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"RING finger protein-7 (RNF7) binds to and regulates the NF-κB inducing activity of the Psoriasis-linked CARMA2sh protein"

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Index

Abstract

Psoriasis is a common immune-mediated inflammatory disease of the skin and joints. It is a chronic and essentially untreatable disease. Although genome-wide association studies have identified over 20 susceptibility loci for psoriasis, very recently only mutations in CARMA2/CARD14 have been shown to cause psoriasis dominantly and with high penetrance. The alternative splicing variant of CARMA2, CARMA2short, is the most prominent CARMA2 isoform expressed in human keratinocytes. Psoriasis-linked CARMA2 gain-of-function mutations lead to unopposed NF-κB activation and induction of inflammatory mediators from keratinocytes. We recently discovered that RNF7 (Ring Finger Protein 7) interacts with CARMA2sh.

RNF7 (RING finger protein-7), also known as RBX2 (RING box protein-2), ROC2 (Regulator of cullins-2), or SAG (Sensitive to Apoptosis Gene) belongs to an evolutionarily conserved gene family with 96% sequence identity between human and mouse. RNF7 is expressed ubiquitously in human tissues with a very high expression in heart, skeletal muscle, and testis, three organs with high levels of oxygen consumption. At the subcellular level, RNF7 is expressed in both cytoplasm and nucleus. Structurally, both human and mouse RNF7 encode a protein of 113 amino acids, of which 12 are cysteine residues. At the carboxyl portion of RNF7 protein, there is a C3H2C3 motif, which chelates two zinc atoms to form the RING domain, a characteristic of a domain with E3 ubiquitin ligase activity. Here we demonstrate that RNF7 is able to dampen the ability of CARMA2sh to induce NF-κB and that CARMA2sh mutants E138A and E142G escape this form of regulation.

Background

Immunity is a state of specific resistance to infection. Specific resistance is directed against a particular type of microorganism and is the single most important characteristic of immunity. The immune system enables the body to recognize a foreign agent as non-self**,** which is something other than a person's own substances (self). The immune system takes a specific action for neutralizing, killing, and eliminating that agent. The action involves nonspecific resistance as well. On occasion, the immune system activity may lead to tissue damage as seen in allergic disorders and other states of hypersensitivity. The immune system's activity is based on its ability to distinguish characteristic proteins or protein-linked components associated with alien substances. Once this distinction has been made, certain lymphocytes are provoked to produce antibodies directed against the foreign matter, while other lymphocytes are sensitized to the invading agent and react with it directly. Thus, there are two major branches of the immune system: antibody-mediated immunity (also known as humoral immunity) and cell-mediated immunity.

The immunity response was explained focusing the attention on the innate response that can be induced by several stimuli and on adaptive response that is lymphocyte mediated giving attention on the mechanisms involved to ensure the immunity. Thirty years ago, the transcriptional factor NF-κB was discovered and during the years it has been validated its role in the immunity and in inflammation. In the first chapter NF-κB was described underling its function activation pathways and the different stimulus that trigger its activation. Moreover, we highlight several proteins involved in NF-κB activation such as CARMA2. This one is a scaffold protein, in which mutation events change the working of this protein leading to the Psoriasis. Then psoriasis was described explaining which is the cause of this skin disease and which factors are involved

and some therapeutic strategies. The second chapter of this work explains the aim of this work. Because CARMA2 protein is involved in this skin disease, it has been used for a yeast two hybrid assay in order to discover new interactors of this protein so the third chapter describes the two hybrid assay principles and all the procedures used for our experiments while in fourth one we talk about our experiments. More exactly RNF7 protein, was described and characterized in CARMA2 pathway.

1.1 NF-κB

NF-κB represents a group of structurally related and evolutionarily conserved proteins that belong to the Rel family. In mammals the family includes 5 proteins that belong to two classes. The first class includes Rel-A (p65), c-Rel and Rel-B proteins that are synthesized as mature products and do not require proteolytic processing. The second group is encoded by the NF-κB1 and NF-κB2 genes, whose products are first synthesized as large precursors, p105 and p100, respectively, that require proteolytic processing to produce the mature p50 and p52 NF-κB proteins. All the members of this family contain the well-conserved Rel homology region which is 300 amino acids long and is involved in DNA binding and dimerization, a NLS, a stretch of basic amino acids and it is the IkB binding site and a trans-activation domain present in c-Rel and p65. The carboxy-terminal half of p100 and p105, instead, contains the ankyrin repeat motif.

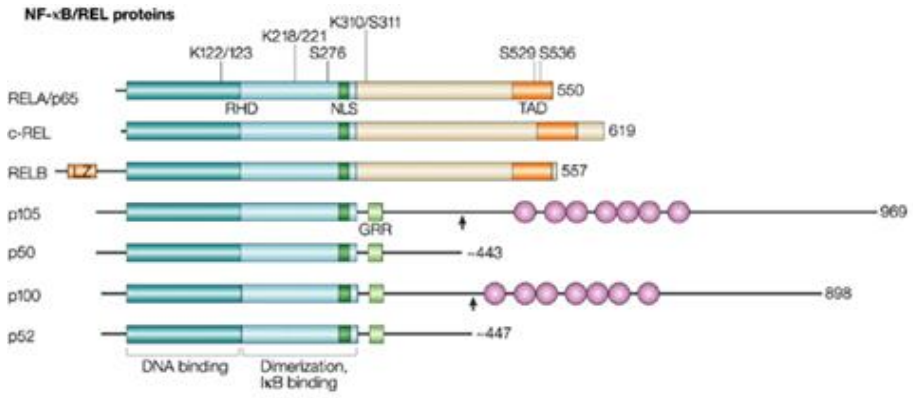

Figure 1. NF-κB family. All the members contain the well-conserved Re1 homology region (RHD) involved in DNA binding and dimerization. The NLS is involved in nuclear translocation. Both p50 and p52 are generated by a proteolytic processing event of the precursor p105 and pl00 possibly involving the glycine "hinge" (GRR) region.

In the Rel/NF-κB family, all the protein can form both homodimers and heterodimers. Each dimer could have a specific function in the signaling. The

major dimers product are: p50/RelA (the canonic dimer) and p52/RelB. Sometimes, homodimers can form but they are inactive. In the case of p50 and p52, they lack the TAD domain so they can't active the transcription indeed they compete with the active dimers for binding at kB sites. Crystallography studies show the interaction between the aspartate residue of p50 and a asparagin belonging to p65 facilitate the DNA binding but in the homodimers this interaction is unfavorable [1].

The NF-κB activity is tightly regulated by the interaction with the inhibitor protein IκB (Inhibitor of κB). IκB proteins can be divided in three functional groups: the typical IκB proteins IκBα, IκBβ and IκBε, which are present in the cytoplasm of unstimulated cells and undergo stimulus-induced degradation and re-synthesis; the precursor protein p100 and p105 which can be processed to form the NF-κB proteins p52 and p50 respectively and that can function as IκB-like proteins; and the atypical IκB proteins IκBζ (encoded by NFKBIZ), BCL-3 (B cell lymphoma 3) and IκBNS (encoded by NFKBID), which are generally not expressed in unstimulated cells but are induced following activation and mediate their effects in the nucleus. The IkBs are characterized by the presence of multiple ankyrin repeats and interact with NF-κB via Rel homology domain (RHD). The IkBs are a small family that contain multiple copies of a 30-33 aa sequence, called ankyrin repeats which mediate the association between IkB and NF-κB dimers. The ankyrin repeats interact with a region in the RHD (Rel homology domain) of the NF-κB proteins and by this mask their NLS and prevent nuclear translocation. Among these proteins, IκBα is the prototypical member and it works as inhibitor masking the nuclear localization signal of NF-κB, sequestering it in an inactive form in the cytoplasm. Upon stimulation, IκB is quickly phosphorylated by the IKK (IκB kinase) complex and it undergoes rapid ubiquitin-mediated proteasomal degradation that results in the release of NF-κB dimers [2].

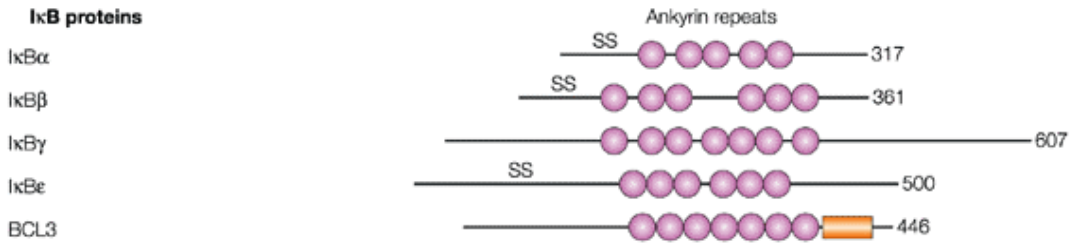

Figure 2. The IKB Family. The distinguish feature of the IKB proteins is the presence of ankyrin repeats whose numbers is different among the members. This domain mediates the binding to Rel domain of NF-κb proteins.

1.1.1 The NF-κB signaling pathways

There are two signaling pathways leading to the activation of NF-κB known as the canonical pathway (or classical) and the non-canonical pathway (or alternative pathway).

The canonical pathway

The canonical NF-κB pathway has been defined primarily in response to TNFα and IL-1 signaling, prototypical proinflammatory cytokines that have important roles in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), asthma, and chronic obstructive pulmonary disease.

Activation of NF-κB dimers is due to IKK-mediated phosphorylation-induced proteasomal degradation of the IkB inhibitor enabling the active NF-κB transcription factor subunits to translocate to the nucleus and induce target gene expression. NF-κB activation leads to the expression of the IkBα gene, which consequently sequesters NF-κB subunits and terminates transcriptional activity unless a persistent activation signal is present. More exactly, binding of ligand to a cell surface receptor, leads to the recruitment of adaptors to the cytoplasmic domain of the receptor. These adaptors in turn recruit the IKK complex which leads to phosphorylation and degradation of the IkB inhibitor. The canonical pathway activates NF-κB dimers comprising of RelA (p65), c-Rel and p50 that are held in the cytoplasm through interaction with these inhibitors [3].

The non-canonical pathway

The non-canonical pathway is responsible for the activation of p100 which is then processed to p52, followed by the translocation of the NF-κB2(p52)/RelB

complex to the nucleus and occurs during the development of lymphoid organs responsible for the generation of B and T lymphocytes. Only a small number of stimuli are known to activate NF-κB via this pathway and these factors include lymphotoxin B and B cell activating factor (BAFF). This pathway uses an IKK complex that comprises two IKKα subunits, but not NEMO. In the non-canonical pathway, ligand induced activation results in the activation of NF-κB-inducing kinase (NIK), which phosphorylates and activates the IKKa complex, which in turn phosphorylates p100 leading to the processing and liberation of the p52/RelB active heterodimer. In contrast to p100, p105 undergoes constitutive cleavage to produce p50, whether p105 can undergo inducible processing remains a contentious issue [4].

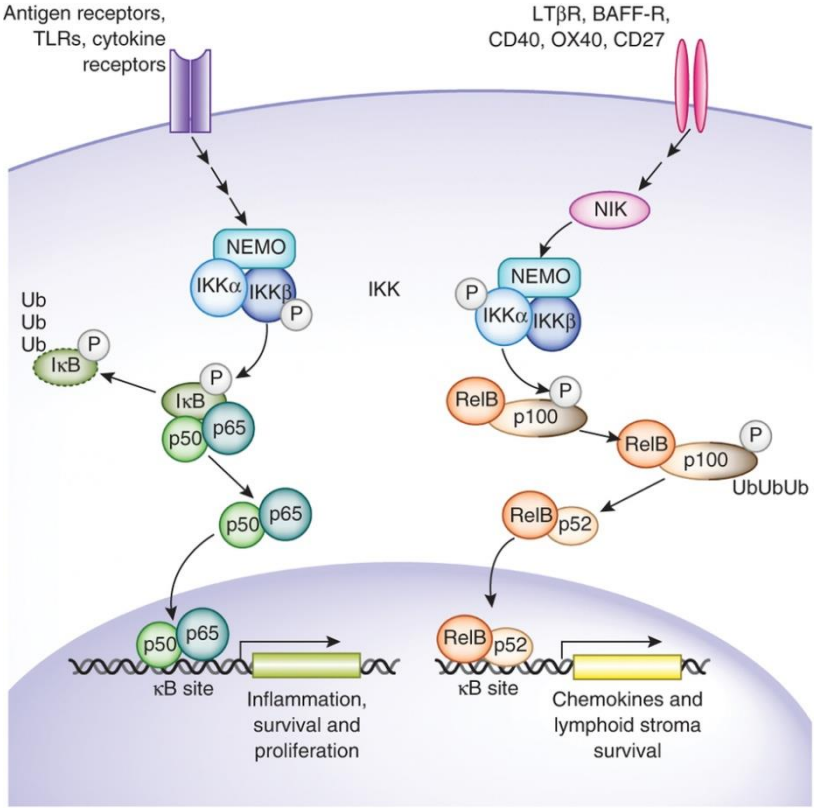

Figure 3. The canonical and non-canonical NF-κB pathways. The canonical pathway induced by signals including antigens, TLR ligands and cytokines such as TNF uses a wide variety of signaling adaptors to engage and activate the IKKβ subunit of the IKK complex. IKKβ phosphorylation of classical IκB proteins bound to NF-κB dimers such as p50-p65 results in ubiquitination (Ub) of IκB and proteasome-induced degradation. This allows

NF-κB to enter the nucleus where it binds specific DNA sequences (κB sites) involved in controlling the transcription of genes encoding functions as diverse as inflammation, cell survival and cell division. The noncanonical pathway.) engaged by members of the TNF-like family of cytokines requires NIK to activate IKKα, which then phosphorylates p100 (NF-κB2), triggering its proteasomal processing needed for the activation of p52-RelB dimers. Among its functions, this specific NF-κB heterodimer controls gene expression crucial for lymphoid organogenesis.

After IkB degradation, NF-κB shows its NLS, so it can move in the nucleus and it can bind kB site, that contains a repeated consensus sequence, 5'-GGGRNYYYCC-3' (where R is a purine, Y is a particular pyrimidine and N is a generic base), and it can recruit the transcriptional apparatus and direct the target gene expression. The NF-κB interaction with the DNA has been described through crystallography studies. In particular, the p50 handle, envelopes one of two filaments of the double strand, interacting with a sequence of 5 bp, 5'-GGGRN-3'. The monomer p65 instead, can bind the DNA with its handle that recognizes a sequence of 4bp :5'YYCC-3'. The binding at these sequences level, determinates the activation of several genes:

- genes of the immune response (cytokine, chemokine receptor, adhesion molecules);
- genes that encode for molecule involved in NF-κB (negative feedback);
- anti-apoptotic genes (cIAP, BCLXL, cFLIP);
- genes that encode for molecule involved in the cellular cycle (cyclin D1, cMYC).

1.1.2 Differential NF-κB activation

Numerous pathways lead to the activation of NF-κB. Almost universally, these pathways proceed via activation of IKK, degradation of IκB, and enhancement of the transcriptional activity of NF-κB. However, there is significant variability in fundamental aspects of these pathways, for example, TNFα signaling via TNFR1 results in the rapid activation of IKK and nearly complete degradation of IκBα within 10 min, whereas signal-induced degradation of IκBα through the TCR takes nearly 45 min. Furthermore, individual NF-κB responses can be characterized as consisting of waves of activation and inactivation of the various NF-κB family members. This is because of sustained activation of the IKK complex, and its selectivity for different IκBs, as well as the differential regulation of IκB expression by NF-κB dimers. The baseline complement of NF-κB complexes present in a cell can therefore influence the nature of the transcriptional response to a given stimulus.

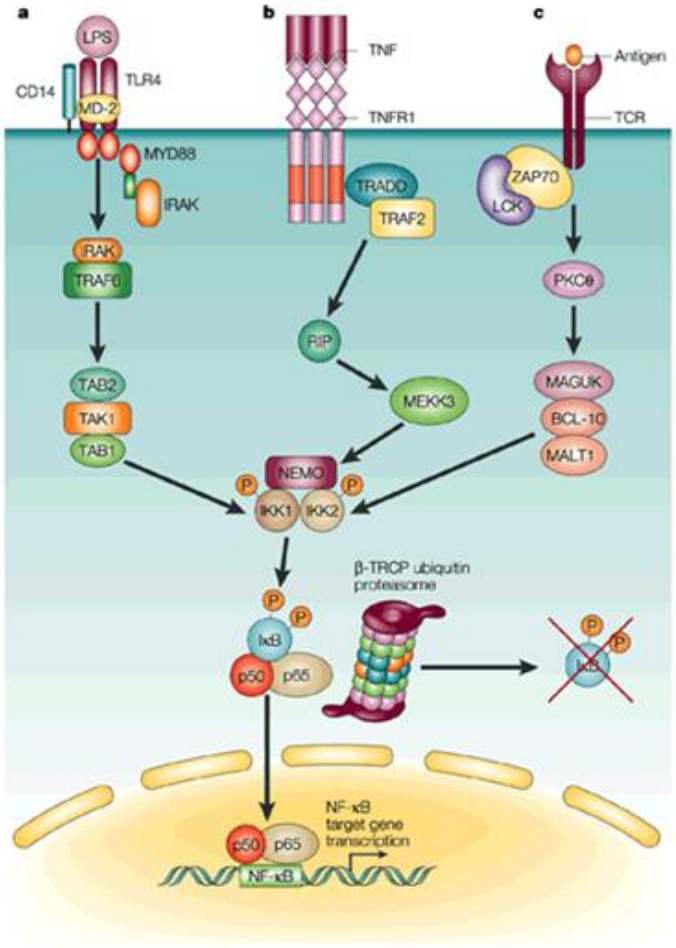

Figure 4. Main NF-κB pathways: TLR/IL-1R pathway, TNFR pathway and TCR/BCR pathway.

1.1.3 The IKK complex

The IKK plays a pivotal role in the NF-κB activation. It is composed of three subunits, IKKa (IKK1), IKKb (IKK2), and IKKγ (also known as NF-κB essential modulator, NEMO). IKKa and IKKb are the catalytic subunits of the complex, sharing 52% overall sequence identity and 65% identity in their catalytic domains. The third subunit, IKKγ/NEMO, is the regulatory subunit and is not related to the catalytic subunits. IKKa and IKKb have similar primary structures and contain protein kinase domains at their Ntermini, and leucine zippers (LZ) and helix–loop–helix (HLH) motifs in their C-terminal portions. IKKγ does not contain a recognizable catalytic domain, but is composed mostly of three large a-helical regions, including an LZ. The IKK complex in cells can be purified as a 700-900 kDa complex that is a molecular weight higher respect to the sum of the molecular weights of the three subunit; so, it is possible that the various combinations of subunits can form higher order complexes though multimerization probably associating with other unknown protein components.

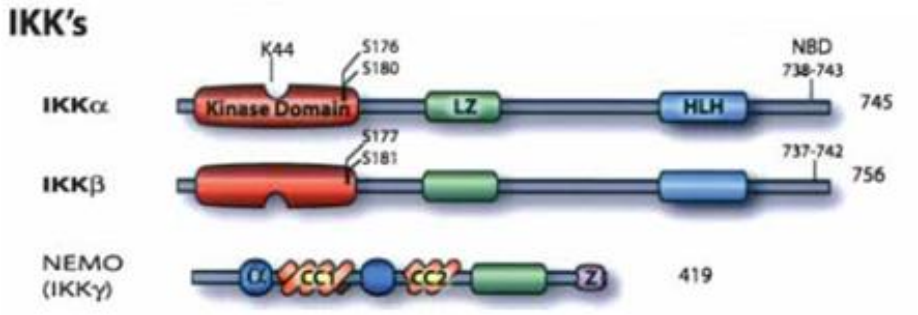

Figure 5. Schematic representation of IKK subunits. The number of amino acids in each protein is indicated on the right. LZ, Leucine Zipper domain; HLH, helix-loop-helix; Z, Zinc-finger domain; CC1/2, coiled-coil domains; NBD, NEMO binding domain; α, α-helical domain.

The activation of the IKK complex depend on phosphorylation on two specific serine residues within the catalytic domain of IKKα and IKKβ.

In most canonical NF-κB signaling pathways, including those that are activated by the pro-inflammatory cytokines TNF and IL-1 and by various microbial products, IKKβ is necessary and sufficient for the phosphorylation of IκBα, although there is increasing evidence of a role for IKKα in this process. Even when IKKα is dispensable for IκBα phosphorylation, it can function as an important regulator of the NF-κB dependent transcriptional response through non-IκB substrates, such as NF-κB itself, transcriptional co-activators and co-repressors. IKKγ is required for all canonical NF-κB signaling pathways, including those that are catalyzed by IKKα. By contrast, the non-canonical NF-κB pathway proceeds through the induction of p100 processing and the release of p52-containing dimers, and this process is thought to depend primarily on IKKα.

1.2 The CBM complex

CARMA1, BCL10 and Malt 1 form the CBM complex that is a scaffold for the TRAF 6 and TAK1 binding [5]. About CARMA protein, there are some isoforms: CARMA1, CARMA2 and CARMA3. These three protein share a similar structural domain and display a distinct expression profile: CARMA1 is expressed in spleen, liver and peripheral lymphocytes,CARMA2 is expressed in the placenta and skin and CARMA3 is expressed in embryonic lung, liver and kidney The CARMA1 protein (also called Card 11 or Bimp2) contains various protein-protein interaction domains that contribute individually to the coordinated binding of specific interaction partners of the antigen receptor-induced NF-κB and JNK signaling pathway. CARMA1 contains multiple protein interaction domains that are important for its function. The N-terminal part of this protein is structurally characterized by the presence of a caspase recruitment domain (CARD) that binds to the CARD of Bcl-10 and a coiled coil motif that binds to MALT1. The C-terminal part of CARMA1 contains structural motif that are characteristic for proteins of the MAGUK family; it comprises a PDZ domain (named after its presence in the proteins PSD95, DLG and ZO-1), a Src homology 3 (SH3) domain that is important for binding to lipids and a GUK domain (guanylate kinase) that mediates binding to PDK1. The PDZ domains typically target these proteins to the plasma membrane by binding to the cytoplasmic carboxyl terminus of transmembrane proteins, whereas the SH3 domain is involved in an unusual intramolecular interaction with the GUK domain that does not involve a typical proline-rich SH3 target motif The N-terminal and the C-terminal parts are separated by a linker region that was recently identified as target of phosphorylation events that control the conformation of CARMA1 and the accessibility of its CARD motif [6][7].

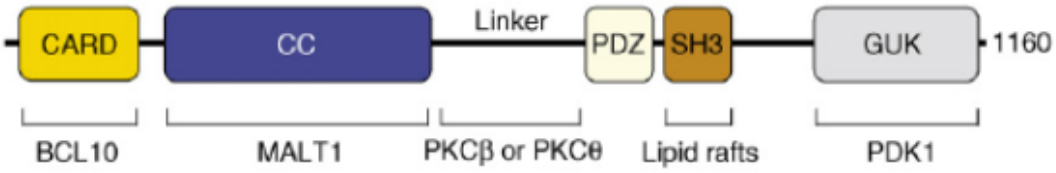

Figure 6. Domain structure and identified binding partners of CARMA1.

BCL10 is a ubiquitous protein. It is 32 kDa protein characterized by an amino-terminal CARD motif and a Ser/Thr rich carboxyl terminus of unknown function. The gene encoding BCL10 is subject to chromosomal translocation in mucosa-associated lymphoid tissue and this translocation correlates with BCL10 over-expression. Moreover, mice deficient in BCL10 show impaired BCR and TCR- induced NF-κB activation and cell proliferation. These findings indicate that BCL10 overexpression might be linked to constitutive NF-κB activation and cell proliferation of MALT B-cell lymphomas. BCL10 is a positive regulator of lymphocytes proliferation because it links the stimulation through the lymphocytes antigen receptor to the NF-κB activation. Experiments on cell lines, especially the genetically modified mice production and deficient for BCL10 gene, indicate that BCL10 is necessary for a correct immune response. In fact, mice knock out for BCL10 are immunodeficient for the lymphocytes inability to proliferate after antigenic proliferation because of the NF-κB no activation. Moreover, BCL10 is important for the embryonic development and for the correct closure of the neural tube. So, BCL10 is required for the correct functioning of IKK complex and NF-κB activation in different cell lines [8].

Figure 7. Structural domains of BCL10.

MALT1 is a caspase-like protein related to so-called metacaspase found in plants, fungi and protozoa, was recently identified as a protein that might be involved in transmission of the signal from BCL10 to the IKK complex. Structurally, MALT1 is characterized by an amino-terminal death domain, a homotypic interaction domain related to the CARD, followed by two immunoglobulin-like domains and a carboxy-terminal caspase-like domain (its catalytic activity has not yet defined) where there is the TRAF6 binding site. MALT1 interacts directly with BCL10 and activates IKK through the NEMO ubiquitination. This event occurs at lysine 63 of NEMO zinc finger and the lysine replacement with an arginine causes a reduction of NEMO ubiquitination by MALT1.

Figure 8. MALT1 molecular structure.

The CARMA proteins mediated activation pathway of NF-κB depends strictly on the correct formation of the trimeric CBM complex, an event that appears to be regulated by a complex array of phosphorylation events modulating CARMAs association with BCL10 and MALT1.

In resting cells, CARMAs adopt an inactive conformation that is stabilized by an intramolecular interaction between the CARD and the linker region, thereby blocking CARD-dependent protein-protein interactions. Stimulation induced phosphorylation of the Ser552 and Ser645 linker residues may weaken this interaction, possibly by electrostatic repulsion, inducing an open conformation of CARMAs. In this conformation, the CARD motif and potentially the coiled coil domain are accessible for homophylic interaction with the CARD domain of BCL10, that is constitutively associated with MALT1[9].

Although the CARMA proteins have a crucial role in this signaling pathway and, therefore, in the correct regulation of NF-κB, inducing stimuli and the exact series of events that allow the assembly of the CBM complex are yet not fully elucidated, especially for CARMA2 and CARMA3. In fact, the best studied and known model of activation of this complex is that involving CARMA1 that act downstream B and T cell receptors and that is activated by antigen binding. However, the connection between upstream signaling events and the NEMO-IKK complex is represented by the CBM complex. In fact, upon TCR or BCR stimulation, CARMA1 is recruited into the lipid rafts, also known as immunological synapse, where all the kinases and adaptor proteins involved in the signaling are already recruited and activated. The CARMA1 translocation depends on its MAGUK domain that is believed to link proteins to the cytoplasmic membrane and on its inducible interaction with an adaptor protein called ADAP (adhesion and degranulation promoting adapter protein). Next, CARMA1 physically associates with PKCθ in T cells or PKCβ in B cells and this association is dependent on its linker region. This region is phosphorylated by PKCs in a signal dependent manner on two putative phosphorylation sites, Ser552 and Ser645. This phosphorylation results in CARMA1 conformational changes that allow interaction with BCL10 and MALT1 and, therefore, the assembly of CBM complex. CARMA1, in addition, promotes the activating phosphorylation of BCL10 by RIP2 (Receptor interacting protein 2), a Ser/Thr kinase that carries a CARD domain at its carboxy terminus, also known as RICK, CARDIAK or CCK. CARMA1 directly associates with NEMO, and modulates the polyubiquitination of NEMO upon TCR stimulation. It has been shown that NEMO can specifically recognize Lysine 63 (K63)-linked polyubiquitin chains, and becomes polyubiquitinated upon activation of NF-κB signaling cascades These two properties of NEMO seem to be required for activation of the IKK complex. It is also possible that K63-linked NEMO polyubiquitination allows NEMO oligomerization through cross-recognition by its own ubiquitin-binding domain and leads to generation of the high molecular

weight IKK complex. Interestingly, several studies suggest that K63-linked polyubiquitination of NF-κB signaling components, such as RIP1, IRAK, BCL10, and MALT1 might be recognized by NEMO following stimulation of different receptors. Indeed, it has been shown that TCR ligation leads to K63-linked polyubiquitination of BCL10 at Lys31 and Lys63 in the CARD domain, and this polyubiquitin chain can be recognized by NEMO [7]. Thus, polyubiquitinated BCL10 may be involved in a signal-dependent IKK redistribution and possibly activation. The CBM complex most likely serves as a molecular platform to recruit signaling components responsible for the K63-linked polyubiquitination of NEMO. Indeed, activation of the IKK complex is not only dependent on IKK phosphorylation but also on CARMA1-dependent NEMO modification. Initially, the physical interaction of NEMO with CARMA family members was identified by the yeast two-hybrid screening and. confirmed by coprecipitation experiments in mammalian cells Shambharkar *et al*. revealed that NEMO is polyubiquitinated in a CARMA1-dependent manner upon TCR engagement, although the mechanism by which CARMA1 regulates NEMO polyubiquitination is not fully defined. One study suggests that the CBM complex recruits TRAF6 (TNF receptor associated factor 6), and TRAF6 induces K63-linked polyubiquitination of NEMO upon TCR stimulation. Alternatively, MALT1 may induce NEMO ubiquitination at the Lys399 residue. Therefore, it remains to be determined whether MALT1-dependent polyubiquitination of NEMO is functionally important for TCR-induced responses. In addition, although TRAF6 has been suggested to mediate TCR-induced polyubiquitination of the IKK complex, T cell-specific deletion of TRAF6 does not impair TCR-induced NF-κB activation, suggesting that either there is a redundant mechanism for TRAF6-mediated activation of IKK or TRAF6 is not involved in the activation of IKK. Because CARMA1-dependent NEMO polyubiquitination is required for IKK activation, revealing the mechanism by which CARMA1 regulates NEMO polyubiquitination should provide further insight about the regulation of the IKK complex. Similar events

downstream GPCRs transduce activation signal to NF-κB through CARMA3, whereas little is known about CARMA2 activating receptors, nevertheless this model of activation could presumably be extended to it [7,9,10,11].

1.2.1 BCL10 ubiquitination

For NF-κB activation, BCL10 requires a ubiquitination event on the lysine 63. This event requires the MALT1 paracaspase and E2 ubiquitin ligase. After cellular stimulation, BCL10, binds the tissue specific CARMA protein through CARD-CARD interaction. In this way, the E2 ubiquitin ligase complex, lies next to Nemo that is associated to CARMA region (Ile 600-Leu 800). The proximity of Nemo to E2 complex facilitates the Nemo ubiquitination on the lysine 63. Ubiquitination of BCL10 by the MALT1-binding ubiquitin ligase TRAF6 may regulate the assembly of CBM complex. Ubiquitinated BCL10 may recruit the IKK complex via the ubiquitin binding domain of the IKK subunit NEMO, and this may promote further IKK activation by TRAF6-dependent ubiquitination (Sun et al. 2004; Wu and Ashwell 2008). Additional BCL10 ubiquitination events may target BCL10 for lysosomal or proteasomal degradation upon its IKK mediated phosphorylation (Scharschmidt et al. 2004; Lobry et al. 2007). Together with CARMA1, BCL10 is required for the activation of the protease activity of MALT1 (Rebeaud et al. 2008), which contributes to NF-κB activation in an IKK independent manner.

1.2.2 CARMA2/CARD14 protein

Whereas CARMA1 and CARMA3 have been identified as crucial components of signal transduction pathways that lead to activation of NF-κB, little is known about the function of CARMA2/CARD14. Originally identified as a 1004 amino acid residues protein functioning as molecular scaffold for the assembly of multiprotein complexes at specialized regions of the plasma membrane, CARMA2 was shown to be able to activate NF-κB and to induce BCL10 phosphorylation. Recently, Scudiero et al. reported the existence of two different splice variants of CARMA2[12]. One variant contain the same translational start of full length CARMA (CARMA2FL) but includes an additional exon (alternative exon 15) containing an alternative stop codon, this transcript is predicted to encode for a polypeptide corresponding to amino acids 1-740 of CARMA2FL, and was named CARMA2short (CARMA2sh). The second variant mRNA skips exons 1-4, contains an alternative translational start codon in exon 5 and includes an additional exon containing an alternative stop codon. The CARMA2 isoform encoded by this splice variant lacks the CARD domain, includes amino acids Met238-Val618 of CARMA2FL, contains an unique carboxy terminus and was named CARMA2cardless (CARMA2cl) [12].

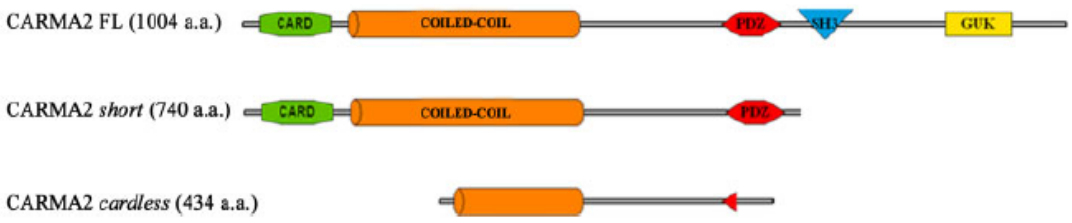

Figure 9. Schematic representation of CARMA2FL, CARMA2sh, and CARMA2cl isoforms.

The expression pattern of these two newly identified transcripts is different; in fact, while CARMA2sh appears to be expressed in various normal or tumor cell

lines, including human fetal brain and leukocytes, CARMA2cl appears to be expressed only in HeLa cells. This suggest that the functions performed by the corresponding polypeptides might be different, namely CARMA2sh would play a biological function common to many cell types, whereas the function of CARMA2cl may be more cell-type specific. In addition, the two isoforms behave differently compared to CARMA2FL. In fact, while CARMA2sh, similarly to CARMA2FL, retains the ability to activate NF-κB, CARMA2cl is unable to promote NF-κB activity, thus confirming that the N-terminal CARD domain of CARMA proteins is essential for NF-κB signaling. In addition, CARMA2cl, lacking the CARD domain, is also unable to establish homophylic molecular interactions with the other CARD-containing signaling proteins that generally act downstream of CARMAs, including BCL10.

Moreover, both CARMA2sh and CARMA2cl lack the SH3, PDZ and GuK domains, which are essential for membrane localization and lipid rafts recruitment of CARMA proteins. So, it is possible that these two newly identified splice variants may regulate signal transduction pathways of stimuli starting not from the cell membrane, but rather from intracellular compartments.

Regarding the functional activity of these two isoforms, the most evident biological aspect that they appear to affect is apoptosis induced by endoplasmic reticulum stress inducers (tunicamycin or thapsigargin) or translational inhibitors (anisomycin). In fact, ectopic expression of CARMA2sh and partially that of CARMA2cl, confers resistance to apoptosis induced by tunicamycin, thapsigargin or anisomycin in HEK293T and HeLa cells. Contrary, when CARMA2 expression is silenced by small interfering RNA (siRNA), apoptotic response is significantly increased compared to control siRNA.

The mechanism through which CARMA2sh protects cells from programmed cell death could obviously be related to the ability of this protein to induce activation of NF-κB transcription factor, which is well known to regulate transcription of anti-apoptotic genes, but it is evident that other protective mechanisms may exist, since even CARMA2cl partially protects from induced

apoptosis, although unable to activate NF-κB. Thus, one can assume that different CARMA2 isoforms might likely impact other functions of this protein, in addition to regulation of apoptosis and NF-κB activation [12].

1.2.3 MALT1 ubiquitination

MALT1 is a scaffold protein that activates the NF-κB-regulating IKK complex by physically bridging activated CARMA1 with BCL10 and TRAF6 (Thome 2008). The first evidence indicating a role for MALT1 in NF-κB activation came from two studies showing MALT1 and BCL10 synergistically activated NF-κB in 293T cell reporter assays (Uren et al. 2000; Lucas et al. 2001). Moreover, MALT1 plays an important pathogenic role in the development of specific types of B-cell lymphomas. The molecular mechanism engaged by MALT1 to control NF-κB activation in normal and malignant lymphocytes is thus a matter of considerable interest. Recent studies suggest that MALT1-dependent NF-κB activation is mediated by both scaffold and a protease functions (Thome 2008). The scaffold function of MALT1 depends upon its multiple protein-protein interaction domains (Uren et al. 2000; Lucas et al. 2001; Sun et al. 2004; Noels et al. 2007). MALT1 contains a central caspase-like domain, together with a death domain (DD), three immunoglobulin domains and a C-terminal extension. Interestingly, the MALT1 region C-terminal to its protease domain contains multiple ubiquitination sites that can be ubiquitinated by TRAF6 and thereby contribute to the physical recruitment of the IKK complex.

1.3 CARMA2 in psoriasis

CARMA2 has an important role for psoriasis development. Psoriasis is a chronic, inflammatory disease of the skin and other organs. It affects approximately 2% of individuals of European descent, and in up to 30% of cases, it is associated with chronic inflammatory psoriatic arthritis (see later). Jordan et al. identified rare, gain-of function mutations in this protein and 16 in two large multiplex families affected by Mendelian forms of psoriasis and psoriatic arthritis. These mutations are responsible for the elusive psoriasis susceptibility locus 2 (PSORS2) in chromosomal region 17q25[13].

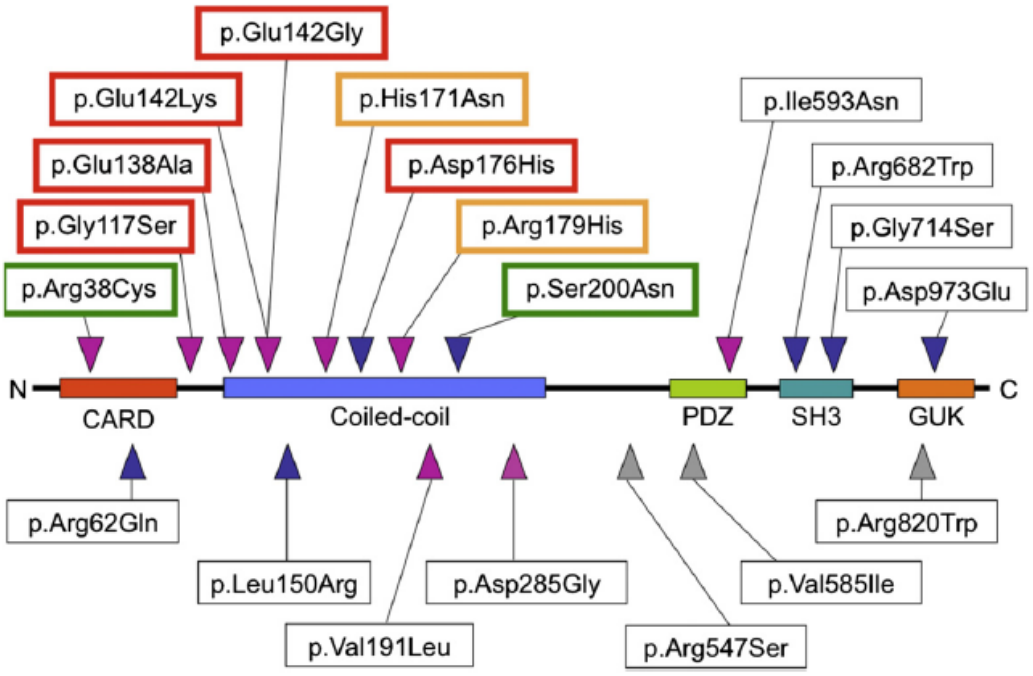

Figure 10. CARD14 Protein Domains and Locations of Amino Acid Substitutions.

Most of this pathogenic alterations were enriched in the coiled-coil domain of CARD14. This domain is predicted to be involved in the oligomerization of CARD14 with other proteins and the formation of its active conformation. Some coding polymorphisms in CARD14, rs11652075 (p.Arg820Trp), and c.599G>A

(p.Ser200Asn) were associated with psoriasis in several large cohorts. In the two largest psoriasis cohorts, evidence of association between psoriasis and rs11652075 increased when rs11652075 was conditioned on PSORS1. Two rare variants, c.424G>A (p.Glu142Lys) and c.425A>G (p.Glu142Gly), were identified in cases but not in controls and manifested as overtly causing of disease. Compared with wild-type CARD14sh, they significantly enhanced NF-κB activation (4.03- and 5.00-fold enhancement, respectively), and they clustered with p.G117Ser and p.Glu138Ala. The c.424G>A (p.Glu142Lys) variant was identified in a Caucasian male who was diagnosed with psoriasis at 42 years of age and who responded well to treatment with UV light and a topical mixture of corticosteroid and a vitamin D analog. The c.425A>G (p.Glu142Gly) variant was found in a Caucasian male who was diagnosed with psoriasis in infancy and whose father also had psoriasis. He showed a partial remission of psoriasis with methotrexate treatment. It is noteworthy that after these variants were stimulated with TNF-α, levels of NF-κB activation induced by these variants and the pustular-psoriasis substitution, p.Glu138Ala, decreased at the 24 hr mark. Both of these variants lie in the coiled-coil domain of CARD14, as does the de novo pustular-psoriasis substitution, p.Glu138Ala. Compared with wild-type CARD14sh, a third variant, p.Asp176His (rs144475004), leads to a little enhanced NF-κB activation.

So, it might lie below the NF-κB-activation threshold required for disease. Other variants such as p.Arg38Cys and p.Ser200Asn exhibited significantly less NF-κB activation than did wild-type CARD14sh. Previous studies have shown that decreased activation of NF-κB, much like increased activation, can induce inflammation and epidermal hyperplasia. It might be interesting to examine clinical features, such as inflammation after skin wounding, of individuals with these variants. However, it should be noted that the p.Arg38Cys and p.Ser200Asn substitutions did not induce expression of the pathogenic psoriasis signature when transfected into keratinocytes. Two variants, p.His171Asn and p.Arg179His, required stimulation with TNF-a to achieve maximal levels of

NF-κB activation. The p.Arg179His substitution was observed in two unrelated cases from Toronto and one control. The cases included a female who was diagnosed with psoriasis at 40 years of age and a male who was diagnosed with psoriasis at 64 years of age and who had a family history of psoriasis. The female responded well to oral and topical steroids, but the male was not treated. The p.His171Asn alteration was seen in two unrelated psoriasis- and psoriatic-arthritis-affected individuals from Newfoundland and was not seen in controls. One individual was diagnosed with psoriasis at 40 years of age and psoriatic arthritis at 41 years of age and had a family history of psoriasis. The second individual was diagnosed with psoriasis at 55 years of age after being diagnosed with psoriatic arthritis at 53 years of age. The identification of the c.511C>A (p.His171Asn) variant in only the Newfoundland population suggests that it arose as a result of a founder effect in this population.

The altered transcriptome signature with pathologic substitutions included upregulation of psoriasis-specific transcripts SOD2, IL6, CSF2, IL8, MMP9, BRF2, CCL20, SLC7A2, OLR1, IL36G, GBP2, TNFAIP2, and TNF. Expression of these molecules is expected to be an early event in the pathogenesis of psoriasis. Many of these genes have been implicated in immune-system development. However, BRF2 is implicated in the development of squamous cell carcinoma of the lung. 3 This suggests that it might have global effects on the transcriptional profile of squamous cell epithelia in general and might help elicit a wound-healing or regenerative response in psoriatic keratinocytes. Despite the dramatic effects of some CARD14 variants as keratinocyte transfectants, there was a wide range of phenotypes, even among individuals who carried the same substitution. This suggests that in many instances, the variable phenotypes are likely to be due to genetic background and/or environmental factors. Similarly, age of onset and response to treatment differed among individuals with the putative pathogenic variants from the coiled-coil domain (p.Glu142Lys, p.Glu142Gly, and p.Glu138Ala).

However, there might be some genotype-phenotype correlations because the pustular-psoriasis substitution, p.Glu138Ala, led to the most severe phenotype (in terms of both clinical presentation and increased NF-κB activation) relative to that produced by wild-type CARD14sh. The child with this alteration presented with a spectrum of plaque-type lesions, but she mostly presented with pustular lesions. This implies that some forms of plaque psoriasis might be pathogenetically linked to pustular psoriasis at the severe end of the disease spectrum. The child's lesions also exhibited a pronounced infiltration of neutrophils. The observation that the p.Glu138Ala alteration led to the most severe clinical phenotype and induced the greatest increase in NF-κB activation and upregulation of psoriasis-associated transcripts suggests that the phenotype of psoriasis could, in some cases, be predicted by the detection of pathogenically increased levels of NF-κB activation and signaling. Even CARD14 missense variants such as rs11652075 c.2458C>T (p.Arg820Trp) and c.599G>A (p.Ser200Asn) were associated with psoriasis in cohorts of European ancestry. More exactly, in the Asian cohort, the c.2458T>C polymorphism was also associated with psoriasis, while c.599G was monomorphic [14].

2. Aim of the work

As described previously, NF-κB is an important transcription factor with pleiotropic action, whose activity can be induced by a wide variety of stimuli and is finely regulated by several mechanisms of signal transduction.

In fact, several stimuli can induce the activation of NF-κB, such as virus infection, cytokines, bacterial lipopolysaccharide, TCR and BCR binding-antigens, X- and UV-ray, nitric oxide and DNA damage. The obvious consequence is that a huge number of physiological processes, from lymphocytes proliferation and differentiation to cytokines production or cell cycle regulation and even apoptosis, can be regulated by the activity of this transcription factor, that promotes (or represses) expression of many different genes. For these reasons, NF-κB plays a pivotal role in the maintenance of the correct homeostasis of human organism. In fact, deregulation of this transcriptional activity has been shown to be involved in many important diseases, from asthma to other chronic inflammatory diseases, such as Crohn's disease or ulcerative colitis, or autoimmune diseases, such as rheumatoid arthritis.

In addition to the roles that NF-κB plays in inflammatory diseases, constitutive activation of the NF-κB pathway is involved in some forms of cancer such as leukemia, lymphoma, colon cancer and ovarian cancer. Mutations that can lead to such tumors include those that inactivate IκB proteins as well as amplifications and rearrangements of genes encoding the NF-κB transcription factor subunits. However, more commonly it is thought that changes in the upstream pathways that lead to NF-κB activation become deregulated in cancer. Consequently, it is easy to understand why unraveling the intricate mechanisms that govern activation and function of NF-κB transcription factor is a desirable goal in the scientific world. Moreover, recent studies show the role of NF-κB in

the development of psoriasis. More exactly it triggers a skin inflammation due to the presence of T cells in the skin and activation of immune-related pathways. As previously said, mutation of CARMA2 protein leads to psoriasis so, aim of this work was to identify and characterize new molecular partners of CARMA2, in order to put at least one of the missing pieces in the puzzle that constitute CBM complex mediated NF-κB signaling activation pathways. Also it would be interesting to understand how this protein is involved in the psoriasis. To this aim, in our laboratory were performed two screenings, using the two-hybrid system technology, using CARMA2 as bait. In fact, identification of new molecular partners of CARMA2 could shed light on the mechanism of action and the biological role of this protein, and to verify, also, whether CARMA2 has a peculiar activity in the cell or if it shares some redundant functions, although tissue-specific, with other members of the CARMA family of scaffold proteins.

3. Materials and Methods

Two-hybrid screening

The two-hybrid screening system was used to identify new molecular interactors of CARMA2. In this screening, the first 300 amino acids of CARMA2, i.e. a peptide containing the CARD domain and a part of the coiled coil domain, were used as bait to screen a Peripheral Blood Lymphocytes (PBL) cDNA library cloned in the pGAD10 vector. The screenings were conducted using the MATCMAKER GAL4 Two-Hybrid Sistem 3 provided from Clontech and the mutant AH109 *S.Cerevisiae* yeast strain as host. All the procedures described were performed in sterile conditions and with sterile materials and reagents.

Baits preparation

The cDNA coding for the baits was amplified through PCR reactions using primers containing the restriction sites for the enzymes EcoRI and XhoI (NEB). cDNA fragments were cloned in the shuttle vector pGBKT7 (Clontech), previously digested with EcoRI and XhoI, dephosphorylated at the 5'-end with treatment with CIP (Calf Intestinal Phosphatase, NEB) and purified with phenol-chloroform. The DNA ligation reactions were performed using the T4 DNA ligase (NEB), at 16°C for 4 h in a 10µl total volume. Control reactions were performed in the same manner without adding cDNA fragments (adding, instead, water). 5µl of sample and control reactions were transformed in *E.Coli* DH5α competent cells, plated on selective LB medium containing kanamycin and grown O.N. at 37°C. Colonies were screened for fragments insertion through digestion with cloning enzymes on the purified cDNA.

Fusion libraries preparation

Library was purchased from Clontech transformed in *E.Coli*. Library was amplified through growth on selective ampicillin-containing medium. Colonies were collected, concentrated on a tube bottom and lysed with an hypoosmotic solution. Nucleic acids were, then, precipitated in dry ice with isopropanol and at -80°C with 96% EtOH and NaAc 3M. RNA was removed with Lithium Chloride and degraded through treatment with ribonucleases at 30°C for 30 min. Proteins were removed through phenol-chloroform extractions and a wash with chloroform. Purified plasmids were resuspended with water.

Baits transformation

The *S. Cerevisiae* mutant yeast strain AH109 was plated on a rich solid medium (YPD) added with adenine and leaved at 30°C for some days until colonies appeared. One colony was collected and cells were resuspended in a tube with 11 ml of liquid YPD+ADE. 1ml of this suspension was mixed in a tube with 9 ml of fresh YPD+ADE. Both tubes were incubated O.N. at 30°C with shaking at 150 rpm. The next morning, cells from the diluted inoculum were collected through centrifugation and washed twice with sterile water. The pellet was resuspended in a mix of 400µl water, 50µl LiAc (stock 10X; 1M, pH 7.5) and 50µl Tris-EDTA (TE, stock 10X, pH 7.5). 50µl of this solution were mixed with 5µl of DNA carrier and 4µl of library DNA. Then, 300µl of a solution made up of 1,2ml 50% PEG, 150µl LiAc and 150µl TE were added. The resulting solution was incubated at 30°C for 30 min at 150rpm. Then, 35µl of DMSO were added to the solution and the resulting mix was incubated at 42°C in a water bath for 15 min. After that, yeast cells were collected through centrifugation, resuspended with 100µl of water and plated on a minimal medium (SD) added with adenine, histidine and leucine. Medium lacked tryptophan that was used as selective marker for transformation.

Library transformation

One of the yeast colonies positive for transformation with bait (i.e. pGBKT7 vector), was collected and resuspended in 100 ml of SD added with 1 ml His 100x, 1 ml Leu100x and 2 ml Ade100x and incubated O.N. at 30°C with shaking at 250rpm. Next morning, the inoculums was added with 1liter of YPD+ADE. Yeast cells were grown at 30°C for 4 h at 250rpm. Cells were collected through centrifugation and washed once with 20 ml of water. The pellet was resuspended with 4 ml of water and added mixed to LiAc and TE to a final volume of 5 ml and transferred in a flask. To this solution were added 1,5 ml DNA carrier, 400/800 µg library cDNA and 40 ml of a solution made up of 32 ml 50% PEG, 4 ml LiAc and 4 ml TE. The resulting solution was incubated at 30°C for 45 min with mild shaking. Then, the solution was added with 5 ml of DMSO and incubated at 42°C in a water bath for 15 min. Cells were collected and plated on SD without adding amino acids or nucleotides in order to select cells that were positive for bait-prey interactions in which the transcriptional activity restored. Plates were incubated at 30°C until colonies appeared.

DNA extraction from yeast cells

Colonies that grew on minimal SD were positive for both transformations. They acquired the ability of grow in absence of tryptophan and leucine as they carried both pGBKT7 and pACT2 (or pGAD10). In addition, in these colonies, restoring of transcriptional activity of GAL4, conferred the ability to grow in absence of histidine and adenine, thanks to transcription of reporter genes. Positive colonies were re-streaked on fresh SD plates and incubate at 30°C in order to eliminate some false positive and amplify the positive clones. Obtained colonies were, then, dissolved in 500µl of H2O and then lysed with lyticase from Arthrobacter Luteus (sigma) incubated 30 minutes at 37°C. Subsequently glass beads were added to allow a mechanic disruption of yeast and incubated

10 minutes at 95°C. The samples obtained were used for pcr. The primer used were:
Fwd CTATTCGATGATGAAGATACCCCACCAAACC
Rev GTGAACTTGCGGGGTTTTTCAGTATCTACGATT

The PCR products were sequenced and analysed using BLAST algorithm at the NCBI web site. The ones with a correct frame were digested and cloned in expression vectors.

In vitro assays

Cell Culture

The mammalian cells used in this work were HEK293, HeLa Jurkat murine embryonic fibroblast (MEF) cells. These cells were cultured, respectively, in Dulbecco's modified Eagle's (HEK293 and HACAT) medium supplemented with 10% FBS (Fetal Bovine Serum) and antibiotics (penicillin-streptomycin). NHEK cells were grown in 5% CO2 at 37°C in defined Keratinocyte Growth Medium (KGM) (LONZA) containing bovine pituitary extract (BPE), human epidermal growth factor (hEGF), insulin, hydrocortisone, transferrin, epinephrine, and antibiotic, GA-1000, at proprietary concentrations as determined by the manufacturer.

Calcium-Phosphate Transfection Protocol

HEK293 were transfected by calcium phosphate precipitation, a chemical-based transfection. Cell culture was grown in a monolayer until a 30-40% of confluence before transfection. Calcium-phosphate co-precipitate changed in base of cells number. For a single sample in a 6-well plate the specific amount of DNA was dissolved in 220 µl of sterile distilled water, then, 250 µl of 2x

Hepes-Buffered Saline (HBS) was added to the mixture at room temperature. 30 µl of Calcium Chloride 2M was added dropwise, swirling gently to mix well. After 20 minutes of incubation at room temperature, precipitates were added to cells, that were subsequently incubate for 18-24 hours at 37 °C in a humidified incubator in an atmosphere of 5% CO_2.
For 10 mm plates the procedure is equal but change the precipitation reaction mixture that has been doubled in volume.

Co-Immunoprecipitation assays

For co-immunoprecipitation experiments, transfected cells were lysed in lysis buffer and immunocomplexes to ProteinA/G (agarose beads) bound to the antibody overnight at 4°C. Then, the samples are washed with lysis buffer without inhibitor, boiled and resolved by SDS/PAGE and analysed by immunoblot assay.

Protein Extraction and Western Blot

About 24h after transfection or plating, cells were washed in PBS (Phosphate Buffered Saline), to remove exceeding medium, scraped and centrifuged at 6000 rpm for 5 minutes at 4°C. Then, cellular pellet was resuspended in Lysis Buffer (150 mM NaCl, 20 mM Hepes, pH 7.4, 1% Triton X-100, 10% glycerol and a mixture of protease inhibitors). After 30 minutes of lysis in ice, samples were centrifuged at 13000 rpm for 5 minutes to separate insoluble fraction, containing membranes and cellular organelle, from soluble fraction. This fraction was transferred into clean tubes and mixed with Sample Buffer (250 mM Tris-Hcl, pH 6.8; 40% Glycerol; 8% SDS; 20% β-mercaptoethanol; 0,01% bromophenol blue), boiled at 95°C for 5 minutes and resolved by SDS-PAGE.

Proteins separated by SDS-PAGE, were transferred onto nitrocellulose membrane, and incubated with primary antibodies (O.N. at 4°C) followed by horseradish peroxidase-conjugated secondary antibodies incubation (1h at R.T.) (Amersham BioSciences). Blots were developed using the ECL system (Amersham BioSciences).

Luciferase Assay

The luciferase assay has been used for measuring the changes in the NF-κB activation in different condition. For this assay a reporter gene has been used. Reporter genes have their protein product that can be directly or indirectly quantified. In this case, the luciferase system has been used. The luciferase is a small firefly protein that splits its substrate, the luciferin through a ATP-dependent reaction. This reaction generates light at 562 nm. Luciferin belongs to a class of light-emitting biological pigments, found in several organisms, that cause bioluminescence (firefly, dinophlagellate, snail, bacteria). The light intensity can be measured by the luminometer. For this assay, the cells have been co-transfected with a plasmid DNA containing the construct and with a vector containing the luciferase reporter gene under the control of a NF-κB responsive promoter (pNF-κB-luc, CLONTECH). The level of luciferase expressed depend on the capacity of the construct to activate NF-κB. For the normalization of the measured values than the transfection efficiency, the RSV-βGal has been transfected. The β-galactosidase is constitutively synthesized in the transfected cells because it is under the control of a strong promoter. So the reading of the light emitted by splitting of its substrate (galacton), indicates the transfection efficiency After 24h from transfection, the cells have been washed with PBS, centrifuged and lysed with Reporter Lysis Buffer (Promega). Then, pre soluble fractions have been red at the luminometer for the β-galactosidase and luciferin. The luciferase values have been normalized with respect to the transfection efficiency (β-gal values) and used for the average calculation.

Plasmids

Constructs used for transfection experiments were cDNA or portions of them cloned in the mammalian expression vector pCDNA4 HisMax with FLAG or HA epitope. Cloning of gene was performed using correct restriction sites and enzymes, controlling the reading frame of DNA sequence. Constructs were amplified in bacteria cells (E.Coli, DH5α) and purified using the GeneElute Maxiprep Kit provided from Sigma-Aldrich. Constructs used for the two-hybrid screening were cloned in pGBKT7 and pACT2 (or pGAD10 or pGADT7rec) expression vector, as provided from manufacturer.

Antibodies

Primary antibodies recognizing the FLAG or HA epitopes were provided from Sigma-Aldrich. Primary antibody directed against RNF7 was provided from ProteinTech.

Delete Mutants

The truncated forms of RNF7 and CARMA2 were generated by PCR. Oligonucleotides used for amplification were designed on the full-length sequences of proteins and restriction sites were added at 5’ or 3’ of oligos. PCR products were cloned in the pCDNA4 HisMax expression vector using correct restriction enzymes and site and controlling the reading frame of the DNA sequence.

RING-dead mutant

Four oligos partially overlapping in the point of mutation was designed. The RNF7 cDNA cloned in pCDNA4 HisMax and resuspended in a solution containing water, Klenow Buffer (NEB) and the four specific oligos were

denaturated at 100°C for 10 min. Then sample was incubated in ice for about 10 min to allow DNA annealing. 1µl of Klenow polymerase (NEB) was added and the polymerase reaction was performed at 37°C O.N. Klenow polymerase activity was stopped with incubation at 75°C for 20 min. DNA was digested with DpnI restriction enzyme (NEB) to eliminate template DNA. DNA was purified through phenol-cloroform extraction and precipitated with NaAc 3M and 96%EtOH at -80°C. DNA was resuspended with 10µl water and transformed in *E.Coli* DH5α cells. positive colonies were amplified and plasmidic DNA was extracted and analyzed for the mutation insertion through sequencing.

Real-time PCR

Total RNA was isolated from cells or tissues using TRIzol reagent (Invitrogen). Expand High Fidelity PCR system (Roche) was used to amplify RNF7. The reverse transcriptase reaction was performed using 1 µg of total RNA in a 20 µl reaction and 1 µl of the resulting cDNA was used in the subsequent amplification step along with 300 nM of each primer. The following oligos were utilized to amplify RNF7: *fwd*: 5'-TCAAGCTGAAAACAAACAAGAGG-3' and *reverse* 5'-GTAGGGCACTGGATTACAAGATC-3'. The relative transcription level was calculated by using the ΔΔCt method. Real-time PCR reactions were performed in triplicate by using the SYBR Green PCR Master Mix (Qiagen) in a 7900HT sequence-detection system (Applied Biosystems).

RNA interference

The shRNA (pLKO vectors) directed against RNF7 was from Sigma Aldrich and used as recommended by the manufacturer.

4. Results

In order to identify new molecular interactors of CARMA2, we performed the yeast two hybrid assay. More exactly we used the N-terminal domain of CARMA2sh (CARMA2 1-300, containing the CARD domain and a part of the coiled coil domain) as bait to screen a human peripheral blood lymphocytes cDNA library. This screening led to the identification of several positive clones, few of which were in the correct reading frame or presumably interesting for our field of investigation. Among these, a cDNA coding for the human RING finger protein 7 drew our attention.

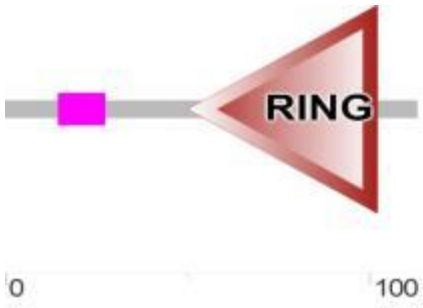

Figure 11. RNF7 molecular structure

RNF7 (RING finger protein-7), also known as RBX2 (RING box protein-2), ROC2 (Regulator of cullins-2), or SAG (Sensitive to Apoptosis Gene) belongs to an evolutionarily conserved gene family with 96% sequence identity between human and mouse and 55% between human and yeast [15]. RNF7 is expressed ubiquitously in human tissues with a very high expression in heart, skeletal muscle, and testis, three organs with high levels of oxygen consumption. At the subcellular level, RNF7 is expressed in both cytoplasm and nucleus [16]. Human *RNF7* is mapped onto chromosome 3q22–24 with three splicing variants and two family pseudogenes [17], whereas mouse *RNF7* was mapped onto chromosome 9 with three exons and two introns [17]. Structurally, both human and mouse *RNF7* encode a protein of 113

amino acids, of which 12 are cysteine residues [16]. At the carboxyl portion of RNF7 protein, there is a $C_3H_2C_3$ motif, which chelates two zinc atoms to form the RING domain (Fig. 7), a characteristic of a domain with E3 ubiquitin ligase activity [15].

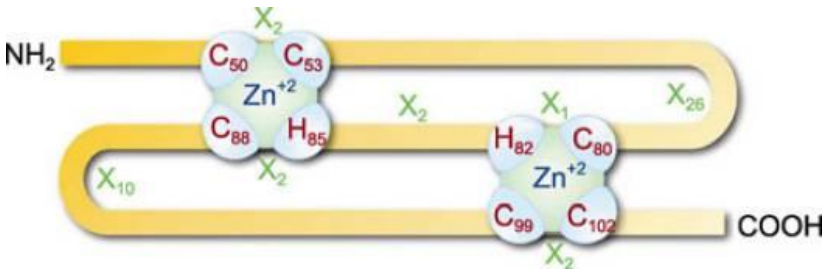

Fig.12 A schematic presentation of the RING finger domain on RNF7
Shown is the C3H2C3 RING finger domain that binds to two zinc atoms in a cross-braced arrangement with the codon number of each cysteine or histidine residue indicated. The X stands for any amino acid, whereas the numeric number stands for the number of amino acids between two cysteine or histidine residues.

Initially, the cDNA coding for RNF7 was cloned in pcDNA3 expression vector and transfected in HEK 293 cells to assess the expression. Next we confirmed the interaction with CARMA2 through a coimmunoprecipitation assay. To this aim we transfected RNF7 fused with HA epitope in HEK293 cells with plasmids expressing the short form of CARMA2 fused with FLAG epitope. Cell Lysates

were mixed to agarose beads conjugated with an antibody against the HA epitope (fig.13).

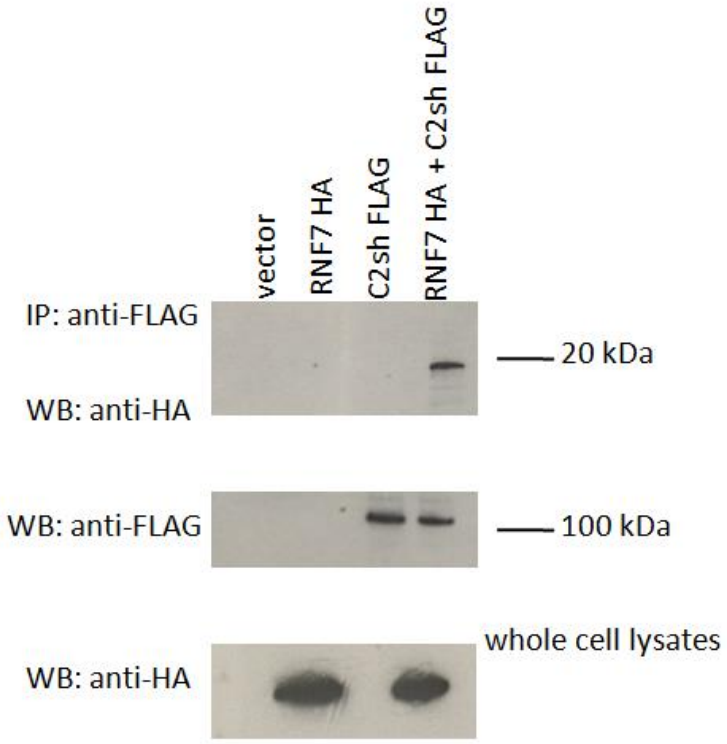

Fig.13 RNF7 binds to CARMA2 HEK293 cells were co-transfected with RNF7-HA and CARMA2-FLAG. Cell lysates were immunoprecipitated (*IP*) with anti-FLAG antibody and analysed by immunoblot probed with anti-HA for RNF7. C2 has a molecular weight of about 95 kDa while RNF7 is about 17 kDa.

Then we performed a luciferase assay, a functional assay that allows to understand the role of RNF7 on NF-κB activation. More exactly, it gives a quantitative measure of the variations in NF-κB activation due to overexpression of RNF7, an enzymatic assay with reporter gene was used. In this case, we used the luciferase system, a firefly protein that cleaves its substrate, luciferin, in a ATP-dependent reaction, producing light at 562 nm. Intensity of emitted light can be easily measured by an appropriate instrument, the luminometer. Because we wanted to evaluate the activity of a transcription factor, cells were co-transfected with the plasmidic DNA having the construct of interest and with a vector containing the luciferase reporter gene under the

control of a promoter responsive to NF-κB (pNF-κB-luc, CLONTECH). The more the construct of interest is able to activate NF-κB, the more luciferase is expressed and the stronger is the intensity of emitted light. In addition, for every experimental point, also the RSV-βGal DNA construct was transfected to normalize activation values measured respect to transfection efficiency. β-galactosidase is constitutively synthesized in transfected cells as it is under the control of a strong promoter (RSV); so, measure of emitted light following cleaving of its substrate (Galacton) indicates the efficiency of transfection. It's clear that CARMA2sh is a NF-κB activator and RNF7 is not, but, when they are transfected together there is a NF-κB inhibition while this not happens for CARMA2sh mutants (fig.14). More interestingly the effect of RNF7 on CARMA2sh is dose dependent (fig.15)

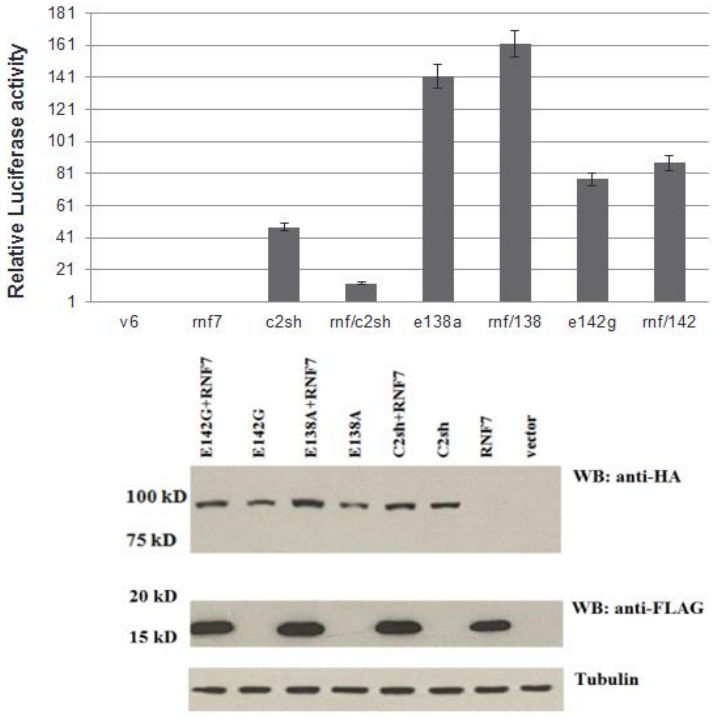

Fig.14 RNF7 dampens the activation of NF-κB mediated by CARMA2sh All the reported results and graphs are representative of at least three independent experiments.

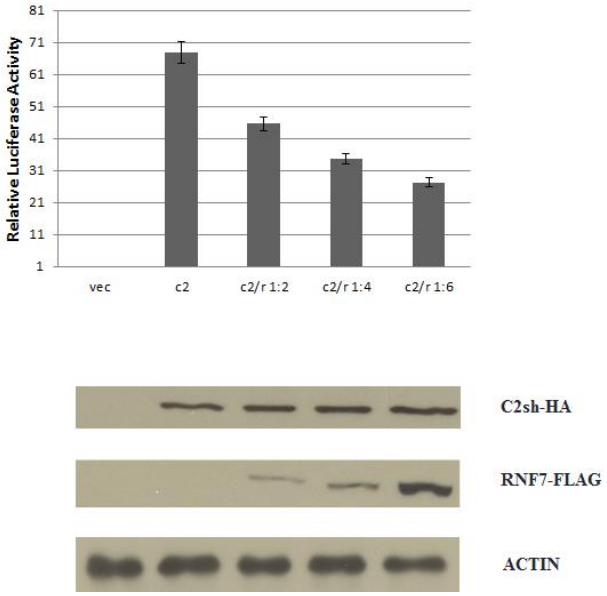

Fig 15. The effect of RNF7 is dose dependent HEK293 were transfected with CARMA2sh and crescent dose of RNF7.

We then investigated on the minimal interaction region between RNF7 and CARMA2sh. So, we co-transfected CARMA2sh domains HA-tagged (CARD, Coiled Coil and PDZ) with RNF7 FLAG-tagged. We immunoprecipitated HA-tag and immunoblot for FLAG-tag and we found that RNF7 is able to bind CARD and PDZ domains of CARMA2sh but not its coiled coil (fig.16). Then we cloned in pcDNA3 just the RING domain of RNF7 and performed the same experiment. What we found is that RING domain is able to bind CARMA2sh but we were not able to see any interaction with its domains alone (fig.17).

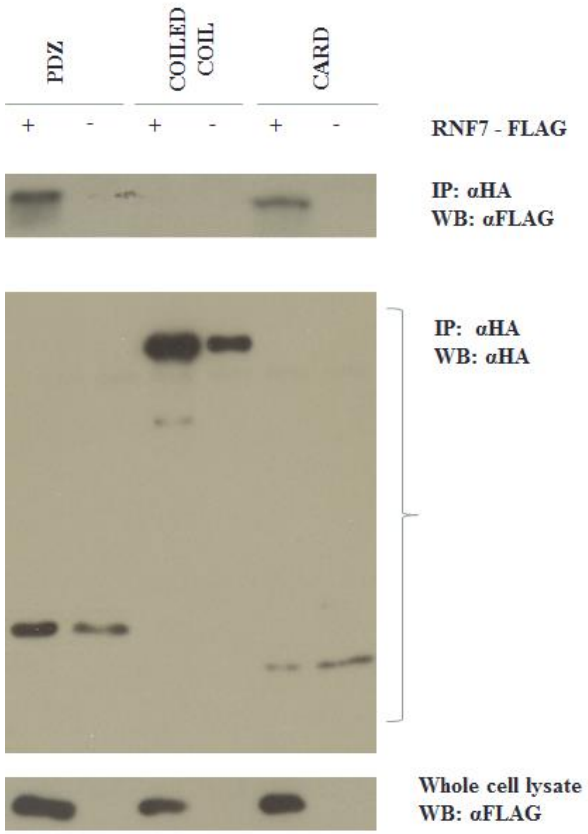

Fig 16. RNF7 interacts with CARD and PDZ domains of CARMA2sh HEK293 were transfected with CARMA2sh domains HA-tagged and with or without RNF7 FLAG-tagged. Lysates were then mixed with protein G beads and anti-HA mouse antibody.

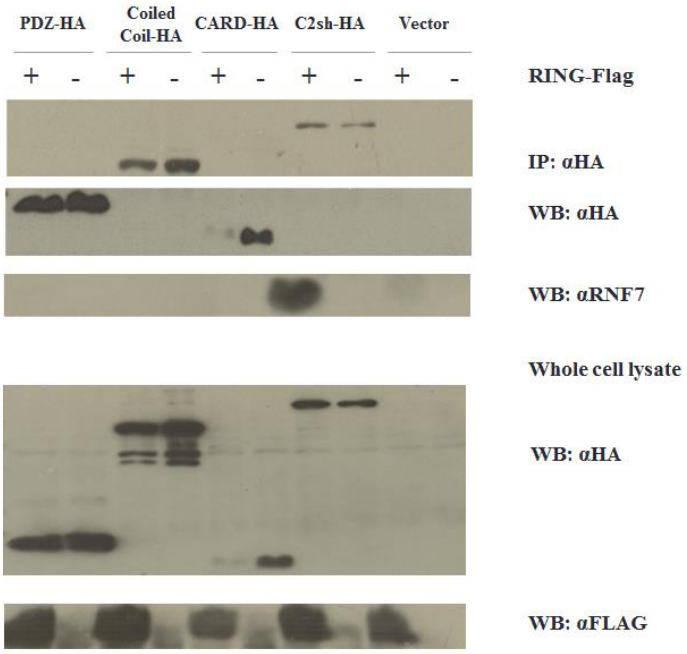

Fig 17. RING domain of RNF7 is able to interacts with CARMA2sh but not its domains alone HEK293 were transfected with CARMA2sh domains HA-tagged and with or without RING domain of RNF7 FLAG-tagged. Lysates were then mixed with protein G beads and anti-HA mouse antibody.

Since CARMA2 belongs to the CARMA family of proteins together with CARMA1 and CARMA3 we wanted to verify whether RNF7 effect is specific for CARMA2sh or it affects also the other two members of this family. So we co-transfected HEK293 cells with CARD domains of CARMA1, CARMA2 and CARMA3, together with RNF7. We found that the inhibitory effect of RNF7 affects all three members of this family of proteins [fig.18].

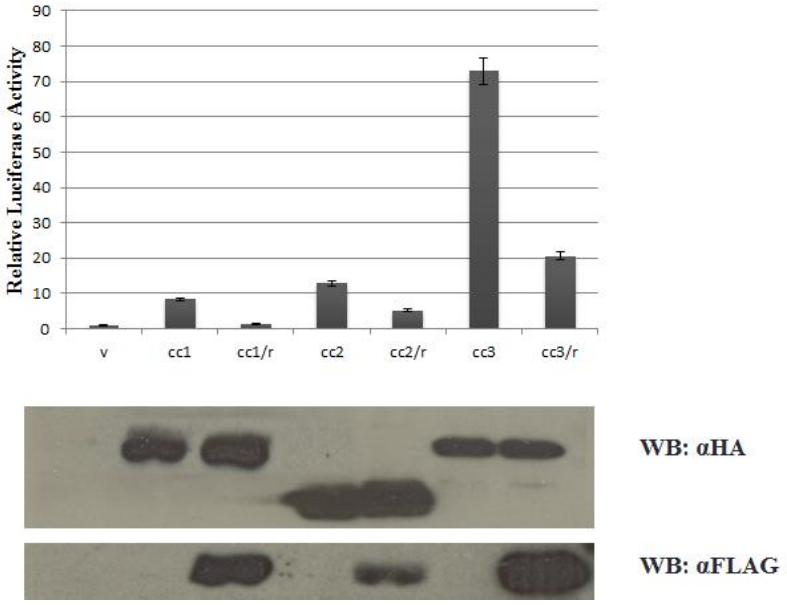

Fig. 18 Effect of RNF7 on CARMA family of protein members HEK293 were transfected with CARD domain of CARMA1, CARMA2 and CARMA3 together with RNF7 and construct vectors for luciferase assay.

Next, we examined whether RNF7 could lead to the degradation of some proteins of this pathway but we found nothing (data not shown). So, we just focused on the ubiquitination status of BCL10, MALT1 and NEMO.

We transfected RNF7-FLAG and CARMA2sh alone and together and we immunoprecipitated for endogenous BCL10, MALT1 and NEMO. Regarding BCL10, RNF7 do not decrease the ubiquitination caused by CARMA2sh (fig. 19).

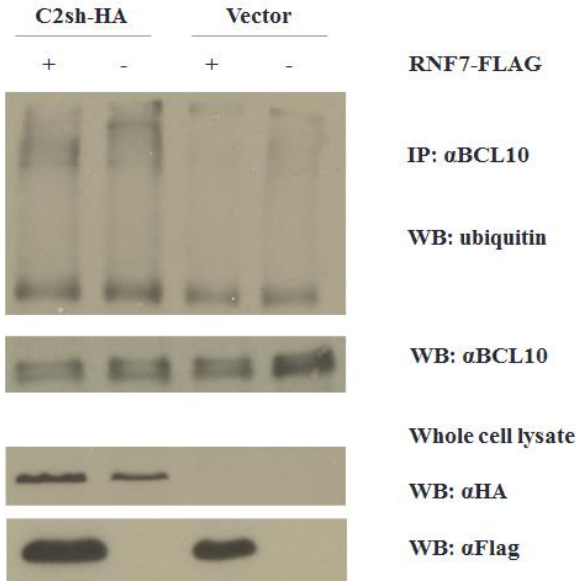

Fig. 19. BCL10 ubiquitination HEK293 were transfected with Ubiquitin, CARMA2sh HA-tagged and with or without RNF7 FLAG-tagged. Lysates were then mixed with protein G beads and anti-BCL10 antibody. We then immunoblotted for anti-p4D1 (ubiquitin) antibody.

Things change for MALT1 and NEMO. In the presence of both CARMA2sh and RNF7 the ubiquitination of MALT1 and NEMO decrease respect to that of CARMA2sh alone (fig. 20-21). Moreover, RNF7 is not able to dampen the ubiquitination status of NEMO mediated by mutants of CARMA2sh. More interestingly, it seems that CARMA2sh is unable to bind to NEMO in the presence of RNF7.

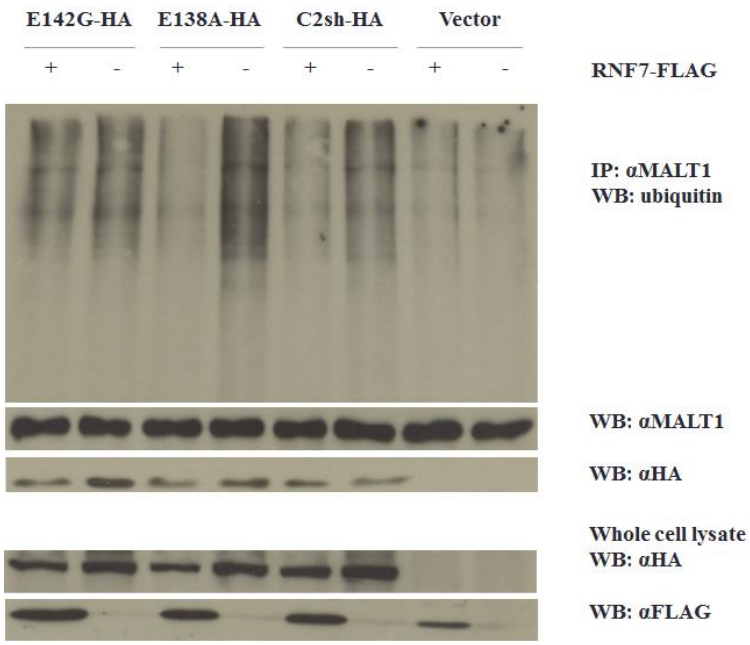

Fig. 20 MALT1 ubiquitination HEK293 were transfected with ubiquitin and CARMA2sh HA-tagged with or without RNF7 FLAG-tagged. Lysates were then mixed with protein G beads and anti-MALT1 mouse antibody.

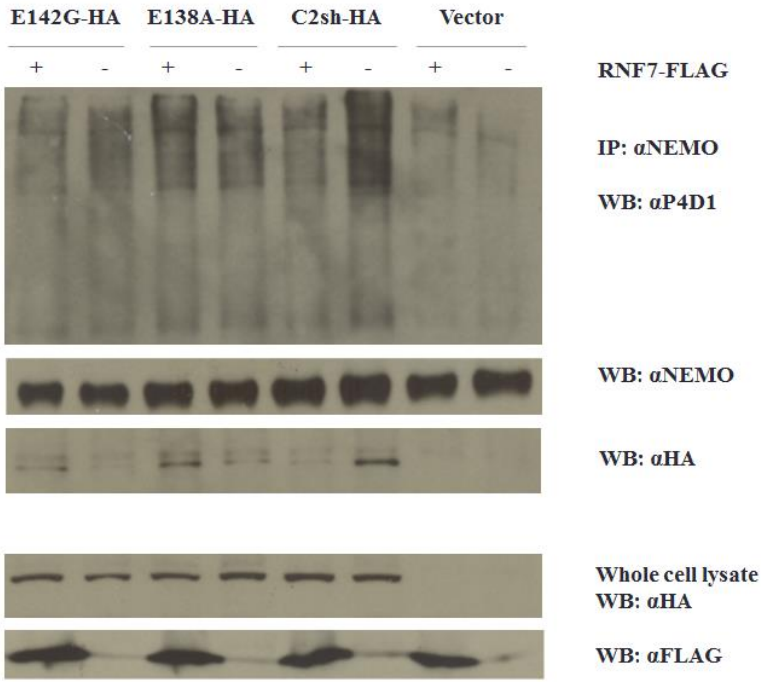

Fig. 21 NEMO ubiquitination HEK293 were transfected with Ubiquitin and CARMA2sh HA-tagged with or without RNF7 FLAG-tagged. Lysates were then mixed with protein G beads and anti-BCL10 antibody. We then immunoblotted for anti-p4D1 (ubiquitin) antibody.

To state whether the RING domain is involved in this form of regulation of RNF7 on CARMA2sh we made a dead-RING mutant of RNF7 into which the RING domain is disrupted by changing 2 cysteines (C99 and C102) into serine [18]. Then we transfected this mutant into HEK293 with CARMA2sh and immunoprecipitated lysates for NEMO. What we observed was the same effect of RNF7 WT (fig. 22).

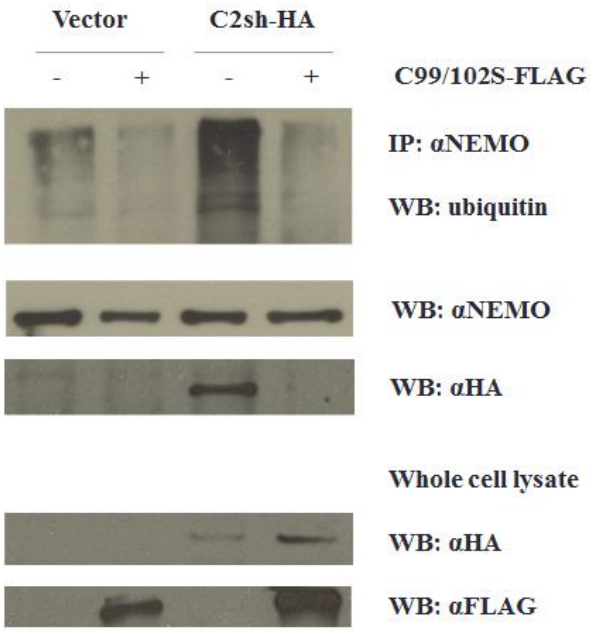

Fig. 22 Ubiquitination of NEMO in presence of RNF7 RING mutant HEK293 were transfected with Ubiquitin and CARMA2sh HA-tagged with or without RNF7 mutant FLAG-tagged. Lysates were then mixed with protein A beads and anti-NEMO antibody. We then immunoblotted for anti-p4D1 (ubiquitin) antibody.

We confirmed this data also by luciferase assay (fig. 23).

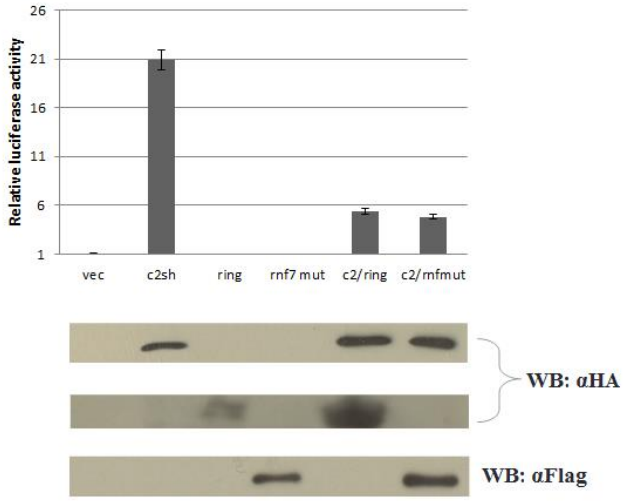

Fig. 23 Luciferase assay with RNF7 mutant and RING domain HEK293 were transfected with pNF-κB-luc the RSV-βGal and CARMA2sh HA-tagged with RNF7 mutant FLAG-tagged or RING domain HA- tagged.

After these preliminary studies directed to characterize RNF7 and its relationship with CARMA2, we focused our attention on the role of this protein could have in psoriasis disease.

We used human immortal keratinocyte and primary keratinocytes from child foreskin (NHEK). We infected these cells with lentiviral constructs coding for RNF7. After the infection next, we stimulated these cells with PESC (PBS, E.Coli, S.Aureus, C.Valida) and IL-1b alone for 12h to evaluate the inflammatory cytokine and chemokine levels. Then we extracted the mRNA from these cells and performed a qRT-PCR. Before to perform this quantitative PCR, we tested several housekeeping genes and we used the more stable one as normalizer. For our experiments we used the Actin, while the genes analyzed are CCL20, CCL5, IL-6, IL-8, HIF1a.

Hacat infected with lentiviral vector expressing for RNF7 and treated with stimuli show a decreased expression level for genes analyzed (fig. 24). Moreover treated controls show an increased expression level of RNF7 (fig 25).

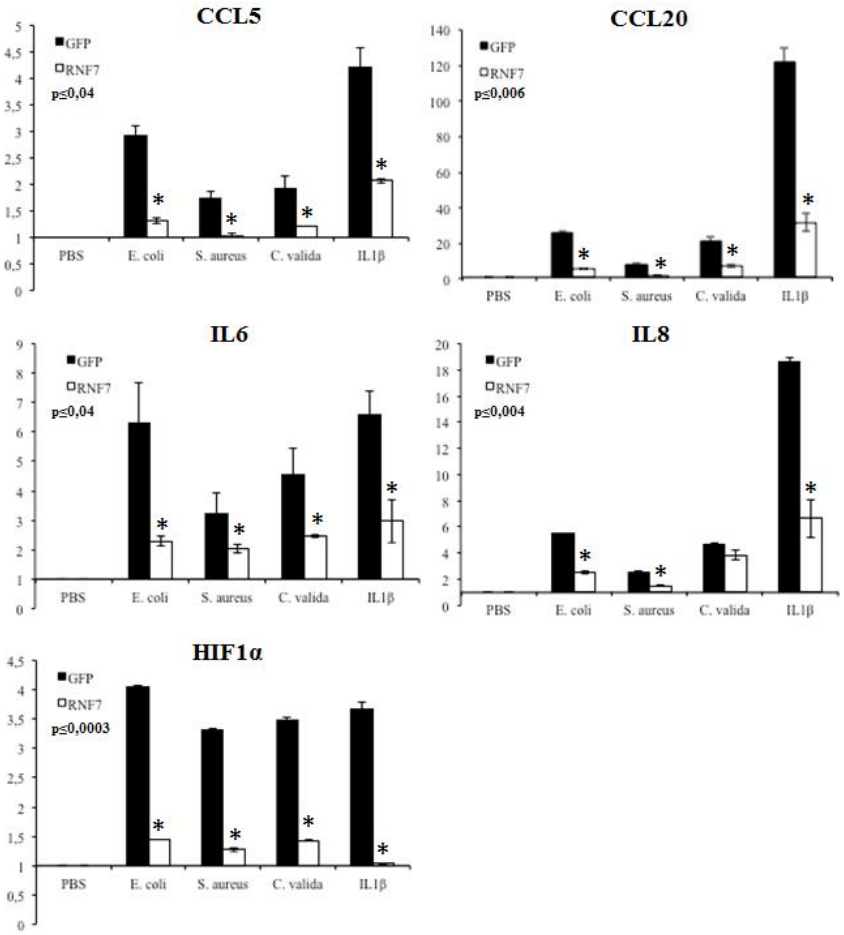

Fig. 24 Expression level of cytokines and chemokines in Hacat HACAT cells were infected with lentiviral vector expressing for RNF7 and GFP. Cells were then treated with stimuli and mRNA was extracted after 12h. These results are representative of three different experiments.

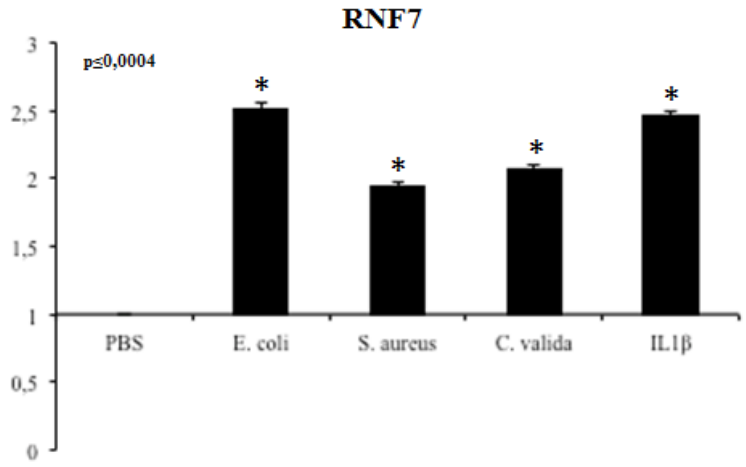

Fig. 25 RNF7 expression in treated Hacat

We then performed the same experiment in NHEK by interfering RNF7 (fig.26). As we expected we noticed increased expression of analysed genes in treated NHEK that don't express RNF7 anymore (fig. 27).

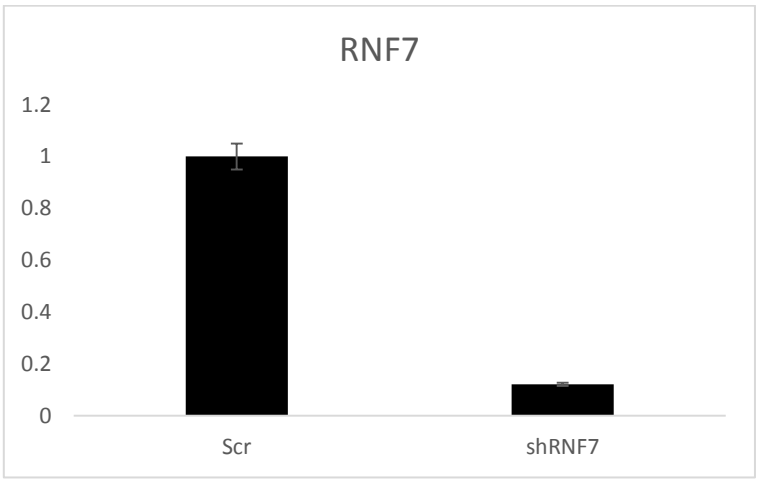

Fig. 26 Reduction of the RNF7 expression level in NHEK infected with the shRNA

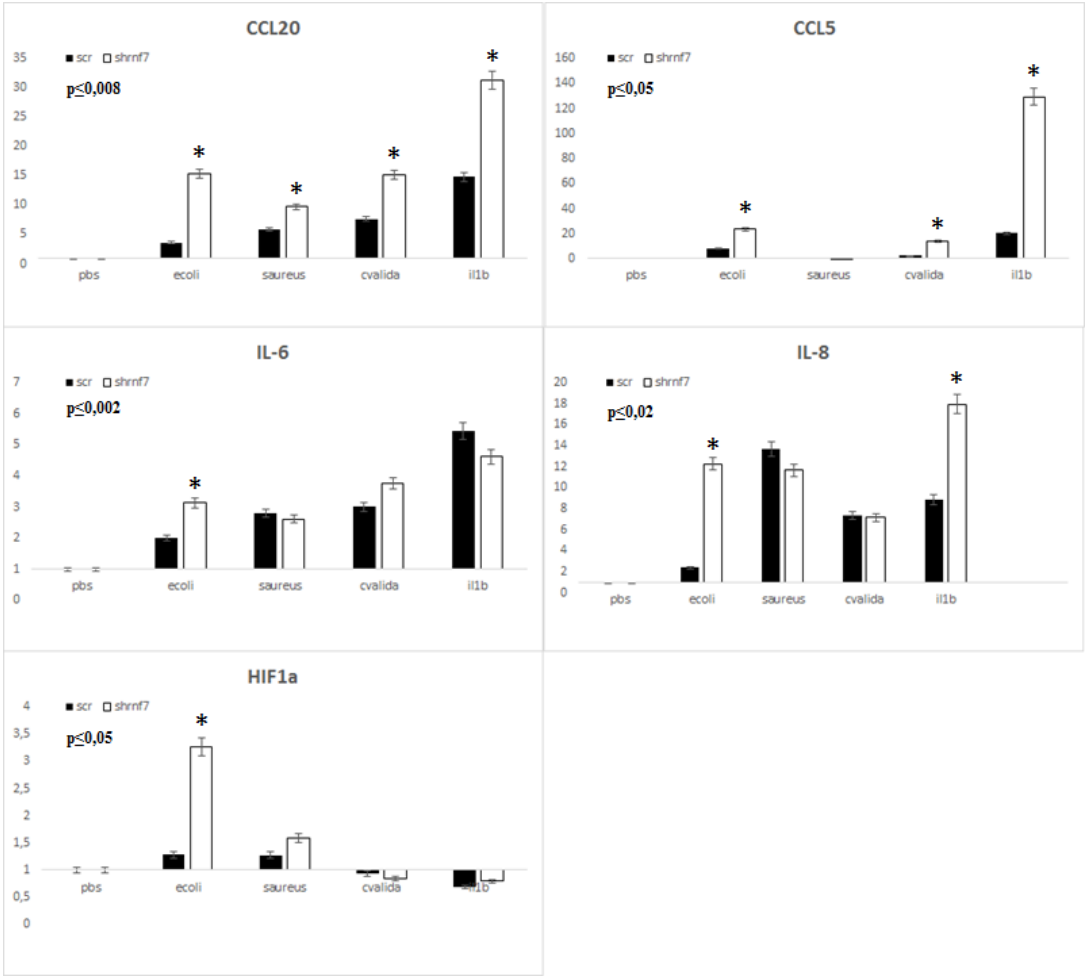

Fig. 27 qRT-PCR on treated NHEK interfered for RNF7 NHEK cells were infected with lentiviral vector expressing a shRNA for RNF7. Cells were then treated with stimuli and mRNA was extracted after 12h. These results are representative of three different experiments.

5. Discussion

CARMA2 is a scaffold protein involved in the NF-κB activation pathway [10][12]. In the last years, it comes out that mutations in this protein leads to the psoriasis [14]. This one is a skin disease in which there is an inflammation of the skin triggered by an increased presence of inflammatory cytokine and chemokine and T-cell [19]. The aim of this work was to try to shed light on the molecular events that govern NF-κB transcriptional activity and to gain more insight in the molecular partners that influence activity of f CARMA2. Two-hybrid screenings allowed us to identify a new molecular interactor of CARMA2: the ring finger protein 7 RNF7. Regarding RNF7, we showed that it specifically interacts with the CARMA2 short form and specifically with its CARD and PDZ domains. As proposed by Ryan R. McCully and Joel L. Pomerantz CARD proteins can oligomerize to form CBM complex and how they do this could explain why RNF7 binds just CARD and PDZ domains of CARD14 (fig. 28) [20].

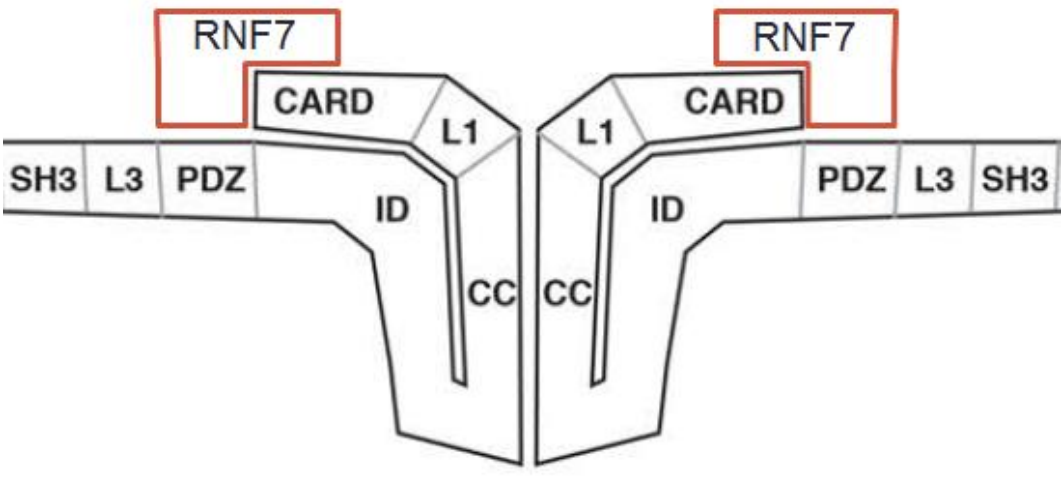

Fig. 28 The model how RNF7 could bind to CARMA2sh

So, we transfected RNF7-FLAG tagged together with CARMA2sh-HA and FLAG tag. We immunoprecipitated for HA and immunoblotted for FLAG to see whether RNF7 was able to block somehow CARMA2sh oligomerization but CARMA2sh kept oligomerizing. In addition, RNF7 exerts a strong inhibitory effect on the CBM complex mediated NF-κB activation. These results suggest that RNF7 could, with a not better identified mechanism, modulate the function of this important transcription factor. We also searched for degradation of some well known proteins of this pathway. We transfected RNF7 together with CARMA2sh and immunoblotted lysates for MALT1, BCL10, IkBa, NEMO, TRAF6 and TRAF2 but no degradation of sort was detected. Despite the same effect of RNF7 on CARMA2sh was confirmed by luciferase assay also for CARD domain of CARMA1 and CARMA3, its mechanism of action still remains not perfectly clear. Moreover, we are studying the relation between RNF7 and CARMA2 mutants in the psoriasis pathogenesis. As it was already been said, mutations in CARMA2 are involved in psoriatic phenotype and E138A and E142G mutations escape somehow this form of regulation mediated by RNF7 so this can be a crucial point which leads to psoriasis pathology. To better characterize the role of RNF7 in CARMA2sh pathway, we infected immortalized keratinocyte with RNF7 and primary keratinocytes with shRNA for RNF7 and we analyzed the levels of some cytokines and chemokines involved in the inflammation. We found that the expression of these are decreased thanks to RNF7 under stimuli and when RNF7 is knocked down in NHEK these cytokines increase.

6. Conclusions

There is no question that understanding how the NF-κB pathway influences, and it is influenced by different signaling pathways will provide crucial insight into the regulation of immune responses. As systemic blockade of NF-κB will lead to adverse effects, elucidating the pathways that selectively activate and shape NF-κB responses may represent a clinically relevant first step in the design of specific inhibition of NF-κB in chronic inflammatory diseases. Extensive research efforts have been devoted to characterizing the mechanisms that activate NF-κB in various scenarios. Nevertheless, it seems that understanding of how specificity is conferred to NF-κB gene signatures is still just beginning. In this work, we reported the identification of a new protein involved in the signaling pathways leading to NF-κB activation, RNF7. This protein revealed able to interact with the splicing variant of CARMA2, CARMA2sh. Although many other investigations have to be performed, we showed that ectopic expression of RNF7 is able to reduce NF-κB activation mediated by CARMA2, through a not yet defined mechanism. This result is very interesting because it could introduce a therapeutic strategy for psoriasis. Data regarding RNF7 presented in this work are sufficiently encouraging to induce us to better investigate this protein function. It is interesting that RNF7 dampens the NF-κB transcriptional activity mediated by CARMA2sh but not by its mutants. From the functional point of view, RNF7 is able to mediate this effect not by addressing to proteasome some of the proteins involved in the NF-κB pathway through degradative ubiquitination due to its E3 ligase activity. In particular, it would be interesting to define the molecular mechanisms and partners that modulate its activity on NF-κB, that could give us further insight in this new field of investigations. RNF7 is able to dampen cytokines and

chemokines expression in treated human immortalized keratinocytes (HACAT) and human primary keratinocytes (NHEK) interfered for RNF7 and treated with some stimuli showed an increased expression of inflammation of the same cytokines and chemokines. Some questions remain unsolved, for example how Carma2sh mutants can escape this form of control. These are preliminary data, more must be done to give a role to this protein in this pathology.

7. Acknowledgments

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9. List of Publications (2014-2017)

"Functional characterization of zebrafish (Danio rerio) Bcl10."
Mazzone P, Scudiero I, Ferravante A, Paolucci M, D'Andrea LE, Varricchio E, Telesio G, De Maio C, Pizzulo M, Zotti T, Reale C, Vito P, Stilo R.
PLoS One. 2015 Apr 7;10(4):e0122365. doi: 10.1371/journal.pone.0122365. eCollection 2015.

"The Dishevelled, EGL-10 and pleckstrin (DEP) domain-containing protein DEPDC7 binds to CARMA2 and CARMA3 proteins, and regulates NF-κB activation."
D'Andrea EL, Ferravante A, Scudiero I, Zotti T, Reale C, Pizzulo M, De La Motte LR, De Maio C, Mazzone P, Telesio G, Vito P, Stilo R.
PLoS One. 2014 Dec 26;9(12):e116062. doi: 10.1371/journal.pone.0116062. eCollection 2014.

"Functional Characterization of Porcine (Sus scrofa) BCL10"
Pellegrino Mazzone, Ivan Scudiero, Angela Ferravante, Marina Paolucci, Luca E. D'Andrea, Ettore Varricchio, Gianluca Telesio, Maddalena Pizzulo, Tiziana Zotti, Carla Reale, Pasquale Vito, Romania Stilo
Open Journal of Immunology, 2015, 5, 64-71 Published Online June 2015 in SciRes.
http://www.scirp.org/journal/oji http://dx.doi.org/10.4236/oji.2015.52007

"CARMA2sh and ULK2 control pathogen-associated molecular patterns recognition in human keratinocytes: psoriasis-linked CARMA2sh mutants escape ULK2 censorship."
Pasquale Vito, Ivan Scudiero, Luca Egildo D'Andrea, Pellegrino Mazzone, Angela Ferravante, tiziana zotti, Gianluca Telesio, Gabriele De Rubis, Carla Reale, maddalena pizzulo, muralitharan konar, and Romania Stilo
Cell Death & Disease – Nature