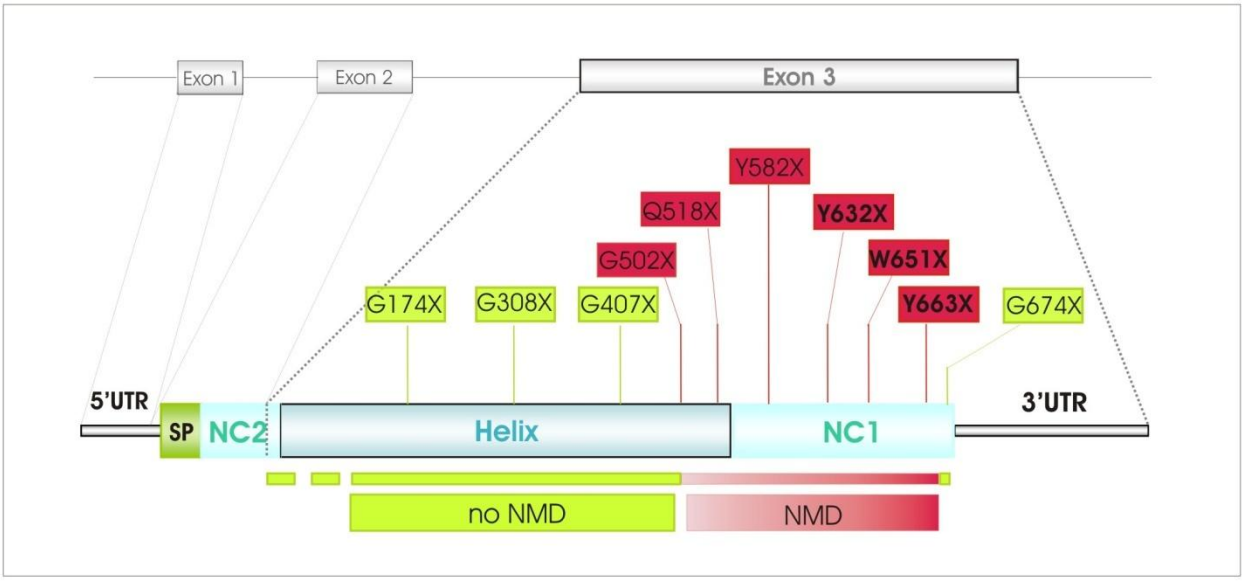


Figure 3.5 Effect of PTC mutations on last-exon NMD and their distribution along the coding region for collagen X. Diagram of the intron-exon structure and the location of PTC mutations inside the mRNA and protein sequence. PTC mutations are indicated above the mRNA/protein structure. The effect of each mutation is indicated by color, red rectangles represent mutations that undergo last-exon NMD, green rectangles indicate mutations that do not elicit NMD. The range of NMD is shown underneath the diagram, where strong decay caused by a PTC is indicated by a dark shade of red, remote NMD by a lighter shade.

3.2 ROLE OF SPECIFIC CONSERVED SEQUENCES IN THE 3'UTR IN MEDIATING LAST-EXON NMD

In my studies, the mutations distributed along the coding region of the collagen X mouse gene construct did not cause nonsense-mediated mRNA decay when close to the 3'end, but elicited NMD in the vicinity of the 5'end of the collagen X gene.

More specifically, the mutation immediately upstream of the normal termination codon did not cause NMD (G674X). Mutations that were close to the 3' end of the NC1 domain (Y663X, W651X, Y632X, Y582X), caused strong NMD in those clones that were not treated with CHX. PTC mutations Q518X and G502X, that lay just 5' of coding sequence for the NC1 domain, caused only mild NMD. Mutations further upstream and inside the COL1 domain (G407X, G308X and G174X) ceased to cause NMD (refer to Figure 3.5).

These results suggested that a trigger, other than a downstream exon junction complex, must be responsible for this last-exon NMD in collagen X. Furthermore, the trigger, or downstream sequence element (DSE) must be only functional for PTC mutations within approximately 500-550kb of the normal stop codon, leaving mutations upstream in NC1 domain, COL1 domain and most likely NC2 domain in an out-of-range zone for NMD. Such DSEs that could be an equivalent to mammalian EJC's have been found in intronless genes in yeast, where they are responsible for EJC-independent NMD (refer to chapter 1.2.2). Furthermore, Amrani et al [73] demonstrated the significance of a properly configured 3'UTR for message stability and translation termination in yeast. PTC's that elicited NMD in yeast constructs ceased to do so when they were flanked by a properly configured 3'UTR. This led to the assumption that the downstream trigger or DSE responsible for last-exon NMD in collagen X may lie within the 3'UTR.

3.2.1 Deletion of specific regions of homology within the 3'UTR

Col10a1 has a long 3'UTR (993bp). Bioinformatic analysis revealed that it contained three regions of homology strongly conserved across species (human, mouse, rat, dog, cow, chimp), suggesting that these may have an important biological role. The first region of homology, region I (23bp, 76bp downstream of the start of the 3'UTR), showed

91% identity to UTR sequence alignment from human and mouse. Region II (117bp, 384bp downstream), showed 87% homology, and region III (79bp, 875bp downstream) showed 86% homology between species. Region III contains a 13bp AU-rich element (ARE), which have been shown to have a role in regulating mRNA stability in other genes[76].

To establish if the 3'UTR is involved in last-exon NMD, 3'UTR regions were deleted in the mouse col10a1 construct containing the Y632X mutation, which normally undergoes last-exon NMD (refer to chapter 3.3.2).

3.2.2 Results

3.2.2.1 Deletion of the 3'UTR in the col10a1 gene construct

To test if a DSE controlling NMD competency was within the 3'UTR, the whole 3'UTR was deleted. If a DSE were present inside the 3'UTR, the deletion of this region would result in mutant mRNA stability in spite of the Y632X mutation present in the gene construct.

The Y632Xdel3'UTR deletion was engineered into the col10a1 gene construct using splicing-by-overlap extension (refer to section 2.2.2). The ColXFlag vector containing the Y632X mutation was used as template. Constructs were stably transfected into mouse MCT chondrocytes, and treated in the absence or presence of CHX prior to RNA extraction. Reverse transcription was performed, and a 389bp product containing the Y632Xdel3'UTR deletion was amplified in PCR. To assess NMD, the allele-specific primer extension assay was performed, utilizing radioactively labelled nucleotides and the 18mer extension primer MXSN632. In the assay, two [α-^{32}P]-dCTP's bound to the endogenous allele or one [α-^{32}P]-dATP to the mutant allele. The level of endogenous collagen X was used as internal control to the mutant collagen X levels. NMD was assayed by comparison of the mutant/endogenous collagen X ratios of those clones treated in the presence to those treated in the absence of the NMD inhibitor, CHX. Protection levels varied from 0.35 to 1.86 with a median 1.01 fold protection from NMD in those clones treated with CHX. This demonstrated that the Y632Xdel3'UTR deletion stopped the NMD that was normally caused by the Y632X mutation. To further explore the DSE within the 3'UTR, subsequently smaller sections of the 3'UTR were deleted.

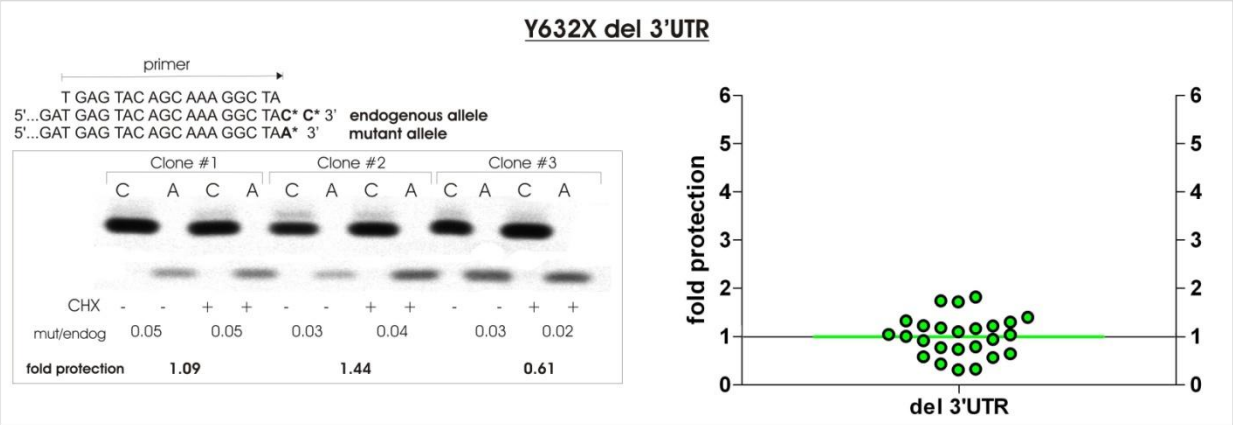


Figure 3.6 Y632Xdel3'UTR. Primer extension analysis of PCR-amplified cDNA fragments. The construct contained the Y632X mutation, which elicits last-exon NMD in the col10a1 gene construct. Primer extension reaction was performed utilizing the 18mer MXSN632F primer, which bound 5' and one base short of the Y632X mutation. Radioactively labelled nucleotides were used, that were complementary to either the endogenous or the mutant sequence. Two [α-^{32}P]-dCTP'S bound to the endogenous allele (C), one [α-^{32}P]-dATP bound to the mutant allele (A). Endogenous collagen X was used as internal control for expression levels to the mutant collagen X (mut/endog). To assess NMD, mRNA levels of clones treated in the presence of CHX were compared to the levels of clones treated in the absence of the NMD inhibitor (fold protection). Protection levels ranged from 0.35 to 1.86 with a median 1.01fold protection in those clones treated with CHX. These data demonstrated that the region responsible for last-exon NMD must lie within the 3'UTR, since its deletion stopped the Y632X mutation from eliciting NMD.

3.2.2.2 Deletion of the regions of homology in the 3'UTR, Y632Xdel1, Y632Xdel2 and Y632Xdel3

Y632Xdel1 was deleted in the col10a1 gene construct containing the Y632X mutation. The 23bp region (+76 to +99bp, 91% homology between species) was deleted using splicing-by-overlap extension, whose 2° reaction spanned the whole 3'UTR and the exon 3 sequence containing the Y632X mutation. To assess NMD, cells were treated in the presence or absence of the NMD inhibitor, CHX on the 7th day. RNA was then extracted and converted to cDNA by RT-PCR. A 389bp cDNA product was amplified and used as template in the following allele specific primer extension assay. Here, two [α-^{32}P]-dCTP's annealed to the endogenous collagen X produced by the MCT chondrocytes that was used as internal control. Or, one [α-^{32}P]-dATP bound to its specific sequence. To determine the effect of the Y632Xdel1 deletion, cDNA levels of clones treated in the presence of CHX were compared to those grown in its absence.

The detected ratios +CHX/-CHX varied from 0.19 to 3, with a median ratio of 1.37. These data showed that the Y632X mutation present in this construct still elicited last-exon NMD. The first region of homology is therefore not responsible for last-exon NMD.

The second candidate region to be involved in last-exon NMD was region 2. This 117bp region (+384 to +499bp) showed 87% homology between mouse and human sequences. Again using the Y632X-containing construct as the template, region 2 was deleted To determine the effect of the deletion on the last-exon NMD that the Y632X mutation elicited, cells were treated with or without CHX. A 389bp fragment was amplified by RT-PCR and PCR, using the PCR primers MXSN632F and MX5R. The 18mer extension primer MXSN632F was used in the one-cycle reaction. Radioactively labelled nucleotides were used that bound only to their specific allele, i.e. two [α-^{32}P]-dCTP's bound to the endogenous collagen X sequence and one [α-^{32}P]-dATP annealed to its complementary mutant allele. To investigate the effect of the deletion on the last-exon NMD caused by the Y632X mutation, +CHX/-CHX cDNA ratios were examined. Ratios of mutant to endogenous collagen X were calculated, since endogenous collagen X served as internal control (mutant/endongenous). The calculated ratios varied from 1.12 to 3.22. The median was calculated to be 1.31. Deletion of the second region of homology within the 3'UTR did not stop the NMD caused by the Y632X

mutation present in the construct. Region 2 therefore is not responsible for last-exon NMD.

The third region potentially involved in mediating last-exon NMD was the 79bp (+875 to +950) region 3. This region showed an 86% homology between human and mouse sequences. Furthermore, region three contained the highly conserved 13bp AU-rich element (refer to chapter 4.3.3). As in the other deletions, the Y632Xdel3 deletion was engineered into the ColXFlag vector containing the Y632X mutation that elicits last-exon NMD. A 389bp fragment containing the Y632X mutation was amplified by RT-PCR and PCR utilizing primers MXSN632F and MX5R. To assay the effect of the deletion on the last-exon NMD, that the Y632X mutation elicits, allele specific primer extension was performed. The 18mer extension primer MXSN632F bound 5' and one base short of the mutation, and the radioactive nucleotide [α-^{32}P]-dCTP bound specifically to the endogenous allele, the nucleotide [α-^{32}P]-dATP to the mutant allele. NMD was assessed by comparison of the cDNA levels of clones treated with or without CHX (+CHX/-CHX). These ratios varied from 0.4 to 2.13 with a median of 0.8 and a mean of 0.9.
These data demonstrated that with the deletion of region 3, the NMD caused by the Y632X mutation was inhibited. Therefore, the region responsible for NMD must either be region 3 in itself, or a shorter section of region 3, e.g. the AU-rich element. Subsequently, two more sections of region 3 were deleted.

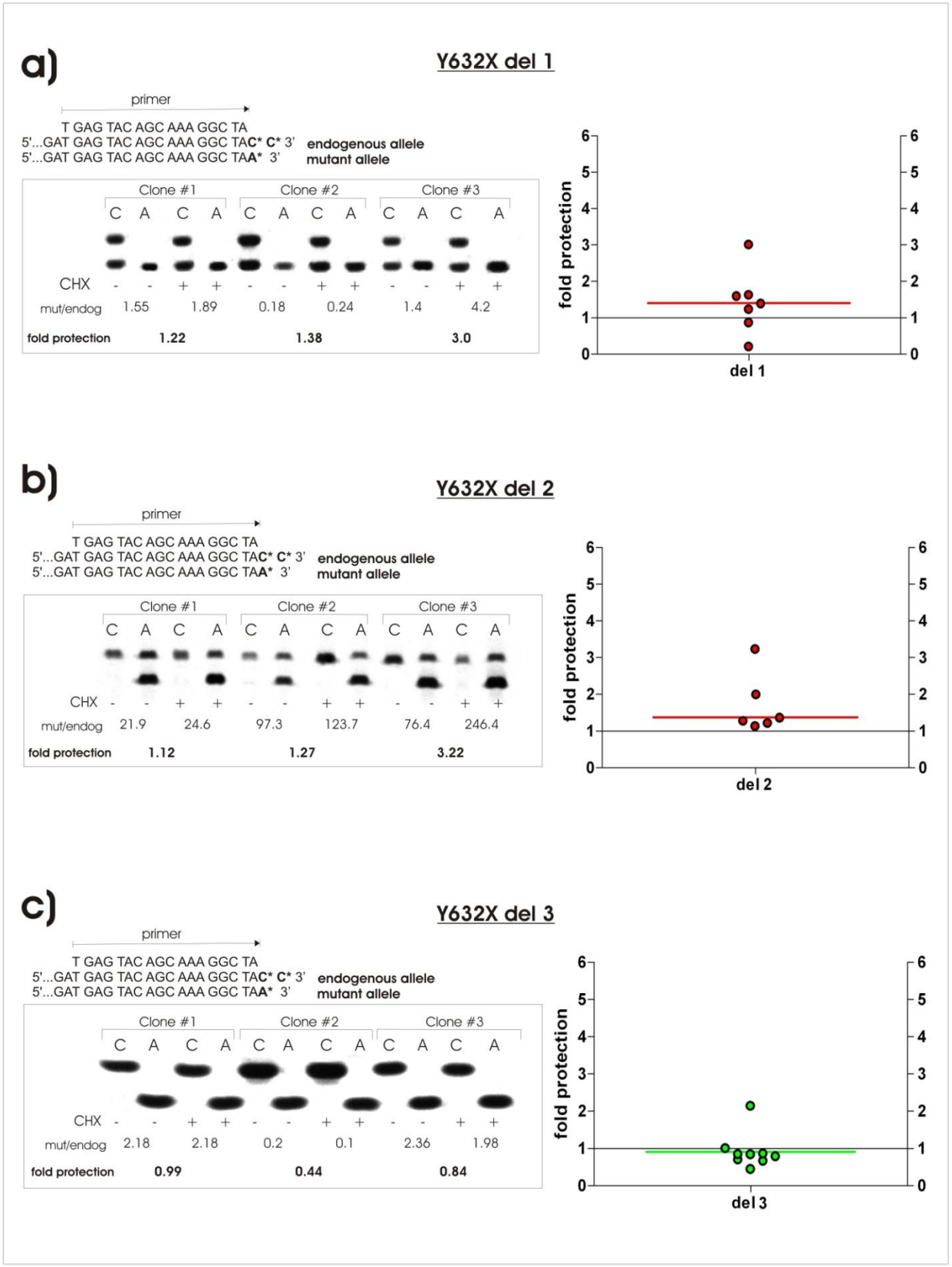

a)
Y632X del 1
primer
T GAG TAC AGC AAA GGC TA
5'...GAT GAG TAC AGC AAA GGC TAC* C* 3' endogenous allele
5'...GAT GAG TAC AGC AAA GGC TAA* 3' mutant allele
Clone #1
Clone #2
Clone #3
C A C A C A C A C A C A
CHX - - + + - - + + - - + +
mut/endog 1.55 1.89 0.18 0.24 1.4 4.2
fold protection 1.22 1.38 3.0
fold protection
del 1
b)
Y632X del 2
primer
T GAG TAC AGC AAA GGC TA
5'...GAT GAG TAC AGC AAA GGC TAC* C* 3' endogenous allele
5'...GAT GAG TAC AGC AAA GGC TAA* 3' mutant allele
Clone #1
Clone #2
Clone #3
C A C A C A C A C A C A
CHX - - + + - - + + - - + +
mut/endog 21.9 24.6 97.3 123.7 76.4 246.4
fold protection 1.12 1.27 3.22
fold protection
del 2
c)
Y632X del 3
primer
T GAG TAC AGC AAA GGC TA
5'...GAT GAG TAC AGC AAA GGC TAC* C* 3' endogenous allele
5'...GAT GAG TAC AGC AAA GGC TAA* 3' mutant allele
Clone #1
Clone #2
Clone #3
C A C A C A C A C A C A
CHX - - + + - - + + - - + +
mut/endog 2.18 2.18 0.2 0.1 2.36 1.98
fold protection 0.99 0.44 0.84
fold protection
del 3

Figure 3.7 Primer extension assay of 3'UTR deletions. Regions of homology between species in the 3'UTR were deleted to determine their involvement in mediating last-exon NMD. Using the ColXFlag construct containing the Y632X mutation which elicits last-exon NMD, three regions of homology were deleted. Primer extension was performed, and two [α-^{32}P]-dCTP's annealed specifically to the endogenous allele, one [α-^{32}P]-dATP bound to the mutant allele. To the left, three representative clones for each mutation are depicted. Endogenous collagen X cDNA levels were used as internal control (mut/endog) and calculated ratios of cDNA levels of clones treated with or without the NMD-inhibitor, CHX (-/+CHX) are shown underneath (fold protection). The extension primer sequence is shown above the clones. On the right, all analysed clones are plotted, and a colored line represents the median of all clones. a) Y632Xdel1. Ratios varied from 0.19 to 3, with a median ratio of 1.37. b) Y632Xdel2 showed ratios from 1.12 to 3.22 with a median of 1.31. c) For the Y632Xdel3 cDNA ratios from 0.4 to 2.13 were calculated, with a median of 0.8.

3.2.2.3 Deletion of two conserved elements inside region 3

Within the third region of homology of the 3'UTR, a 13bp AU-rich element (ARE) was identified. ARE's have been shown in other genes to be involved in regulating the half-life of mRNA[76]. To determine the influence of this ARE in the collagen X 3'UTR, two different deletions were made.

Y632Xdel3b deleted an element spanning the ARE and 19bp downstream sequence. Again, the deletion was made in the construct containing the Y632X mutation eliciting last-exon NMD. The Y632Xdel3b construct was then stably transfected into MCT chondrocytes, treated in the absence or presence of CHX, and RNA extracted. NMD was assessed by allele specific primer extension, where two [α-^{32}P]-dCTPs annealed to the endogenous collagen X sequence, resulting in a radioactively labelled 20mer, or one [α-^{32}P]-dATP elongated the 18mer extension primer, resulting in a radioactive 19mer. Samples were run on a 12% polyacrylamide gel, and mRNA levels of clones treated with CHX compared to the levels of clones not treated. Endogenous collagen X was used as internal control. The calculated ratios varied from 0.6 to 1.4, with a median of 1.03 and a mean of 1.01.
These data showed that the deleted section of region 3 stopped the last-exon NMD caused by the Y632X mutation. Region 3b is involved in last-exon NMD.

To assess whether the whole of region 3b or solely the ARE are involved in last-exon NMD, a 3'UTR construct was made deleting the ARE. The Y632XdelARE deletion was introduced into the col10a1 gene construct containing the Y632X PTC mutation. Y632X causes last-exon NMD *in vivo*[46] and in the mouse transfection system (refer to chapter 3.3.2). Deletion of a region involved in eliciting last-exon NMD would therefore result in inhibition of NMD caused by Y632X. Again, cells were treated in the presence or absence of the NMD inhibitor, CHX. In the allele specific primer extension, two [α-^{32}P]-dCTP's annealed to the endogenous sequence, one [α-^{32}P]-dATP elongated the mutant allele. Ratios were calculated of clones treated with CHX to those treated without (+CHX/-CHX). The ratios calculated for Y632XdelARE varied from 0.11 to 4.26 with a median of 1.67 and a mean of 2.14, demonstrating that deletion only of the ARE did not inhibit the NMD caused by Y632X. Region ARE is therefore by itself not involved in last-exon NMD.

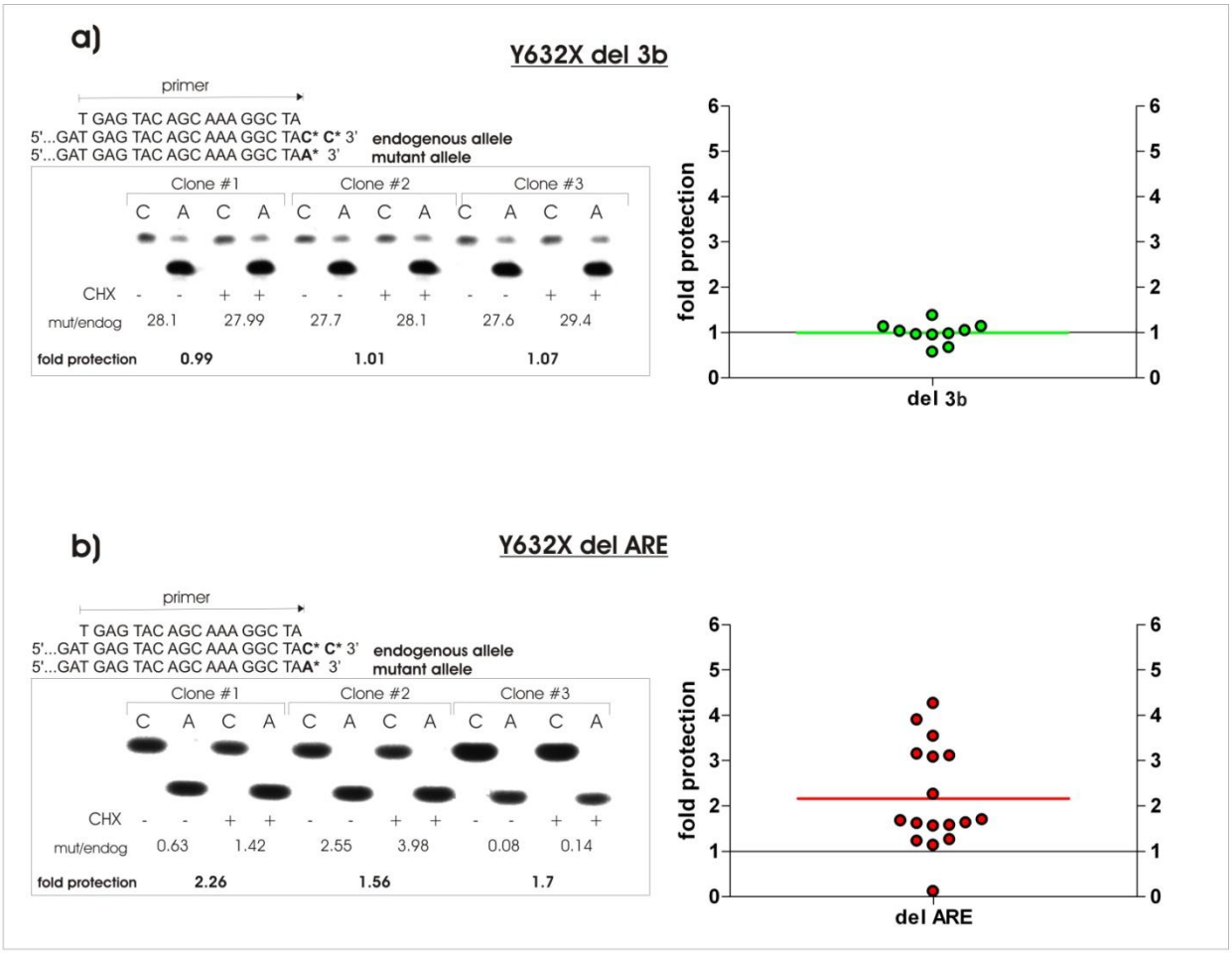


Figure 3.8 Allele specific primer extension assay of specific deletions in the third region of homology of the 3'UTR. A construct containing the Y632X PTC mutation was used as template, that caused NMD *in vivo* and in the transfection system. The extension primer annealed 5' and one base short of the Y632X mutation. Radioactive nucleotides bound specifically to either endogenous (C) or mutant sequence (A). Clones were treated in absence or presence of the NMD inhibitor CHX (-/+). Endogenous collagen X was used as internal control (mut/endog). To the left, three representative clones are depicted underneath the extension primer sequence. The ratio +CHX to –CHX for those clones is shown underneath (fold protection). To the right, all analyzed clones are plotted, the colored line representing the median. **a)** Y632Xdel3b deleted a region spanning the ARE and 19bp downstream sequence. The +CHX/-CHX ratios varied from 0.6 to 1.4, with a median of 1.03, showing that region 3b is involved in last-exon NMD **b)** Y632XdelARE deleted the 13bp AU-rich element found in region 3. Ratios varied from 0.11 to 4.26 with a median of 1.67, showing that region ARE by itself is not involved in last-exon NMD.

3.2.3 Effect of deletion of the three regions of homology within the 3'UTR

Deletion of the three regions of homology within the 3'UTR gives new insights into the possible mechanism underlying last-exon NMD. It was demonstrated that deletion of a section within the 3'UTR stops last-exon NMD, therefore region 3b must be involved in last-exon NMD.

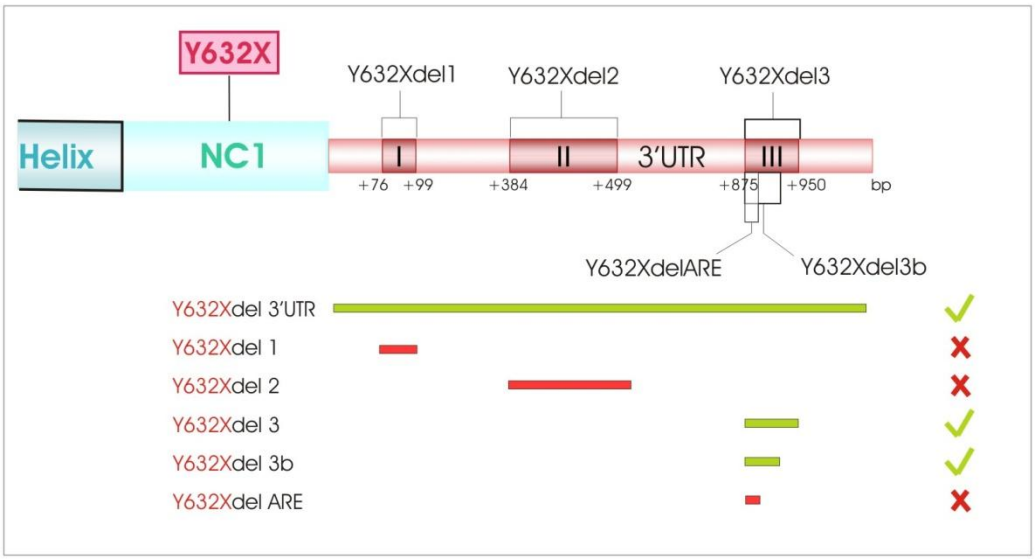


Figure 3.9 Deletion of regions of homology withing the 3'UTR of COL10A1 and their effects on NMD-triggering of the Y632X mutation. Deletion of the whole 3'UTR, Y632Xdel3'UTR stops the last-exon NMD caused by Y632X. Deletion of two regions of homology, Y632Xdel1 and Y632Xdel2 does not have any effect on NMD while deletion of Y632Xdel3 again inhibits NMD. Deletion of the smaller region Y632Xdel3b still inhibits NMD while deletion of the yet smaller Y632XdelARE does not have an effect on NMD.

Chapter Four

Discussion

4.1 INTRODUCTION

Nonsense and frameshift mutations cause premature termination codons. Studies on cartilage biopsies of SMCD patients revealed that the PTC mutations at codons 611, 632 and 663 in collagen X cause degradation of the mutant mRNA in a process called nonsense-mediated mRNA decay[42, 46, 77], a highly complex surveillance mechanism for mRNA. One of the basic principles of mammalian NMD mechanisms known to date is their dependence on downstream exon-exon junctions that are marked by a protein complex after transcription. However, all mutations found in SMCD patients were found in a region coded for by exon 3, the last exon in collagen X which is not followed by a downstream exon-exon junction. Thus, collagen X SMCD mutations should not be susceptible to NMD.

These data raised several interesting questions on SMCD nonsense mutations.
Firstly, why were there no mutations found in coding sequences for 5' NC1 regions, COL1 and NC2 domains. What different effect on mRNA would these mutations exert?
And secondly, how could the PTC mutations found in SMCD patients cause NMD if they were located within the last exon? Which region/mechanism is responsible for specifying this last-exon NMD?

4.2 EFFECT OF POSITION OF THE PREMATURE TERMINATION CODON MUTATIONS ON COL10A1 *mRNA IN CHONDROCYTES*

In SMCD patients' chondrocytes, the detected PTC mutations caused decay of the collagen X mRNA. Interestingly, all nonsense mutations were found in the last exon, where they should be immune to NMD (refer to chapter 1.2.1). Moreover, all mutations were confined to the 3' end of exon 3, which encodes the 3' region of the NC1 domain. So far, no mutations were found closer to the 5'end of the NC1 domain, inside the COL1 domain (helix) or within the NC2 domain, which are also coded for by exon 3. Mutations further upstream of the first known exon 3 mutation (T590fsX604) might have a serious deleterious effect and would therefore cause a different phenotype or even prevent implantation or lead to early embryonic abortion. Another possibility is that these more 5' mutations do not have any negative effects at all, but are well tolerated.

To address the first question, the subject of PTC mutation localisation confined to the NC1 domain, PTC mutations were engineered into a mouse gene construct. The mutations were distributed along exon 3, covering some of the known SMCD mutations locations as well as upstream regions. Here, no SMCD nonsense mutations have been found to date. Possibly, mutations upstream of the known mutations show a different pathology to the known mutations. Thus, they could either be tolerated e.g. by translation reinitiation. Or, if these mutations did not elicit last-exon NMD, they could cause a different or much more severe phenotype.

4.2.1 PTC mutation G674X does not cause nonsense-mediated decay

The G674X PTC mutation was engineered into the *Col10a1* gene construct seven amino acids upstream of the natural stop codon at amino acid 681. When mutant/endogenous levels of clones treated with CHX were compared to those not treated with the NMD inhibitor, ratios from 0.18 to 1.01 resulted in the eight clones that were analyzed. The median protection level was 0.95, indicating that treatment with CHX did not alter the collagen X mRNA expression level in the mouse MCT chondrocytes. The G674X mutation does therefore not elicit last-exon NMD in the mouse transfection system.

In 1993, Warman et al discovered a 13bp deletion in the collagen X gene in a large mormon kindred. This deletion led to a frameshift and a PTC at codon 674. It was thought that this mutation prevented association of the mutant polypeptide during trimer formation, resulting in a decreased amount of normal protein [15].

In a recent paper by Tsang et al [5], a transgenic mouse was made harboring this 13bp deletion that caused the PTC at codon 674. Tsang et al found that in the mouse the mutant protein was expressed, thereby preventing proper protein folding and causing ER stress. In this case, the unfolded protein response (UPR), a highly conserved network activated by inhibition of protein folding in the endoplasmic reticulum (ER) was activated (reviewed in [6]). The UPR caused the cells to re-enter the cell cycle and to revert to a less mature state where the mutant protein was reduced. Thereby apoptosis, which can be induced through the UPR when prolonged ER stress occurs, was avoided. These findings confirm my findings on NMD in G674X, where mutations in this region do not undergo NMD.

4.2.2 SMCD mutations Y663X, W651X and Y632X cause nonsense-mediated decay

Upstream of the G674X mutation that does not elicit NMD, PTC mutations Y663X, W651X and Y632X were engineered into the collagen X construct. These mutations were of particular interest. In SMCD patients, codons 663, 651 and 632 have been identified as PTC bearing locations.

Y663X lies at -54bp from the natural stop codon. When mutant/endogenous levels of clones treated with CHX were compared to those not treated, ratios of 0.71 to 64.86 were calculated. A median protection level from NMD of 3.56 could be calculated of all clones that were analyzed. In a representative clone harboring the Y663X mutation, collagen X mRNA levels would be about 3.6 times higher when NMD was prevented by CHX than when it was not prevented.

Therefore, the Y663X mutation elicits last-exon NMD in the mouse transfection system.

Recent finding by Ho et al [77] display similar findings. In one SMCD patient harboring the Y663X mutation, they found 36% mutant mRNA in the growth plate hypertrophic zone, and 64% wild-type collagen X mRNA. The zone was disorganized and expanded. When they in vitro translated the mutant mRNA, truncated collagen X chains resulted that were unable to assemble and interfered with the assembly of normal α1(X) chains into trimers. These results were interpreted as a gain-of-function effect as the molecular background in this SMCD patient. However, the results also suggest that a certain amount of mutant mRNA had already decayed, since only 36% mutant mRNA were present in the growth plate cartilage. Seemingly, the mutation does not lead to complete NMD, and the remaining mutant mRNA is able to elicit the UPR. Thus, cells return to an earlier state of differentiation and express lower levels of collagen X (refer to [5]).

In SMCD patients bearing the Y632X or the Y611X mutation, the mutant collagen X mRNA was completely decayed, and the resulting common effect was functional haploinsufficiency of collagen X [46] [42] [82].

The results show, that even though NMD occurs for a certain mutation, the molecular basis may vary between mutations. If incomplete NMD occurs for a mutation, as seems to be the case in the studies by Ho et al, the resulting phenotype could be a mix of functional haploinsufficiency and the gain-of function effect of the remaining mutant collagen X mRNA.

W651X resides at -90bp upstream from the stop codon at AA681. The collagen X mRNA ratios resulting of CHX treatment varied from 1.25 to 5.36. The median protection level from NMD by CHX was 2.13. A typical clone harbouring the W651X mutation would show higher levels of collagen X mRNA in clones where NMD was inhibited than in those where it was not inhibited, evidencing the NMD-causing effect of the W651X mutation. This mutation was discovered in a SMCD patient by McIntosh et al [83].

The effect of the Y632X mutation on mRNA stability was important to establish in the mouse transfection system, since this mutation elicits complete last-exon NMD *in vivo*[46]. Thus, it could be used as a positive control to the mouse transfection system. When ratios were calculated of clones treated with CHX to those not treated, protection levels resulted varying from 0.78 to 23.22. The median protection level from NMD of 1.86 clearly showed that the Y632X mutation in the mouse transfection system instigates nonsense-mediated decay.

These results concur with those from the SMCD patients' growth plate cartilage extracts[46]. However, the Y632X mutation in SMCD patients causes complete NMD of the mutant allele, whereas it only reduces the amount of mutant allele to a minimum of 4.31% of the original amount and the average clone reduced the mRNA level to about 20.8% of the mRNA level of clones treated with CHX.

If NMD was identified in the mouse transfection system, it was interpreted as significant, even though the mRNA was not completely decayed. Most likely, the deviance reflects the different conditions under which the mutations exert their effect on mRNA in the MCT cells. *In vitro* culture conditions imitate the *in vivo* conditions of cartilage, but they are not identical. Also, MCT cells that are grown at their permissive temperature of 33°C [78] transform into a chondrocyte-like phenotype, but they are stably transfected cells and the characteristics of a chondrocyte might change slightly from those altered conditions. Furthermore, mutations that were found to elicit last-exon NMD in SMCD patients' cartilage extracts did not elicit NMD in bone cells or lymphoblasts (refer to chapter 1.3 and reference [42]), confirming tissue-dependency of the NMD effect.

4.2.3 Stop codons Y582X, Q518X and G502X elicit nonsense-mediated decay

The three stop mutations Y582X, Q518X and G502X cluster around the NC1 domain/COL1 domain border (refer to Figure 2.1). The collagen X mutation closest to the NC1/COL1 border that was found in SMCD patients was the missense mutation Y582D [84], which lies at -297bp upstream of the normal stop codon. In the same position, at codon 582, a stop mutation was engineered into the mouse transfection system (Y582X). For the Y582X mutation, ratios of mRNA levels of clones treated with the NMD-inhibitor CHX to those not treated varied from 0.18 to 5.68. The median protection level from NMD by CHX was 1.38. Therefore, collagen X mRNA in a typical clone harboring the Y582X PTC mutation undergoes last-exon NMD.

Q518X lies at -489bp from the normal stop codon, inside the coding region for the COL1 domain (helix). The calculated ratios of mRNA levels from clones treated with CHX to those not treated ranged from 0.29 to 2.29, and a median protection level of 1.16 was found, demonstrating that Q518X elicits NMD. Thus, the representative clone for the Q518X PTC mutation would be protected from NMD by CHX, but only to a very small degree.

G502X resides -537bp upstream of the normal stop coden, inside the coding region for the COL1 domain. The +CHX/-CHX ratios of those clones varied from 0.4 to 4.78. The typical clone harboring the G502X mutation would show a 1.168 fold protection from NMD. The G502X mutation induces remote last-exon NMD .

To date, no mutations have been found as far upstream from the bona fide termination codon as Q518X and G502X. The finding that these mutations undergo only mild NMD could suggest that the mutant mRNA undergoes only mild NMD *in vivo* as well. The mutant protein would, at least to a certain degree, be produced. Thus, its interference with normal α1(X) chains would inhibit correct trimer formation. Here, the predominant effect in those mutations might be to activate the UPR and a gain-of function effect could result.

4.2.4 Stop codons G407X, G308X and G174X cease to cause NMD

Stop codons G407X, G308X and G174X reside in the region of *col10a1* that codes for the COL1 domain (helix). To date, in SMCD patients, no mutations were found in this region.

G407X resides -822bp upstream of the normal stop codon. Under CHX treatment the clones displayed +CHX/-CHX ratios that ranged from 0.3 to 1.03. The typical clone containing the G407X mutation would show a 0.88 fold protection from NMD. Thus, the G407X premature stop mutation does not elicit last-exon NMD in the mouse transfection system.

G308X resides at -1119bp from the natural stop at amino acid 681. The calculated ratios to assess last-exon NMD varied form 0.31 to 1.07. The median protection level by CHX from last-exon NMD was 0.92. Therefore, the G308X mutation does not elicit NMD.

G174X lies at -1521bp from the natural stop codon. When clones grown in the presence of CHX were compared to clones treated without the NMD-inhibitor, ratios from 0.18 to 1.01 resulted. The median protection level was 0.95. Thus, the G174X PTC mutation does clearly not elicit last-exon NMD in the mouse transfection system.

Interestingly, of the three nonsense mutations that reside within the COL1 domain (helix), none elicited last-exon NMD. The possible effects of this are discussed in detail below (refer to section 4.2.5).

4.2.5 The PTC mutations show a 3' to 5' polarity of NMD

Mutations engineered into the *col10a1* coding sequence showed variable effects of last-exon NMD. In the primer extension analysis, PTC mutations Y663X, W651X, Y632X and Y582X which reside in the col10a1 region coding for the NC1 domain clearly elicited last-exon NMD. These results match the results found in SMCD patients cartilage for the PTC mutation Y632X *in vivo*. All four mutations resided in the region of the *col10a1* gene coding for the NC1 domain (helix), which is also the only region of *COL10A1* in which mutations were found in SMCD patients.

PTC mutations Y518X and G502X, which reside just inside the *col10a1* region that codes for the COL1 domain (helix) do also elicit last-exon NMD. However, the protection level from NMD is very low.

Stop mutations G407X, G308X and G174X that were engineered into the *col10a1* sequence further upstream from the natural stop codon inside the COL1 domain ceased to elicit last-exon NMD.

Overall the susceptibility to NMD in the PTC mutations engineered into the mouse gene construct showed a gradient, with the highest susceptibility rates closest to the 3'end of the NC1 domain and the lowest rates close to the NC1 domain/COL1 domain border further 5' from the normal stop codon. Inside the COL1 domain PTC mutations are no longer susceptible to last-exon NMD (refer to Figures 3.5 and 4.1).

A gradient in NMD was also found by Harries et al[61], who reported a 5' to 3' polarity in NMD susceptibility in the hepatocyte nuclear factor-1 beta gene with the most 5' regions having the highest NMD. These workers suggested that the mechanism may involve translation re-initiation downstream of a PTC mutation, and that the distance from the start codon was also involved in determining whether a PTC mutation caused NMD. The gradient found in collagen X mRNA is in the opposite direction with highest decay rates at the 3'end. A possible explanation for this could be that there is an out-of-reach limit of the NMD machinery which could involve the 3' end of the collagen X gene. A possible role for the 3' region of the gene in specifying *col10a1* NMD is explored in detail in section 4.3.

However, in recent studies on the 3' to 5' polarity of this section of the collagen X mRNA, Tan et al were not able to show this polarity in NMD susceptibility [85]. When examining higher numbers of those clones that showed polarity it was found that the suscenptibility to NMD was given for mutations close to the 3' end of the gene (down to Y632X, as also shown in my research), while those mutations closer to the 5' end did not show this susceptibility (Y582X, Q518X and G502X).

To get concluding results on this matter, futher studies on this region are currently conducted utilizing real time PCR for even higher accuracy (Bateman et al, unpublished data).

The observation of the effects of these mutations sheds light on the functionality of NMD as well as the question, why no mutations were found in upstream NC1 and COL1 regions in SMCD patients. Mutations inside the COL1 domain are immune to last-exon NMD. Therefore, the stop codons would be recognized and a truncated protein be produced. This would result in a very large amount of mutant protein in these cells. In patients harboring mutations within the COL1 domain, the missing effect of NMD would

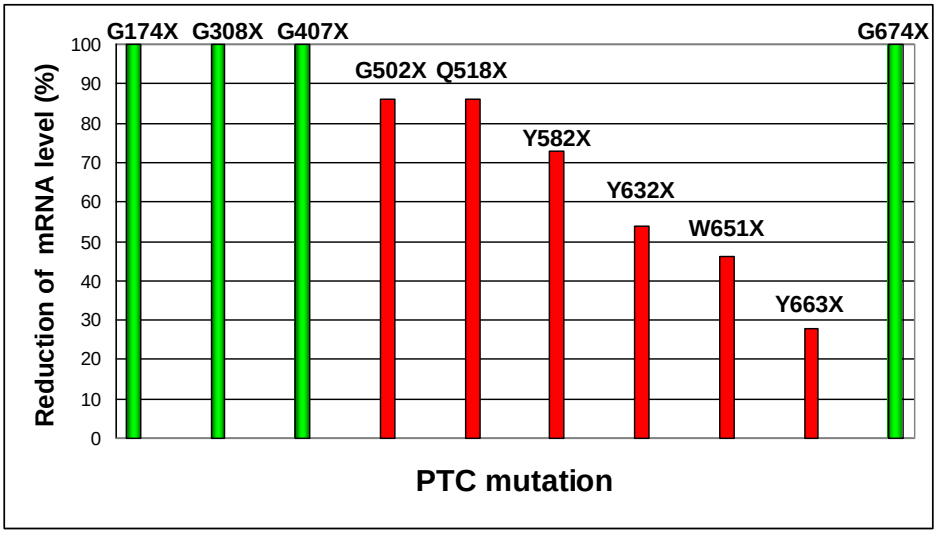
mean a high load of truncated protein within the cell, causing strong ER stress. It can be conjectured that the ER stress in this case would turn the UPR in the direction of apoptosis rather than the return of the cell to an earlier state in the cell cycle, as seems to be the case with the Y663X mutation[77]. Apoptosis of those cells would cause severe disruption of the growth plate.The phenotype resulting from the either dominant-negative or gain-of-function effect of these truncated proteins might vary greatly from the phenotype found in SMCD patients as yet. The effect of the mutations may be extremely deleterious, by interfering with basic embryonic functions like bone development, thus preventing the viability of the embryo.

Another possibility would be for the mutations to not have an effect on the mRNA at all. It has been observed in other genes that some mutations can be tolerated by means of stop codon readthrough [69]. Here, the efficiency of translation termination is regulated by proteins like UpF1, UpF2 and UpF3. Their absence allows suppression of translation termination. This leaky termintion at PTC mutations antagonizes NMD, leading to a nonsense suppression phenotype [86]. This would mean that humans with those PTC mutations would present a completely normal phenotype and the mutation never be detected.

It is also possible that the cells have a mechanism to reinitiate translation (reviewed in [87]). Translation reinitiation is a mechanism by which the termination complex is disassembled to prevent cells from the effects of translational errors. In this case, the mutations within the helix would also be tolerated by the cell.

The existence of eight imperfections within the Gly-X-Y triplet repeat sequence of the collagen X helix suggests that translation reinitiation or stop codon readthrough exist in collagen type X.

Table 4.1 Median reduction of mRNA level (%) caused by each PTC mutation. Columns indicate the median percentage (% of 100 percent in clones treated with CHX) to which mRNA levels were reduced in clones not treated with CHX. Green columns represent PTC mutations that did not elicit NMD, red columns represent PTC mutations that elicited NMD.



4.2.6 Future studies on SMCD mutations

The studies on collagen X NMD have raised further questions, e.g. what effect the mutations within the helical region, that fail to elicit NMD have on protein assembly. And, ultimately, what effect the mutations have on the phenotype.

Observation of the collagen X expression of the transfected cells would help determine the effect of each mutation on the collagen X protein. It would also be of interest to define if the cells produce a truncated protein. Mutations that failed to elicit NMD would cause a truncated protein that would be likely to interfere with trimer assembly, and activate the UPR. To detect whether the UPR was caused by the mutant mRNA, levels of key components of the UPR like IRE1, PERK, BiP and ATF6 could be examined in those cells.

Furthermore, a transgenic mouse system would be able to substantiate the effect of each mutation on the phenotype. The transgenic mouse system would be able to provide an answer to the question why no mutations have been found upstream of the NC1 domain, if mice showed either a completely normal phenotype or a very profound SMCD phenotype.

4.3 ROLE OF CONSERVED SEQUENCES IN THE 3'UTR IN MEDIATING LAST-EXON NMD

The finding that mutations distributed along the last exon of the collagen X gene showed a gradient in their susceptibility to nonsense-mediated decay strengthened the theory of an all new mechanism involved in collagen X's last-exon NMD. Since PTC mutations closest to the 3' end of the NC1 domain underwent the strongest NMD, the 'machinery' responsible for NMD was suspected to be close to its effect. The 3'untranslated region (3'UTR) is the most likely candidate region, since it has been shown to be involved in mRNA stability and translation termination in yeast[73].
The recent discovery of a polarity in NMD[61] (refer to section 4.2.5), supports the idea of a (cis-acting?) element like the 3'UTR to be responsible for NMD. With this polarity, a certain distance from such an element could make the NMD-mechanism less efficient or terminally inefficient, depending on the distance of a PTC to a triggering machinery within the 3'UTR (refer to chapter 1.2.2).

Furthermore, recent studies revealed the role of the 3'UTR in mediating nonsense-mediated decay independent of exon-exon junctions in the immunoglobulin-µ gene[88]. Buhler et al were able to demonstrate that a PTC in the Ig-µ gene construct elicited NMD independently of a downstream splicing event and therefore of a downstream EJC. (However, the level of decay caused by a PTC was about 7fold decreased when an EJC was present in the downstream sequence). They were also able to show that the distance between a PTC and the 3'UTR determines an mRNA's susceptibility to NMD.

These data suggest that the 3'UTR is a good candidate region for specifying last-exon NMD in *Col10a1*. To assess which part of the 3'UTR might be involved in mediating last-exon NMD in collagen X, bioinformatic analysis of the *COL10A1* 3'UTR sequence was performed. The analysis revealed the presence of three regions of homology, which are conserved throughout species (human, mouse, dog, cat, chimp, cow). Furthermore an AU-rich element (ARE) was detected in the third region of homology. ARE's are found in the 3'UTR of many genes [89] and are characterized by their ability to induce rapid deadenylation-dependent mRNA decay[76].

To address the second question raised by the uncommon localisation of collagen X mutations in SMCD patients, which region might be responsible for last-exon NMD (refer to chapter 4.1), the three regions of homology inside the 3'UTR were deleted separately. To assess the effect of each deletion on last-exon NMD, mutagenesis was performed on a construct containing a collagen X PTC mutation that elicited last-exon NMD *in vivo* and *in vitro*, Y632X.

4.3.1 Deletion of the 3'UTR in the col10a1 *gene construct stops NMD*

To establish whether any of the regions of homology or another region inside the 3'UTR were involved in mediating last-exon NMD, the whole 3'UTR was deleted in a construct that contained the Y632X PTC mutation. Thus, if any region inside the 3'UTR were responsible for NMD in collagen X, the Y632X PTC would cease to elicit last-exon NMD.

The protection levels of the 25 analyzed clones ranged from 0.35 to 1.86, with a median protection of 1.01. The protection level in a typical clone harboring the Y632X PTC mutation but not the Y632Xdel3'UTR deletion, the protection level was 1.86. This demonstrated that deletion of the 3'UTR inhibits the NMD caused by the Y632X mutation present in the construct.

Interestingly, in clones harboring the Y632Xdel3'UTR deletion, mutant levels of collagen X were lower than endogenous levels, while they were notedly higher in clones that solely accomodated the Y632X mutation. The reason for this is most likely the involvement of the 3'UTR in mRNA stability. The 3'UTR has been repeatedly shown to participate in mRNA stability (surveyed in [90]). Since the deletion of the 3'UTR did not result in complete instability and therefore degradation of the mRNA, the effect of the 3'UTR deletion on NMD could still be determined. The median protection level in those clones harboring the Y632Xdel3'UTR deletion is clearly lower than in clones harboring just the Y632X mutation and therefore confirms the involvement of the 3'UTR in mediating last-exon NMD.

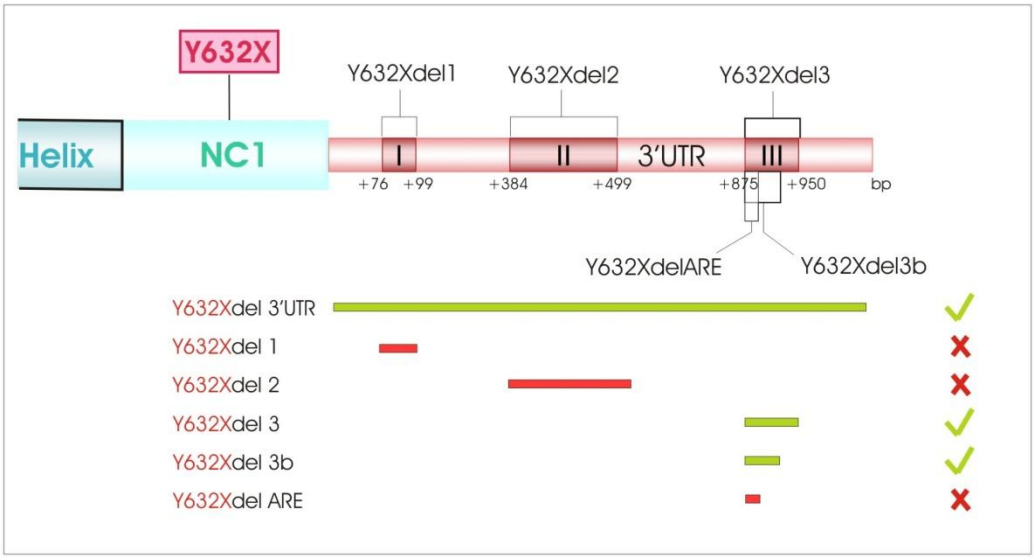


Figure 4.1 Deletion of regions of homology withing the 3'UTR of *COL10A1* and their effects on NMD-triggering of the Y632X mutation. Deletion of the whole 3'UTR, Y632Xdel3'UTR stops the last-exon NMD caused by Y632X. Deletion of two regions of homology, Y632Xdel1 and Y632Xdel2 does not have any effect on NMD while deletion of Y632Xdel3 again inhibits NMD. Deletion of the smaller region Y632Xdel3b still inhibits NMD while deletion of the yet smaller Y632XdelARE does not have an effect on NMD.

4.3.2 Deletion of the three regions of homology within the 3'UTR prevents NMD

Having established the involvement of the 3'UTR in mediating last-exon NMD, the next step was to determine which specific region inside the 3'UTR was responsible for NMD. Three homologous regions inside the 3'UTR that were identified by bioinformatic analysis were therefore deleted separately (refer to Figure 2.1).

In a first step, region I of homology was deleted in the construct containing the Y632X PTC mutation. The calculated ratios for this deletion showed protection levels varying from 0.19 to 3.0 with a median protection of 1.37.

The data indicated that in the typical clone harboring the Y632Xdel1 deletion the Y632X PTC mutation would still be able to elicit last-exon NMD. Therefore, the first region of homology inside the 3'UTR was clearly not involved in eliciting last-exon NMD.

In a second step, the second region of homology was deleted, Y632Xdel2 (refer to Figure 2.1). This region showed 87% homology between species. Calculated protection levels from NMD varied from 1.12 to 3.22, the median level was 1.31. Last-exon NMD still occurred in spite of the region 2 deletion, which signified that region 2 was not involved in mediating last-exon NMD.

The third deletion that was performed in the construct containing the Y632X PTC mutation was the deletion of region 3. This third region of homology between species contained an AU-rich element. AU-rich elements are *cis*-acting elements responsible for mRNA decay e.g. in cytokines, nuclear transcription factors, proto-oncogenes and growth factors [91]. Therefore region 3 was a likely candidate region to be involved in last-exon NMD. The calculated ratios of protection from NMD ranged from 0.4 to 2.13, the median protection was 0.8. Therefore, deletion of the third region of homology resulted in inhibition of the last-exon NMD elicited by the Y632X PTC mutation, indicating that in region 3 the sequence involved in the activation of last-exon NMD had been identified.

4.3.3 Deletion of two conserved elements inside region 3. The identification of a potential NMD downstream regulatory element?

With the deletion of region 3, the area involved in eliciting last-exon NMD had been identified. Still, region 3 spanned 79bp, and it was not to be expected that the whole sequence was involved in mediating last-exon NMD. Therefore, two smaller regions were deleted inside region 3. The first was a sequence that contained the ARE and 19bp of downstream sequence, the second deletion affected solely the 13bp AU-rich element (ARE).

The Y632Xdel3b deletion was performed in a construct containing the Y632X PTC mutation which was known to elicit last-exon NMD *in vivo* and *in vitro*. The deletion spanned the AU-rich element identified in region 3 plus 19bp downstream sequence. This 32bp region showed one of the highest homologies between the six species (human, mouse, rat, dog, cow, chimp) found in the 3'UTR of collagen X. When all

clones were analyzed, ratios ranging from 0.6 to 1.4 could be found. The median value was 1.03. This data indicated that region 3b was involved in mediating last-exon NMD.

To determine whether the whole 32bp region 3b was involved in last-exon NMD or if it was only the 13bp ARE, this region was deleted separately.
As in the other deletions, the construct containing the Y632X PTC mutation was used as template for the Y632XdelARE deletion. When all samples were analyzed, the ratios ranged from 0.11 to 4.26. A representative clone would show a 1.67fold protection from NMD. This indicated that deletion of the ARE did not alter the degrading effect of the Y632X PTC mutation. Therefore the AU-rich element alone was not involved in eliciting last-exon NMD.
This was an interesting finding, since the expected result for this experiment was for the ARE to be the region involved in mediating last-exon NMD, as it had been shown to be in numerous other studies[89]. Instead, deleting the 32bp region containing the ARE stopped NMD, while deleting only the ARE did not alter the level of protection by CHX.

Therefore the 19bp of region 3b that exclude the ARE region are involved in the NMD pathway. Deletion of these 19bp inside the 3'UTR completely inhibits nonsense-mediated decay.

4.4 A THEORY ON LAST-EXON NMD IN COLLAGEN X - 3'UTR MEDIATED NMD

The discovery of a 19bp region in the *Col10a1* 3'UTR is a novel finding that suggests a possible mechanistic basis for last-exon NMD. How this region specifies NMD competency is not known and is the subject of further study. However, it seems plausible that one or more known or unknown NMD factors could bind to this region of the 3'UTR, giving collagen X last-exon NMD independence of the exon-exon boundaries that NMD in other genes depends on (please refer to chapter 1.2.1).
In yeast genes, PTC definition does not depend on exon-exon boundaries either (refer to chapter 1.2.2). Downstream destabilizing elements (DSEs) have been identified, as well as other cis- and trans-acting factors. Most recently it has been shown that eIF4e (eukaryotic initiation factor 4e) and Cbc1p (Cap binding complex 1p)-bound pre-mRNAs are targeted for NMD[92]. However, in mammalian cells mRNAs that are bound by

eIF4e do not detectably undergo NMD. Therefore, this might be one of the factors involved in determination of PTCs in the last exon in yeast[92] and possibly also in last-exon NMD of collagen X mRNA.
Since mutations further upstream of the G502X mutation are immune to last-exon NMD, the factors involved in collagen X last-exon NMD specify NMD within a limited 3' region of the gene. Mutations that are closer to the 3'UTR decay to a much higher degree than do mutations further away from the stop codon, and they completely cease to undergo NMD from a certain distance on (refer to chapter 4.2.5). An explanation for this polarity might be that the higher distance from the NMD-determining region and/or protein complex lowers the efficiency of NMD, until a point is reached in the 5' region of the gene that is effectively "out-of-reach" of the last-exon decay machinery.

It is of interest that a similar mechanism has been observed in mammalian cells, where binding of Staufen1 (Stau1) to Upf1 (up-frameshift 1) elicits mRNA decay when bound downstream of a termination codon. Stau1 interacts with certain 3'UTR regions, rendering these mRNAs to so-called Stau1-mediated mRNA decay[93]. Binding of similar NMD factors to the 19bp region inside the 3'UTR of collagen X might also destabilize the mRNA. To confirm the involvement of the 19bp region, the secondary structure of the whole 3'UTR was analyzed using pairing constraints (Mfold, Burnet Institute[94], refer to chapter 2.2.2). It was found that the 3'UTR can fold in at least 28 different variations. However, the 19bp region involved in last-exon NMD showed only four different folding options, in all of which the region was localized to a sidearm of the folded 3'UTR (refer to Figure 4.2). If this region binds an NMD factor (eIF4e, Cbc1p, Stau1?), it could interact with the coding region of the collagen X gene and specify a premature termination codon. This proposal requires experimental testing by characterisation of the putative binding factors to this region.

Validation of the 3'UTR mediated decay theory could be acquired by further research on the binding proteins of this region, and possibly of the mutated region inside the exon. The proteins that have been found to date to be involved in NMD are supposably involved in 3'UTR mediated decay as well. On the other hand, there may be different proteins that have not yet been found to be part of the NMD pathway. Electromobility shift assay (EMSA) could be used to determine whether proteins bind to the 13bp region of the 3'UTR (or other regions within the 3'UTR). To determine whether these

proteins are novel proteins or known NMD effectors or even AU-rich binding proteins, supershift assays can be performed using antibodies to known NMD factors. Novel interacting proteins can be identified using two dimensional electrophoresis and mass spectronomy. Furthermore, since *Col10a1* NMD is chondrocyte specific it will be crucial to determine if the proteins that interact with the 3' UTR are only produced by chondrocytes. The role of the identified 3' UTR binding proteins can be directly tested by repressing expression by RNA interference. All this would open the possibility of approaching the genetic background of SMCD as well as NMD by testing inhibition or enhancement of the 3'UTR mediated NMD pathway in a mouse system and examination of the resulting effects on the phenotype.

This new knowledge on the NMD pathway, 3'UTR mediated NMD, raises new questions on the molecular mechanisms of SMCD and other diseases caused by mutations within the last exon.

Furthermore, investigation on those mutations in the last exon of collagen X that do not elicit NMD is required for new insights into the resulting phenotypes. The finding that they are probably out of reach of the 3'UTR mediated NMD machinery leads to the conclusion that a resulting phenotype is not caused by haploinsufficiency but rather suggests a dominant negative or gain of function effect of the truncated protein resulting from the premature stop codon. Therefore the cartilage phenotype might be different to the SMCD phenotype, and not yet identified as a collagen X related disorder. To find the phenotype associated with these mutations, a transgenic mouse system could be used, the arising phenotype leading to the phenotype caused by those mutations in humans. Knowledge on the molecular causes of the disease will greatly improve research on diagnostic and therapeutic possibilities.

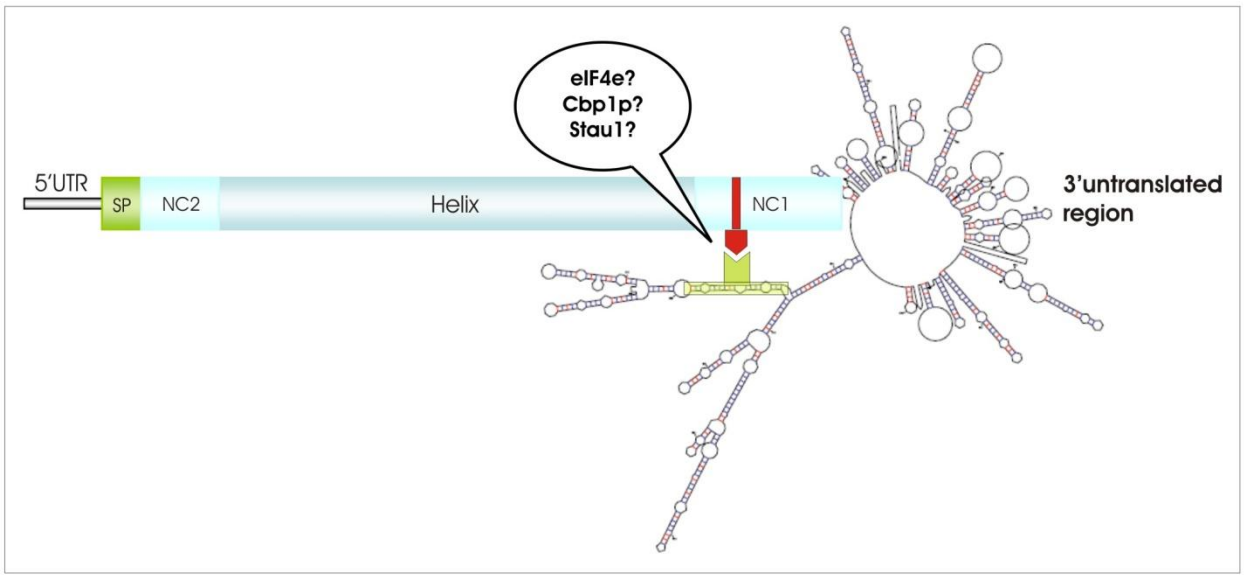


Figure 4.2 3'UTR mediated NMD theory on last-exon NMD. The figure shows one of four possible secondary structures of the 3'UTR mRNA. The 19bp region involved in last-exon NMD resides inside a sidearm (marked in light green), thus interacts with NMD factors (eIF4e, Cbp1p, Stau1?) bound to a PTC inside the NC1 domain (green and red boxes). The structure allows survey of major parts of the NC1 domain. However, regions further upstream will not be scanned and premature termination codons not be detected. The NC domains are shown in light green, the helix in blue. The signal peptide (SP) is depicted in dark green, the 5'UTR by a grey line

.

Chapter Five

Abstract

Genetic disorders are a major cause of human disability and disease. It has been estimated that 5-10% of the population is affected by an inherited disorder during their lifetime, and a startling 70% of pediatric admissions involve a genetic component. Considering this level of influence on human disability and the multifactorial genesis of most diseases, studies on the mechanisms of genetic disease, especially those relating to its fundamental mechanisms will have increasing relevance to almost every known disease.

The mutations that can lead to genetic disorders are various, but one recurrent type is called a nonsense mutation. Nonsense mutations are known to be responsible for one third of all inherited diseases as well as many oncologic diseases. A nonsense mutation, caused by either a deletion, insertion or a point mutation, results in the generation of a stop codon in the mRNA before the genuine stop codon that signals the termination of translation of the mRNA into the appropriate full length protein. The theoretical consequence would be the synthesis of a shorter protein which could exert a dominant negative effect and have serious consequences for cell function.

To avoid these possibly deleterious effects of nonsense mutations, the human body has developed an elegant quality control surveillance mechanism called nonsense mediated mRNA decay (NMD). This pathway detects the premature termination codons (PTC) inside the mRNA and renders the mutation-containing mRNA to NMD. Thus, the cells can be protected from the effect of a PTC. For the NMD machinery to be efficient, a signal is required that allows the machinery to distinguish between the normal and the premature stop codon.

In previous studies on the NMD pathway, basic principles of NMD were developed. The signal to differentiate a PTC from the normal stop was discovered to be the connection point between exons where introns had been removed, the so called exon-exon junctions (EJC). They need to be found downstream of a PTC for the determination of prematurity. Since the last exon is not followed by an EJC any more, PTC's in the last exon of a gene cannot be detected by the NMD machinery and will therefore result in a truncated protein.

However, when the genetic background of the cartilage disease Schmid Metaphyseal Chondrodysplasia (SMCD) was analyzed, it was discovered that the mutations in the last exon of collagen X that are causative for SMCD did indeed undergo NMD – impossible according to the known NMD rules. Furthermore, when the mutations were analyzed more closely it was found that the mutations lost this ability to

trigger NMD in the last exon when they were located at a greater distance from the normal stop codon.

These findings exposed a new complexity of the NMD principles. Obviously, last-exon NMD required a completely different mechanism to detect PTC's than did mutations in the other exons of genes. To identify this mechanism, the close by region of the 3'untranslated region (3'UTR) was analyzed. It was found that, upon deleting a small region of the 3'UTR, NMD in the last exon of collagen X could no longer be triggered. This brought up an all new theory of 3'UTR mediated NMD, that might still involve known NMD factors such as eIF4e or Stau1, but could also include yet unknown proteins.

Further investigation into the 3'UTR mediated NMD will offer insights into a broader spectrum of the principles of NMD. An approach to the new questions arising from this research would be to test the possibility of inhibition or enhancement of the 3'UTR mediated NMD pathway in a mouse system and the resulting effects on the phenotype. Furthermore, investigation on those mutations in the last exon of collagen X that do not elicit NMD will shed light on the resulting phenotype of these mutations. Possibly, they result in a different disease that has yet to be connected with collagen X. The knowledge on the genetic background of this disease will greatly improve the scientific possibilities of research in this field.

In the future, this research might allow us to intervene therapeutically in the genetic components of SMCD and other diseases caused by some form of NMD in humans and ameliorate or even prevent the detrimental effects arising from these genetic diseases.

Chapter Six

Zusammenfassung

Genetische Erkrankungen verursachen einen großen Anteil aller bekannten Erkrankungen und sekundären Behinderungen. In Schätzungen liegt der Anteil der von genetischen Erkrankungen betroffenen Bevölkerung bei 5-10%, sogar 70% der stationären pädiatrischen Aufnahmen sollen eine genetische Komponente beinhalten. Aufgrund dieses großen Einflusses auf Behinderungen und Erkrankungen sowie die multifaktoriellen Ursachen vieler anderer Erkrankungen werden Studien zu den Ursachen genetischer Erkrankungen – besonders diejenigen Studien, die grundlegende Mechanismen erforschen – zunehmend an Relevanz gewinnen.

Verschiedene Typen an Mutationen können zu genetischen Erkrankungen führen. Ein häufiger Mutationstyp ist die Nonsense Mutation. Nonsense Mutationen verursachen sowohl ein Drittel aller Erbkrankheiten als auch viele onkologische Erkrankungen. Sie verursachen – ob durch eine Deletion, Insertion oder Punktmutation –ein Stopkodon in der mRNA. Es liegt vor dem eigentlichen Stopkodon, welches das Ende der Translation der mRNA in das funktionstüchtige komplette Protein signalisiert. Theoretisch würde hieraus die Synthese eines verkürzten Proteins resultieren, das dominant negative Effekte auslösen und somit schwerwiegende Konsequenzen für die Zellfunktion haben kann.

Um diese möglicherweise zerstörerischen Effekte von Nonsense Mutationen zu verhindern hat der menschliche Körper einen eleganten Überwachungsmechanismus entwickelt: Nonsense-mediierten mRNA Zerfall (nonsense mediated mRNA decay, NMD). Dieser Signalweg erkennt diese vorzeitigen Stopkodons (premature termination codons, PTCs) innerhalb von mRNA und unterwirft sie NMD-Zerfall. Die Zelle kann so vor dem Effekt einer PTC geschützt werden.

Die Effizienz des NMD-Zerfallsmechanismus ist abhängig von einem verlässlichen Signal, das die Unterscheidung zwischen einem nomalen und einem frühzeitigen stop Kodon erlaubt.

Frühere Studien konnten grundlegende Prinzipien des NMD Signalpfads darstellen. Ein PTC wird vom regulären Stopkodon mithilfe des so genannten Exon-verbindungs-Komplexes (exon junction complex, EJC) unterschieden. Dieser stellt den Verbindungspunkt zwischen den einzelnen Exonen nach Entfernung der Introns dar. Wird ein EJC stromabwärts eines Stopkodons erkannt, gilt dieser Stop als verfrüht.

Liegt ein PTC jedoch im letzten Exon und folgt diesem somit kein weiterer EJC, kann der Stop nicht als verfrüht erkannt werden und resultiert in einem verkürzten Protein.

Als jedoch genetische Grundlagen der Knorpelerkrankung Schmid Metaphysäre Chondrodysplasie (SMCD) analysiert wurden zeigte sich, dass die ursächlichen Mutationen sämtlich im letzten Exon lagen und dennoch den NMD-Signalpfad auszulösen vermochten – den bekannten NMD-Prinzipien zufolge unmöglich. Darüber hinaus zeigte sich bei genauerer Analyse, dass diese Mutationen bei größerer Distanz zum regulären Stopkodon keinen NMD-Zerfall mehr auslösten.

Diese Ergebnisse legten eine neue Komplexität der NMD Prinzipien dar. Offenbar war ein vollständig anderer Mechanismus notwendig um PTCs im letzten Exon zu erkennen als er es für Mutationen in den anderen Exonen von Genen war. Um diesen zu indentifizieren wurde die benachbarte 3'nicht translatierte Region (3' untranslated region, 3'UTR) untersucht. Es zeigte sich, dass die Deletion eines kleinen Abschnitts der 3'UTR NMD-Zerfall im letzten Exon unterband. Hieraus wurde die vollständig neue Theorie des 3'UTR mediierten NMD-Zerfalls entwickelt, an deren Signalpfad bekannte NMD-Faktoren wie eIF4e oder Stau1 beteiligt sein könnten, darüber hinaus jedoch auch bis dato unbekannte Proteine.

Weiterführende Forschung im Bereich des 3'UTR mediierten NMD-Zerfalls wird Erkenntnisse über ein deutlich breiteres Spektrum der NMD Prinzipien liefern. Ein möglicher Zugang könnte die Erforschung möglicher Inhibition oder Verstärkung des 3'UTR mediierten NMD-Zerfallswegs in einem Maussystem sein, und daraus resultierende phänotypische Veränderungen. Möglicherweise resultieren sie in einem völlig anderen Phänotyp, der bisher nicht mit dem SMCD Phänotyp in Verbindung gebracht werden konnte.

Weiterführende Forschung in diesem Bereich könnte zukünftig therapeutische Interventionsmöglichkeiten in die genetischen Komponenten der Schmid Metaphysären Chondrodysplasie und anderer Erkrankungen aus dem NMD Formenkreis denkbar machen und möglicherweise die Beeinträchtigungen, die aus dieser genetischen Erkrankung resultieren lindern helfen.

TABLE OF TABLES AND FIGURES

LITERATURE

[1] Hall CM. International Nosology and Classification of Constitutional Disorders of Bone (2001). Am J Med Genet 2002; 113:65-77.
[2] Horton WA, Hecht JT. Chondrodysplasias: General Concepts and Diagnostic and Management Considerations. In: Connective Tissue and its Heritable Disorders. Wiley-Liss I '(Wiley-Liss I)'Wiley-Liss Is)'. 2002. p.^pp. 901-908.
[3] von der Mark K. Structure, biosynthesis and gene regulation of collagens in cartilage and bone. In: Dynamics of bone and cartilage metabolism. Orlando: Academic Press; 1999. p.^pp. 3-29.
[4] Kielty CM, Grant ME. The Collagen Family: Structure, Assembly, and Organization in the Extracellular Matrix. In: Connective Tissue and Its Heritable Disorders. 2002. p.^pp. 159-221.
[5] Tsang KY, Chan D, Cheslett D *et al.* Surviving Endoplasmic Reticulum Stress Is Coupled to Altered Chondrocyte Differentiation and Function. PLoS Biol 2007; 5:e44.
[6] Schroder M. The unfolded protein response. Mol Biotechnol 2006; 34:279-90.
[7] Mundlos S, Olsen BR. Heritable diseases of the skeleton. Part II: Molecular insights into skeletal development-matrix components and their homeostasis. Faseb J 1997; 11:227-33.
[8] Mundlos S, Olsen BR. Heritable diseases of the skeleton. Part I: Molecular insights into skeletal development-transcription factors and signaling pathways. Faseb J 1997; 11:125-32.
[9] Emery AH, Rimoin DL. Principles and Practice of Medical Genetics. Edinburgh: Churchill Livingstone; 1990. p.
[10] Schmid F. Beitrag zur Dysostosis enchondralis metaphysaria. Monatsschrift Kinderheilkunde 1949; 97:393-397.
[11] Lachman RS, Rimoin DL, Spranger J. Metaphyseal chondrodysplasia, Schmid type. Clinical and radiographic delineation with a review of the literature. Pediatr Radiol 1988; 18:93-102.
[12] Elliott AM, Field FM, Rimoin DL, Lachman RS. Hand involvement in Schmid metaphyseal chondrodysplasia. Am J Med Genet A 2005; 132:191-3.
[13] Savarirayan R, Cormier-Daire V, Lachman RS, Rimoin DL. Schmid type metaphyseal chondrodysplasia: a spondylometaphyseal dysplasia identical to the "Japanese" type. Pediatr Radiol 2000; 30:460-3.
[14] Bateman JF, Wilson R, Freddi S *et al.* Mutations of COL10A1 in Schmid metaphyseal chondrodysplasia. Hum Mutat 2005; 25:525-34.
[15] Warman ML, Abbott M, Apte SS *et al.* A type X collagen mutation causes Schmid metaphyseal chondrodysplasia. Nat Genet 1993; 5:79-82.
[16] Elima K, Eerola I, Rosati R *et al.* The mouse collagen X gene: complete nucleotide sequence, exon structure and expression pattern. Biochem J 1993; 289:247-253.
[17] Nerlich AG, Kirsch T, Wiest I *et al.* Localization of collagen X in human fetal and juvenile articular cartilage and bone. Histochemistry 1992; 98:275–281.
[18] Grant WT, Wang GJ, Balian G. Type X collagen synthesis during endochondral ossification in fracture repair. J. Biol. Chem. 1987; 262:9844–9849.
[19] Reichenberger E, Aigner T, von der Mark K *et al.* In situ hybridization studies on the expression of type X collagen in fetal human cartilage. Dev. Biol. 1991; 148:562–572.
[20] Schmid TM, Linsenmayer TF. Stucture and Function of Collagen Types. In: Maynes R, Burgeson RE '(Maynes R, Burgeson RE'(Maynes R, Burgeson REs)'. New York: Academic Press; 1987. p.^pp. 223-259.
[21] Adams SL, Pallante KM, Niu Z *et al.* Rapid induction of type X collagen gene expression in cultured chick vertebral chondrocytes. Exp Cell Res 1991; 193:190-7.
[22] Vuorio E, de Crombrugghe B. The family of collagen genes. Annu Rev Biochem 1990; 59:837-72.
[23] Reginato AM, Jimenez SA. Biochemical characterization of the native tissue form of type X collagen from embryonic chick sternal cartilage and identification of a chymotrypsin-sensitive site within its triple-helical domain. Biochem J 1991; 273(Pt 2):333-8.

[24] Schmid TM, Linsenmayer TF. Chains of matrix-derived type X collagen: size and aggregation properties. Connect Tissue Res 1989; 20:215-22.
[25] Myllyharju J, Kivirikko KI. Collagens, modifying enzymes and their mutations in humans, flies and worms. Trends Genet 2004; 20:33-43.
[26] Kwan AP, Cummings CE, Chapman JA, Grant ME. Macromolecular organization of chicken type X collagen in vitro. J Cell Biol 1991; 114:597-604.
[27] Kielty CM, Hulmes DJ, Schor SL, Grant ME. Embryonic chick cartilage collagens. Differences in the low-Mr species present in sternal cartilage and tibiotarsal articular cartilage. FEBS Lett 1984; 169:179-84.
[28] Zhang Y, Chen Q. The noncollagenous domain 1 of type X collagen. A novel motif for trimer and higher order multimer formation without a triple helix. J Biol Chem 1999; 274:22409-13.
[29] Frischholz S, Beier F, Girkontaite I *et al.* Characterization of human type X procollagen and its NC-1 domain expressed as recombinant proteins in HEK293 cells. J Biol Chem 1998; 273:4547-55.
[30] Chan D, Cole WG, Rogers JG, Bateman JF. Type X collagen multimer assembly in vitro is prevented by a Gly618 to Val mutation in the alpha 1(X) NC1 domain resulting in Schmid metaphyseal chondrodysplasia. J Biol Chem 1995; 270:4558-62.
[31] Chan D, Weng YM, Hocking AM *et al.* Site-directed mutagenesis of human type X collagen. Expression of alpha1(X) NC1, NC2, and helical mutations in vitro and in transfected cells. J Biol Chem 1996; 271:13566-72.
[32] Bogin O, Kvansakul M, Rom E *et al.* Insight into Schmid metaphyseal chondrodysplasia from the crystal structure of the collagen X NC1 domain trimer. Structure (Camb) 2002; 10:165-73.
[33] Thomas JT, Boot-Handford RP, Grant ME. Modulation of type X collagen gene expression by calcium beta-glycerophosphate and levamisole: implications for a possible role for type X collagen in endochondral bone formation. J Cell Sci 1990; 95 (Pt 4):639-48.
[34] Kirsch T, von der Mark K. Ca2+ binding properties of type X collagen. FEBS Lett 1991; 294:149-52.
[35] Kwan KM, Pang MK, Zhou S *et al.* Abnormal compartmentalization of cartilage matrix components in mice lacking collagen X: implications for function. J Cell Biol 1997; 136:459-71.
[36] Jacenko O, Chan D, Franklin A *et al.* A dominant interference collagen X mutation disrupts hypertrophic chondrocyte pericellular matrix and glycosaminoglycan and proteoglycan distribution in transgenic mice. Am J Pathol 2001; 159:2257-69.
[37] Schmid TM, Mayne R, Jeffrey JJ, Linsenmayer TF. Type X collagen contains two cleavage sites for a vertebrate collagenase. J Biol Chem 1986; 261:4184-9.
[38] Makitie O, Susic M, Ward L *et al.* Schmid type of metaphyseal chondrodysplasia and COL10A1 mutations-findings in 10 patients. Am J Med Genet A 2005; 137A:241-248.
[39] Ikegawa S, Nakamura K, Nagano A *et al.* Mutations in the N-terminal globular domain of the type X collagen gene (COL10A1) in patients with Schmid metaphyseal chondrodysplasia. Hum Mutat 1997; 9:131-5.
[40] den Dunnen JT, Antonarakis SE. Nomenclature for the description of human sequence variations. Hum Genet 2001; 109:121-4.
[41] Chan D, Ho MS, Cheah KS. Aberrant signal peptide cleavage of collagen X in Schmid metaphyseal chondrodysplasia. Implications for the molecular basis of the disease. J Biol Chem 2001; 276:7992-7.
[42] Bateman JF, Freddi S, Nattrass G, Savarirayan R. Tissue-specific RNA surveillance? Nonsense-mediated mRNA decay causes collagen X haploinsufficiency in Schmid metaphyseal chondrodysplasia cartilage. Hum Mol Genet 2003; 12:217-25.
[43] Chan D, Freddi S, Weng YM, Bateman JF. Interaction of collagen alpha1(X) containing engineered NC1 mutations with normal alpha1(X) in vitro. Implications for the molecular basis of schmid metaphyseal chondrodysplasia. J Biol Chem 1999; 274:13091-7.
[44] Wilson R, Freddi S, Chan D *et al.* Misfolding of collagen X chains harboring Schmid metaphyseal chondrodysplasia mutations results in aberrant disulfide bond formation,

intracellular retention, and activation of the unfolded protein response. J Biol Chem 2005; 280:15544-52.
[45] Wallis GA, Rash B, Sykes B *et al.* Mutations within the gene encoding the alpha 1 (X) chain of type X collagen (COL10A1) cause metaphyseal chondrodysplasia type Schmid but not several other forms of metaphyseal chondrodysplasia. J Med Genet 1996; 33:450-7.
[46] Chan D, Weng YM, Graham HK *et al.* A nonsense mutation in the carboxyl-terminal domain of type X collagen causes haploinsufficiency in schmid metaphyseal chondrodysplasia. J Clin Invest 1998; 101:1490-9.
[47] Frischmeyer PA, Dietz HC. Nonsense-mediated mRNA decay in health and disease. Hum Mol Genet 1999; 8:1893-900.
[48] Kan Z, Rouchka EC, Gish WR, States DJ. Gene structure prediction and alternative splicing analysis using genomically aligned ESTs. Genome Res 2001; 11:889-900.
[49] Lewis B, Grenn R, Brenner S. Evidence for the widespread coupling of alternative splicing and nonsense-mediated decay in humans. Proc Natl Acad Sci U S A 2003; 100:189-192.
[50] Moriarty PM, Reddy CC, Maquat LE. Selenium deficiency reduces the abundance of mRNA for Se-dependent glutathione peroxidase 1 by a UGA-dependent mechanism likely to be nonsense codon-mediated decay of cytoplasmic mRNA. Mol Cell Biol 1998; 18:2932-9.
[51] Schell T, Kulozik AE, Hentze MW. Integration of splicing, transport and translation to achieve mRNA quality control by the nonsense-mediated decay pathway. Genome Biol 2002; 3:REVIEWS1006.
[52] Lykke-Andersen J. mRNA quality control: Marking the message for life or death. Curr Biol 2001; 11:R88-91.
[53] Singh G, Lykke-Andersen J. New insights into the formation of active nonsense-mediated decay complexes. Trends Biochem Sci 2003; 28:464-6.
[54] Le Hir H, Moore MJ, Maquat LE. Pre-mRNA splicing alters mRNP composition: evidence for stable association of proteins at exon-exon junctions. Genes Dev 2000; 14:1098-108.
[55] Ishigaki Y, Li X, Serin G, Maquat LE. Evidence for a pioneer round of mRNA translation: mRNAs subject to nonsense-mediated decay in mammalian cells are bound by CBP80 and CBP20. Cell 2001; 106:607-17.
[56] Maquat LE. Nonsense-mediated mRNA decay in mammals. J Cell Sci 2005; 118:1773-6.
[57] He F, Li X, Spatrick P *et al.* Genome-wide analysis of mRNAs regulated by the nonsense-mediated and 5' to 3' mRNA decay pathways in yeast. Mol Cell 2003; 12:1439-52.
[58] Mendell JT, Medghalchi SM, Lake RG *et al.* Novel Upf2p orthologues suggest a functional link between translation initiation and nonsense surveillance complexes. Mol Cell Biol 2000; 20:8944-57.
[59] Chiu SY, Lejeune F, Ranganathan AC, Maquat LE. The pioneer translation initiation complex is functionally distinct from but structurally overlaps with the steady-state translation initiation complex. Genes Dev 2004; 18:745-54.
[60] Lejeune F, Ishigaki Y, Li X, Maquat LE. The exon junction complex is detected on CBP80-bound but not eIF4E-bound mRNA in mammalian cells: dynamics of mRNP remodeling. Embo J 2002; 21:3536-45.
[61] Harries LW, Bingham C, Bellanne-Chantelot C *et al.* The position of premature termination codons in the hepatocyte nuclear factor -1 beta gene determines susceptibility to nonsense-mediated decay. Hum Genet 2005:1-11.
[62] Maquat LE. Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. Nat Rev Mol Cell Biol 2004; 5:89-99.
[63] Dahlberg JE, Lund E, Goodwin EB. Nuclear translation: what is the evidence? Rna 2003; 9:1-8.
[64] Maquat LE. NASty effects on fibrillin pre-mRNA splicing: another case of ESE does it, but proposals for translation-dependent splice site choice live on. Genes Dev 2002; 16:1743-53.
[65] Wang J, Gudikote JP, Olivas OR, Wilkinson MF. Boundary-independent polar nonsense-mediated decay. EMBO Rep 2002; 3:274-9.
[66] Callis J, Fromm M, Walbot V. Introns increase gene expression in cultured maize cells. Genes Dev 1987; 1:1183-200.

[67] Le Hir H, Nott A, Moore MJ. How introns influence and enhance eukaryotic gene expression. Trends Biochem Sci 2003; 28:215-20.
[68] Wang W, Czaplinski K, Rao Y, Peltz SW. The role of Upf proteins in modulating the translation read-through of nonsense-containing transcripts. Embo J 2001; 20:880-90.
[69] Keeling KM, Lanier J, Du M *et al.* Leaky termination at premature stop codons antagonizes nonsense-mediated mRNA decay in S. cerevisiae. Rna 2004; 10:691-703.
[70] Maquat LE. Nonsense-mediated mRNA decay: A comparative analysis of different species. Curr Gen 2004; 5:175-190.
[71] Gonzalez CI, Ruiz-Echevarria MJ, Vasudevan S *et al.* The yeast hnRNP-like protein Hrp1/Nab4 marks a transcript for nonsense-mediated mRNA decay. Mol Cell 2000; 5:489-99.
[72] Gonzalez CI, Bhattacharya A, Wang W, Peltz SW. Nonsense-mediated mRNA decay in Saccharomyces cerevisiae. Gene 2001; 274:15-25.
[73] Amrani N, Ganesan R, Kervestin S *et al.* A faux 3'-UTR promotes aberrant termination and triggers nonsense-mediated mRNA decay. Nature 2004; 432:112-8.
[74] Cao D, Parker R. Computational modeling and experimental analysis of nonsense-mediated decay in yeast. Cell 2003; 113:533-545.
[75] Delpy L, Sirac C, Magnoux E *et al.* RNA surveillance down-regulates expression of nonfunctional kappa alleles and detects premature termination within the last kappa exon. Proc Natl Acad Sci U S A 2004; 101:7375-80.
[76] Wilusz CJ, Wormington M, Peltz SW. The cap-to-tail guide to mRNA turnover. Nat Rev Mol Cell Biol 2001; 2:237-46.
[77] Ho MS, Tsang KY, Lo RL *et al.* COL10A1 nonsense and frameshift mutations have a gain-of-function effect on the growth plate in human and mouse metaphyseal chondrodysplasia type Schmid. Hum Mol Genet 2007.
[78] Lefebvre V, Garofalo S, de Crombrugghe B. Type X collagen gene expression in mouse chondrocytes immortalized by a temperature-sensitive simian virus 40 large tumor antigen. J Cell Biol 1995; 128:239-45.
[79] Carter MS, Doskow J, Morris P *et al.* A regulatory mechanism that detects premature nonsense codons in T-cell receptor transcripts in vivo is reversed by protein synthesis inhibitors in vitro. J Biol Chem 1995; 270:28995-9003.
[80] Noensie EN, Dietz HC. A strategy for disease gene identification through nonsense-mediated mRNA decay inhibition. Nat Biotechnol 2001; 19:434-9.
[81] Ito T, Suzuki T, Lim DK *et al.* A novel quantitative receptor autoradiography and in situ hybridization histochemistry technique using storage phosphor screen imaging. J Neurosci Methods 1995; 59:265-71.
[82] Wilson R, Freddi S, Bateman JF. Collagen X chains harboring Schmid metaphyseal chondrodysplasia NC1 domain mutations are selectively retained and degraded in stably transfected cells. J Biol Chem 2002; 277:12516-24.
[83] McIntosh I, Abbott MH, Francomano CA. Concentration of mutations causing Schmid metaphyseal chondrodysplasia in the C-terminal noncollagenous domain of type X collagen. Hum Mutat 1995; 5:121-5.
[84] Bateman JF, Freddi S, McNeil R *et al.* Identification of four novel COL10A1 missense mutations in schmid metaphyseal chondrodysplasia: further evidence that collagen X NC1 mutations impair trimer assembly. Hum Mutat 2004; 23:396.
[85] Tan JT, Kremer F, Freddi S *et al.* Competency for nonsense-mediated reduction in collagen X mRNA is specified by the 3' UTR and corresponds to the position of mutations in Schmid metaphyseal chondrodysplasia. Am J Hum Genet 2008; 82:786-93.
[86] Weng Y, Czaplinski K, Peltz SW. Identification and characterization of mutations in the UPF1 gene that affect nonsense suppression and the formation of the Upf protein complex but not mRNA turnover. Mol Cell Biol 1996; 16:5491-506.
[87] Janosi L, Ricker R, Kaji A. Dual functions of ribosome recycling factor in protein biosynthesis: disassembling the termination complex and preventing translational errors. Biochimie 1996; 78:959-69.
[88] Buhler M, Steiner S, Mohn F *et al.* EJC-independent degradation of nonsense immunoglobulin-mu mRNA depends on 3' UTR length. Nat Struct Mol Biol 2006; 13:462-4.

[89] Bakheet T, Frevel M, Williams BR *et al.* ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. Nucleic Acids Res 2001; 29:246-54.
[90] Misquitta CM, Chen T, Grover AK. Control of protein expression through mRNA stability in calcium signalling. Cell Calcium 2006.
[91] Chen CY, Shyu AB. AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem Sci 1995; 20:465-70.
[92] Gao Q, Das B, Sherman F, Maquat LE. Cap-binding protein 1-mediated and eukaryotic translation initiation factor 4E-mediated pioneer rounds of translation in yeast. Proc Natl Acad Sci U S A 2005; 102:4258-63.
[93] Kim YK, Furic L, Desgroseillers L, Maquat LE. Mammalian Staufen1 recruits Upf1 to specific mRNA 3'UTRs so as to elicit mRNA decay. Cell 2005; 120:195-208.
[94] Burnet Institute. mfold.burnet.edu.au/pairs. In: p.^pp.