Novel functions of chromatin-bound $\text{I}_\kappa\text{B}_\alpha$ in oncogenic transformation

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The nuclear factor-$\kappa$B (NF-$\kappa$B) signalling pathway participates in a multitude of biological processes, which imply the requirement of a complex and precise regulation. IxB (for Inhibitor of kappaB) proteins, which bind and retain NF-$\kappa$B dimers in the cytoplasm, are the main contributors to negative regulation of NF-$\kappa$B under non-stimulation conditions. Nevertheless, increasing evidences indicate that IxB proteins exert specific nuclear roles that directly contribute to the control of gene transcription. In particular, hypophosphorylated IxB$\beta$ can bind the promoter region of TNF$\alpha$ leading to persistent gene transcription in macrophages and contributing to the regulation of the inflammatory response. Recently, we demonstrated that phosphorylated and SUMOylated IxB$\alpha$ reside in the nucleus of the cells where it binds to chromatin leading to specific transcriptional repression. Mechanistically, IxB$\alpha$ associates and regulates Polycomb Repressor Complex activity, a function that is evolutionary conserved from flies to mammals, as indicate the homoetic phenotype of Drosophila mutants. Here we discuss the implications of chromatin-bound IxB$\alpha$ function in the context of tumorigenesis.

The nuclear factor-$\kappa$B (NF-$\kappa$B) family of transcription factors has an important role in the regulation of biological processes such as immunity and inflammation, apoptosis, stress response or ageing (Hayden and Ghosh, 2004). This family is composed of five members: RelA (p65), RelB and c-Rel, and the precursor proteins NF-$\kappa$B1 (p105) and NF-$\kappa$B2 (p100) that can be processed into p50 and p52, respectively. Nuclear factor-$\kappa$B signalling factors function as homo- and hetero-dimers that activate or repress gene expression in a context-dependent manner. Under resting conditions, most NF-$\kappa$B resides in the cytoplasm bound to particular inhibitory proteins of the inhibitor of kappaB (IxB) family, IxB$\alpha$, IxB$\beta$ or IxB$\epsilon$, which generate an additional degree of complexity. Phosphorylation of IxB at specific serine residues by the IxB kinase (IKK) complex induces its ubiquitynation and subsequent proteasomal degradation, thus releasing the NF-$\kappa$B factor leading to its nuclear translocation and gene transcription (Beg et al, 1992). However, recent studies have provided evidence that IxB$\alpha$ proteins are more than NF-$\kappa$B inhibitors. In 2010, Rao and colleagues demonstrated that following LPS stimulation, hypophosphorylated newly synthesised IxB$\beta$ binds p65 and c-Rel at the promoter region of specific genes such as TNF$\alpha$ and protects the NF-$\kappa$B factor from IxB$\alpha$ association and chromatin leading to persistent activity (Rao et al, 2010). The same mechanism operates at the IL1-$\beta$ promoter (Scheibl et al, 2010).

Some years ago, we found that in fibroblasts nuclear IxB$\alpha$ associates with NF-$\kappa$B-independent gene promoters, such as hes1 and herp2, which are general regulators of cell differentiation, correlating with their transcriptional repression. TNF$\alpha$ treatment induced IKK recruitment to these particular genes leading to IxB$\alpha$ dissociation and transcriptional activation (Aguilera et al, 2004). Importantly, IKK was found constitutively bound to hes1 and herp2 promoters in colorectal cancer cells associated with increased gene expression (Fernandez-Majada et al, 2007a). Now, we have demonstrated that in keratinocytes there is a fraction of phosphorylated and SUMOylated IxB$\alpha$ (PS-IxB$\alpha$) that binds to chromatin through direct association with histones leading to NF-$\kappa$B-independent gene regulation (Mulero et al, 2013). Interestingly, nuclear PS-IxB$\alpha$ seems to be crucial for proper skin homeostasis, which might explain the severe skin phenotype of IxB$\alpha$-deficient mice that die 5 days after birth because of a massive skin inflammation likely associated with a defective barrier function (Beg et al, 1995; Klement et al, 1996; Rebholz et al, 2007; Table 1). In addition, our results strongly suggest that aberrant accumulation of cytoplasmic IxB$\alpha$ in keratinocytes promotes squamous cell carcinoma (SCC).
Mulero et al, 2013), which is in agreement with the oncogenic phenotype of ectopic IκBz-SR expression in the skin (van Hogerlinden et al, 1999, 2004).

IκBz, AN OLD CYTOPLASMIC PROTEIN WITH A NOVEL NUCLEAR FUNCTION, OR VICE-VERSA

IκBz regulates epidermal homeostasis. Mammalian epidermis consists of four different layers: basal, spinous, granular and cornified. The proliferating basal layer of the epidermis contains most of the stem and progenitor cells of this tissue, which then undergoes a differentiation process that culminates in their fully matured and enucleated as they reach the surface layer, thus generating the cornified layer of keratinocytes. However, this is not an easy task for a tissue that is permanently exposed to a plethora of external insults such as UV radiation, extreme temperature variations and chemical exposure among others. The success of the skin differentiation process depends on the integration of intrinsic cellular programmes with external signals including the response of the immune system. Recently, we found that PS-IκBz is predominantly distributed in the nucleus of keratinocytes bound to the promoter of genes such as HOX and IRX (Mulero et al, 2013), which control skin differentiation and proliferation but are also involved in the developmental regulation. Once in the chromatin, PS-IκBz facilitates the recruitment of polycomb repressive complex 2 to gene promoters and dictates their competence to be induced following TNFz stimulation, thus establishing a mechanistic link between inflammatory signals and skin homeostasis. Remarkably, this role for IκBz as a regulator of polycomb function is operating during Drosophila development since mutations in Cactus (the IκBz orthologue in flies) enhanced the homeotic phenotype of Pc (Polycomb) mutations. These results together with the identification of IκBz orthologues in the worm Caenorhabditis elegans that does not have a real NF-κB pathway suggest that this newly identified function of PS-IκBz is an ancestral function previous to the specialisation of the immune system.

Nuclear IκBz as a tumour suppressor in the skin. Indicative of a tumour-suppressor function for PS-IκBz, immunohistochemical analysis of a panel of human skin samples demonstrated that IκBz remains nuclear in the keratinocytes of benign skin lesions such as elastosis, psoriasis, acinic keratosis and Bowen disease, but is specifically lost in the more malignant lesions such as SCC. Further analysis of a cohort of 112 patients with urogenital SCC at different stages of tumour progression showed that in samples corresponding to invasive and metastatic SCC, IκBz was totally excluded from the nucleus but accumulated in the cytoplasm (Mulero et al, 2013), suggesting that beyond loss of nuclear IκBz, cytoplasmic IκBz (likely SUMOylated-IκBz) might have additional pro-tumorigenic functions, which will be investigated. This possibility is in agreement with the skin phenotype of different mice strains ectopically expressing the IκBz super-repressor mutant (IκBz-SR), which develop SCC-resembling tumours in the skin (Dajee et al, 2003), 2004; Fernandez-Majada et al, 2007), 2007b; Hoberg et al, 2004), thus promoting a global inflammatory response. A recent study by Dajee et al (2003) demonstrated that mutant RASV12 failed to generate tumours when expressed in primary human keratinocytes (that were growth arrested), but it efficiently induced tumours resembling human SCC when co-expressed with IκBz-SR. Based on others and our results, we speculate that cytoplasmic accumulation of IκBz in skin tumours might sequester nuclear co-repressors and HDACs (Aguilera et al, 2004; Fernandez-Majada et al, 2007a, 2007b; Hoberg et al, 2004), thus promoting a global miss-regualtion of gene transcription. In this sense, specific HDAC proteins contain SUMO-binding elements that could favour their binding to PS-IκBz either in the nucleus or the cytoplasm (model in Figure 1). Whether the cytoplasmic IκBz that is found in SCC samples is SUMOylated and/or phosphorylated remains to be addressed.

More recently, a knock-in mouse containing an IκBz protein with a mutated nuclear export sequence that is mostly localised in the nucleus of most cells has been characterised. These mutant mice show impaired canonical and alternative NF-κB pathways in the mature B cells with the absence of secondary lymphoid organs, but they display functional skin with no evidence of critical barrier defects (Wuerzberger-Davis et al, 2011). However, a detailed analysis of mutant skins from 7- to 8-week-old animals revealed significant

Table 1. Skin phenotypes associated to IκBz mutant mouse models

| Model                                        | Phenotype                                                                                  | Reference                          |
|----------------------------------------------|-------------------------------------------------------------------------------------------|------------------------------------|
| Conventional IκBz-deficient mice             | Increase on proliferative keratinocytes relative to differentiated cell types accompanied | Beg et al, 1995; Klement et al,    |
|                                              |   by a widespread dermatitis. Skin inflammation associated to high levels of IL-1β and    | 1996; Rebholz et al, 2007          |
|                                              |   IFN-γ in the dermis and infiltration of CD8 + T cells and Gr-1 + neutrophils in the      |                                    |
|                                              |   epidermis                                                                                 |                                    |
| Keratin5 promoter- IκBz-deficient mice       | Abnormal proliferation of keratinocytes without epidermal inflammation                    | Rebholz et al, 2007                |
| Double IκBz- and TNFz-deficient mice         | Viable more than 3 months. Skin phenotype rescued                                           | Shih et al, 2009                   |
| Keratin5 promoter- IκBz-SR transgenic        | Severe macroscopic phenotype characterised by flaky skin, hair loss, dysplasia of the     | van Hogerlinden et al, 1999;       |
|                                              |   dermis and development of SCC and inflammatory response                                  | Seitz et al, 1998; van Hogerlinden  |
|                                              |                                                                                           | et al, 2004                         |
| Human keratinocytes expressing IκBz-SR and/or | IκBz-SR alone showed mild hyperplasia. Co-expression of RAS and IκBz-SR produced large    | Dajee et al, 2003                   |
| RAS Gly12Val transplanted into CB-17 scid     | neoplasms resembling SCC                                                                  |                                    |
| mice                                          |                                                                                           |                                    |
| NfkbiaNES/NESS mice harbouring a triple       | Defect on secondary lymphoid organ formation and impaired B-cell                         | Wuerzberger-Davis et al, 2011;     |
| mutation in the NES                           |   maturation. Expansion of the proliferative compartment and reduction of the            | Mulero et al, 2013                  |
|                                              |   layers in the skin                                                                        |                                    |

Abbreviations: IκB = inhibitor of kappaB; IL = interleukin; INF = interferon; NES = nuclear export sequence; SCC = squamous cell carcinoma; SR = super repressor.
differences with wild-type mice including increased proliferation index, expansion of the K14-positive basal layer, and reduction on the thickness of the intermediate K10-positive skin layer (Mulero et al., 2013). Future experiments should address the question of whether constitutively nuclear IkBaB results in altered Hox and Irx expression in the basal or supra-basal layer of keratinocytes, and whether it protects these cells from undergoing tumorigenesis under specific transformation procedures or even with ageing.

**IkB kinase alpha (IKKz) modulates IkBaB localisation in keratinocytes.** It is worth mentioning, that not only IkBaB but also the IKKz displays a predominantly nuclear distribution in the skin (Marinari et al., 2008; Zhu et al., 2007). Loss of IKKz associates with altered proliferation and differentiation of epidermal keratinocytes (Hu et al., 1999; Sil et al., 2004), whereas mutations that resulted in IKKz loss or in the generation of truncated proteins that failed to interact with chromatin led to the development of skin papillomas and SCC (Liu et al., 2006; Park et al., 2007). Conversely, chemically induced skin carcinogenesis is reverted by overexpression of wild-type IKKz (Liu et al., 2006). Our recent results indicated that active IKKz induced a partial accumulation of SUMOylated-IkBz in the cytoplasmic compartment of keratinocytes, and IKK activation correlates with cytoplasmic accumulation of IkBaB in human SCC samples (Mulero et al., 2013). These results open the possibility that IkBaB miss-regulation might contribute to the phenotypes observed downstream of IKKz alterations, although it remains to be studied how IKKz regulates IkBaB distribution. Because chromatin-bound IkBaB is already phosphorylated in the residues known to be targets of canonical IKK activity, and we found that this phosphorylation is IKK independent, we speculate that nuclear IKKz might induce PS-IkBz chromatin release by regulating specific editing enzymes (i.e., phosphatase and desumoylase), scaffold proteins or proteins associated with the repressive PS-IkBz complex such as histones or members of the polycomb repressive complex 2, resulting in a reduced affinity of IkBaB with chromatin.

**Different IkBaB populations include different post-translational modifications.** Several publications previously established the link between SUMOylation and transcriptional repression by specific transcription factors such as CtBP, KAP1 or Sp3 (reviewed in Garcia-Dominguez and Reyes (2009)). SUMOylated-IkBz had previously been identified in different types of cells, however, no specific functions for this modified version of the molecule had been ascribed. In 1998, Deserreiro and colleagues detected the existence of endogenous SUMO-1-modified IkBaB in a panel of cancer cell lines, being SUMO-1 linked to lysine 21 of IkBaB. Because lysine 21 is involved in the signal-mediated degradation of IkBaB (following poly-ubiquitination), SUMO-1-IkBz was resistant to degradation and led to reduced NF-kB activity in these cells. Interestingly, SUMOylation was reduced in IkBaB mutant proteins mimicking serine 32 and 36 phosphorylation that is a mark for degradation (Deserreiro et al., 1998). Accumulation of SUMO-1-bound IkBaB is also detected downstream of adenosine signalling during hypoxia and reoxygenation (Liu et al., 2009), although its functional contribution in this system is unknown. Recently, it was shown that IkBaB was modified by SUMO-2/3 but also by a combination of SUMO and ubiquitin chains (Culver et al., 2010) resulting in an IkBaB molecule that can still be regulated by degradation following NF-kB activation (Aillet et al., 2012). The use of particular cell types, and the variations in the technical approaches (including the reagents used for stabilising SUMO chains and for detection) can explain the different results obtained by each group, however, it is still possible that several modifications coexist in particular IkBaB subpopulations leading to functional specificities. Interestingly, SUMOylable proteins are characterised by the presence of a specific FKPe/D motif, which is the case of IkBaB but not of other IkB orthologues such as IkBaB (Kracklauer and Schmidt, 2003). An extended consensus has also been reported, in which SUMOylation is imposed by negatively charged residues (including phosphorylated aminoacids) downstream of the FKPe/D motif (Hietakangas et al., 2003). Thus, one could speculate that IkBaB SUMOylation is subsequent to its phosphorylation at serines 32 and/or 36 by specific kinase/s other than IKKβ (Mulero et al., 2013). Thus, it is of crucial importance to identify such kinases, investigate how they are regulated under specific conditions, different tissues or particular processes, and evaluate their contribution to IkBaB SUMOylation and function. Finally, studying the contribution of SUMO modifications to IkBaB function is not an easy job as mutants that fail to be SUMOylated (i.e., the K21-22 mutant) are also unable to be ubiquitinated and degraded, thus leading to strong NF-kB phenotypes. To circumvent this problem, we are currently generating SUMO-IkBz fusion proteins, which will be tested for their chromatin- or NF-kB-related activities.

The discovery that PS-IkBz exerts a nuclear function that affects NF-kB-independent transcription in response to NF-kB-related stimuli such as TNFz is also a temptation to re-examine previous results that might have misinterpreted because of the most prominent role of cytoplasmic IkBaB. A putative involvement of PS-IkBz in other types of cancer should also be studied; mainly in those that are associated with aberrant or chronic IKK activity such as the case of inflammation-related ones.

On the other hand, it is possible that specific elements of the chromatin-bound IkBaB complex are directly involved in IkBaB SUMOylation. For example, Pc2, one of the mammalian orthologues of the *Drosophila* Polycomb (PRC1) that we found as functionally associated with IkBz/Cactus during *Drosophila* development, is a SUMO E3 ligase (Kagey et al., 2003). Moreover, it is not just IkBaB but other chromatin components such as the histones and elements of the PRC complex including EZH2 and SUZ12 are capable to be SUMOylated, which might impact on their capacity to recruit transcriptional repressors at specific genomic regions, which will be addressed in a near future.

**Future therapeutic perspectives based on IkBz.** The importance of investigating the mechanisms involved in IkBaB and PS-IkBz regulation and their functional relevance in tumour progression is evidenced by the fact that over 250 000 patients are diagnosed with...
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SICC in the world each year. Squamous cell carcinoma is the second most common skin cancer that arises in any region of the body but more frequently in areas exposed to the UV light. Although infrequent (about 10% of the cases), the appearance of metastasis in SCC patients has a very bad prognosis because the absence of treatments, especially in aged patients. Moreover, to date there are no good biomarkers that predict metastatic SCC potential beyond the previous appearance of local relapse, tumour staging or the presence of MYC amplifications (Toll et al., 2009). We are currently investigating whether detection of cytoplasmic IκBz or PS-IκBz, or/and nuclear-active IKKz identifies the small fraction of SCC patients that will develop tumour relapse or metastasis. Those patients could be then selected for more aggressive therapies, but also as candidates for future personalised treatments based on IκBz/IKKz-based therapies.

CONCLUSIONS

Initially, IκB proteins were described as cytoplasmic regulators of the NF-κB pathway; however, several studies focused on IκBz and IκBβ demonstrated the existence of specific nuclear roles for both proteins. First, it was found that IκBz contains a functional nuclear localisation signal that is essential for the efficient post-activation termination of NF-κB signalling by facilitating chromatin release of p50-p65 dimers (Arenzana-Seisdedos et al., 1997). However, IκBz can be retained bound to the chromatin at specific promoter regions through its interactions with histones H2A and H4, thus playing an essential contribution to skin differentiation. We propose that PS-IκBz can exert both pro-oncogenic and tumour-suppressor functions, which need to be investigated in detail. In contrast, nuclear IκBβ is mainly associated with the regulation of the inflammatory response, through the maintenance of persistent cytokine expression in macrophages. The finding that this polycomb-associated IκBz function is conserved in the Drosophila development, together with and the identification of IκB homologues in worms lacking a truly NF-κB pathway suggests that this is an ancestral function that might exert a more general contribution to the cellular physiology. Finally, the identification of specific post-translational modifications that are restricted to chromatin-bound IκBz converts its modifying enzymes and PS-IκBz in attractive targets for novel therapeutic strategies specific for particular IκBz-associated pathologies and patients.

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