Notch3 and pre-TCR interaction unveils distinct NF- κ B pathways in T-cell development and leukemia

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Notch signaling plays a critical role in T-cell differentiation and leukemogenesis. We previously demonstrated that, while pre-TCR is required for thymocytes proliferation and leukemogenesis, it is dispensable for thymocyte differentiation in Notch3-transgenic mice. Notch3-transgenic premalignant thymocytes and T lymphoma cells overexpress pT α /pre-TCR and display constitutive activation of NF- κ B, providing survival signals for immature thymocytes. We provide genetic and biochemical evidence that Notch3 triggers multiple NF- κ B activation pathways. A pre-TCR-dependent pathway preferentially activates NF- κ B via IKK β /IKK α /NIK complex, resulting in p50/p65 heterodimer nuclear entry and recruitment onto promoters of Cyclin D1, Bcl2-A1 and IL7-receptor- α genes. In contrast, upon pT α deletion, Notch3 binds IKK α and maintains NF- κ B activation through an alternative pathway, depending on an NIK-independent IKK α homodimer activity. The consequent NF- κ B2/p100 processing allows nuclear translocation of p52/RelB heterodimers, which only trigger transcription from Bcl2-A1 and IL7-receptor- α genes. Our data suggest that a finely tuned interplay between Notch3 and pre-TCR pathways converges on regulation of NF- κ B activity, leading to differential NF- κ B subunit dimerization that regulates distinct gene clusters involved in either cell differentiation or proliferation/leukemogenesis.

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Introduction

NF- κ B transcription factors play an evolutionarily conserved and critical role in triggering the transcription of a variety of genes involved in inflammatory and immune responses and in the control of cell proliferation, differentiation and apoptosis (Karin *et al*, 2002; Karin and Lin, 2002). In mammalian cells, the NF- κ B family is composed of five members, RelA (p65), RelB, c-Rel, NF- κ B1 (p50 and its precursor p105), and NF- κ B2 (p52 and its precursor p100), and exists as a heterogeneous collection of homodimers and heterodimers (Ghosh and Karin, 2002). The activity of NF- κ B homo- and heterodimers is tightly controlled through the association with inhibitory proteins of the I κ B family that sequester them into the cytoplasm. The pathways that lead to NF- κ B activation depends on components of the I κ B kinase (IKK) complex, which contains two catalytic subunits, IKK α and IKK β and a regulatory IKK γ /NEMO (Hayden and Ghosh, 2004). NEMO, like IKK β , is required for signaling through the canonical NF- κ B pathway. The critical event in initiating this pathway is the IKK β -dependent phosphorylation of I κ B α to trigger its ubiquitin-dependent degradation and induce nuclear entry of a ubiquitous NF- κ B dimer, p65:p50. Recent work has yielded some knowledge of an alternative pathway based on a regulated processing of the NF- κ B2/p100 precursor protein, allowing the resulting p52 fragment to translocate to the nucleus in association with some other NF- κ B proteins (mainly RelB) (Senftleben *et al*, 2001; Dejardin *et al*, 2002; Yilmaz *et al*, 2003). This pathway depends on NF- κ B-induced kinase (NIK) that specifically phosphorylates and activates IKK α , and upon activation recruits IKK α into a multiprotein complex with p100. This alternative pathway plays a crucial role in controlling the development, organization and function of secondary lymphoid tissue (Senftleben *et al*, 2001; Dejardin *et al*, 2002; Derudder *et al*, 2003; Bonizzi *et al*, 2004). However, its specific role in thymus development has not been addressed, as yet. The relative role of the canonical and non-canonical NF- κ B activation pathways in regulating the molecular mechanisms involved in T-cell differentiation, proliferation and/or neoplastic transformation are yet to be elucidated. In a previous report, we demonstrated that T-cell leukemia/lymphoma arising in transgenic mice overexpressing an I κ B-driven constitutively active intracellular domain of Notch3 (Notch3-IC), displays a persistent high expression of pre-T-cell receptor (pre-TCR) pT α chain and an I κ B α -dependent constitutive activation of NF- κ B (Bellavia *et al*, 2000). Pre-TCR signaling has recently been shown to lead to the activation of canonical NF- κ B pathway, independently on ligand binding (Aifantis *et al*, 2001) and, more recently, we demonstrated that PKC θ might mediate such an activation, since deletion of PKC θ in Notch3-IC transgenic mice, decreases the I κ B α -dependent activation of NF- κ B while reducing the incidence of leukemia (Felli *et al*, 2005). Finally, we observed that deletion of pT α gene mostly abrogated the

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development of leukemia/lymphoma in Notch3 transgenic mice, while the constitutive activation of Notch3 was able to rescue the blockage of the pre-TCR check-point by promoting immature DN thymocytes to progress toward a more differentiated phenotype (e.g. acquisition of CD4, CD8 and TCR β expression) in the absence of pT α /pre-TCR (Bellavia *et al*, 2002). We report here that activated Notch3 signaling triggers multiple NF- κ B activation pathways in Notch3-IC transgenic mice. Abrogation of pT α /pre-TCR function drastically reduces the activation of the canonical pathway, by switching on a NIK-independent alternative pathway activation that is strictly dependent on IKK α activity, essential for p100 processing to p52 (Bonizzi and Karin, 2004). This allows Notch3 to keep NF- κ B constitutively active despite the absence of membrane associated pre-TCR, leading to transcriptional regulation of different p52/RelB complex-dependent target genes specifically involved in thymocyte survival and differentiation.

Together, these observations suggest that a finely tuned interplay between Notch3 and pre-TCR signaling pathways converges on the regulation of NF- κ B activity and may direct T cells toward either further differentiation or neoplastic transformation outcomes.

Results

Persistence of Notch3-triggered NF- κ B activity in the absence of a functional pT α /pre-TCR

We previously observed that thymocytes from Notch3-IC transgenic mice display dysregulated high expression of pT α /pre-TCR chain and constitutive NF- κ B activity (Bellavia *et al*, 2000). Furthermore, we demonstrated that combined overexpression of pT α and Notch3 in transgenic

mice, triggers important molecular events, some of which are NF- κ B-mediated and cooperate in sustaining Notch3-induced lymphomagenesis (Bellavia *et al*, 2002; Talora *et al*, 2003; Felli *et al*, 2005). In order to investigate the possible role of pT α /pre-TCR in mediating Notch3-dependent NF- κ B constitutive activity, we monitored the ability of Notch3 to regulate NF- κ B activity in the absence of pT α , by utilizing previously established Notch3-IC/pT α ^{-/-} double-mutant mice (Bellavia *et al*, 2002). Electrophoretic mobility shift assay (EMSA) was performed with nuclear extracts from freshly isolated unfractionated thymocytes obtained from 4-week-old wild type (wt) and Notch3-IC transgenic (N3-IC) and 6–8 weeks old Notch3-IC/pT α ^{-/-} double-mutant (N3-IC/pT α ^{-/-}) mice, ages at which the thymocyte subset distribution is similar with respect to CD4 and/or CD8 expression in all the mice (Figure 1A). As shown in Figure 1B, NF- κ B DNA-binding activity is significantly increased in Notch3 mice, but, interestingly, it is still higher in Notch3/pT α ^{-/-} double mutant, with respect to wt mice, suggesting that pre-TCR is dispensable for Notch3-induced NF- κ B activation. However, Figure 1C shows that while in Notch3-IC thymocytes most of DNA-bound NF- κ B complexes are p50/p65, as previously described (Bellavia *et al*, 2000), in double-mutant thymocytes we observed a decreased supershift of p65, accompanied by an increased supershift of p50 and p52 and a displaced RelB binding, that suggest the increased generation of p50/p50 inhibitory homodimers and p52/RelB heterodimers. Figure 2 further support this hypothesis, showing a decrease of p50 nuclear translocation accompanied by a sustained higher nuclear translocation of p52 in Notch3-IC/pT α ^{-/-} with respect to Notch3-IC transgenic and wild-type thymocytes (Figure 2A). While Notch3-IC/pT α ^{-/-} and Notch3-IC thymocytes display a higher nuclear translocation of RelB with

Figure 1 Notch3 constitutively activates NF- κ B and the balance between different dimeric complexes depends on the presence of pre-TCR. (A) CD4⁺ and/or CD8⁺ subset distribution (detected by CD4 versus CD8 two-color FCA) of thymocytes from 4-week-old wt and Notch3-IC transgenic mice (N3-IC), and 8-week-old Notch3-IC/pT α ^{-/-} mice (N3-IC/pT α ^{-/-}); (B) EMSA of NF- κ B complex in nuclear extracts from freshly isolated thymocytes of wt, N3-IC transgenic and N3-IC/pT α ^{-/-} mice. (C) The nuclear extracts were also incubated in the absence (–) or presence (+) of antibodies against individual NF- κ B proteins (p50, p65, p52 and RelB, left panel; c-Rel, right panel) to characterize the complexes. An unrelated antibody was used as a negative control (not shown).

Figure 2 Nuclear translocation of different NF- κ B complexes in Notch3 transgenic and Notch3/pT α ^{-/-} mice. **(A)** Western blot analysis of p52, p100, p50 and RelB in cytoplasmic and nuclear extracts from thymocytes of wt, N3-IC transgenic and N3-IC/pT α ^{-/-} double-mutant mice. In total, 50 μ g of protein was loaded into each lane. **(B)** Immunofluorescence with anti-p52 antibody (red) and Hoechst staining (blue) of freshly isolated thymocytes from wt, Notch3 transgenic and N3-IC/pT α ^{-/-} mice. **(C)** Nuclear extracts from freshly isolated thymocytes from 4-week-old wt and Notch3 transgenic mice, and 8-week-old N3-IC/pT α ^{-/-} mice were revealed by Western blot with an anti-phospho-p65 (left panel). Whole-cell extracts (400 μ g) of freshly isolated thymocytes was immunoprecipitated with mouse monoclonal anti-p65 or rabbit polyclonal anti-p50 antibodies and revealed by Western blot with anti-phospho-p65 (right panels). **(D)** p52/p100 mRNA expression was assayed by RT-PCR from freshly thymocytes derived from wt, Notch3 transgenic and N3-IC/pT α ^{-/-} double-mutant mice, β -actin was used as loading control. **(E)** p100 processing in thymocytes derived from wt, Notch3 transgenic and N3-IC/pT α ^{-/-}. Upper panel, graphic representation of p100 processing as determined by densitometry; lower panel, pulse-labeled cells chased for 3 h.

respect to wt (Figure 2A). The immunofluorescence staining directly shows the increased nuclear translocation of p52 (Figure 2B). Furthermore, a decreased amount of phosphorylated p65 is observed in nuclear and total extracts of Notch3/pT α ^{-/-} double mutant with respect to Notch3-IC transgenic thymocytes (Figure 2C), leading to the generation of a decreased amount of functionally active heterodimers composed of phosphorylated p65 bound to p50 in Notch3/pT α ^{-/-} double-mutant thymocytes (Figure 2C lower right panel). Interestingly, we also observed a significant increase of the p52 precursor, p100, nuclear translocation in Notch3/pT α ^{-/-} double mutant with respect to Notch3-IC transgenic thymocytes (Figure 2A). p100 and p52 increased translocation could also be supported by an increased p100 synthesis, since increased p100 mRNA levels were observed in double-mutant thymocytes (Figure 2D). Finally, a pulse-chase assay indicated that p100 processing was also increased in Notch3/pT α ^{-/-} double mutant with respect to Notch3-IC transgenic thymocytes (Figure 2E), which further supports the increased generation of p52.

The possible participation of c-Rel to the NF- κ B complex in thymocytes of different mice was also analyzed by EMSA. The EMSA shown in Figure 1C (right panel) reveals that NF- κ B complexes do not contain significant amount of c-Rel subunit bound to DNA. This observation is in agreement with

a previous report showing that c-Rel DNA-binding activity is very weak in thymocyte extracts (Weih *et al*, 1994).

Together, these data suggest that Notch3 signaling is able to constitutively activate both the canonical and alternative NF- κ B activation pathways in thymocytes, and that the relative balance between the two pathways depends on the presence or absence of a functional pre-TCR.

Notch3 is able to trigger an NIK-independent IKK α -dependent alternative pathway of NF- κ B activation in the absence of pT α /pre-TCR

Stimuli that lead to activation, nuclear translocation and DNA binding of NF- κ B heterodimers impact on IKK complex, by regulating IKK assembling and activation. In the canonical NF- κ B signaling pathway, the activated IKK complex, predominantly acting through IKK β in an IKK γ -dependent manner, catalyzes phosphorylation, polyubiquitination and subsequent proteosomal degradation of I κ B α . We previously showed that I κ B α degradation is a critical event in the activation of NF- κ B in Notch3-IC transgenic thymocytes (Bellavia *et al*, 2000). Here we show that I κ B α degradation is absent in Notch3-IC/pT α ^{-/-} mice, since its protein levels are similar to wild-type thymocytes and significantly higher than in Notch3-IC thymocytes (Figure 3A). The reduced I κ B α degradation observed in Notch3-IC/pT α ^{-/-} thymocytes does

Figure 3 Notch3 regulates the assembling and activation of different IKK complexes, depending on the presence of pre-TCR. (A) Western blot analysis of whole-cell extracts (50 μ g) from freshly isolated thymocytes probed with anti-I κ B α , IKK β and anti-IKK α . The β -tubulin expression was used as loading control. (B) *In vitro* kinase assay using both GST-I κ B α (left panel) and GST-p100 (right panel) as exogenous substrates performed on IKK α immunoprecipitation from cell lysates of thymocytes from 4-week-old wt and Notch3 transgenic mice, and 8-week-old N3-IC/pT α ^{-/-} mice. The upper panels show a graphic representation of both I κ B α and p100 phospho-substrates amount as determined by densitometry. (C) Total cell extracts (400 μ g) derived from wt, N3-IC and N3-IC/pT α ^{-/-} thymocytes were immunoprecipitated with mouse monoclonal anti-IKK α antibody and revealed in Western Blot with both anti-IKK α and anti-IKK β antibodies. The upper panel shows a graphic representation of the IKK α /IKK β complex amount as determined by densitometry. (D) Upper panel, Western blot analysis of whole-cell extracts (50 μ g) from freshly isolated thymocytes probed with an anti-NIK antibody. Lower panel, total cell extracts (400 μ g) derived from wt, N3-IC and N3-IC/pT α ^{-/-} thymocytes, immunoprecipitated with anti-NIK and revealed in Western blot with anti-Notch3 antibody. (E) Total cell extracts (400 μ g) derived from wt, Notch3 and N3-IC/pT α ^{-/-} thymocytes were immunoprecipitated with mouse monoclonal anti-IKK α antibody and revealed in Western blot with both anti-NIK antibody (upper panel) and anti-Notch3 antibody (lower panel). (F) Western blot analysis of whole-cell extracts (50 μ g) from freshly isolated thymocytes probed with anti-phospho-IKK α . The β -tubulin expression was used as loading control. (G) I κ B α stability analysis in thymocytes derived from wt, Notch3 transgenic and N3-IC/pT α ^{-/-} by cycloheximide (CHX) treatment (right panel). The β -tubulin expression was used as loading control. Graphic representation of I κ B α protein half-lives as determined by densitometry (left panel).

not depend on protein resynthesis but possibly to an increased stability. Indeed, blocking protein synthesis with cycloheximide (CHX) for 3 and 6 h, I κ B α was more rapidly degraded in Notch3-IC transgenic with respect to double-mutant thymocyte extracts in which, however, a lower degradation rate is appreciated (Figure 3G).

In keeping with these observations, the levels of IKK β are significantly decreased in thymocytes from Notch3-IC/pT α ^{-/-} with respect to Notch3-IC mice (Figure 3A, middle panel). In contrast, the lower panel of Figure 3A shows that the levels of IKK α are similar in both Notch3-IC transgenic and Notch3-IC/pT α ^{-/-} double-mutant thymocytes. Together, these observations prompted us to study the composition of IKK complex in different mice. Thymocyte extracts were immunoprecipitated with anti-IKK α , followed by immunoblotting with IKK β and IKK α . Figure 3C shows a decrease of IKK α /IKK β complex formation in Notch3-IC/pT α ^{-/-} thymocytes with respect to

Notch3-IC cells, while showing a slight increase of IKK α revealed by immunoblotting with IKK α itself, in the same extracts. The IKK α -mediated phosphorylation of specific IKK complex substrates, I κ B α and p100, were also assessed and the panel B of Figure 3 shows a decreased I κ B α phosphorylation, associated to an increased p100 phosphorylation in Notch3-IC/pT α ^{-/-} thymocytes, further supporting the increased function of an IKK α -dependent alternative NF- κ B pathway in the absence of a functional pre-TCR (Figure 3B).

It has been previously shown that the activation of the alternative pathway mainly depends on NF- κ B-inducing kinase (NIK) that specifically phosphorylates and activates IKK α , and, upon activation, recruits IKK α into a multiprotein complex, allowing p100 processing to p52 (Xiao *et al*, 2004). We therefore examined the protein expression levels of NIK and its binding to IKK α in thymocyte extracts obtained from different mice. The Figure 3 shows that thymocytes from

Notch3-IC transgenic thymocytes display higher levels of NIK (Figure 3D) as well as increased IKK α /NIK complex (Figure 3E), when compared to wild-type and double-mutant thymocytes.

Thus, unexpectedly, in thymocytes from double-mutant mice, in which we observe a preferential activation of an IKK α -dependent alternative pathway, the protein levels of NIK, as well as its interaction with IKK α , are decreased (Figure 3E). On the other hand, we observed an increased nuclear translocation of p100 (Figure 2A), which has been shown to be also able, in cooperation with activated IKK α , to sustain the processing of p100 to generate p52 (Qing and Xiao, 2005).

The reduced interaction between IKK α and NIK, showed in double-mutant thymocytes extracts (Figure 3E), may also explain the reduction of IKK β bound to IKK α in IP experiment shown in Figure 3C. Indeed, it has been previously shown that the activation of IKK β , required to form a functional IKK α /IKK β heterodimer, requires the recruitment of NIK by IKK α (O'Mahony *et al*, 2000).

It has been recently reported that Notch1 associates with p56lck and PI3K kinases and increases their phosphorylation in activated T cells (Sade *et al*, 2004). In order to investigate whether Notch3 was able to act similarly with respect to IKK α , we examined the possible interaction between Notch3 and the IKK α kinase. Figure 3E shows that Notch3 forms a complex with IKK α in the extracts of thymocytes obtained from both Notch3 and Notch3-IC/pT α ^{-/-} mice, suggesting that this unmodified contact is pT α /pre-TCR independent. Interestingly, the immunoblotting with an anti-phosphoIKK antibody of thymocyte extracts showed a significant increased phosphorylation in extracts from double-mutant thymocytes (Figure 3F). Given the decrease of IKK α /IKK β complex formation in Notch3-IC/pT α ^{-/-} thymocytes with respect to Notch3-IC cells (Figure 3C), this observation further supports the possible increased activation/phosphorylation of IKK α in double-mutant thymocytes, despite a decreased IKK α /NIK interaction.

Altogether our observations suggest that Notch3 is able to activate canonical and alternative NF- κ B pathways by regulating the assembling and activation of different IKK complexes. Both these pathways convey on constitutive NF- κ B activation, but differ each other, the first one requiring the presence of a pT α /pre-TCR receptor, which leads mainly to heterodimer IKK α /IKK β complex formation. The second one is instead pre-TCR-independent and possibly results in the formation of IKK α /IKK α homodimer. Importantly, IKK α forms a complex with Notch3 independently on the presence

of pre-TCR (Figure 3E, lower panel), but only the presence of pre-TCR allows NIK to participate at the Notch3-IKK α complex (Figure 3E, upper panel and Figure 3D, lower panel).

The Notch3-induced recruitment of different NF- κ B heterodimers on gene promoters depends on the presence of pre-TCR

It has been recently reported that the two NF- κ B activation pathways differentially activate a number of genes (Bonizzi and Karin, 2004). The canonical pathway has been suggested to be mainly involved in the transcriptional control of genes involved in the innate immune response, but also in lymphoid cell proliferation and protection from apoptosis, while the alternative pathway plays a central role in the expression of genes involved in the development of secondary lymphoid organs.

The results reported above demonstrate that in the absence of pT α /pre-TCR, Notch3 specifically activates an IKK α -dependent alternative NF- κ B activation pathway. Interestingly, we previously reported that enforced expression of Notch3 is effective in inducing T-cell leukemia only in the presence of a functional pre-TCR (Bellavia *et al*, 2002). On the other hand, in the absence of pT α , Notch3 is able to rescue the blockage of the pre-TCR check-point by forcing immature DN thymocytes to progress toward a more differentiated phenotype, while it is not able to restore the total thymocyte yield (Bellavia *et al*, 2002). The failure of Notch3-IC/pT α ^{-/-} double-mutant mice to drive sufficient thymocyte population expansion, despite a significant rescue of T-cell differentiation, supports a model in which Notch3 and pre-TCR signals cooperate in a nonredundant manner to regulate either proliferation or differentiation of thymocytes and allowed us to hypothesize that the Notch3-induced differential activation of these two pre-TCR-dependent and -independent NF- κ B pathways may play a specific role in either thymocyte proliferation/leukemogenesis or differentiation.

To explore this hypothesis we compared the expression levels of genes known to be responsive to NF- κ B transcriptional regulation and differentially involved in these processes in thymocytes obtained from wild type, Notch3-IC mutant and Notch3-IC/pT α ^{-/-} double-mutant mice. We considered cyclin D1, known for its growth-promoting function in several cancers, Bcl2-A1, an antiapoptotic gene previously described as a pre-TCR-induced and Notch3-increased regulator of thymocyte survival (Bellavia *et al*, 2000; Mandal *et al*, 2005); IL7R α , known to play a critical role in early thymocyte development (Peschon *et al*, 1994). Figure 4 shows that all of these genes are significantly upregulated (from 3.5- up to

Figure 4 NF- κ B target genes are differentially regulated by Notch3 depending on the presence or absence of pre-TCR. (A) Cyclin D1, (B) Bcl2 A1 and (C) IL7 receptor alpha expression was analyzed by RT-PCR from primary thymocytes derived from wt, Notch3 transgenic and N3-IC/pT α ^{-/-} double-mutant mice. β -Actin was used as loading control.

Figure 5 Notch3 induces the recruitment of different NF- κ B complexes on gene promoters, depending on the presence of pre-TCR. Schematic representation of (A) cyclin D1, (B) Bcl2 A1 and (C) IL7 receptor α promoter regions assessed by chromatin immunoprecipitation are indicated. Primary T cells derived from Notch3 and N3-IC/pT α ^{-/-} mice were processed by using antibodies directed against p65, RelB, p52, and RNA polymerase II (pol II) or no antibody as control. Immunoprecipitated DNA was analyzed by PCR with (A) cyclin D1, (B) Bcl2 A1 and (C) IL-7 receptor α promoter-specific primers. The amount of input promoter DNA is also shown. The results are representative of three similar experiments.

7.5-fold of increase) in thymocytes of transgenic with respect to wild-type mice. In contrast, such an effect was variably influenced by the absence of pre-TCR. Indeed, while the levels of cyclin D1 (Figure 4A) completely returned to the wild-type levels, the levels of IL7R α (Figure 4C) were further increased whereas the levels of Bcl2A1 (Figure 4B) remained at an intermediate level in thymocytes from Notch3-IC/pT α ^{-/-} double mutant with respect to Notch3-IC transgenic and wild-type mice.

To address whether the different genes are direct targets for different pre-TCR-dependent or -independent NF- κ B dimers, we performed chromatin immunoprecipitation (ChIP) experiments (Saccani and Natoli, 2002).

Figure 5 shows that the recruitment of p65, responsible for the strongest transcriptional potential of canonical NF- κ B complex (Chan *et al*, 2005), to the promoters of those different genes is readily detected in chromatin extracts of Notch3 transgenic thymocytes, further supporting the hypothesis that in the presence of pre-TCR Notch3 preferentially activates the canonical NF- κ B pathway. In contrast, in the absence of pre-TCR, p65 is never recruited to the different gene promoters, once again supporting the hypothesis that p65 does not bind DNA in the absence of pre-TCR, possibly because it cannot be phosphorylated (Figure 2C). Under these conditions, p52 appears to be the main NF- κ B transcriptional complex component, since this protein is always recruited to the promoter of different genes in thymocytes from Notch3/pT α ^{-/-} double-mutant mice, confirming the constitutive processing of p100 induced by the activation of Notch3 in the absence of pre-TCR. However, while RelB is also recruited on the promoter of IL7R α and Bcl2A1 genes in thymocytes from double-mutant mice, it is not recruited onto the cyclin D1 promoter (Figure 5A). This suggests an increased formation of activatory RelB/p52 heterodimers, thus justifying the increased IL7R α and Bcl2A1 mRNA levels with respect to the wild-type samples. On the other hand, p52, in the absence of RelB, might form only inhibitory homodimers in cyclin D1

promoter justifying the lack of increase of cyclin D1 mRNA levels in double-mutant thymocytes. Interestingly, the fact that the IL7R α mRNA levels are higher in thymocytes from double-mutant mice in which only RelB/p52 dimers are recruited to the promoter, with respect to transgenic thymocytes in which both p50/p65 and RelB/p52 are recruited may suggest some antagonism between the different heterodimers. However, we cannot rule out that the final transcriptional effect on different gene expression depends from the interaction with transcription factors other than NF- κ B.

Discussion

Our findings show that Notch3 is able to trigger both canonical and alternative NF- κ B activation pathways, known to determine selective activation of different NF- κ B dimers, dependent on IKK β or IKK α kinase activity respectively. We demonstrate that the ability of Notch3 to trigger either NF- κ B pathways depends on the presence or the absence of pT α /pre-TCR function, respectively. Therefore, we hypothesize that each pathway, by differentially regulating several genes, could mediate both the T-cell oncogenic (Bellavia *et al*, 2000) and/or differentiative (Bellavia *et al*, 2002) potential of Notch3.

The Notch3-induced pre-TCR-dependent NF- κ B pathway might be mediated by PKC θ since our previously reported observations showed that PKC θ is a downstream target of Notch3-signaling and that its Notch-dependent membrane translocation and activation, via Lck, require a functional pre-TCR in order to trigger the IKK β -dependent NF- κ B canonical pathway (Felli *et al*, 2005). Most importantly, deletion of PKC θ decreases such an activation as well as the incidence of leukemia (Felli *et al*, 2005).

In the present report, we provide genetic and biochemical evidence that Notch3, when devoided of the functional cooperation with pT α /pre-TCR signaling, is able to induce constitutive activation of an alternative NF- κ B pathway and

Figure 6 Speculative model of the mechanisms by which Notch3 induces the activation of different NF- κ B pathways. **(A)** Outline of the molecular events triggered by Notch3 in the presence of a functional pre-TCR. Signals arising from Notch3 and pT α /pre-TCR cooperation mainly involve the β and γ subunits of the IKK complex, via PKC θ , resulting in degradation of I κ B α , nuclear translocation and DNA binding of p50/RelA heterodimers. These molecular events are strictly associated to canonical NF- κ B activation pathway and result in prosurvival/proliferative gene activation. In the presence of pre-TCR, Notch3 also increases the association between IKK α and NIK, possibly leading to a further increase of the overall NF- κ B activation, via the non-canonical pathway. **(B)** In the absence of pre-TCR Notch3 switches on another pathway, directly interacting with and upregulating IKK α function, which enhances the phosphorylation level of p52 precursor, p100, and results in a higher p52/RelB heterodimer nuclear translocation. As a consequence, p52/RelB heterodimers accumulate in the nucleus and regulate genes that are crucial for the survival and differentiation of thymocytes.

suggest that this may have biological consequences. Indeed, we previously reported that deletion of pT α gene was able to abrogate tumor development in Notch3-IC transgenic mice (Bellavia *et al*, 2002), but, in contrast, Notch3 was able to overcome the differentiative blockade of thymocytes due to the absence of pre-TCR.

Overall, these data, together with our previous observation that Notch3 is able to transcriptionally activate pT α (Talora *et al*, 2003), allow us to speculate that Notch3 may sequentially activate firstly the alternative pathway of NF- κ B to sustain thymocyte survival and early intrathymic differentiation steps before the appearance of pT α , then, being able to trigger the transcription of pT α , it may cooperate with pre-TCR to activate the canonical pathway of NF- κ B, mediate the thymocyte expansion and allow further differentiation.

The alternative pathway, independent of IKK β and IKK γ , acts in a way strictly dependent on IKK α homodimer, which represents the direct target of Notch3 in the absence of pre-TCR. Indeed, in a pT α /pre-TCR deprived environment, Notch3 forms a complex with IKK α kinase, independently on NIK. This interaction results in enhanced IKK α kinase activity followed by a higher p100 substrate processing, and release of p52/RelB complex. Moreover, a high nuclear translocation of p52/RelB is observed, which was also supported by DNA-binding assay showing that NF- κ B complex is mainly composed of p52 and RelB subunits in Notch3-IC/pT α ^{-/-} double-mutant thymocytes. This specific NF- κ B composition may be responsible for the restored T-cell development, observed in Notch3-IC/pT α ^{-/-} double-mutant mice, beyond the DN developmental stage at which thymocytes are usually blocked in pT α ^{-/-} mice not overexpressing Notch3 (Fehling *et al*, 1995; Bellavia *et al*, 2002). In keeping with this hypothesis, many reports describe that the heterodimer p52/

RelB is selectively recruited to the IKK α -dependent gene promoters of genes playing a crucial role in the organization of secondary lymphoid organs (Bonizzi *et al*, 2004; Bonizzi and Karin, 2004). Accordingly, we observed that in chromatin extracts of thymocytes of Notch3/pT α ^{-/-} double-mutant mice, p52/RelB heterodimer is recruited to the promoter of the prosurvival Bcl2A1 gene and of the IL7 α gene, which has been shown to play a crucial role in the early stages of intrathymic T-cell differentiation, while it is not recruited to the cyclin D1 promoter, which is more specifically associated to the triggering of cell proliferation in different cancers. RelB could also be complexed with p50 in Notch3-IC/pT α ^{-/-} thymocytes. However, it has been previously shown that in response to some stimuli (i.e. TNF- α), the association of p100 with RelB/p50 dimers in the nucleus increases, leading to the specific inhibition of RelB DNA binding (Derudder *et al*, 2003). The significant increase of p100 together with the decrease of p50 nuclear translocation, we observed in double-mutant cells, when compared to transgenic thymocytes, allowed us to exclude that RelB/p50 dimers are recruited to gene promoters in Notch3-IC/pT α ^{-/-} thymocytes. Upregulation of p100 might additionally display a proapoptotic and antioncogenic function (Wang *et al*, 2002) in Notch3/pT α ^{-/-} double-mutant mice and antagonize in this way tumorigenesis.

Overall, our previous (Bellavia *et al*, 2000; Felli *et al*, 2005) and present data suggest that pT α /pre-TCR can act as positive mediator in Notch3-induced canonical NF- κ B activation pathway, leading to the generation of p50/p65 heterodimer. Indeed, we show here an increase of I κ B α substrate phosphorylation as well as of phosphorylated p65 nuclear translocation in Notch3-IC transgenic with respect to wt and Notch3/pT α ^{-/-} double-mutant animals. The abrogation of

pT α /pre-TCR function instead drastically reduces the activation of the canonical pathway, by switching on the activation of an alternative pathway that is strictly dependent on IKK α activity and independent on NIK. Therefore, we provide a model (Figure 6) by which Notch3 keeps NF- κ B constitutively active both in the presence and in the absence of membrane associated pre-TCR. Either condition may result in the preferential generation of different IKK complexes, differentially requiring the cooperation of NIK and resulting in the activation of p50/p65 and/or p52/RelB complex followed by the transcriptional regulation of different NF- κ B target genes, which may direct thymocytes toward further differentiation or neoplastic transformation outcomes.

Materials and methods

Mice

The generation and typing of Notch3-IC transgenic (Bellavia *et al*, 2000) and Notch3-IC/pT α ^{-/-} double-mutant (Bellavia *et al*, 2002) mice have been described.

Immunoprecipitation

Freshly isolated thymocytes were lysed in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA pH 8.0, 30 mM NaF, 2 mM Na-pyro-Phosphate, 1 mM Na-orthovanadate (Na₃VO₄), Protease inhibitor cocktail tablets (Roche, Penzberg, Germany). In total, 300–500 μ g of total proteins in lysates from each sample were immunoprecipitated with antibodies against p50 (sc-8414 Santa Cruz Biotechnology, Inc.), p65 (#3034, Cell Signaling), IKK α (IMG-136, IMGENEX) and NIK (sc-7211, Santa Cruz Biotechnology, Inc.) conjugated to A/G plus-agarose (sc-8014, Santa Cruz Biotechnology, Inc.).

Immunoblotting

For immunoblotting, proteins were resolved by 10% SDS-PAGE and blotted onto nitrocellulose membrane. Blots were incubated with the indicated primary antibodies, carefully washed and after incubation with horseradish peroxidase-labeled goat-antirabbit or goat-antimouse (Amersham, Arlington Heights, IL), developed with the ECL detection system (Amersham). Blots were incubated with antibodies directed against IKK α (IMG-136, IMGENEX), IKK β (IMG-159, IMGENEX), p50 (#06-886, Upstate), p65 (#3034, Cell Signaling), p52/p100 (sc 298, SantaCruz Biotechnology Inc.), p-IKK α /p (Thr 23-R) (sc-21660-R, Santa Cruz Biotechnology, Inc.), RelB (sc-c19, Santa Cruz Biotechnology, Inc.), I κ B α (sc-4094, Santa Cruz Biotechnology, Inc.), NIK (sc-7211, Santa Cruz Biotechnology, Inc.) and Notch3 (sc-20 Santa Cruz Biotechnology, Inc.). The levels of phospho-p65 in the immunoprecipitated samples were detected using an anti-phospho-p65 (#3031, Cell Signaling).

RT-PCR analysis

Total RNA was isolated from thymocytes in guanidine isothiocyanate and further processed for reverse transcriptase (RT)-PCR as previously described (Bellavia *et al*, 2000). PCR was performed using the following primers:

IL7- α Forward, 5' CGAGTGAATGCCTAACTC 3';
 IL7- α Reverse, 5' GCGTCCAGTTGCTTTAC 3';
 p100 Forward, 5' GCCTGGATGGCATCCCCG 3';
 p100 Reverse, 5' CTTTCACTGGAGGCACCT 3';
 Bcl2-A1 Forward, 5' ATTCCAACAGCCTCCAGATATG 3';
 Bcl2-A1 Reverse, 5' GAAACAAATATCTGCAACTCTGG 3';
 Cyclin D1 Forward, 5' CGTGCAGAAGGAGATTGTGC 3';
 Cyclin D1 Reverse, 5' GTCTGCTGTCTCATCCGC 3'.

Cell labelling and pulse-chase assay

Freshly isolated thymocytes were starved for 1 h in DMEM lacking methionine and cysteine and followed by metabolically labelling for 45 min with 350 μ Ci ³⁵S-labeled methionine/³⁵S-labeled cysteine. The pulse-labeled cells were chased for 3 h in complete DMEM supplemented with 1 mg/ml cold methionine/cysteine and lysed in HNTG buffer (50 mM HEPES pH 7.5, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 10% glycerol, supplemented with a

protease inhibitor cocktail). The radiolabeled p100 and p52 were isolated by immunoprecipitation using anti-p52 (sc-298, Santa Cruz Biotechnology Inc.), fractionated by SDS-PAGE and visualized by autoradiography.

Cycloheximide assay

To inhibit protein synthesis freshly isolated thymocytes were cultured in RPMI in the presence of 10 μ g/ml CHX for various time lengths. Total cell lysates were fractionated by SDS-PAGE and subjected to immunoblot analysis with anti-I κ B α (sc-847, Santa Cruz Biotechnology Inc.).

Generation of recombinant pGEX plasmids

Glutathione S-transferase (GST)-NF- κ B2 (p100) fusion protein was created by insertion of p100 nucleotides encoding amino acids 1–899 into the pGEX-4T vector (Pharmacia, Uppsala, Sweden) between EcoRI sites in frame with GST. The fusion protein construct uses the p100 stop codon.

Purification of GST-p100 proteins from Escherichia coli

GST fusion protein expression and purification were performed essentially as described by Smith and Johnson (1988). Briefly, overnight cultures of *E. coli* (BL21 strain) transformed with either pGEX-4T or pGEX-4T recombinants were diluted 1/10 in LB medium with ampicillin (100 μ g/ml Sigma Chemical Co., Poole, Dorset, UK) and incubated at 37°C. After 4 h of growth isopropyl- β -D-thiogalactopyranoside (IPTG; Sigma Chemical Co., Poole, Dorset, UK) was added at a final concentration 0.5 μ mol/l, and the cultures were incubated for 4 h at 30°C. The bacterial cultures were pelleted by centrifugation at 5000 g for 5 min at 4°C and resuspended in 1/10 volume of NTEN (20 μ mol/l Tris pH 8, 100 μ mol/l NaCl, 1 μ mol EDTA, 0.5% NP-40). The bacteria were then lysed by mild sonication and centrifuged at 10 000 g for 10 min at 4°C. An aliquot of 25 μ l of glutathione-Sepharose 4B (1:1 vol/vol in NTEN containing 1% powdered milk; Pharmacia) was added to each milliliter of the bacterial supernatant, and the suspension was gently rocked for 30 min at 4°C. The glutathione-Sepharose beads were then washed three times with NETN and GST-fusion proteins were eluted with 15 μ mol/l of reduced glutathione (Sigma) in 50 μ mol/l Tris-HCl, pH 8. For analysis eluted proteins were loaded onto SDS-polyacrylamide gels and visualized by Coomassie blue staining.

IKK- α kinase assay and immunoblotting analysis

Thymocytes were lysed in buffer containing 20 mM Tris pH 7.6, and 0.5% NP-40, 0.25 M NaCl, 3 mM EDTA, 3 mM EGTA, 20 mM NaF, 2 mM Na₃VO₄, 1 mM dithiothreitol, 100 μ g/ml leupeptin, 20 μ g/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMFS) and immunoprecipitated with anti-IKK α (IMG136 IMGENEX) conjugated to protein A/G plus-agarose (sc-8014, Santa Cruz Biotechnology, Inc.). The immunoprecipitates were incubated with a kinase buffer master mix supplemented with 20 μ M ATP, 5 μ Ci of [γ -³²P]ATP and 3 μ g of GST-I κ B α substrate (sc-4094, Santa Cruz Biotechnology, Inc.) or 3 μ g GST-p100 substrate at 30°C for 30 min. The samples were analysed by 10% SDS-PAGE and the phosphorylation status of GST-I κ B α and GST-p100 substrates was detected by autoradiography.

Electrophoretic mobility shift assay

Nuclear extracts were prepared as previously described (Bellavia *et al*, 2000). Unfractionated thymocytes were derived from 5 weeks old wt and Notch3-IC transgenic mice and from 6 to 8 weeks old Notch-IC/pT α ^{-/-}. α ³²P dATP-labeled double-stranded oligonucleotide spanning the NF- κ B site (5'-GATCCAACGGCAGGGGAATT CCCCTCTCCTTA-3') was incubated with 5 μ g of nuclear extract at RT for 20 min with, 2 μ g of poly(dI-dC) in 50 mM NaCl, 10 mM Tris pH 7.5, 1 mM DTT and 20% glycerol. Band shifts were resolved on non-denaturing 4% polyacrylamide gel. Antibodies against p50 (sc-114X), p65 (sc-7151), RelB (sc-226), c-Rel (N sc-70) (Santa Cruz Biotechnology, Inc.), and p52 (#413, Upstate Biotechnology, Lake Placid) were incubated for 20 min at RT before the binding reaction.

Chromatin immunoprecipitation assay

Protein complexes were crosslinked to DNA in living nuclei by adding formaldehyde (Sigma, Inc.) directly to freshly isolated thymocytes to a final concentration of 1%. Crosslinking was allowed to proceed for 10 min at 37°C and was then stopped by the addition of glycine to a final concentration of 0.125 M. Crosslinked

cells were washed twice with phosphate-buffered saline containing PMSF 1 mM and pelleted. Nuclei were extracted with a 20 mM Tris pH 8, 3 mM MgCl₂, 20 mM KCl buffer containing protease inhibitors, pelleted by microcentrifugation and lysed by incubation in SDS lysis buffer (1% sodium dodecyl sulfate, 10 mM EDTA, 50 mM Tris-HCl pH 8.1), containing protease inhibitors. The resulting chromatin solution was sonicated for 15 pulses of 15 s at 80% power to generate 300–1000 bp DNA fragments. After microcentrifugation, the supernatant was diluted 1:10 with a dilution buffer (0.01% sodium dodecyl sulfate, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl, containing protease inhibitors), precleared with Salmon Sperm DNA/Protein A agarose (#157, Upstate Biotechnology), and divided into aliquots. In total, 5 μ g of antibody (anti-p52 sc-298, anti-p65 sc-h286, anti-RelB sc-226, anti-Pol II sc-899, Santa Cruz Biotechnology Inc.) was added to each aliquot of chromatin and incubated on a rotating platform for 12–16 h at 4°C. Antibody–protein–DNA complexes were isolated by immunoprecipitation with Salmon Sperm DNA/Protein A agarose (#157, Upstate Biotechnology). Following extensive washing, bound DNA fragments were eluted and analyzed by subsequent

PCR using primers specific for the cyclin D1, Bcl2 A1 and IL7 α mouse promoters.

Immunofluorescence

Cytospin preparations of thymocytes from wt, Notch3-IC transgenic and double-mutant Notch3-IC/pT α ^{−/−} mice were fixed in 4% paraformaldehyde for 20 min at room temperature, incubated in 0.2% Triton X-100 to permeabilize cells and then incubated in blocking buffer (PBS with 3% BSA). Primary antibody was a mouse monoclonal anti-p52 (sc-7386, Santa Cruz Biotechnology Inc.). Secondary antibody was Texas-RED-conjugated anti-mouse Ig (Jackson Immuno Research Laboratories). All slides were examined by confocal microscopy.

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References

- Aifantis I, Gounari F, Scorrano L, Borowski C, von Boehmer H (2001) Constitutive pre-TCR signaling promotes differentiation through Ca²⁺ mobilization and activation of NF- κ B and NFAT. *Nat Immunol* **2**: 403–409
- Bellavia D, Campese AF, Alessi E, Vacca A, Felli MP, Balestri A, Stoppacciaro A, Tiverton C, Tatangelo L, Giovarelli M, Gaetano C, Ruco L, Hoffman ES, Hayday AC, Lendahl U, Frati L, Gulino A, Screpanti I (2000) Constitutive activation of NF- κ B and T-cell leukemia/lymphoma in Notch3 transgenic mice. *EMBO J* **19**: 3337–3348
- Bellavia D, Campese AF, Checquolo S, Balestri A, Biondi A, Cazzaniga G, Lendahl U, Fehling HJ, Hayday AC, Frati L, von Boehmer H, Gulino A, Screpanti I (2002) Combined expression of pT α and Notch3 in T cell leukemia identifies the requirement of preTCR for leukemogenesis. *Proc Natl Acad Sci USA* **99**: 3788–3793
- Bonizzi G, Beben M, Otero DC, Johnson-Vroom KE, Cao Y, Vu D, Jegga AG, Aronow BJ, Ghosh G, Rickert RC, Karin M (2004) Activation of IKK α target genes depends on recognition of specific κ B binding sites by RelB:p52 dimers. *EMBO J* **23**: 4202–4210
- Bonizzi G, Karin M (2004) The two NF- κ B activation pathways and their role in innate and adaptive immunity. *Trends Immunol* **25**: 280–288
- Chan C, Li L, McCall CE, Yoza BK (2005) Endotoxin tolerance disrupts chromatin remodeling and NF- κ B transactivation at the IL-1 β promoter. *J Immunol* **175**: 461–468
- Dejardin E, Droin NM, Delhase M, Haas E, Cao Y, Makris C, Li ZW, Karin M, Ware CF, Green DR (2002) The lymphotoxin-beta receptor induces different patterns of gene expression via two NF- κ B pathways. *Immunity* **17**: 525–535
- Derudder E, Dejardin E, Pritchard LL, Green DR, Korner M, Baud V (2003) RelB/p50 dimers are differentially regulated by tumor necrosis factor- α and lymphotoxin-beta receptor activation: critical roles for p100. *J Biol Chem* **278**: 23278–23284
- Fehling HJ, Krotkova A, Saint-Ruf C, von Boehmer H (1995) Crucial role of the pre-T-cell receptor α gene in development of α β but not γ δ T cells. *Nature* **375**: 795–798
- Felli MP, Vacca A, Calce A, Bellavia D, Campese AF, Grillo R, Di Giovine M, Checquolo S, Talora C, Palermo R, Di Mario G, Frati L, Gulino A, Screpanti I (2005) PKC θ mediates pre-TCR signaling and contributes to Notch3-induced T-cell leukemia. *Oncogene* **24**: 992–1000
- Ghosh S, Karin M (2002) Missing pieces in the NF- κ B puzzle. *Cell* **109** (Suppl): S81–S96
- Hayden MS, Ghosh S (2004) Signaling to NF- κ B. *Genes Dev* **18**: 2195–2224
- Karin M, Cao Y, Greten FR, Li ZW (2002) NF- κ B in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* **2**: 301–310
- Karin M, Lin A (2002) NF- κ B at the crossroads of life and death. *Nat Immunol* **3**: 221–227
- Mandal M, Borowski C, Palomero T, Ferrando AA, Oberdoerffer P, Meng F, Ruiz-Vela A, Ciofani M, Zuniga-Pflucker JC, Screpanti I, Look AT, Korsmeyer SJ, Rajewsky K, von Boehmer H, Aifantis I (2005) The BCL2A1 gene as a pre-T cell receptor-induced regulator of thymocyte survival. *J Exp Med* **201**: 603–614
- O'Mahony A, Lin X, Geleziunas R, Greene WC (2000) Activation of the heterodimeric IkappaB kinase alpha (IKK α)–IKK β complex is directional: IKK α regulates IKK β under both basal and stimulated conditions. *Mol Cell Biol* **20**: 1170–1178
- Peschon JJ, Morrissey PJ, Grabstein KH, Ramsdell FJ, Maraskovsky E, Gliniak BC, Park LS, Ziegler SF, Williams DE, Ware CB, Meyer JD, Davison BL (1994) Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* **180**: 1955–1960
- Qing G, Xiao G (2005) Essential role of IkappaB kinase alpha in the constitutive processing of NF- κ B2 p100. *J Biol Chem* **280**: 9765–9768
- Saccani S, Natoli G (2002) Dynamic changes in histone H3 Lys 9 methylation occurring at tightly regulated inducible inflammatory genes. *Genes Dev* **16**: 2219–2224
- Sade H, Krishna S, Sarin A (2004) The anti-apoptotic effect of Notch-1 requires p56lck-dependent, Akt/PKB-mediated signaling in T cells. *J Biol Chem* **279**: 2937–2944
- Senftleben U, Cao Y, Xiao G, Greten FR, Krahn G, Bonizzi G, Chen Y, Hu Y, Fong A, Sun SC, Karin M (2001) Activation by IKK α of a second, evolutionary conserved, NF- κ B signaling pathway. *Science* **293**: 1495–1499
- Smith DB, Johnson KS (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67**: 31–40
- Talora C, Campese AF, Bellavia D, Pascucci M, Checquolo S, Groppioni M, Frati L, von Boehmer H, Gulino A, Screpanti I (2003) Pre-TCR-triggered ERK signalling-dependent downregulation of E2A activity in Notch3-induced T-cell lymphoma. *EMBO Rep* **4**: 1067–1072
- Wang Y, Cui H, Schroering A, Ding JL, Lane WS, McGill G, Fisher DE, Ding HF (2002) NF- κ B2 p100 is a pro-apoptotic protein with anti-oncogenic function. *Nat Cell Biol* **4**: 888–893
- Weih F, Carrasco D, Bravo R (1994) Constitutive and inducible Rel/NF- κ B activities in mouse thymus and spleen. *Oncogene* **9**: 3289–3297
- Xiao G, Fong A, Sun SC (2004) Induction of p100 processing by NF- κ B-informing kinase involves docking IkB kinase α (IKK α) to p100 and IKK α -mediated phosphorylation. *J Biol Chem* **279**: 30099–30105
- Yilmaz ZB, Weih DS, Sivakumar V, Weih F (2003) RelB is required for Peyer's patch development: differential regulation of p52-RelB by lymphotoxin and TNF. *EMBO J* **22**: 121–130