The constitutive NF-κB activity observed in a variety of hematologic and solid tumors has been connected with multiple aspects of oncogenesis, including the regulation of cell proliferation, the control of apoptosis, the promotion of angiogenesis and the stimulation of invasion/metastasis. Such enhanced NF-κB activity occurs through several mechanisms like genetic rearrangements leading to activation of a proto-oncogene (BCL-3) or to the creation of an oncogenic fusion protein (p100ΔC), mutations of inhibitory proteins (IκBα, TRAF3, CYLD), gene amplifications (NIK) or increased IKK activity (McKeithan *et al*., 1987; Cabannes *et al*., 1999; Emmerich *et al*., 1999; Jungnickel *et al*., 2000; Annunziata *et al*., 2007; Keats *et al*., 2007; Rayet and Gelinas, 1999; Kim *et al*., 2006).

Chromosomal translocations of the *nfκb2* gene have been found in various types of human lymphoid malignancies, and more particular in neoplasms derived from mature T-cells, such as mycosis fungoides and Sezary's syndrome (Fracchiolla *et al*., 1993; Migliazza *et al*., 1994; Neri *et al*., 1991; Thakur *et al*., 1994). These rearrangements all cluster within the 3' coding region of the gene and lead to the production of C-terminally truncated p100 proteins, which, in some cases, are fused to heterologous tails. The cardinal feature of these mutants is the lack of the processing inhibitory domain and variable portions of the ankyrin-repeats domain of p100, suggesting that this alteration may be the common mechanism responsible for the constitutive activation of NF-κB2 *in vivo*. As a result of this C-terminal deletion, the p100ΔC mutants are all nuclear, present a defective IκB-like function and, except for p100HB, are constitutively processed into p52. Some of them (Lyt10-Cα, LB40) have been shown to transform mouse or rat embryonic fibroblasts as well as the murine prolymphocytic B-cells FL5.12 *in vitro* (Ciana *et al*., 1997; Qing *et al*., 2007). Moreover, fibroblasts expressing Lyt10-Cα were able to form tumors in immunodeficient mice (Ciana *et al*., 1997). But the first direct evidence of the tumorigenic capacity of an NF-κB2 mutant *in vivo* was provided by the transgenic mice expressing Hut78 (p80HT) in lymphocytes, which developed small B-cell lymphomas with multi-organ metastasis (Zhang *et al*., 2007). However, whereas the oncogenic potential of the NF-κB2 mutants is now well established, it remains unclear how they contribute to the development of lymphomas.

To gain insight into the molecular mechanisms underlying this oncogenic potential, we first tried to identify the protein domains and the related functional properties, required for the tumorigenic activity of one of these lymphoma-

associated p100ΔC proteins, Hut78. For this purpose, we generated, by mutation or deletion, mutants lacking the principal domains of the protein, such as the nuclear localization signal, the glycine-rich region, the ankyrin repeats or part of the RHD, and tested their ability to transform mouse fibroblasts *in vitro* and *in vivo*. We didn't detect any difference in the growth curves between the NIH3T3 infected with p52, Hut78 or its mutants and the control cells. Similar results were observed with Lyt10-Cα- or LB40-transfected Balb cells, moreover lymphocytes isolated from Hut78 transgenic mice didn't exhibit neither enhanced proliferation, but were rather resistant to activation-induced apoptosis (Ciana *et al*., 1997; Zhang *et al*., 2007). Taken together, these results suggest that NF-κB2 truncated proteins have no significant effect on cell proliferation. However, in contrast to Lyt10-Cα- and LB40-transfected cells, whose cloning efficiency was higher than control cells (Ciana *et al*., 1997; Qing *et al*., 2007), neither p52, Hut78 nor its mutants were able to induce foci formation in NIH3T3 cells. Moreover, while injection of the Lyt10-Cα-transfected Balb cells in immunodeficient mice led to tumor formation in all animals within two months (Ciana *et al*., 1997), we couldn't observe any tumor development in immunodeficient mice injected with NIH3T3 cells expressing p52, Hut78 or its mutants during the time of experimentation (12 weeks). The late onset of lymphoma development in the Hut78 transgenic mice (from 41 up to 96 weeks) suggest that the oncogenic potential of Hut78 is low and that malignant transformation requires additional genetic or epigenetic alterations, such as the *c-myc*, *cbl* or p53 rearrangements observed in the HUT78 cell line (Finger *et al*., 1988; Blake *et* Langdon, 1992; Tolomeo *et al*., 1998). This may explain why we failed to demonstrate the tumorigenic ability of Hut78 or one of its mutants in our model.

Comparison by micro-array analysis of the genes expression profiles in NIH3T3 cells infected by the retroviral constructs pBabe-p52, pBabe-Hut78 or by the empty vector pBabe, revealed that *mmp9* was the most strongly induced candidate, in both p52 and Hut78 overexpressing cells. Quantitative real-time PCR analysis and zymography assays confirmed the induction of MMP9 on both the mRNA and protein levels on p52 or Hut78 overexpression in NIH3T3 cells. *mmp9* was also defined as a p52 or Hut78-target gene in lymphoid cells as its expression was increased in Hut78-transfected 164T2 cells and impaired in p52-depleted HUT78 cells. These findings are especially relevant in this lymphoma-associated context as MMP9 expression has been suggested as a marker for distinguishing a benign disease and malign lymphoma (Hazar *et al*.,

2004). Indeed, in non-Hodgkin's lymphomas, constitutive production of MMP9 and its high serum levels are associated with clinical aggressiveness and correlate with tumor grade and poor prognosis (Kossakowska *et al*., 1992, 1996 and 1999; Sakata *et al*., 2004; Vacca *et al*., 2000). These observations in human are consistent with results obtained in some experimental mouse models in which high levels of MMP9 have been found in the serum of T-lymphoma-bearing mice (Aoudjit *et al*., 1997). In patients with mycosis fungoides (MF) in particular, expression of MMP9 mRNA by tumor cells was significantly increased with disease progression, from patch (clustered lymphoid tumor T-cells within epidermis and superficial dermis) to nodular stage (clustered and scattered T-cells within epidermis and dermis in depth) (Vacca *et al*., 2000). Therefore, by inducing *mmp9* gene expression, Hut78 would contribute to the dissemination of T-lymphoma cells to peripheral tissues and their deepening into the dermis. This idea is supported by our invasion assay results, as the p52-depleted HUT78 cells, which display lower MMP9 mRNA and protein levels, were impaired in their ability to migrate through a Matrigel barrier. However, while overexpression of MMP9 in T-lymphoma cells was shown to accelerate the growth of thymic lymphomas (Aoudjit *et al*., 1998a), a recent study using MMP9-deficient mice demonstrated that leukemogenesis, lymphoma growth and dissemination can occur in absence of MMP9 (Roy *et al*., 2007). The authors proposed that if MMP9 is not essential for the development of primary lymphoid tumors and the dissemination of lymphoma cells in peripheral tissues, it can nevertheless increase the tumorigenic potential of lymphoma cells. To further explore the role of p52-induced MMP9 expression in the ability of tumor cells to invade peripheral sites will necessitate the use siRNA strategies targeting either p52, either MMP9 in HUT78 cells and lasting for the period of lymphoma development in mice. Moreover, setting up specific clones overexpressing Hut78, and thus MMP9, during the whole time of experimentation would also allow determining if Hut78 confers a more aggressive behavior to lymphoma cells.

The equilibrium between MMPs and its endogenous inhibitors, TIMPs, was also shown to determine the degree of invasiveness of a given cancer cell (Aoudjit *et al*., 1998b). Reduction of TIMP expression by antisense strategy has also been shown to confer tumorigenic and metastatic properties to non invasive NIH3T3 cells (Khoka *et al*., 1989). Our micro-array data indicate a down-regulation of TIMP4 in p52 or Hut78 overexpressing NIH3T3 cells, which may also account for the role of Hut78 in lymphoma progression by tipping the

balance between MMPs and TIMPs towards invasion. But this part of the work still needs to be investigated.

Under physiological conditions, MMP9 expression, secretion and activity are highly regulated. The proximal promoter of the *mmp9* gene contains several binding sites for transcription factors including AP-1, NF-κB, Sp-1 and Ets, which render this gene expression inducible on exposure to external stimuli, such inflammatory cytokines (Sato and Seiki, 1993; van den Steen *et al*., 2002). Although MMP9 was found overexpressed in various malignancies, the mechanisms that control its expression in tumor cells remain poorly understood. We identified here aberrantly expressed p52 as well as production of p100 truncated mutants that constitutively generate p52, like Hut78 or LB40, as a mechanism responsible for inappropriate expression of MMP9 in tumor cells.

The NF-κB2 truncated protein Hut78 was shown to bind DNA in an unprocessed form and appeared to have acquired intrinsic transactivation properties (Zhang *et al*., 1994; Chang *et al*., 1995). However, our results indicate that overexpression of p52 alone is also able to induce *mmp9* gene expression, which raises the question of whether the transcriptional activity of Hut78 on the *mmp9* gene promoter is mediated directly by Hut78 itself and/or indirectly by the p52 issued from its processing. There are several experimental evidences for p52 being essential for the oncogenic potential of the truncated NF-κB2 proteins. Recently, Xiao and colleagues showed that a processing-deficient mutant of LB40 was unable to induce anchorage-independent cell growth or to regulate a subset of tumor-associated genes, as compared to LB40 or p52, therefore suggesting that the processed p52 functions as the mediator of p100ΔC-induced oncogenesis (Qing *et al*., 2007). These findings are consistent with the increased gastric and lymphoid cell proliferation observed in p100 knock-in mice with constitutive expression of p52, although their early postnatal death may prevent further formation of tumors (Ishikawa *et al*., 1997). However, while transgenic mice with targeted expression of Hut78 display a marked expansion of peripheral B-cell population and develop B-cell lymphoma through induction of the TRAF1 anti-apoptotic pathway, their counterparts expressing p52 in lymphocytes do not develop lymphomas and show only transient TRAF1 upregulation (Zhang *et al*., 2007; Wang *et al*., 2008). Therefore, the role of the constitutive production of p52 in the oncogenic potential of NF-κB2 truncated mutants remains controversial.

Our data support the idea that Hut78-induced MMP9 transcriptional activation occurs through p52 overproduction as LB40, another p100ΔC mutant

which constitutively generates p52, is also able to induce *mmp9* gene expression in NIH3T3 cells, in contrast to p100HB, which is also nuclear, but barely processes into p52. However, these results don't rule out the possibility that Hut78 can specifically transactivate other genes by itself, such as *TRAF1*, which seems required to drive lymphomagenesis *in vivo* (Zhang *et al*., 2007).

Furthermore, we showed that p52 and Hut78 require a functional GRR domain to induce MMP9 transcriptional activation.

The classical NF-κB pathway is well-known for being involved in the induction of MMP9 by various stimuli, such as growth factors, cytokines, and the viral transactivator protein Tax (Bond *et al*., 1998; Esteve *et al*., 2002; Mori *et al*., 2002; Yamanaka *et al*., 2004; Alaniz *et al*., 2004; Ho *et al*., 2007; Rhee *et al*., 2007a, 2007b; Srivastava *et al*., 2007). However, little is known about the role of the NF-κB alternative pathway in the regulation of *mmp9* gene expression. Recently, Connelly and colleagues observed increased levels of MMP9 in the mammary glands of transgenic mice where p100/p52 was up-regulated during pregnancy and lactation (Connelly *et al*., 2007). The fact that MMP9 is induced on LTβ stimulation in NEMO-deficient cells, where the classical NF-κB signaling pathway is not functional, demonstrates the involvement of the NF-κB alternative pathway in MMP9 transcriptional regulation. Therefore, when constitutively activated, this p52-dependent signaling cascade may contribute to the development of hematological disorders, notably through aberrant expression of MMP9.

Several studies have recently provided compelling evidences that the noncanonical NF-κB pathway plays critical role in lymphomagenesis. Two of them, showed that primary multiple myeloma (MM) samples and MM cell lines frequently carry mutations in genes encoding essential NF-κB components, leading mainly to the constitutive activation of the noncanonical NF-κB pathway, with the most common abnormality being inactivation of *TRAF3* (Annunziata *et al*., 2007; Keats *et al*., 2007). Constitutive CD40 signaling in B cells was also shown to lead to the selective activation of the noncanonical NF-κB pathway and the MAPKs Jnk and Erk, whose concerted action promotes B cell lymphomagenesis (Hömig-Hölzel *et al*., 2008). Saitoh and colleagues demonstrated that overexpression of NIK (NF-κB-inducing kinase) contributes to the tumorigenesis of adult T-cell leukemia (ATL) and Hodgkin Reed-Sternberg cells (H-RS) that do not express viral regulatory proteins (Saitoh *et al*., 2008). Taken together, these findings highlight the importance of the

alternative NF-κB pathway in lymphomagenesis, in which aberrantly expressed MMP9 may play a role.

How p52, which is devoid of transactivation domain, is able to induce MMP9 transcriptional activation still remains to be elucidated. We showed a strong interaction between p52/Hut78 and the other NF-κB family members RelA, RelB, p50 and BCL-3, but RNA interference would be helpful to determine which one(s) is(are) essential to trigger p52-induced *mmp9* gene expression.

We nevertheless gained insights into the molecular mechanisms underlying the p52-dependent MMP9 transcriptional regulation by identifying some co-regulators. We indeed found that p52 and Hut78 interact with HDAC3 and that their DNA-binding domain negatively regulates this interaction. As HDAC3 acts as a co-repressor, we suppose that this protein may be specifically removed by p52/Hut78 from specific gene promoters, such as *mmp9*, while co-activators would be simultaneously recruited. The histone H3K4 methyltransferases MLL1 and MLL2 constitute such co-activators, as they are specifically tethered on the MMP9 promoter, but not on the IκBα promoter in NIH3T3 cells overexpressing p52. As a result, the *mmp9* gene promoter is trimethylated on lysine 4 of histone H3 and thus transcriptionally active, while the *IκBα* gene is not. These results suggest that p52 is required for initial targeting of the HMT complex to a specific promoter and further methylation of H3K4 at this locus. However, the mechanisms underlying the recruitment of H3K4 histone methyltransferases to the target genes remain elusive. Several hypotheses have been proposed (Ruthenburg *et al*., 2007). One of these suggests the association of the MLL-like protein with a site-specific DNA-bound transcription factor as it was shown for estrogen receptor α and MLL2 and MLL4 (Mo *et al*., 2006; Dreijerink *et al*., 2006), E2F6 and MLL1 (Dou *et al*., 2006) or the viral transcription factor VP16 and SET1A (Wysocka *et al*., 2003), for example. Demers and colleagues showed that the hematopoietic activator NF-E2 associate with the MLL2/ASH2L complex in erythroid cells and that this methyltransferase complex is recruited to the β-globin locus during erythroid differentiation in a NF-E2-dependent manner (Demers *et al*., 2007). We also found that p52 colocalizes and interacts with ASH2L, but not with the other complex subunits RbBP5 and WDR5. Therefore, in support with Demers' model, we propose that p52 mediates the recruitment of the MLL1/ASH2L or MLL2/ASH2L complex at the promoter of MMP9, while the other complex components such RbBP5 and WDR5 would rather regulate the H3K4

methylation activity. To further assess the importance of MLL1 and MLL2 complexes in p52-induced MMP9 induction, it would be interesting to evaluate the effect of MLL1 and/or MLL2 depletion on *mmp9* gene expression and on the methylation status of its promoter in p52-overexpressing cells.

The chromatin remodeling events associated with p52-induced *mmp9* gene expression might not be limited to H3K4 methylation and other histone modifying enzymes may also play a role. For example, Jmjd3, the histone demethylase responsible for the removal of the repressive H3K27 trimethylation, which was shown to be induced on TNFα or LPS stimulation in an NF-κB-dependent manner, was found to be incorporated in RbBP5-containing complexes (De Santa *et al*., 2007; Li *et al*., 2008). These results strongly support the existence of a cross-talk mechanism between two mutual counter-regulatory histone marks, H3K4me3 and H3K27me3 and led us suppose that other chromatin modifications might also be involved in NF-κB target gene regulation. This reveals the potential complexity of the epigenetic mechanisms modulating NF-κB activity and opens new fields of research, which just start being explored.

In summary, if constitutive production of p52 by Hut78 is probably not sufficient to drive lymphomagenesis alone, it may contribute to lymphoma dissemination through induction of *mmp9* gene expression by tethering selected H3K4 methyltransferases in a promoter-specific manner. Our results offer therefore attractive targets for the treatment of lymphomas which exhibit rearrangements of the *nfκb2* gene.

First, the constitutive production of p52 and its role in MMP9 induction provide the rationale for the use of inhibitors of NF-κB, especially those targeting the alternative pathway like IKKα-selective inhibitors, to treat this pathology. Over 800 natural and synthetic compounds have been shown to inhibit the NF-κB signaling pathways at multiple levels (www.nf-kb.org), usually among other biological effects. Some of these drugs, including bortezomib (Velcade®), thalidomide (Thalidomid®), lenalidomide (Revlimid®) and arsenic trioxide have now proven to be effective in the treatment of multiple myeloma in combination with conventional chemotherapies (Li *et al*., 2008). DHMEQ (dehydroxymethylepoxyquinomicin), which acts in the translocation of NF-κB into the nucleus, LC-1 or pristimerin constitute other promising NF-κB inhibitors for the treatment of adult T-cell leukemia/lymphoma (ATL), chronic lymphocytic leukemia or multiple myeloma (Horie *et al*., 2006; Hewamana *et al*., 2008; Tiedemann *et al*., 2008).

Blocking MMP9 appears to be another potential therapeutic strategy. The frequent overproduction of MMPs in malignant tumors prompted the development of various agents inhibiting the proteolytic activity of these enzymes for cancer therapy. However, most clinical trials using MMP inhibitors have yielded disappointing results (Wojtowicz-Praga *et al*., 1996; Coussens *et al*., 2002; Overall and Lopez-Otin 2002; Pavlaki and Zucker 2003), probably because of their unselectivity and the complex roles that these enzymes play in cancer progression. New MMPs inhibitors with increased selectivity and oral bioavailability have been developed (non-peptido molecules mimicking the cleavage site of MMP substrates, tetracycline derivatives, biphosphonates, carboxylated glucosamine derivatives) and some are now under clinical trials (Lockhart *et al*., 2003; Cianfrocca *et al*., 2002; Falardeau *et al*., 2001; Lacerna and Hohneker, 2003 ; Mendis *et al*., 2006). Nevertheless, in light of their results, Roy and colleagues suggest that treatment with selective MMP9-inhibitors for the treatment of invasive cancer may partially overcome the aggressive behavior of lymphoma cells expressing high levels of MMP9, and will not be sufficient to completely eradicate the dissemination of these cells (Roy *et al*., 2007).

Interestingly, novel strategies based on expression patterns of MMPs in malignant tumors, exploit the catalytic functions of these enzymes for cancer therapy (Folgueras *et al*., 2004). Some of these approaches employ macromolecular carriers that are linked to anticancer drugs released from the carrier by the proteolytic activities of MMPs present in the tumor environment (Mansour *et al*., 2003).

Targeting epigenetic marks represents also an interesting strategy to control the progression of cancer. The abnormal recruitment or overexpression of HDACs observed in many tumors led to the development of HDAC inhibitors, such as romidepsin (depsipeptide) or vorinostat (Zolinza®), which is currently used for the treatment of refractory cutaneous T-cell lymphoma. The increasing evidence for a direct link between histone methyltransferases and cancer bring to focus these families of enzymes as putative targets for cancer therapy (Santos-Rosa and Caldas, 2005) and H3K4 HMT inhibitors might prove useful in this case.

However, most of these drugs are not 100% selective as they often target other proteins or all members of the family rather than an individual one. Inhibition of specific mRNAs by small interfering RNAs constitutes a hopeful technology to achieve maximum selectivity and has proven to be an effective approach against several cancer models (Friedrich *et al*., 2004; Guo *et al*.,

2004). Another major concern comes from the broad and complex roles of these proteins in physiological and pathological process and thus the risk of side effects due to the action of such inhibitors on normal cells. Therefore if our results provide interesting clues for the treatment of lymphomas exhibiting high activity of the alternative NF-κB signaling pathway, better knowledge and understanding of the mechanisms that control NF-κB target gene expression remain necessary to design, in the most rational way, more specific and efficacious anti-cancer drugs and strategies. Moreover, the clinical activity of a single agent will probably be not sufficient and it is likely that their rational combination with each other or with conventional agents will enhance their effectiveness and achieve stronger and more selective responses.