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*J Immunol* 2001; 167:6366-6373; doi: 10.4049/jimmunol.167.11.6366

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The Association of Aiolos Transcription Factor and Bcl-xL Is Involved in the Control of Apoptosis

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We have analyzed the mechanism implicated in the control of the anti-apoptotic role of Bcl-xL. We show that IL-4 deprivation induces apoptosis, but does not modulate Bcl-xL expression. Because Bcl-xL does not promote cell survival in the absence of IL-4, we investigate the mechanism by which Bcl-xL was unable to inhibit apoptosis. Using yeast two-hybrid system, coimmunoprecipitation, and indirect immunofluorescence techniques, we found that Bcl-xL interacts with the transcription factor Aiolos in IL-4-stimulated cells, increasing upon IL-4 deprivation. IL-4 does not promote translocation of Aiolos or Bcl-xL, but induces tyrosine phosphorylation of Aiolos, which is required for dissociation from Bcl-xL. Transfection experiments confirm that cells overexpressing Bcl-xL are able to prevent apoptosis in the absence of IL-4. On the contrary, cells that overexpress Bcl-xL and Aiolos are unable to block apoptosis in the absence of IL-4. We propose a model for the regulation of the Bcl-xL anti-apoptotic role via Aiolos. The Journal of Immunology, 2001, 167: 6366–6373.

Hemopoietic cell lines are dependent on the presence of appropriate cytokine(s) for their continued growth and survival. In the absence of growth factor, they undergo apoptosis. Bcl-2 family proteins serve as critical regulators of pathways involved in apoptosis, acting to either inhibit or promote cell death (1). This family is divided into three subfamilies. The first one comprises anti-apoptotic molecules (Bcl-2-like proteins), the second one includes proteins that are pro-apoptotic (Bax-like proteins) and the third group includes proteins that share only the BH3 domain and are pro-apoptotic (BH3-only proteins) (2, 3). Bcl-2 family proteins homo- and heterodimerize, and the balance between specific homo- and heterodimers is thought to be critical to the maintenance of cell survival or the induction of apoptosis (4). Whereas up- or down-regulation of these proteins may account for the survival of certain cell types in response to extracellular stimuli, it is also possible that survival factors may use other proteins to alter the ability of apoptotic proteins to promote cell survival or death.

An important advance in understanding apoptosis has come from the description of the Bcl-2 gene family. Within this group, Bcl-x gene encodes several alternatively spliced protein isoforms that can enhance or diminish apoptosis after transfection (5–8). The Bcl-xL inhibits cell death upon growth factor deprivation (9). A second Bcl-x isofrom, Bcl-xS, encodes a smaller protein of 170 amino acids that enhances apoptosis (10). The murine Bcl-x gene family has been expanded to include two additional isoforms, which may inhibit apoptosis in B cells (7, 11). Bcl-xL contains a hydrophobic segment at the C-terminal end (12–14) that is believed to serve as a membrane anchor.

The Aiolos transcription factor has been identified as a homologue of the Ikaros transcription factor, whose expression is restricted to the lymphoid lineage. Aiolos homodimers are potent transcriptional activators, whereas the transcriptional activity of Aiolos-Ikaros heterodimers range from low to undetectable. Aiolos was first described in committed lymphoid progenitors and is strongly up-regulated as these progenitors become restricted to T and B lymphoid pathways (15). Aiolos plays an important role as a regulator of B cell differentiation, proliferation, and maturation to an effector state (16). The interplay between these proteins in the regulation of gene expression is further complicated by additional Ikaros isoforms or by other proteins that can sequester either Ikaros or Aiolos in transcriptional inner complexes (15). Aiolos is first detected at low level in double-negative thymocyte precursors and is up-regulated as these progress to double-positive stage. Aiolos expression decreases in splenic T cells. In thymus, Bcl-2 is expressed in double-negative cells and in a few double positives, as well as in nearly all single-positive cells and in mature T cells (17–19). These observations suggest that Aiolos and Bcl-2 expression occur in parallel throughout the lymphocyte differentiation stages. We have recently shown that IL-2 starvation induces Ras/Aiolos association, resulting in apoptotic cell death. One of the functional consequences of Ras/Aiolos interaction is the blockade of Aiolos translocation to the nucleus. In the absence of IL-2, dephosphorylated Aiolos is sequestered in the cytoplasm by Ras. IL-2 stimulation induces tyrosine phosphorylation of Aiolos and dissociation from Ras. We have identified functional Aiolos binding sites in the Bcl-2 promoter. Mutation of these Aiolos binding sites within the Bcl-2 promoter inhibits transactivation of the reporter gene, suggesting a direct control of Bcl-2 expression by Aiolos. Cotransfection experiments confirm that Aiolos induces Bcl-2 expression and prevents apoptosis in IL-2-deprived cells (20). In this study, we propose a model for the control of Bcl-xL anti-apoptotic role via association to the transcription factor Aiolos.

Materials and Methods

Cells and cultures

TS1oAβ is a murine T cell line stably transfected with the human IL-2Rα- and β-chains (21). This cell line responds independently to IL-2, IL-4, or IL-9. Cells were cultured in RPMI 1640 (BioWhittaker, Walkersville,
MD), supplemented with 5% heat-inactivated FCS (Life Technologies, Gaithersburg, MD), 2 mM glutamine, 10 mM HEPES, 0.5 mM arginine, 0.24 mM asparagine, 50 μM 2-ME, and 60 μM IL-4.

**Lymphokines, Abs, reagents, and plasmids**

murine rIL-4 or supernatant of a HeLa subline transfected with pKCRIL-4.neo was used as a source of murine IL-4. Anti-Bcl-x<sub>L</sub> Ab was from Calbiochem (La Jolla, CA) or Transduction Laboratories (Lexington, KY). Mouse pan-Ras was from Oncogene Research Products (Cambridge, MA). Anti-Aiolos polyclonal Ab was generated in our laboratory. Anti-PTyr Ab was from Transduction Laboratories (Lexington, KY) or Transduction Laboratories (Lexington, KY). Mouse pan-Ras Ab was from Boehringer Mannheim, Germany). Peroxidase-conjugated goat anti-rabbit or anti-mouse Ig Ab was from Sigma-Aldrich (St. Louis, MO). Anti-Aiolos Ab was generated in our laboratory, and Cy3-, Cy2-, or Alexa 488-conjugated secondary Abs were from Molecular Probes (Eugene, OR). DEAE-Dextran was from Pharmacia (Uppsala, Sweden) and the Capture-Tec pHook3 kit was from Invitrogen (San Diego, CA). Expression vector pDNA3-Bcl-x<sub>L</sub> was kindly provided by Dr. L. del Peso (Madrid, Spain). Expression vector pCIneo-Aiolos and production of specific anti-Aiolos Ab was previously described (20).

**Transient transfection**

TS1αβ cells were transiently transfected using the DEAE-Dextran method. Cells (10<sup>5</sup> to 10<sup>6</sup>) in exponential growth were washed with TS buffer (25 mM Tris-HCl, 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 0.6 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4). Cells were transfected with pHook3 or pHook3 in combination with Bcl-x<sub>L</sub> or with Bcl-x<sub>L</sub> and Aiolos. 750 μl of TS buffer and 750 μl of freshly prepared DEAE-Dextran (1 mg/ml in TS buffer) were mixed successively with the cells and incubated for 20 min at room temperature, after which 13 ml of RPMI 1640–5% FCS were added. Cells were incubated (1 h at 37°C), centrifuged, and resuspended in 12 ml of RPMI 1640–5% FCS alone or supplemented with 60 μM IL-4. The pHook3 vector drives the expression of a hapten-specific single-chain Ab (sFv) on the surface of transfected cells. Cells expressing the sFV were isolated from the culture by binding to hapten-coated (pHox) magnetic beads and were analyzed.

**Estimation of apoptosis by PI staining**

A total of 2 × 10<sup>5</sup> cells were washed and resuspended in PBS, permeabilized with 0.1% Nonidet P-40, and stained with 50 μg/ml PI immediately before analysis. Samples were analyzed using an Epics XL flow cytometer (Cotax, Miami, FL). Apoptosis was measured as the percentage of cells in the sub-G<sub>1</sub> region of fluorescence scale having a hypodiploid DNA content.

**Immunoprecipitation and Western blotting**

Cells (1 × 10<sup>6</sup>) were IL-4 stimulated or deprived for 12 or 24 h and lysed for 20 min at 4°C in lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, protease and phosphatase inhibitor cocktail). Lysates were centrifuged (20 min, 15,000 rpm, 4°C) and either immunoprecipitated with the corresponding Ab (overnight at 4°C) or centrifuged, and resuspended in 12 ml of RPMI 1640–5% FCS before analysis. Samples were analyzed using an Epics XL flow cytometer. Apoptosis was measured as the percentage of cells in the sub-G<sub>1</sub> region of fluorescence scale having a hypodiploid DNA content.

**Results**

Bcl-x<sub>L</sub> does not promote cell survival in IL-4-deprived TS1αβ cells

We have previously described that Bcl-2 is expressed in IL-2-stimulated cells and Bcl-x<sub>L</sub> in IL-4-cultured TS1αβ cells (22). We asked whether Bcl-x<sub>L</sub> could replace the anti-apoptotic role of Bcl-2 in IL-4-stimulated cells. When IL-4-maintained cells are deprived of lymphokine, they undergo apoptosis (Fig. 1A). As early as 4 h after IL-4 deprivation, around 9% of cells were apoptotic, reaching 35%–40% at 24 h, whereas control IL-4-stimulated cells show no apoptosis.

To study the effect of IL-4 deprivation on Bcl-x<sub>L</sub> expression, TS1αβ cells were IL-4 stimulated or deprived for different periods of time, and total Bcl-x<sub>L</sub> expression was analyzed by Western blot. Bcl-x<sub>L</sub> expression was observed in control IL-4-stimulated cells and was similar throughout the deprivation period analyzed (Fig. 1B). Although similar levels of Bcl-x<sub>L</sub> were detected after IL-4 withdrawal, deprivation induced a strong increase in apoptosis (Fig. 1A). We did not observe expression of Bcl-x<sub>S</sub> (data not shown).
Because Bcl-xL does not promote cell survival in the absence of IL-4, we investigated the mechanism by which Bcl-xL was unable to inhibit apoptosis. Using Bcl-xL as bait in the yeast two-hybrid system, we screened a cDNA library from the TS1αβ cell. Among of the yeast transformants screened, 41 clones were identified that interacted with the Bcl-xL fusion protein. The clones were sequenced. Seven of these were Aiolos, 24 were Bad, six were Bcl-G, and four were Harakiri. The interaction between Aiolos and Bcl-xL proteins is indicated by the induction of LacZ expression (Fig. 2). Neither Aiolos nor Bcl-xL alone restore LacZ expression. We were not able to detect interaction between Ras/Bcl-xL or Aiolos/Raf. Deletion of the BH4 and BH3 domain of Bcl-xL abolishes interaction with Aiolos (Fig. 2). Ras-Raf interaction was used as a positive control. To analyze the cellular distribution of Aiolos, total, nuclear, and cytoplasmic expression of Aiolos was studied in IL-4 stimulated or deprived cells. When IL-4-maintained cells were deprived of lymphokine, total Aiolos expression was not modified throughout the starvation period analyzed, compared with control cells (Fig. 3A). To study the distribution of Aiolos, we performed Western blots of nuclear and cytoplasmic extracts of IL-4-stimulated or -deprived cells. Similar levels of Aiolos were detected in cytoplasmic extracts of IL-4-stimulated or -deprived cells (Fig. 3B). As an internal control of protein fractionation, hybridization with pan-Ras (cytoplasmic marker) is shown, using nuclear proteins as a negative control (Fig. 3B, lane N). As a control of the proper protein fractionation procedure, membrane was probed with anti-histone Ab. Similarly, Aiolos was detected in nuclear extracts of IL-4-stimulated or -deprived TS1αβ cells (Fig. 3C), suggesting that IL-4 deprivation does not induce trafficking of Aiolos from the cytoplasm to the nucleus. Purity of protein fractionation was detected by hybridization with anti-histone Ab (nuclear marker). Cytoplasmic proteins were used as a negative control (Fig. 3C, lane C). The absence of nuclear Bcl-xL expression was shown by probing the blot with anti-Bcl-xL Ab.

**Association of Bcl-xL and Aiolos in vivo**

To analyze whether Bcl-xL and Aiolos could interact in vivo, validating the results obtained in the two-hybrid system, we performed reciprocal communoprecipitation experiments of cytoplasmic proteins under IL-4 stimulation or deprivation of TS1αβ.
cells. Bcl-xL was detected in anti-Aiolos immunoprecipitates of IL-4-stimulated cells, increasing in 12-h and 24-h IL-4-deprived cells (Fig. 4A). Densitometric analysis of Aiolos/Bcl-xL association shows an increase of ~3-fold in IL-4-deprived cells compared with control IL-4-stimulated cells. The specificity of this interaction was confirmed by immunoprecipitation with an irrelevant Ab, anti-IL-2 (Fig. 4A). Similar amounts of Aiolos are shown in the cytoplasm of IL-4-stimulated or -deprived cells (Fig. 4A). Purity of protein fractionation was detected by hybridization with anti-histone Ab, using nuclear extracts as a positive control (Fig. 4A, lane N). In reciprocal experiments, Aiolos was detected in anti-Bcl-xL immunoprecipitates of IL-4-stimulated cells, increasing throughout the deprivation period (Fig. 4B), showing an ~3-fold higher association in IL-4-deprived cells compared with control cells. Bcl-xL protein does not change, as evidenced by hybridization of the membrane with anti-Bcl-xL Ab (Fig. 4B). The specificity of the interaction was tested as above. Aiolos/Bcl-xL interaction was also observed using freshly isolated thymocytes, confirming the results obtained in vitro (Fig. 4A, lane T). We were not able to detect association of Aiolos and the survival molecule Bcl-3 (data not shown). These results show that Aiolos interact with Bcl-xL and that the association increases with the deprivation period. SD of n = 7 is shown for Fig. 4, A and B. The t test for Fig. 4A shows p < 0.001 for 24-h samples and p < 0.01 for 12-h samples. The t test for Fig. 4B shows p < 0.0001 for 24-h samples and p < 0.005 for 12-h samples. To estimate the total Bcl-xL amount associated to Aiolos, supernatant from Aiolos immunoprecipitates (10 x 10^6 cells) corresponding to 15% (Fig. 4C, lane 1, 1.5 x 10^5), 10% (lane 2, 1 x 10^5), 5% (lane 3, 0.5 x 10^5), 2.5% (lane 4, 0.25 x 10^5), and 1.25% (lane 5, 0.12 x 10^5) of total cells was transferred to nitrocellulose and blotted with anti-Bcl-xL Ab. As shown in Fig. 4C, ~10% of the total amount of Bcl-xL was associated to Aiolos. It is interesting to mention that we are estimating the association of cytoplasmic Aiolos and Bcl-xL. We have analyzed in detail by sucrose gradient the cellular distribution of Bcl-xL in IL-4-stimulated cells showing that ~65% of total Bcl-xL is associated to lipid rafts and ~35% is detected in cytoplasm (data not shown).

IL-4 induces tyrosine phosphorylation of Aiolos

To further analyze the mechanism by which Bcl-xL and Aiolos interact in the cytoplasm of IL-4-deprived cells, and given that...
IL-2 induces tyrosine phosphorylation of Aiolos, we analyzed whether IL-4 could induce Aiolos phosphorylation. Tyrosine-phosphorylated Aiolos was detected in Aiolos immunoprecipitates of IL-4-stimulated cells, decreasing throughout the deprivation period analyzed (Fig. 5A). Cytoplasmic immunoprecipitated Aiolos was detected by reprobing the membrane with anti-Aiolos Ab, showing similar levels. To verify that Bcl-xL was associated to unphosphorylated Aiolos, we performed Bcl-xL or Aiolos immunoprecipitation of cytoplasmic proteins upon IL-4-stimulation or -deprivation conditions. Tyrosine-phosphorylated Aiolos was detected in Aiolos immunoprecipitates of control IL-4-stimulated cells (Fig 5B, lane C). Tyrosine-phosphorylated Aiolos was not detected in Bcl-xL immunoprecipitates of IL-4-stimulated or -deprived cells (Fig. 5B). Immunoprecipitated Aiolos and Bcl-xL were detected by reprobing the membrane with anti-Aiolos and anti-Bcl-xL Ab. Densitometric analysis of Western blot showed ~3x more Aiolos/Bcl-xL association in IL-4-deprived cells. This result suggests that IL-4 induces tyrosine phosphorylation of Aiolos, preventing its association with Bcl-xL or, alternatively, that IL-4 may influence the state of Bcl-xL and, consequently, its dissociation from Aiolos.

Because IL-4 deprivation in TS1αβ cells correlates with induction of apoptosis without modification of Bcl-xL expression and increased association of dephosphorylated Aiolos to Bcl-xL, we hypothesized that Bcl-xL would not be able to prevent apoptosis in IL-4-deprived cells because this anti-apoptotic molecule is associated to unphosphorylated Aiolos in the cytoplasm. Cells transfected with Bcl-xL and deprived of IL-4 for 24 h show a strong reduction in the fraction of apoptotic cells compared with IL-4-deprived mock-transfected cells (Fig. 6A). Transfected cells show 2-fold induction of Bcl-xL expression. To confirm that Aiolos transcription factor associates Bcl-xL, preventing its anti-apoptotic role, cells were transfected with pHook3 or pHook3, Bcl-xL, and Aiolos, and then were selected. Cells transfected with Bcl-xL and Aiolos and deprived of IL-4 for 24 h show a percentage of apoptosis comparable to that observed in mock transfectants (Fig. 6A). The fraction of apoptotic cells remains similar in control or transfected cells maintained in IL-4. Expression of transiently transfected Bcl-xL and Aiolos was confirmed by comparison of Bcl-xL and Aiolos protein levels in transfected cells and mock controls (Fig. 6B). Densitometric analysis of Western blot of transfected cells shows an induction of Bcl-xL and Aiolos expression in transfected cells. To conclusively confirm that association of Aiolos to Bcl-xL prevents its anti-apoptotic role, we performed Bcl-xL immunoprecipitation in Bcl-xL/Aiolos-transfected cells. Fig. 7 shows a high level of Aiolos/Bcl-xL association in Aiolos/Bcl-xL-transfected cells compared with mock- or Bcl-xL-transfected cells, as observed upon densitometric analysis of Western blot. Given that association of Aiolos to Bcl-xL in IL-4-deprived cells blocks the anti-apoptotic role of Bcl-xL, we analyzed whether down-regulation of Aiolos expression in IL-4-deprived cells would enhance cell survival by Bcl-xL. Fig. 8 shows that inhibition of Aiolos expression by antisense oligonucleotide treatment enhances cell survival by Bcl-xL in the absence of IL-4. The inhibition of Aiolos expression upon antisense oligonucleotide treatment was estimated by Western blot (Fig. 8). Taken together, these results suggest that Bcl-xL is not able to act as a survival factor in IL-4-deprived cells, because this anti-apoptotic molecule is sequestered by Aiolos and only cells overexpressing Bcl-xL are able to block apoptosis in the absence of IL-4.

**Discussion**

Bcl-x is a member of the Bcl-2 family that plays a key role in the regulation of apoptosis in various cell lineages. Stimulation of TS1αβ cells by IL-4 induces Bcl-xL expression, which was not altered throughout the starvation period analyzed, suggesting that IL-4 deprivation-induced apoptosis proceeds along pathways that do not involve changes in Bcl-xL expression. It is also interesting...
to note that IL-4-stimulated cells do not express the anti-apoptotic molecule Bcl-2 (22).

It has been described in some cellular models that apoptosis induction correlates with down-regulation of Bcl-2, but not Bcl-x, expression (23, 24). Although Bcl-x can be up-regulated in T cells and overexpression of Bcl-xL can enhance T cell survival (11, 14, 25), physiologic expression of Bcl-xL is not sufficient to confer resistance to apoptosis after TCR ligation, because it is expressed equally well in apoptotic and nonapoptotic T cell blasts, suggesting association with other partners (26). The ability of Bcl-xL to protect thymocytes from death signals is counteracted by intracellular inhibitory proteins. There is accumulating evidence that function of Bcl-xL is modulated by several interacting proteins such as Bax, Bad, and Bag1 (12, 27, 28). This is also the case for TS1αβ cells maintained in the presence or absence of IL-4. A significant proportion of Bcl-xL is found to be cytosolic. Hydrophobic analysis of Bcl-xL sequence predicts a transmembrane domain at the C-terminal region (9, 12). The observation that a significant fraction of Bcl-xL is cytosolic suggests either that the C-terminal hydrophobic domain may be hidden within the interior of the protein or that Bcl-x may be involved in binding to other cytosolic factors (29). These observations suggest that cytoplasmic retention may be due to physical association with other proteins or to posttranslational modifications.

Bcl-x can be alternatively spliced to produce two protein isoforms, Bcl-xL and Bcl-xS (7, 9). IL-4-stimulated TS1αβ cells only express the Bcl-xL isoform. Cell death by IL-2 deprivation has been correlated, in some cellular models, with a decrease in the level of Bcl-x (30), but in our experimental system, Bcl-xL protein level was constant after IL-4 deprivation. Although Bcl-xL is expressed after IL-4 stimulation, it seems to be insufficient to promote cell survival because Bcl-xL is also expressed in IL-4-deprived cells. A pathway different from Bcl-2 and Bcl-xL may be triggered by IL-4 to prevent apoptosis. Alternatively, Bcl-xL could not be able to function as an anti-apoptotic molecule because it is physically associated with other molecules in the cytoplasm of
IL-4-deprived cells. IL-4 deprivation induces inhibition of Bcl-3 expression, resulting in apoptotic cell death, which is blocked by Bcl-3 expression. This result suggests that Bcl-3 can replace the anti-apoptotic role of Bcl-x, acting as survival factor in IL-4-deprived TS1αβ cells. Bcl-3 down-regulation presumably results in a change in the regulation of gene or genes important in some aspects of proliferation, differentiation, or survival. Bcl-3 expression is probably controlled by IL-4-regulated transcription factors through binding-site recognition in the promoter region of Bcl-3. It has also found that Bcl-3 is related to genes implicated in cell lineage determination and cell cycle control (31). A correlation has also been demonstrated between Bcl-3 expression and proliferation of B lymphocytes (32). Taken together, these results suggest transcriptional (Bcl-3 expression) and nontranscriptional (Bcl-x/Aiolos interaction) pathways involved in the control of apoptosis in IL-4-stimulated cells. It is also interesting to note that Bcl-x/L interacts with Aiolos in freshly isolated thymocytes, confirming the results obtained in vitro. In addition, we do not rule out that Bcl-x/L and Bcl-3 have a synergistic effect on the control of apoptosis.

We have recently shown that IL-2 starvation induces association of Ras and dephosphorylated Aiolos (20), there is no Bcl-2 expression, and apoptosis is induced as a consequence. IL-4 stimulation does not induce Bcl-2 expression, probably due to the lack of Aiolos translocation to the nucleus, although Aiolos is expressed in the nucleus, suggesting that some induced factor(s) repress Bcl-2 transcription. Alternatively, low nuclear level of Aiolos is unable to induce Bcl-2 expression. IL-2 addition induces tyrosine phosphorylation of Aiolos and dissociation from Ras. Similarly, IL-4 induces tyrosine phosphorylation of Aiolos and dissociation from Bcl-x/L. Our data demonstrate a specific interaction between Aiolos and Bcl-x/L, and no Bcl-3, in IL-4-deprived cells as well as in thymocytes and provide an explanation for the inability of Bcl-x/L to prevent apoptosis. In addition, we suggest that IL-4 plays an important role in the control of Aiolos/Bcl-x interaction. The finding reported in this manuscript proposes a novel role for Aiolos in IL-4-deprived cells as a blocker of Bcl-x anti-apoptotic function through its sequestration by unphosphorylated Aiolos. IL-4-induced tyrosine phosphorylation of Aiolos probably diminishes the affinity of Aiolos for Bcl-x/L, inducing its dissociation. We do not exclude the possibility that phosphorylated Aiolos increases its affinity for other partners or that Aiolos/Bcl-x interaction may also be regulated by other partners. The threshold to apoptosis is controlled by a balance among survival proteins, their inhibitory partners, and the strength of death signals.

Acknowledgments
We thank Dr. L. del Peso (Madrid, Spain) for gifts of reagents used in this study.

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