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ETS2 overexpression in transgenic models and in Down syndrome predisposes to apoptosis via the p53 pathway

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***ETS2* is a transcription factor encoded by a gene on human chromosome 21 and alterations in its expression have been implicated in the pathophysiological features of Down syndrome (DS). This study demonstrates that overexpression of *ETS2* results in apoptosis. This is shown in a number of circumstances, including *ETS2*-overexpressing transgenic mice and cell lines and in cells from subjects with DS. Indeed we report for the first time that the *ETS2* overexpression transgenic mouse develops a smaller thymus and lymphocyte abnormalities similar to that observed in DS. In all circumstances of *ETS2* overexpression, the increased apoptosis correlated with increased p53 and alterations in downstream factors in the p53 pathway. In the human HeLa cancer cell line, transfection with functional p53 enables ETS2 overexpression to induce apoptosis. Furthermore, crossing the *ETS2* transgenic mice with p53$^{-/-}$ mice genetically rescued the thymic apoptosis phenotype. Therefore, we conclude that overexpression of human chromosome 21-encoded ETS2 induces apoptosis that is dependent on p53. These results have important consequences for understanding DS and oncogenesis and may provide new insights into therapeutic interventions.**

INTRODUCTION

Down syndrome (DS), which is caused by an extra copy of chromosome 21, affects all major organ systems including the bone, immune, central nervous and cardiovascular systems (1) and includes a reduced incidence of solid tumours and an increased incidence of childhood leukemias (2,3). Since chromosome 21 was sequenced (4), the major challenge in DS research is to identify the normal function of the 225 predicted genes and, importantly, to determine how overexpression of these genes contributes to the pathophysiological features of DS. Since the pathological features of Down syndrome also occur in the general population, albeit less severely and later in life, elucidation of the function of chromosome 21 genes has important implications for major human diseases.

The gene that encodes ETS2 is located on human chromosome 21q22.3 (5), and has been reported to be overexpressed in brain and fibroblasts from subjects with DS (6,7). *ETS2* is a member of the Ets family of transcription factors, which has been proposed to have important functions in cancer, bone development and immune responses (8,9,12). Its overexpression could therefore contribute to features of Down syndrome related to these functions. It is currently known that Ets proteins recognize GGAA/T core sequences in promoters of target genes which, using *in vitro* model systems, have been found to include stromelysin-1, urokinase plasminogen activator, ICAM-1, PLA_2P, Presenilin-1, Cyclin D1, Jun B, Gadd45, p21/ cip1, p19ARF and p53 (10). While the identification of such target genes implies that ETS2 may be important in normal processes such as cell cycle regulation or tissue remodeling or in diseases such as cancer, little is known about proven *in vivo* functions of ETS2.

Therefore, in order to determine the *in vivo* function(s) of ETS2 and the contribution of its overexpression to the pathophysiological features of Down syndrome, we generated *ETS2* transgenic mice that were previously reported to exhibit craniofacial and bone abnormalities resembling those seen in DS (11). In the present study we further demonstrate that

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moderate overexpression of *ETS2* in the thymus and spleen (not shown) of the transgenic mice and in murine and human cell lines leads to increased apoptosis that requires p53. Importantly, elevated levels of ETS2, an increase in apoptosis and a similar deregulation of the p53 pathway is observed in DS tissues and cell lines, strongly suggesting that increased ETS2 contributes to DS pathogenesis by increasing p53 dependent apoptosis.

RESULTS

Transgenic mice overexpressing *ETS2* display hypocellularity of the thymus and increased apoptosis

The thymi of three independent lines of *ETS2*-overexpressing mice (11), CH1, MR 10.3 and MR 10.5, were examined for several criteria of thymus function. Firstly, the thymi of *ETS2*-overexpressing mice were significantly decreased in size (Fig. 1A). At 4 weeks of age the total cell number of the thymi was reduced by $70 \pm 23\%$ ($n = 14$) in line CH1, $42 \pm 18\%$ ($n = 4$) in line MR 10.3 and $27 \pm 13\%$ ($n = 4$) in line MR 10.5, respectively. Flow cytometric analyses of surface antigen-stained thymocytes indicated that there were significant reductions in all thymocyte subsets in all three lines, particularly in the proportion of $CD^{4+}CD^{8+}$ thymocytes (Table 1, CH1 line shown). Histologically, the thymi of *ETS2* transgenic mice display an ill-defined cortico-medullary boundary, a thin cortex and multiple cysts (Fig. 1B). The increased frequency of pyknotic nuclei, suggestive of apoptosis, prompted us to examine the extent of apoptosis in more detail.

In situ detection and quantitation of apoptosis in cryosections of the thymus (Fig. 1C) of CH1 *ETS2* transgenic mice revealed an increase in TUNEL positive apoptotic cells compared with non-transgenic littermate controls [121 ± 9.3 cf. 70 ± 7.4 ($n = 3$), $P < 0.05$]. Not only was there an increase in apoptosis, but also the apoptotic cells were abnormally distributed in the medulla as well as the cortex of *ETS2* transgenic mice. In contrast, in age-matched thymi of 4-week-old control mice, apoptotic cells were detected only in the cortex, indicative of ongoing negative selection. This pattern of apoptosis was mirrored by the distribution of Ets-2 as determined by immunohistochemistry (Fig. 1D). Similarly, a quantitative increase in apoptosis of isolated thymocytes was observed in all lines. For example, there was a 2-fold increase in apoptosis in the CH1 transgenic line detected by flow cytometry of PI stained cells ($29 \pm 3\%$ in control cf. to $62 \pm 8\%$ in CH1 *ETS-2* transgenic mice, Fig. 1E). This correlated with the 3-fold increase in ETS2 expression in these transgenic thymocytes detected by western blots (Fig. 3). Increased apoptosis in the transgenic mice also was detected by DNA laddering (data not shown).

Thus, we have demonstrated that, in the thymus of the *ETS2* transgenic mouse, the increase in ETS2 correlates with increased levels of apoptosis that could play an important role in the pathological processes described.

Constitutive and inducible overexpression of ETS2 transgenes sensitizes cell lines to apoptosis

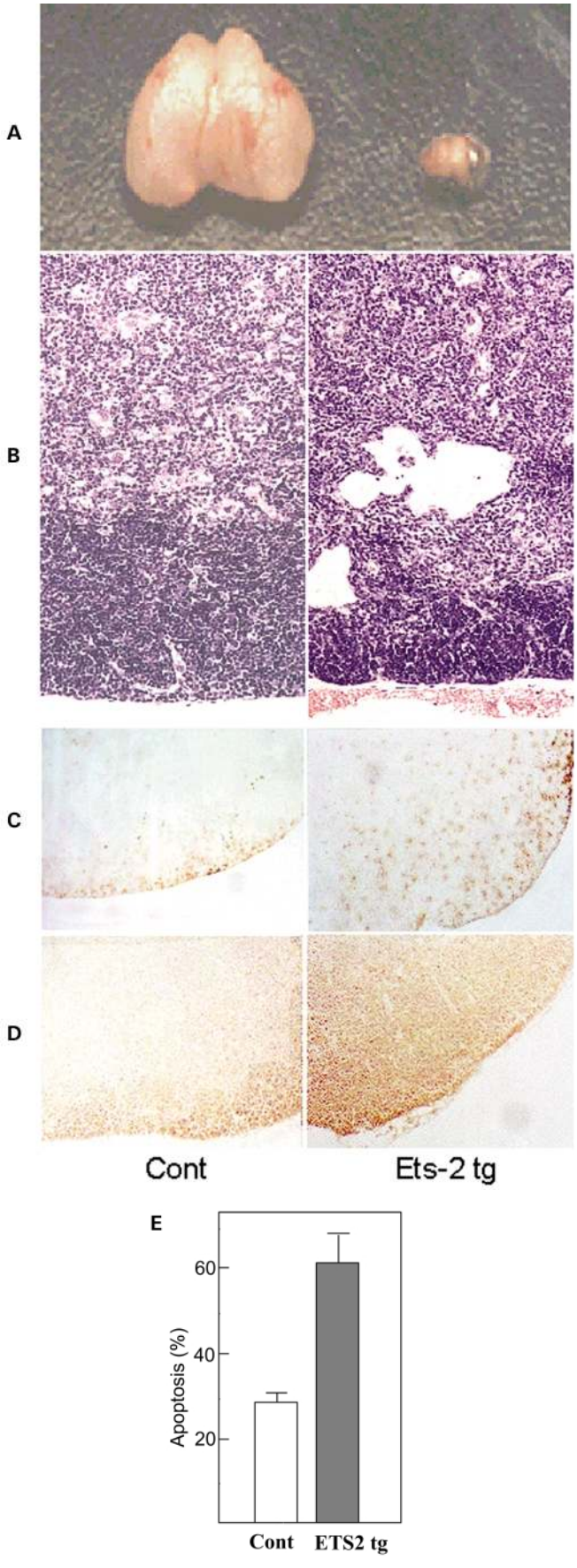
In order to investigate whether ETS2 overexpression predisposes other cell types to apoptosis and to demonstrate a direct correlation between induced expression of the *ETS2* gene and apoptosis, we next examined fibroblast cell lines expressing constitutive and inducible *ETS2* transgenes. First we established that primary embryonic fibroblast cultures from CH1 *ETS2* transgenic mice display increased ETS2 expression as shown by western blot analysis (Fig. 2A). Next we showed that primary embryonic fibroblast cultures from CH1 *ETS2* transgenic mice display increased spontaneous apoptosis in culture, plus markedly increased susceptibility to apoptosis induced by hydrogen peroxide, staurosporin and etoposide (Fig. 2A). This result indicates that the apoptosis was a direct, or cell autonomous, effect of *ETS2* overexpression. In addition, 3T3 fibroblasts that constitutively overexpress either human *ETS2* or murine *Ets2* both displayed an increased level of spontaneous and induced apoptosis (Fig. 2B). In order to demonstrate the acute temporal and concentration dependent nature of *ETS2*-induced apoptosis in a more tightly regulated system, we next tested 3T3 fibroblasts expressing ecdysone-inducible human *ETS2*. Addition of up to 8 μg/ml of the ecdysone analogue muristerone to these *ETS2* Ec-3T3 fibroblasts leads to a dose-dependent induction of ETS2 of up to 5-fold and a dose-related increase in sensitivity to hydrogen peroxide-induced apoptosis (Fig. 2C). The data clearly demonstrate that *ETS2* overexpression leads to apoptosis *in vitro* and *in vivo*; the next compelling question was: which pathway mediates the Ets2-dependent apoptosis?

Table 1. T-cell abnormalities of the thymus of *ETS2* transgenic mice Thymocytes were isolated from the CH1 *ETS2* transgenic at 4 weeks of age. After incubation with fluorescently labeled antibodies against CD^4, CD^8 and CD^3, multicolor flow cytometry was used to quantify the amount of cells expressing these markers. Results are represented as the mean ± SD of at least three independent experiments

T-cell type	Control (4 weeks)	Ets-2 tg (CH1) (4 weeks)
$CD^{4+}CD^{8+}$	$1.04 \pm 0.10 \times 10^8$	$0.32 \pm 0.32 \times 10^8$
$CD^{4+}CD^{8-}$	$1.80 \pm 0.21 \times 10^7$	$0.72 \pm 0.60 \times 10^7$
$CD^{4-}CD^{8+}$	$6.93 \pm 0.91 \times 10^6$	$2.62 \pm 1.96 \times 10^6$
CD^{3+Hi}	$2.49 \pm 0.22 \times 10^7$	$1.14 \pm 0.94 \times 10^7$

Ets-2 overexpression correlates with increased activity of the p53 pathway

Since overexpression of *ETS2* had previously been reported to induce the mRNA levels of the pro-apoptotic molecule, p53 (13), we next tested whether p53 might be involved in the *ETS2* mediated apoptosis discovered in the *ETS2* transgenic thymus and other experimental situations of ETS2 overexpression described above. In each case, increased ETS2 levels were accompanied by increased p53, namely: (a) thymus from *ETS2* transgenic mice (Fig. 3, left panel, CH1 line); (b) 3T3 fibroblasts stably-transfected with murine *Ets2* (Fig. 3 middle panel); and (c) 3T3 fibroblasts transfected with ecdysone-inducible *Ets2* (Fig. 3, right panel, time dependent). Further evidence of activation of the p53 apoptotic pathway was the observation of increased levels of the p53-regulated proapoptotic protein Bax (14,15) and decreased levels of the p53-regulated anti-apoptotic, Bcl2 (16,17) (Fig. 3). Together these data show that the *ETS2*-apoptosis pathway and the p53 pathway are linked.



Down syndrome tissues and cell lines overexpress ETS2, are predisposed to apoptosis and show deregulation of the p53 pathway

We next determined whether the ETS2–p53–apoptosis pathway correlation demonstrated in our transgenic mouse and cell culture models also existed in cells of subjects with DS. Fibroblast cell lines from subjects with Down syndrome displayed increased spontaneous (2-fold), etoposide- (2.5 fold), hydrogen peroxide- (2-fold) and staurosporin- (3 to 4-fold) induced apoptosis compared with age and passage matched control fibroblast cultures (Fig. 4A). Western blot analysis of these fibroblast lines from individuals with DS indicated that ETS2 levels are increased, p53 levels are increased and BCL2 levels are reduced (Fig. 4B). In addition to fibroblasts, other Down syndrome tissues shown to have elevated apoptosis, namely thymus (data not shown) and neurons (18) were also demonstrated to exhibit elevated levels of ETS2, p53 and reduced BCL2 (Fig. 4B). These data from a human genetic disorder mirror the *ETS2* overexpressing transgenic models described above (Figs 1–3). Together the results imply that in DS a correlation exists between elevated ETS2, activation of the p53 pathway and increased apoptosis, which could underlie some of the pathological features of this syndrome.

Genetic demonstration that p53 is necessary for Ets2 induced apoptosis of the thymus

In order to demonstrate that the p53 pathway is necessary for *ETS2* mediated apoptosis *in vivo*, we employed the standard genetic procedure of generating compound crosses of genetically modified mouse lines to prove a genetic linkage. Because the $p53^{-/-}$ mice were on a CBA × C57 BL/6 background, we first backcrossed the CH1 *ETS2* transgenic mice six times to CBA × C57 BL/6 mice then crossed with $p53^{-/-}$ mice (19). The overall thymus size and the total number of viable thymocytes from *ETS2* transgenic $p53^{-/-}$ mice ($1.35 \pm 0.19 \times 10^8$ cells/thymus, $n = 4$) were increased relative to *ETS2* transgenic mice ($0.54 \pm 0.045 \times 10^8$ cells/thymus $n = 3$, $P < 0.05$). Indeed the former values were essentially the same as controls ($1.47 \pm 0.21 \times 10^8$, $n = 8$), suggesting that this part

Figure 1. The thymus of *ETS2* transgenic mice displays severe hypocellularity and increased apoptosis. (**A**) Four-week-old *ETS2* transgenic mice (CH1 line shown) display a marked reduction in size of the thymus. (**B**) H&E staining of paraffin embedded thymus sections of a control and a CH1 *ETS2* transgenic mouse shows the thin cortical layer and multiple cysts in the *ETS2* transgenic thymus. (**C**) Immunohistochemical detection of apoptosis in the thymus of 3-week-old control and CH1 *ETS2* transgenic mice. Detection of apoptosis in 10 µm cryosections of the thymus (right panel) using the TUNEL based method (ApopTag, Oncor). Brown staining decorates cells with fragmented DNA, indicative of apoptosis. Negative controls, where the TdT enzyme was omitted, were totally free of staining (results not shown). (**D**) Immunohistochemical detection of ETS2 in cryosections of the thymus from 3-week-old CH1 *ETS2* transgenic (right) and control mice (left). Brown staining indicates ETS2 immunoreactivity. Pre-incubation of the Ets2 antiserum with excess recombinant ETS2 resulted in a complete loss of ETS2 staining (results not shown). (**E**) Apoptosis determined by flow cytometry. Isolated washed thymocyte suspensions were incubated for 72 h, after which the amount of apoptosis was determined from the number of sub-Go events detected by flow cytometry of fixed PI stained cells. The results shown are the mean of at least three experiments from the CH1 *ETS2* transgenic in triplicate ± SD.

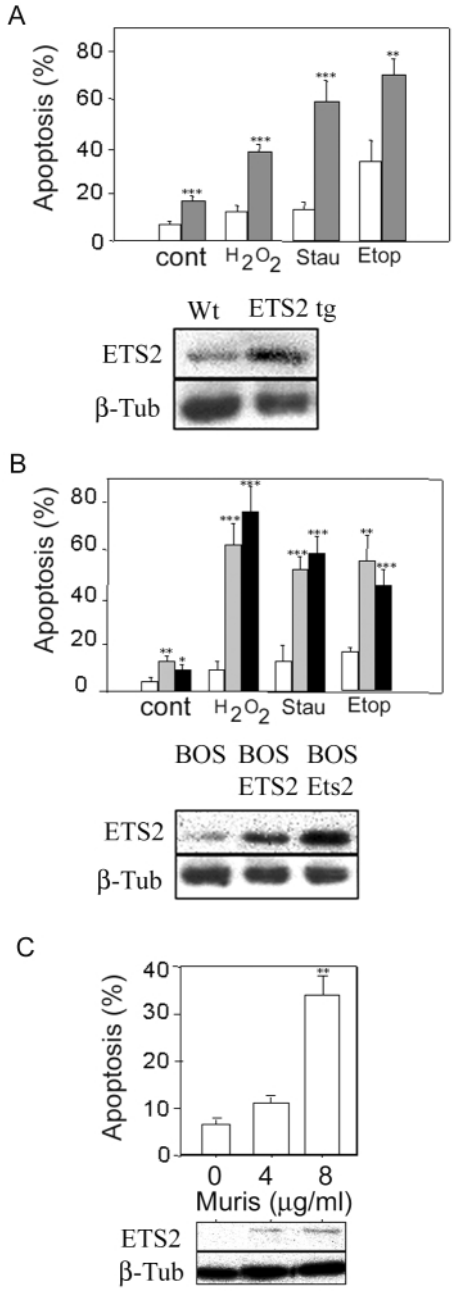


Figure 2. ETS2 overexpressing cell lines show increased apoptosis. (**A**) Primary embryonic fibroblasts established from CH1 *ETS2* transgenic mice (gray) and display an increase in spontaneous, hydrogen peroxide, etoposide and staurosporin induced apoptosis relative to controls (blank). Western blots show elevated levels of ETS2 in untreated cells. (**B**) 3T3 fibroblasts stably overexpressing either human *ETS2* (gray) or murine *Ets2* (black) display an increase in spontaneous, hydrogen peroxide-, etoposide- and staurosporin-induced apoptosis relative to controls (blank). Western blots show elevated levels of human ETS2 and murine Ets2, respectively. (**C**) Ec-*Ets2*-3T3 fibroblasts display a dose-dependent, increased sensitivity to hydrogen peroxide (200 μM, 24 h) induced apoptosis after dose dependent induction of Ets2 by 0, 4 and 8 μg/ml of the ecdysone analogue muristerone for 20 h as demonstrated by western blots. The data are presented as the mean ± SD of at least three separate experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). For the western blot analyses beta-tubulin was used as a loading control.

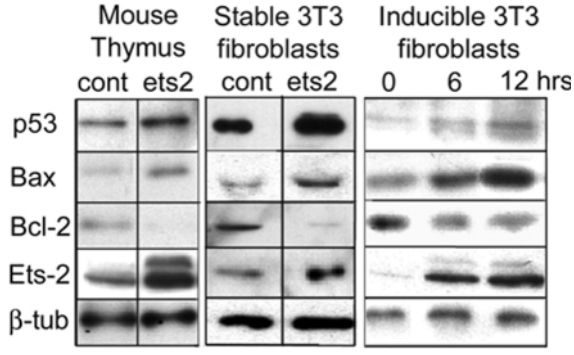


Figure 3. ETS2 overexpression results in increased p53 and Bax and decreased Bcl2 levels. Western blot analysis of protein extracts from 3-week-old thymus from control and CH1 Ets-2 transgenic mice (left panel), 3T3 fibroblasts stably transfected with murine *Ets2* (middle panel) and Ec-*Ets2* 3T3 fibroblasts in which Ets2 expression was induced with 15 μM muristerone for the times indicated (right panel). Proteins were separated on a 10% reducing SDS–PAGE gel, immunoblotted with anti-Ets2, anti-Bax, anti-p53, anti-Bcl2 (Santa Cruz) and anti-β-tubulin (Boehringer) antibodies and visualized with anti-rabbit HRP and anti-mouse HRP, respectively. A representative example is shown.

of the phenotype of *ETS2* transgenic mice had been genetically 'rescued' (Fig. 5A). Furthermore, thymocytes isolated from *ETS2* transgenic $p53^{-/-}$ mice display a significantly reduced rate of spontaneous apoptosis in culture (13 ± 2% apoptosis in *ETS2* transgenic $p53^{-/-}$ mice compared with 33 ± 8% apoptosis in *ETS2* transgenic mice, $P < 0.05$, $n = 3$; Fig. 5B). The size, cell number and the rate of spontaneous apoptosis *in vitro* of the thymus from $p53^{-/-}$ mice are not significantly different from control mice (results not shown) (20). These data demonstrate that p53 is necessary (at least in part) for the increase in spontaneous thymic apoptosis and consequent reduced thymus size in *ETS2* transgenic mice.

Functional p53 is necessary for Ets2-mediated apoptosis of HeLa cervical carcinoma cells

In order to extend the validation of the dependence of *ETS2*-induced apoptosis on a functional p53 to a human cell system and to extend previous data on the possible role of *ETS2* in cancer, we utilized the HeLa cervical carcinoma cell line, which has transcriptionally inactive p53 that cannot activate its downstream target genes (21). Upregulation of Ets2 expression in HeLa cells transfected with ecdysone-inducible *Ets2* did not lead to an increase in spontaneous apoptosis or an increased sensitivity to hydrogen peroxide induced apoptosis (Fig. 6). This is consistent with the above results that, in the absence of functional p53, Ets2 overexpression does not induce apoptosis. Subsequent transfection of these HeLa cells with a wild type p53 led to only a modest increase in spontaneous and hydrogen peroxide induced apoptosis, in the absence of Ets2 overexpression; in agreement with previously published data (Fig. 6) (22). However, when *Ets2* was simultaneously induced in these p53 transfected HeLa cell lines, a dramatic increase in hydrogen peroxide-induced apoptosis occurred from a basal level of 10 to 65% (Fig. 6). We therefore conclude that a functional p53 is necessary for *Ets2*-mediated apoptosis in a human cancer cell line as well as the *in vivo* model systems like the *ETS2* transgenic mouse.

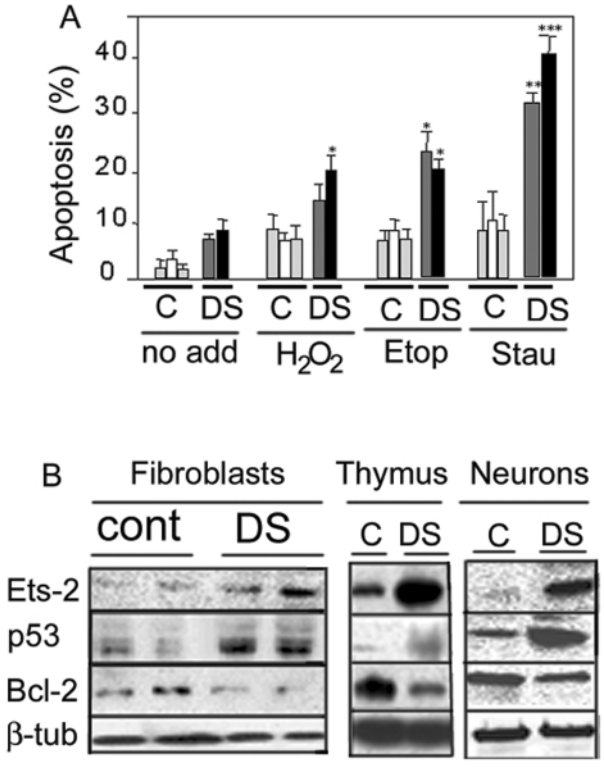


Figure 4. Down syndrome cells display elevated apoptosis, increased Ets2, and deregulation of the p53 pathway. (**A**) Fibroblast cell lines of similar passage number from three control (ATCC CRL1635, ATCC CRL105, ATCC CRL1532) and two DS individuals (ATCC CRL7031, ATCC CRL7090) were cultured at 30% confluence. The cells were subsequently incubated with 200 μM H_2O_2, 5 ng/ml staurosporin or 5 μg/ml etoposide for 24 h. Apoptosis was determined as described in Material and Methods. The results are represented as the mean ± SD of three experiments for each cell line. (**B**) Western blot analysis of protein extracted from two control (ATCC CRL1635, ATCC CRL105) and two DS fibroblast cultures (ATCC CCL84, ATCC CRL7090) and a thymus sample from 3-month-old Down syndrome individual and primary neuronal cultures from pooled aborted DS conceptuses. Blots were probed with Ets2, Bcl2 and p53 antibodies (Santa Cruz). Beta-tubulin and protein concentrations were used to normalize protein loading in each lane. A representative experiment of three is shown.

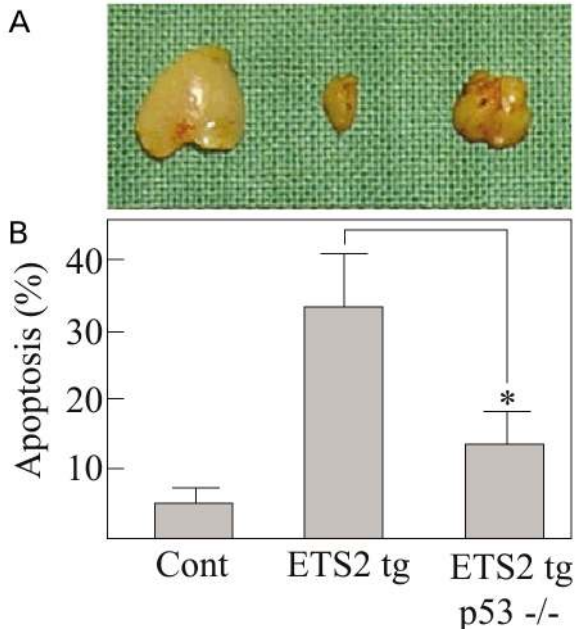


Figure 5. Dependence of ETS2-induced apoptosis on the p53 pathway *in vivo*. (**A**) Thymi and (**B**) the level of apoptosis in isolated thymocyte suspensions, from (left to right): control; CH1 *ETS2* transgenic; and CH1 *ETS2* transgenic crossed with p53 null mice. The results are expressed as the mean ± SD of at least three separate experiments (*$P < 0.05$ relative to the *ETS2* transgenic mice).

DISCUSSION

In this paper we have examined the effects of overexpression of *ETS2* because of its potential role in Down syndrome and related diseases in the general population, such as cancer. *ETS2* overexpressing transgenic mouse lines demonstrate abnormalities in the immune system including hypoplasia of the thymus (Fig. 1, Table 1), spleen (data not shown) and alterations in peripheral blood (not shown) that are similar to those observed in people with Down syndrome (23,24). We demonstrate that the process underlying these changes is an increase in apoptosis. Indeed increased expression of ETS2 predisposes to apoptosis in several defined model systems, including *ETS2* transgenic cell lines and transgenic mice, as well as in fibroblasts, neurons and thymi of subjects with Down syndrome. Furthermore, we demonstrated that cells from individuals with Down syndrome are predisposed to apoptosis, consistent with previous reports that primary neuronal cultures (18) and granulocytes (25) from DS individuals show increased apoptotic cell death and that thymocytes from Ts65Dn mice, a mouse model for DS, display increased apoptosis (26). Therefore, *ETS2*-induced apoptosis could underlie some of the pathophysiological features of Down syndrome by a similar mechanism to that observed in the mouse and cell models systems studied herein.

In order to investigate the mechanism of *ETS2*-induced apoptosis we then investigated whether *ETS2* might induce apoptosis by activation of the p53 pathway since it had previously been shown to transactivate p53 in 3T3 cells (13). In all experiments of *ETS2* overexpression that we investigated, there was an accompanying increase in levels of p53 [including thymus, spleen, fibroblasts and neurons of *ETS2* transgenic mice, *ETS2* transfected cell lines 3T3 and L-cells (not shown) plus DS fibroblasts, thymus and neurons]. Further evidence of the involvement of p53 was that alterations in the levels of p53-regulated apoptotic proteins Bcl-2 and Bax also correlated with Ets2 overexpression. These findings of a correlation between Ets2 overexpression and activation of the p53 pathway while strongly implying a direct involvement, required proof that this was the mechanism of Ets2-induced apoptosis.

Because of the limitations of doing conclusive experiments in humans, we chose two model systems in which to demonstrate that p53 was necessary for *ETS2*-induced apoptosis. Firstly, the *ETS2* transgenic mice were crossed with p53 null mice. The apoptotic thymus phenotype evident in the *ETS2* transgenic mice was 'rescued' in the *ETS2* transgenic $p53^{-/-}$ mice. Thus, in the absence of p53, *ETS2* did not induce apoptosis. The second model was to use the human HeLa cancer cell line in which p53 is not functional. While overexpression of *Ets2* did

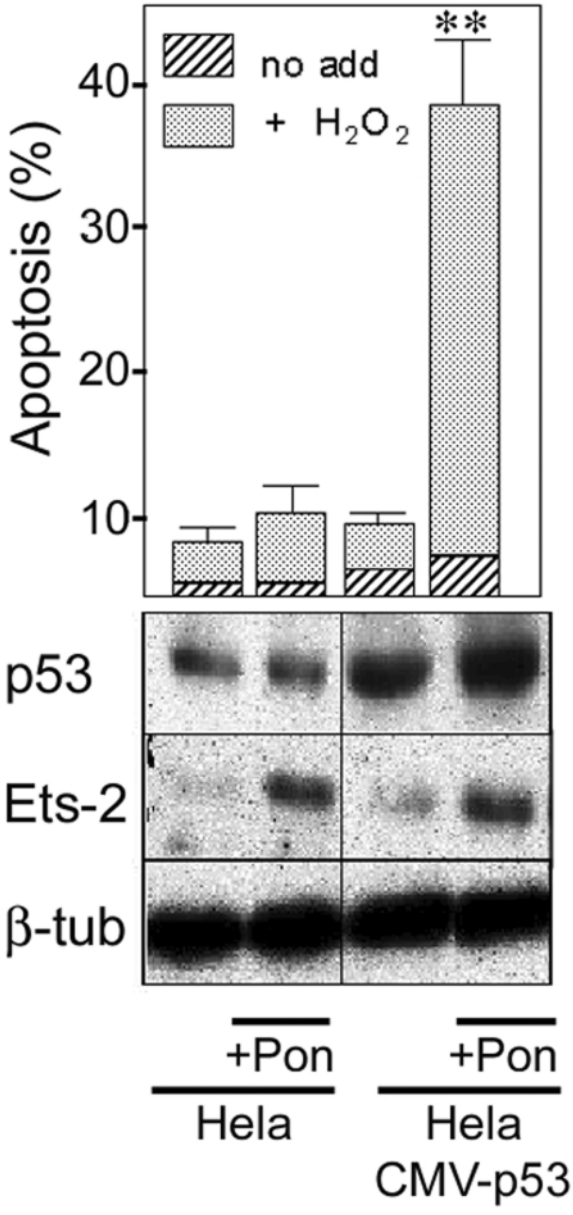


Figure 6. *Ets2*-induced apoptosis of HeLa cells requires functional p53. Apoptosis levels in Ec-*Ets2* Hela cells with ecdysone inducible Ets2 (left panel) and Ec-*Ets2* p53 Hela cells stably transfected with wild-type p53 (right panel). Ponasterone-induced expression of Ets2 in Ec-*Ets2* Hela cells (20 μM ponasterone) leads to only a minor increase in hydrogen peroxide-induced apoptosis (400 μM H_2O_2 for 24 h), whereas ponasterone-induced upregulation of Ets2 in Ec-*Ets2* p53 Hela cells results in a dramatic increase in hydrogen peroxide-induced apoptosis. Identical results were obtained in three independently transfected Ec-*Ets2* Hela cell lines. The results are the mean ± SD of at least three separate experiments (**$P < 0.01$).

not induce apoptosis in these cells, transfection with wild-type p53 enabled *Ets2* overexpression to induce apoptosis. This again demonstrated that *Ets2* required functional p53 for induction of apoptosis and broadened the relevance of *ETS2*-induced apoptosis to the regulation of cancer cells. Interestingly, an increase in p53 and BAX and a reduction in BCL2, which occur in *ETS2* overexpressing cells, have been reported in DS brains at sites of apoptotic cell death (27–29). This suggests that the mechanism of *ETS2*-induced apoptosis elucidated in the transgenic mice and human cell line also applies in DS. The implication of these data is that future therapies that target the p53 pathway could have some benefit in ameliorating some of the pathophysiological features of Down syndrome.

The molecular mechanism responsible for the dependence on p53 for *ETS2*-induced apoptosis has yet to be elucidated. One possibility is that ETS2 transcriptionally upregulates p53. This would be consistent with a previous report in which we demonstrated that overexpression of Ets2 in 3T3 fibroblasts results in increased p53 mRNA, most likely by interaction with several palindromic Ets-binding sites in the p53 promoter (13). A second (not mutually exclusive) possibility is that ETS2 requires p53 protein–protein interaction. This could occur either directly or indirectly since both ETS2 and p53 can bind to p300 (30), an interaction that regulates the stability and apoptosis-inducing properties of p53 (31). These aspects are the subject of ongoing studies.

In addition to the DS features mentioned above, our data may have some relevance to the pathogenesis of cancer. Since individuals with DS also have a reduced incidence of solid tumors (2,3), it was postulated that chromosome 21 might encode one or more tumor suppressor genes (32). Since our data in this paper show that *ETS2* requires p53 for the induction of apoptosis and that DS tissues and cell lines overexpress p53 and *ETS2*, it is tempting to speculate that the overexpression of *ETS2* activates the p53 tumor suppression pathway in DS and thus may contribute to the reduced incidence of solid tumors in DS.

Because of the well-known tumor suppressor activity of p53, our data placing *ETS2* upstream of p53 also raise the question of the role of *ETS2* in oncogenesis in general. Previous data have been paradoxical. On the one hand *ETS2* was shown to be oncogenic in prostate (33,34) and thyroid (35) cancer cells and 3T3 fibroblasts (12), cervical (36) and leukemia cancer cell lines (37–39). On the other hand, overexpression of *ETS2* has been shown to be able to reverse ras-transformation of 3T3 fibroblasts (40,41), induce apoptosis in a prostate cancer cell line (42) and regulate tumor suppressor genes such as PLA2P, p19ARF and p53 (10). Our data helps to resolve the apparent discrepancies over the role of *ETS2* in carcinogenesis. While we clearly demonstrate *in vivo* and *in vitro* that in many cell types *ETS2* is pro-apoptotic, this activity is dependent on the integrity of 'downstream' factors in the pathway such as p53. So, for example, in HeLa cells that have a non-functional p53, upregulation of *Ets2* is not pro-apoptotic while, after transfection with wild-type p53, upregulation of *Ets2* sensitizes HeLa cells to apoptosis. Indeed *ETS2*-overexpressing tumour cells could be selected for defects in p53-dependent apoptotic (or other downstream) pathways and thus be more likely to develop a transformed phenotype. In agreement with this notion, activation of the p53 tumor suppression pathway by activated ras was previously suggested to be a failsafe mechanism that allowed the cell to prevent cell transformation (43–46).

In summary we conclude that ETS2 overexpression results in an upregulation of p53 protein levels, a downregulation of BCL2, and in increase in BAX levels and consequent increased sensitivity to apoptosis. We propose that the overexpression of

ETS2 is an important contributor to the increased susceptibility of DS cells to apoptotic stimuli that might, at least in part, be responsible for the thymic and splenic hypoplasia and conceivably other pathophysiological features shared between *ETS2* transgenic mice and individuals with DS. These results might have even broader implications for the role of *ETS2* and p53 in pathological processes such as oncogenesis.

MATERIALS AND METHODS

Mouse lines

Three independent *ETS2* transgenic mouse lines (CH1, MR 10.3 and MR 10.5) were generated as described previously (11) on a CBA × C57BL/6 background. In these mice the human *ETS2* cDNA is driven by the sheep metallothioneine promoter. The p53 null mice (19) were obtained on a C57BL/6 background. Prior to crossing these mouse strains with the CH1 *ETS2* transgenic mice the mice were backcrossed six times to CBA × C57 BL/6 mice.

Cell lines and culture conditions

3T3 fibroblasts and human control and Down syndrome cell lines were maintained in DMEM containing 10% FCS, 5 μg/ml penicilline and 5 μg/ml streptomycin. L-cells were cultured in RPMI containing 10% FCS 5 μg/ml penicilline and 5 μg/ml streptomycin. Isolated thymocyte suspensions were obtained from freshly removed organs which were subsequently teased through a steel mesh, washed in RPMI, counted and incubated at 5×10^6 cells/ml for 24, 48 and 72 h in RPMI medium with 5 μg/ml β-mercaptoethanol.

For the determination of the sensitivity of cell lines to undergo apoptosis 30–40% confluent cell cultures were either incubated without additions, with 200 μM hydrogen peroxide, 5 μg/ml etoposide or 10 nM staurosporin for 24 h. The amount of apoptosis was subsequently determined by flow cytometry as described below. For determination of apoptosis in Ec-*Ets2*-3T3 and Ec-*Ets2*-L-cells, 30–40% confluent cell cultures were incubated in the presence of the indicated amount of muristerone for 20 h prior to addition of 200 μM H_2O_2 for a subsequent 24 h.

Generation of Ets2-inducible cell lines

Ec-3T3 cells containing the ecdysone receptor fused to a retinoic acid response element were purchased from Invitrogen (San Diego, CA, USA). HeLa cells and L-cells were stably transfected with the ecdysone RXR receptor (Invitrogen, San Diego, CA, USA) to generate Ec-Hela cells. A 1700 bp *Bam*H1 full-length cDNA fragment of murine *Ets2* was cloned into the multicloning site of the pIND vector, linearized and stably transfected into both the Ec-3T3 cells, Ec-HeLa cells. After 4 weeks of selection on 400 μg/ml G418 and 500 μg/ml Zeocin, surviving clones were picked, expanded and tested for Ets2 induction by muristerone. Western blotting with an Ets2-specific antibody (Santa-Cruz, sc-351 X) was used to show a dose dependent induction of Ets2 in both Ec-*Ets2*-3T3 cells, Ec-*Ets2*-HeLa cells after a 20 h incubation with 0, 4 or 8 μg/ml of the ecdysone analog muristerone. Three Ec-*Ets2*-HeLa cell lines that displayed muristerone-inducible Ets2 were subsequently transfected with a CMV-p53 expression that bears a hygromycin resistance marker construct (kindly provided by B. Vogelstein). After 3 weeks of selection in 400 μg/ml hygromycin, surviving Ec-*Ets2*-p53-Hela cells clones were picked, expanded and tested for p53 expression.

Flow cytometry

Multicolor-flow cytometry was performed with a Cytomation MO-Flo flow cytometer (Fort Collins, CO, USA) on single cell thymocyte suspensions using CD4 and CD8 antibodies from BD Pharmingen (San Diego, CA, USA) according to standard protocols.

Western blotting

Western blotting was performed according to standard procedures. Briefly, 15–40 μg of protein was separated on 10% polyacrylamide gels, transferred to Hybond C using semi-dry transfer, blocked with 4% fat-free milk powder, 1% FCS in PBS containing 0.01% Tween-20 and incubated overnight at 4°C with rabbit polyclonal antibodies against p53, Ets2, Bax (Santa Cruz), Bcl2 (Calbiochem) or β-tubulin (Boehringer). After three washings with PBS with 0.01% Tween-20 the membranes were incubated for 1–2 h at room temperature (RT) with appropriate HRP-conjugated secondary antibodies (DAKO, Denmark), followed by another 1 h wash. Super-signal (Calbiochem) chemiluminescent detection of HRP was subsequently used to visualize the cross reacting proteins. Beta-tubulin expression and protein concentration were used to normalize protein loading for each sample.

Immunohistochemistry

Cryosections of 10 μM from thymus and spleen were fixed in PBS containing 4% paraformaldehyde and 0.05% glutaraldehyde for 10 min at RT and washed in PBS. Endogenous peroxidases were inactivated with 3% H_2O_2 for 20 min at RT. Sections were washed in PBS, blocked in CAS block (Zymed, CA, USA) for 30 min and incubated overnight at 4°C with a 1:50 dilution of a commercially available Ets2 antibody (Santa Cruz, C-351 X) in CAS blocking solution. Sections were washed three times in PBS with 0.01% tween-20 and incubated with a 1:100 dilution of anti-rabbit HRP (DAKO, Denmark). Brown immuno-reactivity was detected with DAB as a substrate.

Apoptosis assays

Apoptosis was determined as the amount of cells in the sub-G0 peak after propidium iodide staining of ethanol fixed cells. Briefly, the culture supernatant was collected and centrifuged at 2000 *g* for 5 min. Adherent cells were harvested by trypsiniation and mixed with the culture supernatant and centrifuged another 10 min at 2000 *g*. The pellet was washed once with PBS, resuspended in 90% EtOH and kept overnight at −20°C. Fixed cells were pelleted and stained for at least 1 h with 20 μg/ml propidium iodide in PBS containing 10 μg/ml RNase A. *In situ* detection of apoptosis via TUNEL was performed on

10 μM cryosections from thymus and spleen according to the manufacturer's instructions using the Apoptag kit from Intergen (Oxford, UK). DNA laddering was determined as described in Solovyan and Salminen (47).

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REFERENCES

1. Korenberg, J.R., Chen, X.N., Schipper, R., Sun, Z., Gonsky, R., Gerwehr, S., Carpenter, N., Daumer, C., Dignan, P., Disteche, C. *et al*. (1994) Down syndrome phenotypes: the consequences of chromosomal imbalance. *Proc. Natl Acad. Sci. USA*, **91**, 4997–5001.
2. Satge, D., Sommelet, D., Geneix, A., Nishi, M., Malet, P. and Vekemans, M.A. (1998) tumor profile in Down syndrome. *Am. J. Med Genet.*, **78**, 207–216.
3. Hasle, H., Clemmensen, I.H. and Mikkelsen, M. (2000). Risks of leukaemia and solid tumours in individuals with Down's syndrome. *Lancet*, **355**, 165–169.
4. Hattori, M., Fujiyama, A., Taylor, T.D., Watanabe, H., Yada, T., Park, H.S., Toyoda, A., Ishii, K., Totoki, Y., Choi, D.K. *et al*. (2000) The DNA sequence of human chromosome 21. *Nature*, **405**, 311–319.
5. Sacchi, N., Watson, D.K., Guerts van Kessel, A.H., Hagemeijer, A., Kersey, J., Drabkin, H.D., Patterson, D. and Papas, T.S. (1986) Hu-ets-1 and Hu-ets-2 genes are transposed in acute leukemias with (4;11) and (8;21) translocations. *Science*, **231**, 379–382.
6. Baffico, M., Perroni, L., Rasore-Quartino, A. and Scartezzini, P. (1989) Expression of the human ETS-2 oncogene in normal fetal tissues and in the brain of a fetus with trisomy 21. *Hum. Genet.*, **83**, 295–296.
7. Tymms, M.J. and Kola, I. (1994) Regulation of gene expression by transcription factors Ets-1 and Ets-2. *Mol. Reprod. Devl.*, **39**, 208–214.
8. Remy, P. and Baltzinger, M. (2000) The Ets-transcription factor family in embryonic development: lessons from the amphibian and bird. *Oncogene*, **19**, 6417–6431.
9. Maroulakou, I.G. and Bowe, D.B. (2000) Expression and function of Ets transcription factors in mammalian development: a regulatory network. *Oncogene*, **19**, 6432–6442.
10. Sementchenko, V.I. and Watson, D.K. (2000) Ets target genes: past, present and future. *Oncogene*, **19**, 6533–6548.
11. Sumarsono, S.H., Wilson, T.J., Tymms, M.J., Venter, D.J., Corrick, C.M., Kola, R., Lahoud, M.H., Papas, T.S., Seth, A. and Kola, I. (1996) Down's syndrome-like skeletal abnormalities in Ets2 transgenic mice. *Nature*, **379**, 534–537.
12. Seth, A., Watson, D.K., Blair, D.G. and Papas, T.S. (1989) c-ets-2 protooncogene has mitogenic and oncogenic activity. *Proc. Natl Acad. Sci. USA*, **86**, 7833–7837.
13. Venanzoni, M.C., Robinson, L.R., Hodge, D.R., Kola, I. and Seth, A. (1996) ETS1 and ETS2 in p53 regulation: spatial separation of ETS binding sites (EBS) modulate protein: DNA interaction. *Oncogene*, **12**, 1199–1204.
14. el-Deiry, W.S. (1998) Regulation of p53 downstream genes. *Semin. Cancer Biol.*, **8**, 345–357.
15. Miyashita, T. and Reed, J.C. (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell*, **80**, 293–299.
16. Miyashita, T., Harigai, M., Hanada, M. and Reed, J.C. (1994) Identification of a p53-dependent negative response element in the bcl-2 gene. *Cancer Res.*, **54**, 3131–3135.
17. Miyashita, T., Krajewski, S., Krajewska, M., Wang, H.G., Lin, H.K., Liebermann, D.A., Hoffman, B. and Reed, J.C. (1994) Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression *in vitro* and *in vivo*. *Oncogene*, **9**, 1799–1805.
18. Busciglio, J. and Yankner, B.A. (1995) Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons *in vitro*. *Nature*, **378**, 776–779.
19. Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A. and Jacks, T. (1993) p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*, **362**, 847–849.
20. Benveniste, P. and Cohen, A. (1995) p53 expression is required for thymocyte apoptosis induced by adenosine deaminase deficiency. *Proc. Natl Acad. Sci. USA*, **92**, 8373–8377.
21. Goodwin, E.C. and DiMaio, D. (2000) Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes the orderly reactivation of dormant tumor suppressor pathways. *Proc. Natl Acad. Sci. USA*, **97**, 12513–12518.
22. Haupt, Y., Rowan, S. and Oren, M. (1995) p53-mediated apoptosis in HeLa cells can be overcome by excess pRB. *Oncogene*, **10**, 1563–1571.
23. Larocca, L.M., Piantelli, M., Valitutti, S., Castellino, F., Maggiano, N. and Musiani, P. (1988) Alterations in thymocyte subpopulations in Down's syndrome (trisomy 21). *Clin. Immunol. Immunopathol.*, **49**, 175–186.
24. Murphy, M. and Epstein, L.B. (1992) Down syndrome (DS) peripheral blood contains phenotypically mature CD^{3+} TCR alpha, beta+ cells but abnormal proportions of TCR alpha, beta+, TCR gamma, delta+, and CD^{4+} CD^{45RA+} cells: evidence for an inefficient release of mature T cells by the DS thymus. *Clin. Immunol. Immunopathol.*, **62**, 245–251.
25. Yasui, K., Shinozaki, K., Nakazawa, T., Agematsu, K. and Komiyama, A. (1999) Presenility of granulocytes in Down syndrome individuals. *Am. J. Med. Genet.*, **84**, 406–412.
26. Paz-Miguel, J.E., Flores, R., Sanchez-Velasco, P., Ocejo-Vinyals, G., Escribano de Diego, J., Lopez de Rego, J. and Leyva-Cobian, F. (1999) Reactive oxygen intermediates during programmed cell death induced in the thymus of the Ts(17^{16})65Dn mouse, a murine model for human Down's syndrome. *J. Immunol.*, **163**, 5399–5410.
27. Sawa, A., Oyama, F., Cairns, N.J., Amano, N. and Matsushita, M. (1997) Aberrant expression of bcl-2 gene family in Down's syndrome brains. *Brain Res. Mol. Brain Res.*, **48**, 53–59.
28. Seidl, R., Fang-Kircher, S., Bidmon, B., Cairns, N. and Lubec, G. (1999) Apoptosis-associated proteins p53 and APO-1/Fas (CD95) in brains of adult patients with Down syndrome. *Neurosci. Lett.*, **260**, 9–12.
29. Seidl, R., Bidmon, B., Bajo, M., Yoo, P.C., Cairns, N., LaCasse, E.C. and Lubec, G. (2001) Evidence for apoptosis in the fetal Down syndrome brain. *J. Child. Neurol.*, **16**, 438–442.
30. Jayaraman, G., Srinivas, R., Duggan, C., Ferreira, E., Swaminathan, S., Somasundaram, K., Williams, J., Hauser, C., Kurkinen, M., Dhar, R. *et al*. (1999) p300/cAMP-responsive element-binding protein interactions with ets-1 and ets-2 in the transcriptional activation of the human stromelysin promoter. *J. Biol. Chem.*, **274**, 17342–17352.
31. Grossman, S.R. (2001) p300/CBP/p53 interaction and regulation of the p53 response. *Eur. J. Biochem.*, **268**, 2773–2778.
32. Satge, D., Sasco, A.J., Geneix, A. and Malet, P. (1998) Another reason to look for tumor suppressor genes on chromosome 21. *Genes Chromosomes Cancer*, **21**, 1.
33. Sementchenko, V.I., Schweinfest, C.W., Papas, T.S. and Watson, D.K. (1998) ETS2 function is required to maintain the transformed state of human prostate cancer cells. *Oncogene*, **17**, 2883–2888.
34. Liu, A.Y., Corey, E., Vessella, R.L., Lange, P.H., True, L.D., Huang, G.M., Nelson, P.S. and Hood, L. (1997) Identification of differentially expressed prostate genes: increased expression of transcription factor ETS-2 in prostate cancer. *Prostate*, **30**, 145–153.
35. de Nigris, F., Mega, T., Berger, N., Barone, M.V., Santoro, M., Viglietto, G., Verde, P. and Fusco, A. (2001) Induction of ETS-1 and ETS-2 transcription factors is required for thyroid cell transformation. *Cancer Res.*, **61**, 2267–2275.
36. Simpson, S., Woodworth, C.D. and DiPaolo, J.A. (1997) Altered expression of Erg and Ets-2 transcription factors is associated with genetic changes at 21q22.2–22.3 in immortal and cervical carcinoma cell lines. *Oncogene*, **14**, 2149–2157.
37. Lafage-Pochitaloff, M., Courcoul, M., Simonetti, J., Sainty, D., Dastugue, N., Tabilio, A., Hagemeijer, A. and Birg. F. (1992) Expression of the ETS2 and transferrin receptor genes in Philadelphia-positive chronic myeloid leukemia patients with a reciprocal t(3;21). *Genes Chromosomes Cancer*, **5**, 1–13.

38. Santoro, A., Maggio, A., Carbone, P., Mirto, S., Caronia, F. and Acuto, S. (1992) Amplification of ETS2 oncogene in acute nonlymphoblastic leukemia with t(6;21;18). *Cancer Genet. Cytogenet.*, **58**, 71–75.
39. Dreyfus, F., Picard, F., Gisselbrecht, S., Varet, B., Tambourin, P.E. and Fichelson, S. (1989) ets-1 and ets-2 proto-oncogene expression in human leukemia cells and cell lines. *Nouv. Rev. Fr. Hematol.*, **31**, 217–221.
40. Foos, G., Garcia-Ramirez, J.J., Galang, C.K. and Hauser, C.A. (1998) Elevated expression of Ets2 or distinct portions of Ets2 can reverse Ras-mediated cellular transformation. *J. Biol. Chem.*, **273**, 18871–18880.
41. Wasylyk, C., Maira, S.M., Sobieszczuk, P. and Wasylyk, B. (1994) Reversion of Ras transformed cells by Ets transdominant mutants. *Oncogene*, **9**, 3665–3673.
42. Foos, G. and Hauser, C.A. (2000) Altered Ets transcription factor activity in prostate tumor cells inhibits anchorage-independent growth, survival, and invasiveness. *Oncogene*, **19**, 5507–5516.
43. Lowe, S.W. (1999) Activation of p53 by oncogenes. *Endocr. Relat. Cancer*, **6**, 45–48.
44. Lin, A.W., Barradas, M., Stone, J.C., van Aelst, L., Serrano, M. and Lowe, S.W. (1998) Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes Devl.*, **12**, 3008–3019.
45. Lin, A.W. and Lowe, S.W. (2001) Oncogenic ras activates the ARF-p53 pathway to suppress epithelial cell transformation. *Proc. Natl Acad. Sci. USA*, **98**, 5025–5030.
46. Ries, S., Biederer, C., Woods, D., Shifman, O., Shirasawa, S., Sasazuki, T., McMahon, M., Oren, M. and McCormick, F. (2000) Opposing effects of Ras on p53: transcriptional activation of mdm2 and induction of p19ARF. *Cell*, **103**, 321–330.
47. Solovyan, V. and Salminen, A. (1999) A rapid and efficient method for preparation of genomic DNA suitable for analysis of both high and low molecular weight DNA fragmentation during neuronal apoptosis. *Brain Res. Brain Res. Protoc.*, **4**, 335–340.