# Chapter 2
# Genetic Diseases Affecting the Canonical Pathway of NF-κB Activation

**Abstract** The IKK complex represents the core component of the canonical pathway of NF-κB activation. Any mutation affecting it should therefore impact on NF-κB signaling to some extent. Nevertheless, since IKK-1 and IKK-2 have also been shown to play NF-κB-independent functions, those functions may be affected as well, complicating the picture. Generating further complexity is the location of *NEMO* on the X-chromosome that can cause lyonization-related effects. Over the years, all this predictable intricacy has been confirmed with the discovery of a set of seemingly disparate pathologies, complemented with pathologies caused by mutations of NF-κB subunits regulating exclusively the canonical pathway. They allowed to confirm the essential role of canonical NF-κB activation in innate and acquired immunity or inflammation but also revealed new functions of this pathway.

## 2.1 NEMO-Related Genetic Diseases

NEMO is the regulatory subunit of IKK that allows, though its affinity to polyubiquitinated chains, recruitment of upstream activators and IKK catalytic activation (see Chap. 1). In cells devoid of NEMO activation of NF-κB through the canonical pathway is abolished.

### *2.1.1 Incontinentia Pigmenti*

#### 2.1.1.1 Clinical Features

Incontinentia pigmenti (IP) (OMIM # 308300) is an X-linked genetic disease that is lethal in males early during development, usually before the second trimester [1, 2]. In females, because of the X-inactivation process mitigating the genetic defect, the



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pathology is much more complex and highly variable in presentation. The common feature of the disease is a severe dermatosis that usually starts within two weeks after birth and follows a specific course that covers months/years and can be divided in four distinct, but sometimes overlapping, stages. First, patients exhibit skin blisters and an inflammatory response, accompanied by a massive eosinophilic granulocytes infiltration into the epidermis (Stage I/vesicular stage). Subsequently, verrucous hyperkeratotic lesions develop (Stage II/verrucous stage) then disappear over time, leaving behind areas of hyperpigmentation due to melanin accumulation (Stage III/hyperpigmented stage). These areas, that follow the lines of Blaschko [3], usually vanish by the second decade (Stage IV/atrophic stage), but adults may still exhibit areas of dermal scarring with lack of hair follicle. As will be explained below, this set of events can sometimes re-occur later during lifetime.

In addition to these common skin abnormalities, IP patients can also suffer from odontological, ophtalmological, or neurological problems and in very rare cases of nail dystrophy. Odontological problems are characterized by delayed eruption, oligodontia, agenesis, peg-shaped or malformed teeth, supernumerary teeth, and supplementary cusps and affect more than 80 % of IP patients [4]. Ophtalmological problems that affect approximately 35 % of patients mostly represent abnormalities of the developing retinal vessels [5]. Retinal detachment can be observed as a consequence of a neovascularization following retinal ischemia caused by abnormal peripheral retinal vessels. In the most severe cases, blindness can occur. Neurological abnormalities, found in approximately 30 % of IP cases, start during the first weeks of life, which may concord with the neonatal cutaneous eruption, and include epilepsy, mental retardation, hemiparesis, spasticity, microcephaly, and cerebellar ataxia [6]. In very rare instances, the CNS abnormalities can be fatal, when seizures lead to death due to severe vascular cerebral damages resulting in thalamic hemorrhage, ischemia, and necrosis of both hemispheres.

#### 2.1.1.2 Genetic and Molecular Basis

The gene causing IP has been associated with the chromosomal region Xq28 [7]. It has been demonstrated that *NEMO*, which is located at this position, was the mutated gene. Indeed, a PCR analysis of the *NEMO* locus in cells derived from IP-affected male fetuses revealed an absence of amplified fragment corresponding to exons 4–10 [8]. This loss of exons is caused by a recurrent genomic rearrangement (NEMOdel) affecting approximately 70–80 % of IP patients. It is generated by nonallelic homologous recombination (NAHR) occurring between two repeated MEdium Reiterated 67B (MER67B) sequences located upstream of exon 4 and downstream exon 10 of *NEMO* (Fig. 2.1).

*NEMO* maps in a region with a unique genomic organization, which will be called here the "IP locus". In the centromeric direction, it partially overlaps with the *glucose-6-phosphate dehydrogenase* (*G6PD*) gene. The two overlapping genes share a conserved promoter region (Promoter B), distinct from the specific *NEMO* promoter (Promoter A), which has a housekeeping bidirectional activity [9]. It is

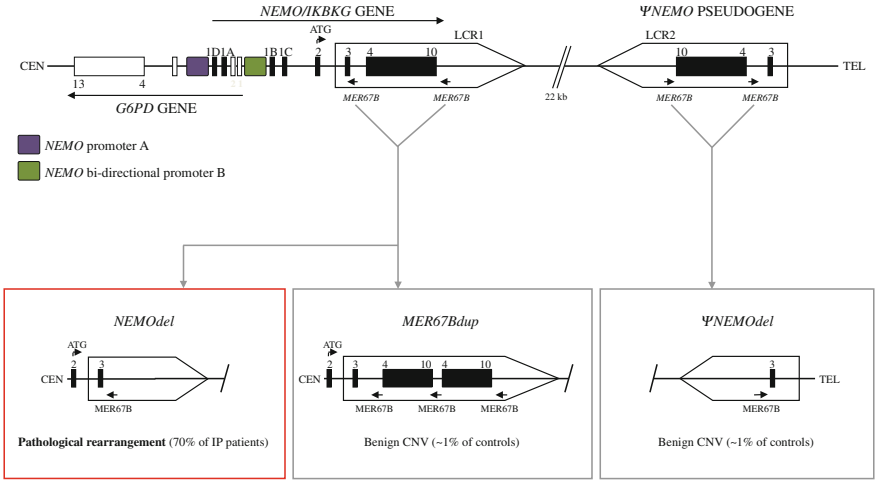


**Fig. 2.1** DNA rearrangements of the *NEMO* and *ψ-NEMO* loci. In the *upper part* of the figure, the genomic organization of the *NEMO* gene and the pseudogene *ψ-NEMO* (IP locus) is shown together with the genomic organization of the neighboring gene *G6PD*, which shares promoter elements with *NEMO*. In the *lower part* of the figure are shown the main rearrangements occurring in the IP locus. Among them is the recurrent one that deletes exon 4–10 of *NEMO* in approximately 70 % of IP patients (*Red frame*). See text for details

worth noting that G6PD is a disease gene causing the X-linked G6PD deficiency (OMIM 305900) [10], the most common enzymopathy in humans. In the telomeric direction, *NEMO* is part of a 35.7-kb segmental duplication (low copy repeats, LCRs) containing also its non-functional truncated copy, *pseudoNEMO* (*Ψ-NEMO*). The two LCRs, covering the functional gene (LCR1) and its pseudogene copy (LCR2), are arranged in an opposite orientation and are highly homologous and prone to recombination [11–13].

Recent findings have demonstrated that the IP locus undergoes NAHR producing either a pathological rearrangement (NEMOdel) or benign variants (MER67Bdup and pseudoNEMOdel) (Fig. 2.1). These non-pathological variants do not interfere with the function of *NEMO* but are considered to be structural variations of the human genome or small copy number variations (CNVs), with low frequencies in the control population (1 %). Genetic evidence has demonstrated that pseudoNEMOdel and MER67Bdup variants should be considered as risk alleles for sporadic IP cases [11].

Aradhya et al. [14] detected evidence for sequence exchange between the LCR1 and LCR2 copies, pointing out that inversion events promoted by their opposite orientation might be responsible for the maintenance of their similarity. Moreover, it has been observed that the recombination events produced by NAHR between the two LCRs repositioned de novo polymorphic alterations arising in the pseudogene such as the exon 4–10 deletion (pseudoNEMOdel) or point mutation, from the pseudogene to the *NEMO* gene, causing de novo IP pathogenic mutations [11–13, 15].

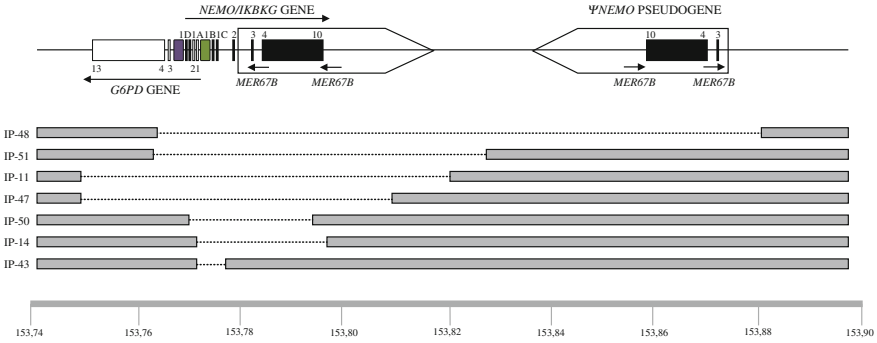


**Fig. 2.2** Chromosomal deletions affecting the *NEMO* gene in IP. The various deletions that have been identified are compiled. In several instances, *G6PD* is also affected. The largest one identified so far (deletion IP-48) also eliminates *ψ-NEMO*. See text for details

In addition to the recurrent event driven by MER67B sequences causing IP in most patients, a high density of repetitive sequences, located in each LCR, gives a genome structure-destabilizing effect predisposing the IP locus to generate novel rearrangements (Fig. 2.2) that eliminate *NEMO*, and sometimes also *G6PD* and *Ψ-NEMO*. In several instances NAHR, causing both benign and pathological alleles [11, 15], and NHEJ (non-homologous end-joining) and Alu–Alu-mediated recombination events, producing either recurrent or nonrecurrent deletions, have been reported [13]. Indeed, the local architecture of the locus with a high frequency of micro-/macro-homologies, tandem repeats, and repeat/repetitive sequences increases its vulnerability to the production of de novo genomic rearrangements through different mechanisms [11, 13, 15]. These events, occurring during both meiosis and mitosis, reveal that the region is prone to generate complex human genomic rearrangements. Taken together, these findings indicate that IP belongs to the class of pathological conditions also known as "genomic disorders" and that the *NEMO*/IP locus must be considered a region of genome instability linked to inherited disease [13].

The recurrent *NEMO* rearrangement in IP results in synthesis of a truncated version of NEMO (aa 1–133 [8]), which, if stable, should only be able to interact with the IKK catalytic subunits but not with its other partners (see Chap. 1). As a consequence, the NF-κB activation process is totally abolished in response to classical inducers of the canonical pathway. Among those inducers is TNF-α, a pro-inflammatory cytokine that has a dual function in triggering either death by apoptosis or survival, this latter fate prevailing only if specific proteins under NF-κB control are synthesized (see Sect. 1.3.3). Accordingly, IP cells are very sensitive to TNF-α-induced programmed cell death, a feature that has important consequences in development of the disease (see below).

Besides the DNA rearrangements affecting the *NEMO* locus, other mutations such as nonsense or frameshift mutations have been reported (Fig. 2.3). Usually, they also result in synthesis of very truncated forms of NEMO devoid of activity. One notable exception is a micro-deletion that removes three nucleotides encoding

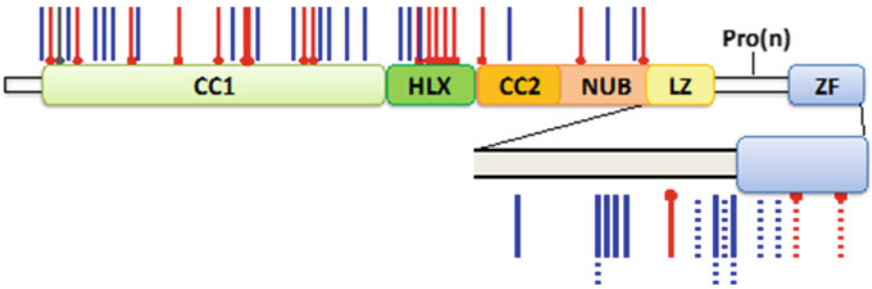


**Fig. 2.3** NEMO truncations resulting from nonsense, frameshift and splicing mutations. *Red dumbbell bars* and *red dotted dumbbell bars* indicate nonsense mutations causing IP and EDA-ID, respectively. *Blue bars* and *dotted blue bars* indicate frameshift and splicing mutations causing IP and EDA-ID, respectively. The *black dotted bar* indicates an ID-related N-terminal truncation of NEMO resulting from a translational reinitiation occurring after a frameshift mutation. Compiled from Refs. [39, 51, 52]. See text for details

amino acid K90 (ΔK90). Mutation ΔK90 has been found associated with a severe form of IP, something not necessarily surprising since it perturbs the interaction of NEMO with the catalytic subunits of IKK [16]. The published structure of the NEMO/IKK interaction domain has confirmed that aa K90 establishes itself a contact with the 732 carbonyl of IKK1/IKK2 [17]. Actually, a mere deletion of one aa occurring at this location should be sufficient to disturb contact points between neighboring amino acids E89, F92, and L93 and residues D738, T735, and F734 of IKK2, respectively.

More interestingly, few missense mutations of *NEMO* have also been identified in IP patients, suggesting that "discrete" molecular abnormalities may be sufficient to generate a full-blown disease (Fig. 2.4). Among them is mutation A323P, which causes a severe form of IP with CNS abnormalities [18]. It has been shown that this mutation affects many NF-κB signaling pathways, such as the ones regulated by IL-1β, TNF-α, LPS, or PMA/ionomycin. Introduction of a proline at this position does not destabilize the protein but rather impairs its ubiquitination, as shown in TRAF6-dependent pathways, or its ability to interact with ubiquitinated partners by affecting the NUB domain. Another mutation producing a mild form of IP, E57K, has been shown to perturb the interaction between NEMO and TRAF6, suggesting the existence of a dual mode of interaction between NEMO and TRAF6 [19]. On one hand, the NUB domain may recognize polyubiquitinated chains of active TRAF6, and on the other hand, the N-terminus of NEMO may directly contact TRAF6. In contrast, the interaction with IKK is not affected.

As said above, chromosome X linkage is an additional parameter that greatly impacts upon the phenotype developed by IP patients. X-inactivation skewing, i.e., inactivation of one chromosome X favored over the other, is often detected. It reveals mechanisms of counterselection that affect cells carrying the *NEMO* mutation. This process is particularly striking in blood cells [20]. In most cases, their genotype is more than 95 % wild type, instead of 50 % in case of a normal X-inactivation process, demonstrating a specific disappearance of the mutated cells

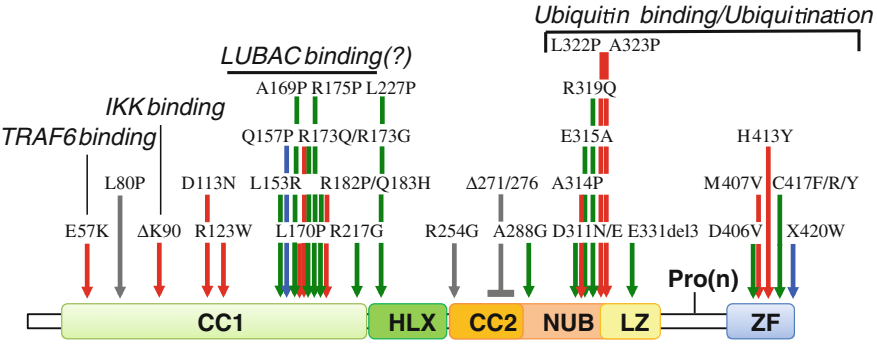


**Fig. 2.4** NEMO missense mutations and short internal deletions of NEMO found in pathology. *Red arrows* indicate IP-related missense mutations, *green arrows* indicate EDA-ID-related missense mutations, *blue arrows* indicate OL-EDA-ID-related missense mutations, and *gray arrows* indicate ID-related missense mutation. A short internal deletion of NEMO associated with ID is also indicated with a *gray bar*. Compiled from Refs. [39, 51, 52]. Formally and putatively (*question mark*) demonstrated molecular defects are also indicated. See text for more details

in this compartment. This elimination process is unfortunately not always as efficient in other cell types or even within the same tissue and can be variable, impacting on the final manifestations of the disease. As a striking example, IP patients carrying the recurrent DNA rearrangement of *NEMO*, therefore the very same genetic defect, can exhibit a phenotype ranging from mild dermatosis, without any additional problems, to the most severe form of the disease including abnormalities of the teeth, eyes, and CNS.

### 2.1.1.3 Pathophysiology

The sequence of events causing IP dermatosis results from an intricate combination of X-linked inactivation-related processes, NF-κB signaling in the skin, and sensibility of NEMO (−) cells to apoptosis. Using various mouse models of the disease, several molecules and cell types participating in IP dermatosis have been identified.

*Nemo* invalidation in the whole mouse generates a phenotype very similar to the phenotype of IP patients [21, 22]. In particular, males die from liver apoptosis at day E12, whereas females survive until birth but, soon after, develop a dermatosis displaying features typical of IP. Similar skin abnormalities have also been observed after invalidating *Nemo* or *Ikk2* specifically in the epidermis (*nemo*$^{epKO}$ and *ikk2*$^{epKO}$) using conditional recombination [23, 24]. This demonstrates that keratinocyte dysfunction is enough to trigger the disease.

The molecular/cellular circuitry involved in skin defect development has been identified to some extent. First, it has been shown that, in both *Nemo*$^{epKO}$ and *Ikk2*$^{epKO}$, several cytokines exhibit an abnormal expression in the skin. This occurs at very early stages (P2/P3), before detecting visible abnormalities. For instance, an

increase of IL-1β synthesis is specifically observed in the epidermis. Since such increase cannot be detected when *Ikk2* KO keratinocytes are cultured ex vivo, this indicates a cell non-autonomous event. Its cause remains unknown, but might be linked to the colonization of skin after birth or some cell fragility (necrosis?) due to NEMO absence. Later on, at P4/P7, an accumulation of TNF-α is detected in the dermis. This second event is crucial for the development of the dermatosis process since crossing the mice with *Tnfr-1* KO mice completely rescues the skin manifestations. It may involve the production of IL-24 by *Nemo* (−) keratinocytes inducing STAT3-dependent synthesis of cytokines and chemokines [25]. This would allow recruitment of immune cells in the skin, among them macrophages [26], dendritic cells, and neutrophils. Importantly, B and T lymphocytes have been shown to be dispensable [24]. The eosinophilic infiltration of the skin resulting in eotaxin accumulation during the vesicular stage and is a feature of IP [27] has not been studied.

From these observations, it has been proposed that the mosaic status of the neonatal skin/epidermis of IP females, composed of cells expressing either wild type or mutant NEMO, is the trigger of the whole dermatosis process. Mutant cells, most likely keratinocytes, would start to over-express pro-inflammatory cytokines such as IL-1β just after birth, and IL-1β would induce TNF-α synthesis by neighboring wild-type cells. This cytokine would in turn both induce an inflammatory process and act on mutant NEMO-expressing cells, causing their death and clearance. This latter event may explain the final atrophic stage of IP dermatosis linked to lesion disappearance. If true, this means that the genetic defect initiating the process in the skin would also be responsible for its shutoff. A paradox is also that an intact NF-κB signaling pathway in surrounding wild-type cells is required for skin abnormalities development.

It is noteworthy that elimination of NEMO (–) cells in the epidermis of IP patients at young ages is not always complete and can resume later on. In some adult IP patients, the skin disease, with its various stages, can be detected again at the same location as the original lesions following episodes of infection [28]. A similar recurrence has been observed using a mouse system allowing inducible *NEMO* invalidation in the skin of adult mice. Upon NEMO deletion in the keratinocytes, all the events recapitulating the IP dermatosis occurred [23].

In contrast to what starts to be deciphered concerning the skin abnormalities in IP patients, very little is still known concerning the events occurring in the eyes or the CNS. Recently, analysis of the eyes of *Nemo* (−) mice has revealed retinal arteriolar abnormalities, including luminal narrowing, endothelial cell hypertrophy, and basement membrane thickening. Surprisingly, neither obvious vaso-occlusion nor neovascularization was observed [29]. Some progress has also been made regarding the primary molecular/cellular event(s) causing CNS abnormalities in IP patients. Since the manifestations of the disease at this level are very diverse, it has been difficult to imagine a single primary defect although micro-vascular ischemia may be a trigger [6]. Supporting this vascular origin of the disease in the brain, Ridder and colleagues [30] have recently reported that NEMO plays a key function in blood–brain barrier integrity by controlling vascular permeability. Mice specifically invalidated for *Nemo* in the brain vessels exhibit abnormalities such as

degenerated microvessels caused by endothelial cell death with only remnants of the extracellular matrix (the so-called string vessels) and edema. Importantly, string vessels have also been observed in the brain of an IP individual and in mice modeling IP. In addition, invalidating *Nemo* in the brain vasculature results in epilepsia, a hallmark of IP.

#### 2.1.1.4 Treatment

Given the extended phenotypic effect of a lack of function of NF-κB in IP patients, it is hard to imaging treating the primary cause, an absence of NEMO or its lack of activity. Instead, efforts are made to attenuate consequences of the disease. A genetic counseling can also be proposed frequently. Indeed, as explained above, a large number of IP patients (approximately 70–80 %) carry the same DNA rearrangement. This allows an easy prenatal testing in case of familial transmission although current figures suggest that this mode of transmission only concerns half of the patients. If testing is possible and IP identified the genetic counseling consists in explaining carefully to the parents the difficulty in fully anticipating the spectrum and extent of abnormalities that will ensue (see Sect. 2.1.1.2).

It has been shown that teeth problems can benefit from orthodontics. Concerning the eyes, ophthalmologic examinations are performed frequently during infancy and early childhood to monitor any deleterious change [31]. CNS abnormalities represent the most challenging issue. Epilepsia almost affects half of the patients with such abnormalities, seizures usually starting early after birth. It has been reported that standard treatment protocols are not always effective. An attempt to treat one IP patient with glucocorticoid has been reported [32]. The cognitive function, especially learning ability, of IP patients can also be surveyed in order to propose some reeducational training [33].

Skin anomalies are usually not specifically treated, mostly because of an incomplete knowledge of the mechanisms/components involved. As said above, TNF-α plays a key role in mouse models of the disease, and anti-TNF-α treatments are available for humans. Nevertheless, it is still uncertain whether inhibiting a process that results in elimination of mutated cells would provide some benefits. Since even a small pool of surviving NEMO (−) cells can re-initiate the disease, keeping those cells alive at young ages with an anti-TNF-α treatment may transform the disease in a chronic one, considering also that skin abnormalities in IP display similarities with psoriasis.

### *2.1.2 Anhidrotic Ectodermal Dysplasia with Immunodeficiency (EDA-ID)*

As explained above, incontinentia pigmenti is caused by lack-of-function mutations of NEMO and affects females. Hypomorphic mutations of *NEMO* that do not

completely inhibit NF-κB activation also exist. They have been found to cause another X-linked pathology, anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) (OMIM # 300291) [34–37]. In this case, the disease affects exclusively males, although in rare occasions affected female exhibit very mild signs of IP. It is characterized by a severe immunodeficiency associated with the impaired development of skin appendages (hair, teeth, and sweat glands).

#### 2.1.2.1 Clinical Features

On the immunodeficiency side, patients suffer from recurrent and life-threatening bacterial and viral infections [38, 39]. The most frequent pathogens are Gram-positive and Gram-negative pyogenic bacteria, such as *S. pneumoniae* and *S. aureus*. Chronic atypical mycobacterial infections are frequently observed. They are associated with the poor prognosis since they progress insidiously and are almost inevitably disseminated when the disease is diagnosed. Some patients also suffer from fungal and viral diseases, including HSV encephalitis. All this is caused, for a large part, by impaired functioning of innate immunity receptors that are well-known NF-κB inducers, such as TLR4 that recognizes LPS from Gram-negative bacteria and other TLRs (see Chap. 1).

Adaptive immunity is also perturbed in EDA-ID patients, but a homogenous clinical and molecular picture is still difficult to draw. A defect in specific antibody production is often observed, with decreased IgG and increased IgA synthesis, and some patients also exhibit a hyper-IgM syndrome. These B-cell defects may be linked to impaired costimulation by CD40. In some rare patients, a T cell impairment has also been reported, and some have claimed that it may explain high sensitivity to infections by mycobacteria. More consistently found is a problem of NK cell activation, despite a normal cell count and proper development [40]. It has been proposed that this impairment may explain recurrent infections of EDA-ID patients with viruses, such as herpesvirus and papillomaviruses. Additionally, a reduced response to virus exposure might result from defective interferon (IFN) synthesis. Indeed, as mentioned above (see Chap. 1), NEMO is an essential component of the pathway specifically responsible for IFN class 1 production.

In a substantial fraction of EDA-ID patients, gut inflammation/colitis has been reported. This does not seem to be caused by mycobacteria or pathogenic bacteria. It is uncertain whether this relates instead to impaired Nod2 signaling and therefore presents similarities to Crohn's disease. The role played by NEMO in homeostasis of the intestinal epithelium (see Sect. 2.1.2.3) may also be evoked.

EDA-ID patients also exhibit an impaired development of skin appendages. This results in sparse hair, missing or scanty eyebrows and lashes, severe oligodontia, and absent or reduced sweating. This observation provided the first demonstration, through a genetic analysis but subsequently confirmed biochemically, that the ectodysplasin (eda)/eda receptor (EDAR) signaling pathway, which is specifically dedicated to formation of hair, teeth, and sweat glands and whose mutations are associated with EDA in humans [41, 42], is connected to IKK/NF-κB activation

through NEMO. Connecting eda/EDAR to the NF-κB pathway requires the adaptor EDARADD (EDAR-associated death domain), whose mutations can cause EDA in humans [43, 44], but also TRAF6 and TAB2/TAK1 that act upstream of IKK [45]. A participation of TRAF2 is also suspected since EDARADD contains a TRAF2-binding sequence in addition to a TRAF6-binding sequence [44].

In rare cases, EDA-ID can be found associated with osteopetrosis, which is characterized by defective RANK signaling and excessive bone formation, and lymphedema, which is caused by impaired VEGFR3 signaling and development of lymphatic vessels [46, 47]. The syndrome is called OL-EDA-ID. Whereas NF-κB is a well-known player in the RANK signaling pathway and is necessary for proper osteoclast function [48], it is still unclear how exactly NEMO/IKK participates in the development of lymphatic vessels. A role of NF-κB in the survival and adhesion properties of endothelial cells composing blood vessels, a developmentally related tissue, is well established. In the lymphatic system, NF-κB might be activated through VEGFR3 [47] and a lack of activation caused by a NEMO mutation would be detrimental. Alternatively, the expression of VEGFR3 itself may be both directly and indirectly regulated by NF-κB. Indeed, it has been shown that Prox1, a lymphatic-specific transcription playing a key role in lymphangiogenesis through VEGFR3 synthesis, is upregulated during inflammation with an involvement of NF-κB [49]. Moreover, the promoter of VEGFR3 contains several NF-κB-binding sites also used during inflammation. A similar mechanism of dual regulation may operate during development. Alternatively, it has been reported that increased VEGF-C synthesis during TNF-α-induced lymphangiogenesis requires NF-κB providing another possible level of regulation for NF-κB by acting a step above on the ligand of VEGFR3 [50].

#### 2.1.2.2 Genetic and Molecular Basis

NEMO mutations causing EDA-ID are less deleterious than the ones causing IP, allowing male survival. They provide an opportunity to learn more about the functions in vivo of the various domains of NEMO since in this case only one chromosome X, which carries the mutation, is expressed. This contrasts with the female situation in which the effect of a mutation has to be considered as potentially moderated by X-inactivation skewing processes (see Sect. 2.1.1.2). Nevertheless, it must be stressed that they represent hypomorphic mutations that may not fully reveal the complete function of a given molecular element. This information can be sometimes obtained by comparing EDA-ID-associated mutations with IP-associated ones. Also worth noting, the occurrence of hypomorphic sets of similar mutations introduces variabilities in NEMO activity that may explain the phenotype heterogeneity of EDA-ID patients.

In contrast to *NEMO* mutations causing IP, which usually result in severe truncations of the NEMO protein, frameshift or nonsense mutations causing EDA-ID often only remove the NEMO ZF. The distinction between IP and EDA-ID remains nevertheless far from clear-cut since similar truncations affecting

the NEMO ZF can also produce IP (Fig. 3.2). Interestingly, blunt deletions of this domain caused by missense deletions result in most cases in EDA-ID, whereas frameshift mutations that append extra amino acid sequences of various lengths at the C-ter of NEMO are more frequently found in IP patients. One explanation may be that extra-polypeptide sequences will impact more severely, by steric hindrance, the overall activity of the C-terminus of NEMO.

The C-terminal part of NEMO is composed of two domains with affinity to polyubiquitin chains, and the ZF represents one of them. The deletions affecting it and causing EDA-ID or IP, as discussed above, impact on the interaction of NEMO with numerous ubiquitinated partners and impair NF-κB activation in several distinct pathways (see Chap. 1). The same has been demonstrated for a set of EDA-ID-associated missense mutations (D406V, M407V, C417R/Y/F) [39, 51, 52]. The other domain with affinity for ubiquitin is the NUB domain and is very often found mutated with amino acids substitutions in EDA-ID patients [39, 51, 52]. This provides very useful information concerning the identity of the ubiquitin-interacting amino acids. Again, the impact of such mutations is supposed to be broad.

Another large set of missense mutations causing EDA-ID is located in the intermediate domain. It has been shown that this domain is required for interaction with LUBAC, more specifically Sharpin, suggesting that functions linked to linear ubiquitination could be the impaired ones. In at least one case, this has been formally demonstrated [53].

It has been reported that an EDA-ID-linked mutation, A288G, located in the CC2 region of NEMO, affects its oligomerization and impairs NF-κB activation in response to TNF-α and LPS [54]. Worth noting, this Gly residue appears near Lys 285, which is modified through K63- and/or linear-linked polyubiquitination, suggesting that this process may also be affected.

Finally, the mutation associated with OL-EDA-ID, X420W, which appends 27 aa at the C-terminus of NEMO, has been shown to strongly destabilize the protein [35]. Nevertheless, it is still unclear whether a simple quantitative defect of NEMO expression is responsible for OL-EDA-ID since other mutations are also associated with reduced NEMO expression [55] without generating osteopetrosis or lymphedema. Most probably, the appended sequence perturbs the ZF function of an already low expressed protein. This hypothesis will be difficult to assess in the mouse since a mutation eliminating the *Nemo* stop codon in this organism would not add the same extra C-terminal sequence as the human one and its length would be much longer. More puzzling, OL-EDA-ID has also been reported to be caused by a Q157P mutation, in a region of NEMO where many other mutations have been shown to cause either EDA-ID or IP.

This last situation confirms the difficulty in establishing in several instances a clear link between a specific kind of mutations and the final phenotype in NEMO-mutated patients. All these observations provide a quite unique and fascinating example of how an X-linked abnormality affecting a pathway with widespread functions, such as the NF-κB pathway, can affect human physiology with a high level of complexity and diverse outcomes.

#### 2.1.2.3 Treatment

EDA-ID patients with suspected infection should be treated with early empirical intravenous antibiotic administration since they do not show increased plasma C-reactive protein concentration and do not mount a fever due to impaired TLR signaling. Usually, patients are supplemented with intravenous or subcutaneous IgG because of their B-cell switching defect. Multidrug antimycobacterial therapy can also be applied in case of mycobacteria dissemination in the organism. Very often, infections of EDA-ID patients can be life-threatening, and the recommended procedure to restore some protective functions is allogenic hematopoietic stem cell transplantation (HSCT) [56–58]. This therapeutic approach remains challenging and is not always successful. This is mostly due to the fact that the defects also include non-hematopoietic tissues. For instance, it has been shown that in patients presenting gut inflammation before transplantation, the problem can be exacerbated by a restored immune system. In mice presenting a well-functioning immune system, a *Nemo* invalidation in the intestinal epithelium leads to severe chronic intestinal inflammation [59]. One may imagine that in transplanted EDA-ID patients correction of the immune compartment but absence of correction of the gut compartment may worsen the situation.

EDA is also by itself a concern, especially when sweating is severely impaired. Because NEMO is the defective component, it is hard to imagine acting at this level. This is unfortunate since treating EDA patients whose condition is caused by eda deficiency might be possible in the future. It has been shown in *tabby* mice, a spontaneous mutant strain defective in murine eda, that injection of EDAR agonists can be used to restore to some extent hair follicle and sweat gland formation [60]. Since the eda/EDAR signaling pathway appears highly restricted to skin appendage development, there might be room for modulating its activity without too much adverse consequences.

### *2.1.3 NEMO-Related Immunodeficiency (NEMO-ID)*

Several male patients suffering from a pure immunodeficiency syndrome not associated with EDA have been reported as mutated in *NEMO* [55, 61–65] (Fig. 2.4). They did not exhibit any specific types of infection that would distinguish them from EDA-ID patients. It is difficult at this stage to fully understand why their *NEMO* mutations do not generate EDA, especially when the same mutation in different families can produce different outcomes at the tooth level for instance [64]. Some immune functions may be more sensitive to specific weak mutations of NEMO than the eda/EDAR pathway. Alternatively, mutations affecting the expression level of the protein may be more deleterious for immunity than for skin appendage development. For instance, a frameshift mutation of NEMO located at amino acid Ala37 and compensated by re-initiation of translation at nearby Met38 produced a less expressed pseudofull length protein causes

immunodeficiency without any signs of EDA [55]. It will be quite interesting to solve this issue since two mutations causing only ID are short deletions (Δ271–276 and Δ353–373) [62, 65] in domains that have not been found associated with any specific functions so far.

## 2.2 IKK2-Related Genetic Disease (Severe Combined Immunodeficiency)

As the subunit of the IKK complex with the strongest catalytic activity IKK2 represents a key player in the activation process of NF-κB (see Chap. 1). Nevertheless, its absence is not as detrimental as the absence of NEMO. Indeed, residual IKK activation can still occur due to the ability of IKK1 to compensate to some extent for the lack of IKK2 activity.

A severe combined immunodeficiency (SCID) characterized by recurrent infections and hypogammaglobulinemia/agammaglobulinemia, but normal B- and T-cell counts was recently identified in several individuals [66–69]. The first ones and the most extensively characterized presented a common genetic origin (Northern Cree ancestry), but no consanguinity [66]. Homozygosity mapping revealed a candidate in a 11.6 Mbp region on chromosome 8. After sequencing the 40 genes located at this locus, only one was shown to exhibit in four distinct cases an homozygous duplication (c.1292dupG) in exon 13 of *IKBKB*, the gene encoding IKK2. This frameshift mutation starting at Q432 was shown to produce a very unstable truncated IKK2 protein (Fig. 2.5) as revealed by Western blotting. Unexpectedly, the expression level of other IKK components, IKK1 and NEMO, appeared also reduced.

Other IKK2 SCID patients were more recently identified carrying homozygous missense mutations (Y107X, R272X, and R286X) in *IKBKB* that result in large truncations of IKK2 [67–69] (Fig. 2.5). Similar to the mutation reported by Pannicke et al., these truncations resulted in a lack of IKK2 expression. When expression of NEMO and IKK1 was examined, conflicting observations were made. Mousallem et al. [68] observed a reduction in NEMO expression but not in IKK1 expression and Nielsen et al. [69] a reduction in neither of the two. The cause of such variability remains difficult to understand.

In line with the severity of the disease, many immune defects were identified in IKK2 SCID patients. They result from a wide range of impaired cell functions.

Although the development of B cells is not affected, many signs of their perturbed differentiation are observed. Cells are almost exclusively naive, with a normal proportion of $CD38^{+}IgM^{+}$ transitional cells but a lack of $CD19^{+}CD27^{+}IgD^{-}$ class-switched-memory cells and $CD38^{+}CD20^{-}$ plasmablasts. When patient-derived B cells are stimulated with CD40L/IL-21, which are usually provided by follicular helper T cell, they do not proliferate and differentiate into

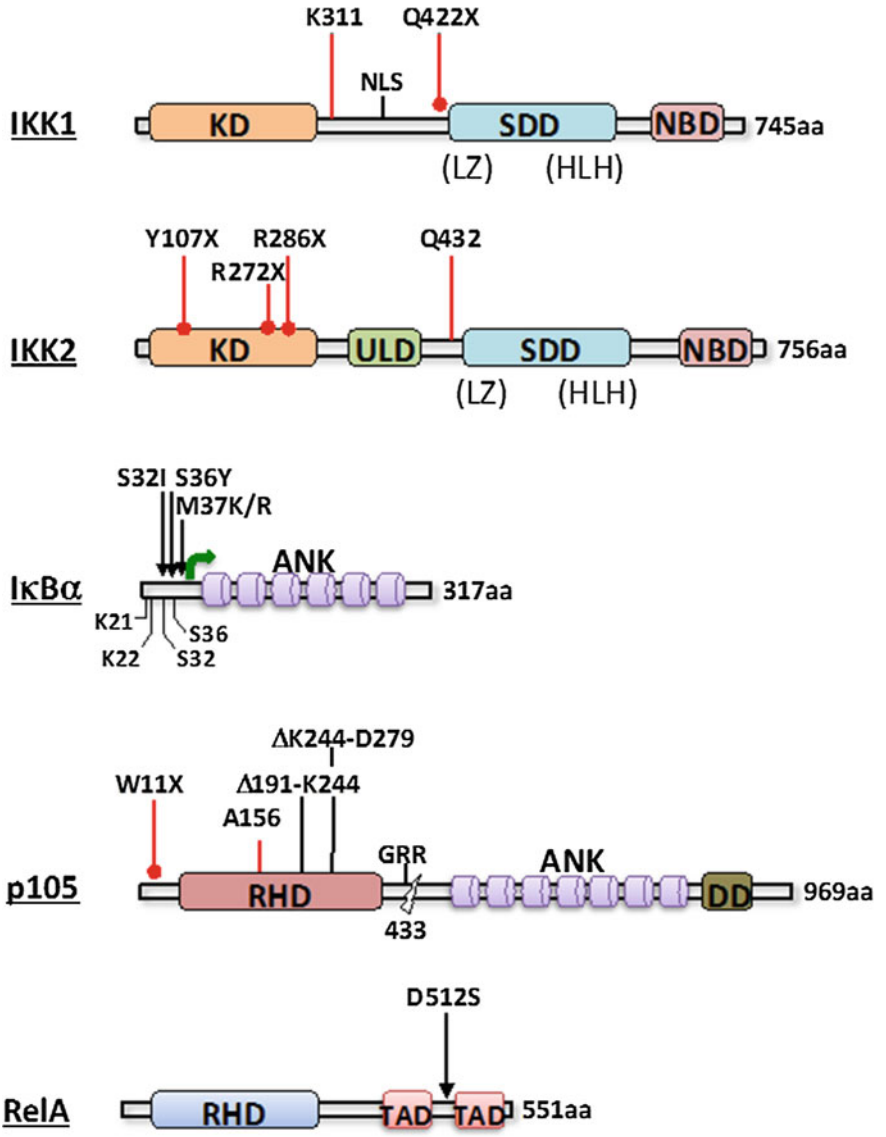


**Fig. 2.5** Mutations of IKK1, IKK2, IκBα, p105, and RelA. The proteins with their functional domains (see Chap. 1) are shown with the positions of the mutations identified in humans. *Red bars* above the protein structures indicate frameshift mutations, *red bars with a dot* indicate nonsense mutations, and *black bars with arrow* indicate missense mutations. A *curved green arrow* indicates a translation re-initiation site. Residues involved in IκBα degradation are indicated below its structure. The cleavage site of p105 used to generate p50 is also shown

plasmablasts. In addition, they do not produce immunoglobulins (Igs). In contrast, proliferation is still observed when induced by the B-cell receptor or the cytosine–guanine dinucleotide polymer CpG, through TLR9, but Ig production is impaired.

Something similar is observed with the T cells. Most CD4 and CD8 cells, whose abundance is normal, express naive antigens such as CD45RA or CD27 despite the exposure of patients to various germs. Moreover, there is an absence of $CD25^{high}FOXP3^{+}$ $CD4^{+}$ Tregs and, more surprisingly, of γδT cells. Expression of activation markers such as CD25 and CD69 is reduced upon exposure of IKK2 SCID T cells to CD3/CD28, and proliferation of the cells is moderately to severely impaired, depending on the patients. In addition, if a little response is observed to soluble or plate-bound, anti-CD3 antibodies phytohemagglutinin mitogenic responses are only moderately reduced. Therefore, the overall activation capacity of T cells derived from IKK2 SCID patients appears reduced to some extent but not abolished.

The number of NK cells in IKK2 SCID patients is reduced and their activation impaired, as shown by decreased CD107a degranulation and IFNγ synthesis [66].

Importantly, several signaling pathways participating in innate immunity were also shown to be affected in IKK2 SCID patients. In fibroblasts, among the most perturbed pathways are the ones responding to TNF-α, LPS (TLR4) and flagellin (TLR5). In contrast, the IL-1β signaling pathway appears less impaired. As a consequence, target genes of NF-κB are differentially affected, depending on the stimulus used, and therefore more or less sensitive to a lack of IKK2 activity.

Although reported in only a single case [67], very mild signs of EDA such as conical teeth may affect IKK2 SCID patients. As explained above (see Sect. 2.2), eda activates NF-κB through the canonical pathway, as demonstrated through the discovery of the EDA-ID pathology caused by hypomorphic mutations of *NEMO*. This would confirm the role of IKK in this developmental pathway. To explain the weakness of the phenotype, some compensation by the catalytic activity of IKK1 may prevent generation of a phenotype as severe as the one caused by *NEMO* mutations.

## 2.3 IKK1-Related Genetic Disease (Cocoon Syndrome)

IKK1 participates in the canonical pathway of NF-κB activation as one of the two catalytic subunits of IKK (see Chap. 1). Nevertheless, its contribution to the kinase activity of this complex appears less essential than the one of IKK2. The situation is different in the non-canonical pathway where it plays a critical function as the kinase inducing the processing of p100. NF-κB-independent functions of IKK have also been proposed, such as control of epidermis development. This last function may explain the overall aspect of IKK1 KO mice (see Chap. 1).

Two cases of fetal encasement malformations occurring in a consanguineous Finnish family were recently reported [70]. Among the numerous abnormalities detected in the fetuses at 12–13 weeks of gestation were defective face, with an abnormal cyst in the cranial region and a large defect in the craniofacial area, an omphalocele and a lobulation defect in the lungs. Poorly developed skeletal muscles were observed as well as seemingly absent limbs, which were bound to the trunk and encased under the skin.

This limb encasement bearing striking homology with the phenotype observed in IKK1 KO mice convinced the authors to sequence the *IKBKA* gene in the two fetuses. An homozygous missense mutation (c.1264C—T) was detected in exon 12, generating a large truncation of IKK1 removing all sequences after its catalytic domain (aa 422–745) including the domains participating in dimerization with IKK2 and interaction with NEMO (Fig. 2.5). This deletion resulted in a strongly reduced expression of the protein. Since a similar pathologic case was previously reported in the literature [71], but not genetically characterized, and qualified as "Cocoon fetus," the term Cocoon syndrome has been proposed by Lahtela et al. [70] to be used for this IKK1-related inherited disease.

More recently, an homozygous splice mutation of IKK1 was identified in a female patient born to a consanguineous family [72]. This mutation is supposed to truncate even further the IKK1 protein than in the Finnish family (Fig. 2.5), but no data regarding its effect on the stability of the protein is available. Again, the phenotype was highly severe and complex with face abnormalities (wide cranial suture and anterior fontanel, bilateral cleft lip and palata, bilateral microphthalmia, etc.), various skeletal abnormalities and extremities exhibiting syndactyly and popliteal webs.

The molecular/cellular basis of the Cocoon syndrome remains poorly defined. Most likely, intermingled defective functions of IKK1 generate this very complex phenotype.

Cranofacial and skeletal abnormalities may reflect the role played by IKK1 as a repressor of fibroblast growth factor (FGF) members [73]. Analyzing the transcriptome of two patients, Lahtela et al. [70] observed a severe reduction in numerous mRNAs, among them the one coding for matrix metallopeptidase 14 (MMP-14), but how their defective expression relates to impaired IKK1 activity remains unclear.

Skin abnormalities, which were first observed after invalidating the *Ikk1* gene in the mouse, are unlikely to reflect NF-κB dysfunction. Besides being very different from the one observed in incontinentia pigmenti, clearly caused by impaired NF-κB activation in the epidermis, they are also characterized by a lack of keratinocyte differentiation which cannot be corrected by activating NF-κB [74]. Actually, as a whole, the Cocoon syndrome displays several similarities with a limb pterygium syndrome (LPS) called lethal-type popliteal pterygium syndrome (LPPS)/Bartsocas-Papas syndrome (BPS) (MIM # 263650) [75]. Interestingly, this specific syndrome has been demonstrated to be caused by mutations of *RIPK4* [76, 77], which codes for a member of the RIP kinase family originally proposed to be an NF-κB activator in PKCβ-dependent pathways [78]. Nevertheless, the original data

were obtained by performing overexpression experiments, which can often generate artefacts. More importantly, popliteal pterygium syndrome can be also caused by mutations of *IRF6*, which encodes an inducible transcription factor located in the cytoplasm [79]. It remains to be determined whether IRF6 activation, which requires a phosphorylation event, is dependent upon IKK1 and/or RIPK4 kinase activity, acting sequentially or in parallel. Since forced expression of RIPK4 in the mouse epidermis corrects the skin defect of *Ripk4*(−/−) mice but not of *Ikk1*(−/−) mice, the former hypothesis is the most likely. Irrespective of the exact molecular relationship existing between IKK1, RIPK4, and IRF6, it has been shown that this module acts on periderm formation [80, 81]. Periderm is a barrier covering the developing epidermis during embryogenesis that is used transiently to prevent adhesion between adhesion competent epithelia. In case of dysfunction, adhesion of multiple soft tissue occurs. This explains the fusion between hind limbs and body wall in modified mice and humans suffering from Cocoon syndrome.

Since this chapter is supposed to deal with NF-κB-related diseases, one may be frustrated by the lack of info concerning the NF-κB field generated by the study of the first identified IKK1-related pathology. Unfortunately, as explained above, the great severity of the Cocoon syndrome and its paucity precludes investigation of NF-κB-related functions of IKK1 in immunity or other processes. Nevertheless, since this disease is caused by pseudo-KO mutations, it cannot be excluded that hypomorphic mutations of IKK1 would generate in humans a less severe condition revealing other functions of this enzyme.

## 2.4 IκBα-Related Genetic Disease (Autosomal-Dominant EDA-ID)

IκBα is the main IκB species regulating the canonical pathway of NF-κB activation and the direct target of the IKK complex (see Chap. 1). Its phosphorylation by IKK induces its degradation by the proteasome and the release of active NF-κB dimers.

In several instances, heterozygous mutations of IκBα impairing its degradation by IKK have been reported to cause a syndrome (autosomal-dominant (AD) EDA-ID, [MIM # 612132]) that share clinical similarities with *NEMO*-related EDA-ID [82–86]. So far, all these mutations modify the serine residues (Ser32, Ser36) that are phosphorylated by IKK upon cell stimulation, the residues nearby the DSGLDS phosphorylation motif, such as Met37, or delete the N-terminus of IκBα (Q9X, W11X, E14X) (Fig. 2.5). In these latter cases, re-initiation of translation occurs at Met37 and thus also eliminates the phospho-acceptor sites. Because of a lack of phosphorylation, IκB cannot be degraded and acts as a dominant-negative protein by accumulating and sequestering NF-κB species such as RelA, c-rel, or p50 in the cytoplasm. This explains why a heterozygous mode of transmission is sufficient to trigger the disease.

Clinically, AD-EDA-ID shares many similarities with EDA-ID but differences also exist. IκBα-mutated patients, such as NEMO-mutated patients, exhibit broad susceptibility to infections, especially those involving pyogenic bacteria. They are also infected by environmental mycobacteria and less frequently by parasites, viruses, and fungi. An impaired cellular response to TLR ligands, IL-1β, IL-18, and TNF-α, is observed. T-and B-cell numbers are normal, but hypogammaglobulinemia is observed with no production of specific antibodies. IκBα-mutated patients also present a severe impairment in TCR signaling with reduced cell proliferation. Several of them were shown to express low numbers of memory CD4 and CD8 T cells and no γδ T cells. This contrasts with EDA-ID-related NEMO patients who are usually not strongly affected in T-cell functions. The difference is likely caused by the fact that NEMO mutations generating EDA-ID are hypomorphic, therefore with remaining NEMO activity, whereas IκBα mutations are true dominant mutations that abolish IκBα degradation upon cell stimulation, blocking more severely the NF-κB activation process.

Recently, Mooster et al. [87] have generated a murine model of AD-EDA-ID by introducing in the mouse *Ikbα* gene a S32I mutation. The overall phenotype mimicked the human pathology, with defective responses to TNF-α or TLRs, but an additional effect of the mutation was observed. Defective secondary lymphoid organogenesis was noticed with a lack of lymph nodes, Peyer's patches, and splenic marginal zones. This suggested an effect of the mutation on the non-canonical pathway of NF-κB activation. Accordingly, stimulation of lymphoid tissue inducer cells and MEFs with β-lymphotoxin was severely impaired because of both defective IκBα degradation and very reduced p100 amount. The observation that IκBα mutants interfere with proper activation of the non-canonical pathway of NF-κB activation may have an important clinical impact. Indeed, this may explain why allogenic hematopoietic stem cell transplantation (HSCT), which is often used to treat EDA-ID patients, is poorly effective in cases of AD-EDA-ID. Most likely, only replacing hematopoietic cells may not be sufficient to fully correct the immune deficiency of AD-EDA-ID patients since lymphoid organogenesis is affected as well. It remains to be firmly established whether AD-EDA-ID patients indeed present defective lymphoid organogenesis. In support of this tonsils and cervical lymph nodes have been noted to be absent in at least one patient.

Mutations of both *NEMO* and *IκBα* as a cause of EDA-ID in humans confirm in vivo the participation of these two components in the same signaling pathways. Nevertheless, how NF-κB signaling is impaired in each case is biochemically quite distinct. In *NEMO*-mutated patients generating EDA-ID, the overall catalytic activity of IKK is reduced but not abolished. Therefore, the various substrates of IKK, among them the members of the IκB family of inhibitors, are putatively affected to the same extent. In the case of mutations affecting IκBα, degradation of the other IκB inhibitors is still properly controlled. The similitude between *NEMO*- and *IκBα*-related EDA-IDs indicates that IκBα is indeed a prime target of IKK in many distinct tissues, among them the hematopoietic compartment and the skin appendages. It also confirms that other properly regulated IκB/NF-κB complexes cannot overcome its function in controlling the activity of specific NF-κB subunits.

## 2.5 P105-Related Genetic Disease (Common Variable Immunodeficiency)

P105 is the precursor of subunit p50 that forms with RelA the main dimeric species controlled by the canonical pathway of NF-κB activation (see Chap. 1).

It has been shown that common variable immunodeficiency (CVID), a heterogenous syndrome characterized by recurrent infections and low antibody levels, can be caused by mutations of *NFKB1*, the gene encoding p105 [88]. In one large family including affected cases over three generations, a large range of highly variable immune response abnormalities was observed. Patients could present an infection-only phenotype or a much more severe condition with lymphoproliferation, lung disease, autoimmune cytopenia, and enteropathy. Moderate to severe hypogammaglobulinemia was also observed.

A genetic linkage study first identified an 18.7-Mbp region on 4q24 as carrying the mutation [89]. Upon whole exome sequencing, a heterozygous mutation of the splice donor site of exon 8 was found, causing aberrant in-frame splicing from exon 7 to exon 9. Elimination of the 159 coding nucleotides of exon 8 results in an internal deletion of 53 aa (Asp191 to Lys244) located in the Rel homology domain (RHD) of p105 (see Fig. 2.5). Patients derived EBV-transformed B cells exhibited a reduction in the amount of both p105 and p50 to approximately 50 %, indicating a severe destabilization caused by the mutation. Only traces of truncated p105 but not of truncated p50 were detected. Consequently, upon PMA/ionomycin treatment, a 50 % reduction in p50 nuclear translocation was observed. From this, it could be concluded that CVID in this family was caused by haploinsufficiency of p50.

Subsequent targeted next-generation sequencing/whole exome sequencing identified two other families with CVID caused by *NFKB1* mutations. Like with the first studied family, a high variability of immune defects was observed. In both cases, the syndrome was associated with haploinsufficiency of p50 also. In the first one, a heterozygous in-frame skipping of exon 9 was found, again introducing a short deletion (Lys244 to Asp279) in the RHD of p105/p50 proteins and their destabilization. In the second one, a heterozygous frameshift mutation affecting amino acid Ala156 of the RHD produced a severely truncated and undetectable p105 protein.

Several individuals of a same family exhibiting a complex syndrome including bone and joints defects, ectodermal dysplasia, hypergammaglobulinemia, and sterile inflammation were also recently reported as displaying a heterozygous mutation in *NFKB1*. This mutation introduces a stop codon (W11E) at the beginning of the coding sequence of p105/p50 [90]. Because of the very short size of the protein produced, this should result in a 50 % reduced expression of the protein like in cases described above. How haploinsufficiency of p50 in this case may generate such complex phenotype, especially involving bones, remains unclear.

## 2.6 RelA-Related Genetic Disease (High Bone Mass Syndrome)

RelA forms with p50 the prototypic and widely expressed NF-κB dimer that is induced following IKK activation (see Chap. 1). It contains, in contrast to p50, a potent transactivation domain (TAD).

A male patient bearing a heterozygous missense mutation of *RELA* substituting in the RelA protein Asp512 with a serine residue was recently identified [91] (Fig. 2.5). He died at very young age from unknown cause precluding any analysis of its immune system. Upon autopsy, bone abnormalities were detected with a high bone mass (HBM) syndrome. In this specific case, HBM would result from increased bone formation rather than more usual osteopetrosis caused by impaired number or function of osteoclasts.

The mutation is located in the linker sequence between TAD1 and TAD2, the two subdomains of RelA TAD. Since induction of RelA transcriptional activity requires phosphorylations of serines and threonines at neighboring residues (Thr505, Ser529, and Ser536), interference in this process may be caused by appearance at aa 512 of a new serine residue. More difficult to understand is the very restricted phenotype of this patient (but again the cause of death was not defined), which concerns exclusively bones with a peculiar form of osteopetrosis and results from a heterozygous mutation. One can only speculate on a possible bone-related gene-specific effect involving either impaired p50/RelA (or even c-rel/RelA) heterodimers or RelA/RelA homodimers [92, 93].

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