[Frontiers in Bioscience 13, 6501-6514, May 1, 2008]

Of alphas and betas: distinct and overlapping functions of STAT3 isoforms

Sarah Dewilde[1], Alessandro Vercelli[2], Roberto Chiarle[3], Valeria Poli[1]

[1]Molecular Biotechnology Center, University of Turin, Via Nizza 52, 10126 Turin, Italy, [2]Department of Anatomy, Pharmacology, and Forensic Medicine, University of Turin, Corso M. D'Azeglio 52, 10126 Turin, Italy, [3]Department of Biomedical Sciences and Human Oncology and CERMS, University of Turin, Via Santena 7, 10126 Turin, Italy

TABLE OF CONTENTS

1. ABSTRACT

STAT3 is a pleiotropic factor activated by many different signals including cytokines, growth factors and oncogenes. It is involved in a striking number of functions and can activate distinct repertoires of genes in different contexts. Like other STAT factors, STAT3 exists in two isoforms generated by alternative splicing, the full length STAT3alpha and the truncated STAT3beta, generally thought to act as a dominant negative factor. However, STAT3beta is not transcriptionally inactive and is able to both activate and repress genes depending on cellular environment. These unique properties of the STAT3beta isoform may contribute to the extraordinary functional complexity of STAT3 physiological and pathological actions, revealed by conditional mutagenesis studies and not yet fully understood. With this in mind, we try here to summarize what is known about the structure and function of the alpha and beta STAT3 isoforms, both *in vitro* and *in vivo*. In addition, we report unpublished data describing the phenotype of mice where the STAT3alpha isoform was specifically ablated.

2. STAT3 IS A MULTIFUNCTIONAL TRANSCRIPTION FACTOR

STAT3 was initially identified as Acute Phase Response Factor (APRF), responsible for activating acute phase gene's promoters in response to IL-6 (1). APRF/STAT3 was then identified as the main STAT factor activated by all members of the IL-6 family of cytokines (2-4). One of STAT3 peculiar features is its capacity to activate different sets of genes in different cell types in response to a striking variety of cytokines, growth factors, hormones and oncogenes (e.g. IL-6 family members, leptin, IL-12, Interferons, IL-10, G-CSF, prolactin, growth hormone, EGF, HGF, bFGF, v-Src, v-Fps, v-Sis). Accordingly, STAT3 is the only family member whose inactivation is early embryonic lethal (5) and STAT3 tissue-specific inactivation has revealed complex and even contrasting roles linked to inflammation, regeneration, growth-factor induced proliferation and energy homeostasis (6). Although STAT3 is generally considered an oncogene, being constitutively phosphorylated in a wide variety of tumors and tumor cell lines where its inactivation triggers growth arrest and cell

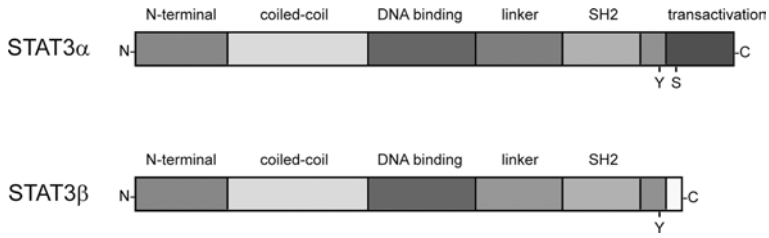

Figure 1. Domain structure of STAT3alpha and STAT3beta. The STAT3alpha protein is composed of several domains: N-terminal, coiled-coiled, DNA-binding, linker, Src homology 2 (SH2) and transcriptional activation domain (TAD). The critical tyrosine motif, Y^{705} (Y), is located between the SH2 and TAD sequences. S^{727} (S) is located within the TAD, and is missing in the STAT3beta splicing variant.

death (7), its role in regulating proliferation is not unambiguous. STAT3 acts indeed as a pro-survival factor through the induction of anti-apoptotic and cell cycle progression genes in most tumoral cells as well as in the regenerating liver and in thymocytes (reviewed in ref. 6). However, STAT3 activation was linked to growth arrest and/or apoptotic cell death in a number of systems, including myeloid cells (8), macrophages (9), IFNbeta-treated pro-B cells (10) and mammary epithelial cells during gland involution (11). In addition, STAT3 is essential for the induction of inflammatory genes in the liver (12), but it also mediates IL-10 anti-inflammatory functions in macrophages, where its inactivation leads to chronic intestinal inflammation (13-15).

An important factor determining STAT3 functional heterogeneity is likely the existence of two alternatively spliced isoforms, the full length STAT3alpha and the truncated STAT3beta, which lacks the carboxy-terminal transcription activation domain (TAD) and is generally considered as a dominant negative form (16, 17). Two other proteolytically generated isoforms, gamma and delta, have been detected in myeloid cells but their physiological relevance remains uncertain. Conventional gene targeting approaches, abolishing the expression of all isoforms, could not discriminate between specific isoform functions. Below we review what is known about the structure, production and functional characteristics of the main STAT3 isoforms, alpha and beta.

2.1. The alternatively spliced isoform STAT3beta is expressed at lower levels but exhibits increased DNA binding activity

STAT3beta is generated by the use of an alternative splicing acceptor site embedded in exon 23 (18). This causes an internal deletion spanning the first 50 nucleotides of exon 23, resulting in a frameshift which introduces 7 alternative amino acid residues followed by a stop codon (Figure 1). The resulting STAT3beta protein lacks the 55 C-terminal residues carrying the STAT3alpha TAD including S727, whose phosphorylation can stimulate transcriptional activity (19). The TAD is replaced by a different seven amino acid tail (16), a feature unique to the beta isoform of STAT3.

The STAT3alpha and STAT3beta proteins are co-expressed in all cell types and tissues examined, with STAT3alpha levels usually greatly exceeding those of STAT3beta (16, 17). However, the ratio of expressed and activated STAT3beta versus STAT3alpha can increase up to 1:1 in hepatocytes and macrophages upon inflammatory stimuli (VP, unpublished observation), as well as in differentiated neutrophils (20, 21). Most cytokines and growth factors can activate both isoforms with similar efficiency (22), and STAT3alpha and beta can form homo- and heterodimers with each other and with STAT1 (23). Interestingly, STAT3beta when overexpressed is constitutively tyrosine-phosphorylated and can bind to DNA and promote transcription in the absence of cytokine treatment (16, 17), likely due to the increased half life of the tyrosine phosphorylated dimer (22). Deletion of the STAT3alpha C-terminal portion enhances both DNA binding activity and dimer stability, suggesting that the acidic C-terminal domain may destabilize STAT3alpha dimers (23). In addition, the unique STAT3beta 7 amino acid tail may also contribute to STAT3beta features since it was shown to trigger prolonged nuclear retention of phosphorylated STAT3beta (24). These data help explaining why STAT3beta contribution to the overall STAT3 DNA binding activity often exceeds its relative abundance (25).

2.2. STAT3alpha and beta transcriptional activity

The STAT3alpha C-terminal TAD is thought to act via interaction with co-activators such as CBP/p300 (26). Because of its increased DNA binding activity and dimer stability in the absence of the TAD, STAT3beta was proposed to be a dominant negative factor. Indeed, this isoform is devoid of trans-activating capacity on a number of recognized STAT3alpha target genes, on which, when overexpressed, it is often able to exert a dominant negative effect. For example, STAT3beta could revert the transcriptional activation of Bcl_{XL}, $p21^{WAF1/CIP1}$ and cyclin D1 promoters induced by STAT3alpha upon c- or v-Src stimulation, respectively (27, 28). In addition STAT3alpha, but not STAT3beta, is involved in mediating IL-10 functions in macrophages, induces SOCS3 in response to IL-6 in mouse embryonal fibroblasts (MEFs) (29) and can associate with SP1 on the ICAM-1 promoter to induce expression in ischemic-reperfused myocardium and vascular endothelium (30). Finally, activation of STAT3alpha, but not of STAT3beta, increases IL-8 synthesis after stimulation of human microvascular endothelial cells with oxidized phospholipids (31).

However, STAT3beta activities have not only been linked to repression. Depending on promoter or

cellular context, STAT3beta can be an equally potent transcriptional activator as STAT3alpha. For example, both isoforms can activate the $p27^{Kip1}$ gene upon G-CSF stimulation in myeloblastic cells (32), as well as the alpha1-anti-chymotrypsin and alpha2-macroglobulin promoters in hepatoma cells, but not in COS cells (33). In addition, STAT3beta is able to induce transcription of liver genes during the early phases of inflammation in mice devoid of the alpha isoform (29). Finally STAT3beta, but not STAT3alpha, can cooperate with c-Jun to activate an alpha2-macroglobulin-Thymidine Kinase promoter fusion in murine teratocarcinoma and COS cells (17). However, the interaction with c-Jun involves the STAT3 N-terminal coiled-coil region, present in both the alpha and beta isoforms (34), which can indeed equally well participate in the formation of a ternary complex (enhanceosome) on the alpha2-macroglobulin promoter involving both STAT3 and AP1 binding sites (35). This suggests that not only transcriptional activity but also interactions with other factors can be dictated by promoter structure. Interestingly, while STAT3alpha-c-Jun cooperation leads to repression of Fas transcription in human melanoma cells, STAT3beta can induce Fas expression (36). Whether this was a direct consequence of STAT3beta transcriptional activity or simply the effect of competition with STAT3alpha was not investigated.

TAD-mediated recruitment of co-activators such as CBP/p300 and NcoA/SRC1a (37, 38) can be enhanced by S727 phosphorylation (26). How does STAT3beta, which lacks the TAD including S727, activate transcription? It is tempting to speculate that STAT3alpha-specific target genes may be activated by direct STAT3alpha-dependent recruitment of co-activators. On the other hand, both STAT3alpha and beta may be able to activate a common subset of target genes by participating in the formation of higher hierarchy complexes, which would in turn provide the optimal surface to recruit co-activators and the basal transcriptional machinery. Depending on promoter composition, specific subsets of target genes might even be solely responsive to STAT3beta. Accordingly, transcriptional activation by either isoform appears to depend on the cell type and the specific stimuli, as shown by the observation that the alpha-2 macroglobulin promoter can be induced only by STAT3alpha in EGF-stimulated COS-7 cells, while upon c-Src activation the same promoter was equally well induced not only by STAT3beta, but also by a mutant form of STAT3alpha where S727 was mutated to alanine (STAT3 S727A) (39). In contrast, S727 phosphorylation was required for transcription from alpha2 macroglobulin, JunB and ICAM-1-IRE promoter constructs in response to IL-6 (26, 40, 41). Overall, these data support the importance of enhanceosome composition in determining STAT3alpha or beta trans-activating potential, with the requirement for TAD and/or S727 phosphorylation depending on promoter and cellular context. Interestingly, pre-established S727 phosphorylation was reported to negatively regulate Y705 phosphorylation (42). STAT3beta, lacking S727, would be insensitive to this negative control, at least partly explaining its constitutive Y705 phosphorylation.

2.3. STAT3beta opposes STAT3alpha transforming activities

STAT3 is constitutively active in many tumoral cells and primary tumors and is therefore considered an oncogene, acting via the induction of target genes involved in apoptosis and proliferation and triggering angiogenesis, tissue invasion and immune evasion (43, 44). Accumulating evidence shows that STAT3beta has inhibitory effects on STAT3alpha oncogenic activity. Indeed, STAT3beta could suppress STAT3-mediated transformation and $p21^{WAF1/CIP1}$, cyclin D1 and Bcl_{XL} expression downstream of v-Src (28, 45). Of note, v-Src-induced transformation could also be suppressed by overexpression of a STAT3 S727A mutant form (46), thus implying S727 phosphorylation in v-Src-mediated cellular transformation. In addition, STAT3beta over-expression decreased Bcl_{XL} expression and promoted apoptosis in IL-6-dependent multiple myeloma cells (47), counter-acted HGF/Met-mediated anchorage-independent growth both *in vitro* and *in vivo* (48), and inhibited cell growth of ovarian, breast and colon cancer cells (49-51). Finally, STAT3beta transfection suppressed *in vivo* growth of B16 human melanoma cells xenografts (52), possibly involving the production of soluble factors (53). Accordingly, STAT3beta could up-regulate the expression of pro-inflammatory cytokines in cancer cells, and supernatants from STAT3beta-transfected B16 melanoma cells were able to induce the activation of macrophages, granulocytes and dendritic cells, correlating with anti-tumoral activity (54). In contrast however, transplantation of bone marrow cells over-expressing STAT3beta in mice led to formation of an aggressive T cell leukemia, similarly to cells expressing a constitutively active form of STAT3alpha (V. Sexl, personal communication), underscoring once again the extreme degree of cell specificity of STAT3 isoform functions and suggesting a potential pro-oncogenic role of STAT3beta in hematopoietic cells.

2.4. The relative levels of STAT3 isoforms are highly regulated during myeloid differentiation

G-CSF engagement of G-CSFR triggers both STAT3alpha and STAT3beta recruitment and activation (55) and the role of STAT3 in granulocytic proliferation, differentiation and maturation has been intensely studied, yielding contrasting results. STAT3 was required for G-CSF-dependent differentiation of granulocytic cell lines (ref. 56 and references therein), and mice carrying a truncated G-CSF receptor lacking the STAT docking sites displayed impaired G-CSF-dependent proliferation and granulocytic differentiation (57). In contrast, STAT3 deletion in bone marrow progenitors caused neutrophilia and increased proliferation in response to G-CSF, suggesting a role for this factor in regulating steady state neutrophil production, which may be mediated by the induction of SOCS3, and excluding its requirement for *in vivo* granulopoiesis (8, 58, 59). Again by conditional mutagenesis, STAT3 was however recently

Table 1. Primers

Name	Sequence
Ex20B	GCGGGCCATCCTAAGCACAAAG
Ex21B	CCTCCTTGGGAATGTCGGGG
In20B1	CACCTGCCGCAAATGTATTAACG

shown to play a crucial role in emergency granulopoiesis and mature neutrophil chemotactic responses, where SOCS3 was dispensable (56).

Distinct roles of the STAT3 isoforms in myeloid cell proliferation, survival and differentiation may partly help reconciling these contrasting results. Indeed, the STAT3alpha:beta ratio is highly regulated in myeloid cells, consistently decreasing with cell maturation and activation (20, 21, 60). In addition, STAT3 gamma and delta, two further isoforms generated by proteolytic cleavage and identified exclusively in myeloid cells, also showed increasing levels and phosphorylation during differentiation (21, 60). Interestingly, STAT3alpha S727 phosphorylation was immediately down-regulated upon differentiation, suggesting that activation of STAT3alpha and serine phosphorylation may not be required at the differentiation stage (21). Moreover, STAT3 DNA binding activity appeared to contain predominantly STAT3beta in G-CSF-responsive primary human myeloid cells and myeloid cell lines, while DNA binding activity of both STAT3alpha and beta was activated in human myeloid leukemia cell lines refractory to G-CSF-induced differentiation (60). However, only the alpha isoform could improve survival of neutrophil progenitor cells in response to G-CSF when over-expressed in the 32Dcl3 cell line (61). The specific role of STAT3beta in this context is probably not that of a negative regulator, since its overexpression did not alter the kinetics of G-CSF-mediated neutrophil differentiation or p27 induction in 32D/G-CSF-R cells, but could rather alleviate inhibition of p27 transactivation by a dominant-negative STAT3 mutant (32).

In conclusion, the contrasting phenotypes of the different STAT3 or G-CSFR mutants may stem from the fact that ablation of both isoforms abolished the expression of functionally distinct polypeptides. Indeed, STAT3alpha and STAT3beta clearly play distinct roles in mediating G-CSF-mediated survival, proliferation and differentiation, with STAT3alpha involved primarily in promoting cell survival and STAT3beta in promoting differentiation.

2.5. *In vivo* evidence for STAT3alpha and STAT3beta specific functions

Recently, mice with specific ablation of either isoform were generated (25). Mice lacking STAT3beta showed no apparent developmental abnormalities but were more sensitive to endotoxic shock, exhibiting increased mortality due to acute tubular necrosis (25) and increased tissue damage in response to LPS (29). Specific deletion of STAT3alpha showed that STAT3beta is not a dominant negative factor, since it could rescue the embryonal lethality of a STAT3 null mutation and was able to induce the expression of specific STAT3 target genes such as the acute phase genes in the liver (29). Interestingly, both isoforms contribute to the known anti-inflammatory functions of STAT3 by different mechanisms: while STAT3alpha was solely responsible of mediating the IL-10 anti-inflammatory effects on macrophages, STAT3beta down-regulated the production of anti-inflammatory cytokines possibly by activating IL-10 production (29). Accordingly, mice specifically lacking STAT3alpha but still expressing STAT3beta were more resistant to endotoxic shock than STAT3 null mice. However, STAT3alpha has nonredundant roles such as modulating cellular responses to IL-6, inducing SOCS3 gene expression in primary embryonal fibroblasts and promoting postnatal survival. Indeed, STAT3alpha$^{-/-}$ mice did survive until birth, in contrast to STAT3-null mice (29). However, they all died within the first 24 hours. Characterization of STAT3alpha$^{-/-}$ newborn mice phenotype is described below (Section 4).

3. MATERIALS AND METHODS

3.1. Production and genotyping of STAT3alpha$^{-/-}$ mice

Mice were maintained in the transgenic unit of the University of Turin (Turin, Italy) with a 12-hour light – 12-hour dark cycle and were provided food and water *ad libitum*. Procedures involving animals and their care were in conformity with national and international laws and policies and were approved by the Faculty Ethical Committee. The generation of STAT3alpha$^{-/-}$ mice used in this study was previously described (29). Genotypes were determined by DNA amplification from tail biopsies using the primers shown in Table 1.

3.2. Histological Analysis

Whole newborn mice were chilled on ice or tissues were dissected, fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. The samples were sectioned (whole pups, 5 micrometer-thick sections; tissues, 8 micrometer-thick sections) and stained with hematoxylin and eosin for morphological analysis. Degree of lung atelectasis, presence of exudate and congestion were evaluated in blind. TUNEL staining on thymus sections was carried out with the In situ Cell Death Detection Kit, TMR red (Roche Diagnostics, Monza, Italy), following manufacturer's instructions. The number of TUNEL-positive cells in three fields (original magnification x400) was counted in blind for each sample.

3.3. Recombinant cytokines

Recombinant human IL-6 was purified from bacteria as previously described (12). Human soluble IL-6 receptor was derived from supernatants of HEC 293T cells transfected with a human sIL-6R cDNA construct as previously described (62).

3.4. RNA analysis

Total RNA from STAT3alpha$^{-/-}$, STAT3alpha$^{wt/-}$ and STAT3$^{wt/wt}$ newborns (n=4) was prepared with the RNeasy Mini kit (Qiagen, Crawley, UK) according to manufacturer's protocol, quantified by spectrophotometry, treated with RNase-free DNAse (Ambion) and subjected to reverse transcription using random primers and the Reverse Transcriptase kit (Promega, Madison, WI, USA). The primers used for semiquantitative PCR analysis are shown in Table 2. PCR products were fractionated on a

Table 2. Primers

Name	Forward	Reverse
SP-A	GCAAACAATGGGAGTCCTCAGC,	TCCTCGGGGCAGCAATGTG
SP-B	AGTGTGAACAGTTTGTGGAACAGC	AGAGGTGTGGGGTTTGGAAGC
SP-C	CTGGCATCGTTGTGTATGACTA	AGCGAAAGCCTCAAGACTAGGG
SP-D	CAAAGGTGAAAGCGGGCTTCC	TCTGATAGTGGGAGAAGGCAACC
VEGF-A	CAGTCCTTAATCCAGAAAGCCT	AGAAAATGGCGAATCCAGTCCC
GAPDH	AGAAGGTGGTGAAGCAGGCATC	CGGCATCGAAGGTGGAAGAGTG

2,5% agarose gel, stained with Ethidium Bromide and quantified with Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA, USA). For quantitative PCR analysis, pre-formulated taqman assays were used according to manufacturer's instructions: GFAP Mm00546086_m1, Nes Mm00450205_m1, Vim Mm00449201_m1, 18S 4326313E (Applied Biosystems, Foster City, CA, USA). Fold induction was calculated by the deltadeltaCt method. Data were normalized to 18S ribosomal RNA levels.

3.5. Cresylviolet staining and neuron counts

Newborn mice were transcardially perfused with 4% paraformaldehyde in phosphate buffer, their heads postfixed, dehydrated, decalcified in 3% HNO3, and included in paraffin (modified from ref. 63). Serial sections, 8 micrometer thick, were stained with 0.5% cresylviolet (Sigma-Aldrich) in 2 % acetic acid. Total neuron numbers in the nodose-petrosal ganglia were counted in blind.

3.6. Western Blot analysis

Tissues were homogenized and proteins extracted in NP40 lysis buffer (50mM TRIS pH 7.4, 10% glycerol, 1% NP-40, 150mM NaCl, 2mM EDTA, 2mM DTT, 0.4mM Na3VO4, 10mM NaF, 0.5mM PMSF, 0.1 Unit/mL aprotinin, 4 microgram/mL pepstatin, and 10 microgram/mL leupeptin), proteins were cleared by centrifugation and concentration was measured using the Bradford method. Proteins were fractionated by 10% SDS-PAGE and transferred to nitrocellulose membrane for immunoblotting. Antibodies against GFAP (Dako, Glostrup, Denmark; 1:1000) or ERK (Santa Cruz Biotechnology, Palo Alto, CA, USA; 1:1000) were used.

3.7. *In vitro* astrocyte culture and immunofluorescence

P0 newborns were decapitated and their heads placed in HEPES-buffered HBSS with 100 U/mL penicillin and 100 microgram/mL streptomycin. The cerebral cortex was fragmented, the sediment removed and the cells were plated on poly-L-lysine-coated coverslips in DMEM containing 5% horse serum, 100 U/mL penicillin and 100 microgram/mL streptomycin, with or without IL-6 (500 ng/uL) and soluble IL-6 receptor (1x). Cell staining was performed using an antibody against GFAP (DAKO, Glostrup, Denmark; 1:200), revealed by a TRITC-labeled anti-rabbit secondary antibody (Sigma), and the nuclear staining agent Hoechst (Sigma-Aldrich). The same antibodies were used for staining brain sections.

3.8. DiI staining

PFA-perfused mice were fixed in paraformaldehyde and treated as previously described (64). Briefly, brains were dissected, coronally cut and 1,1'-ioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes, Eugene, Oregon, USA) crystals were deposited into the corpus callosum of each hemisphere. After 7 days diffusion of the dye at room temperature, the brain parts were included in 4% agar and 100 micrometer-thick sections were analyzed by fluorescence microscopy using a TRITC filter set.

4. RESULTS AND DISCUSSION: PHENOTYPIC CHARACTERIZATION OF STAT3alpha$^{-/-}$ NEWBORN MICE

4.1. Perinatal lethality

We have previously generated STAT3alpha$^{-/-}$ mice by substituting the wild type STAT3 allele with a knocked-in allele that can only express the STAT3beta isoform (29). In contrast to STAT3$^{-/-}$ embryos, STAT3alpha$^{-/-}$ mice developed to term and were born from heterozygous matings at near-mendelian percentage (21% STAT3alpha$^{-/-}$, 54% STAT3alpha$^{-/wt}$, 25% STAT3$^{wt/wt}$, n=296). However, they invariably died between 8 and 24 hours after birth. STAT3alpha$^{-/-}$ newborns were initially indistinguishable from their heterozygous (STAT3alpha$^{-/wt}$) or wild type (STAT3$^{wt/wt}$) littermates. At birth, their body weight (g 1.3 plus/minus 0.1 versus 1.3 plus/minus 0.2 of wt controls, n=68) and body length (cm 2.5 plus/minus 0.1 versus 2.5 plus/minus 0.2 of wt controls, n=68) were normal. In contrast STAT3$^{SA/-}$ mice, expressing only one copy of a mutant STAT3alpha allele where Serine 727 was replaced by an Alanine, showed at birth a 13% reduction in body weight that was ascribed to decreased IGF-1 serum levels (65). Therefore STAT3beta, but not the STAT3 SA mutant, is apparently able to guarantee normal IGF1 expression levels.

STAT3alpha$^{-/-}$ pups invariably went through respiratory crises during which they became cyanotic and gasped for air. Sometimes they were able to recover shortly only to suddenly die afterwards. Although also control littermates occasionally showed similar crises, these were rarely lethal. There was never any trace of milk in the stomachs of the STAT3alpha$^{-/-}$ newborns, even when competing pups were removed. Death could not however be directly ascribed to starvation, since STAT3alpha$^{-/wt}$ or STAT3$^{wt/wt}$ pups never died before the first 24 hours, even when starved due to separation from their mothers immediately after birth. Under these conditions, STAT3alpha$^{-/-}$ pups still died at similar rates. STAT3alpha$^{-/-}$ newborns were able to swallow milk when directly delivered in their mouths, suggesting that pharynx muscle control was not totally compromised.

In an attempt to identify the causes of lethality, whole STAT3alpha$^{-/-}$ pups were collected at the time of

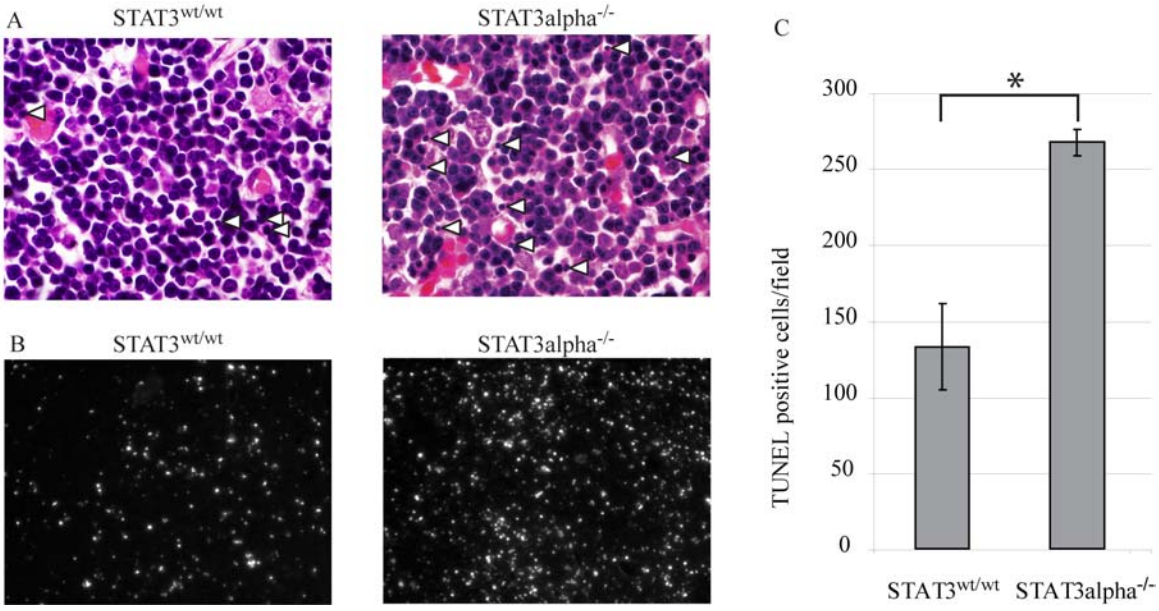

Figure 2. Increased number of apoptotic thymocytes in STAT3alpha$^{-/-}$ mice. A. H&E stained thymi from STAT3$^{wt/wt}$ and STAT3alpha$^{-/-}$ newborns, 10h after birth (original magnification, x1000). Arrowheads indicate thymocytes with pyknotic nuclei. Images are representative of 6 independent samples. B. Thymi from two STAT3alpha$^{-/-}$ and three STAT3$^{wt/wt}$ mice were sectioned and stained for apoptotic nuclei by TUNEL reaction (original magnification, x400). C. Number of TUNEL-positive cells in the thymus of STAT3alpha$^{-/-}$ newborns relative to wildtype littermates. *p< 0.01

Table 3. Lung atelectasis in STAT3alpha$^{-/-}$ newborns

Degree of lung atelectasis	-	+	++
STAT3$^{wt/wt}$ (n=7)	4 (57%)	3 (43%)	0 (0%)
STAT3alpha$^{-/wt}$ (n=15)	8 (53%)	5 (33%)	2 (13%)
STAT3alpha$^{-/-}$ (n=10)	1 (10%)	4 (40%)	5 (50%)

Lung atelectasis rating was performed blindly on ten STAT3alpha$^{-/-}$ newborns and their control littermates. Where no lung collapse was present, lungs were rated - . Where expansion of the lungs was incomplete and interalveolar septae thick, severe atelectasis was indicated as ++, while in newborns where symptoms of atelectasis were minor or localized only to a limited portion of the lung, atelectasis was rated +.

death, together with age-matched STAT3alpha$^{-/wt}$ or STAT3$^{wt/wt}$ littermates, and subjected to histological analysis upon fixation and serial sectioning. No major abnormalities were detected, with only two exceptions: the presence of high numbers of cells with pyknotic nuclei in the thymus, and variable degrees of lung atelectasis, as described below.

4.2. Increased thymic apoptosis

Thymi from STAT3alpha$^{-/-}$ newborns showed a 2-fold increase in the number of TUNEL-positive thymocytes as compared to wild type littermates (Figure 2). However, thymocytes derived from STAT3alpha$^{-/-}$ pups and cultured for 24 or 48 hours did not show any increase in the rate of apoptosis, either spontaneous or induced by dexamethasone or UV treatment, as assessed by Annexin V staining and flow cytometry analysis (data not shown). This observation suggests that the increased apoptotic death detected in the thymus of the STAT3alpha$^{-/-}$ newborns was not due to a cell-autonomous defect. Indeed, a role for STAT3 in supporting the feeder functions of thymic epithelial cells (TEC) has been proposed (66). Our results suggest that this is probably a unique property of the STAT3alpha isoform.

4.3. Defective airways expansion

As assessed by blind scoring, nine out of ten STAT3alpha$^{-/-}$ newborns analyzed by histological staining displayed variable degrees of lung atelectasis (5 severe, 4 mild), with reduced lung size due to incomplete expansion of the alveoli and thick alveolar septae (Figure 3A and Table 3). In contrast, more than 50% of both heterozygous and wild type control pups had normally expanded lungs and, when present, atelectasis was mostly mild (Table 3). The degree of lung congestion and the presence of alveolar exudate were also rated but did not significantly differ between genotypes.

Successful adaptation to air breathing at birth depends on correct lung development including optimal production of pulmonary surfactant, which is required to reduce surface tension at the alveolar air-liquid interface (67). Indeed, disorders of surfactant metabolism such as defective expression of surfactant proteins SP-B and SP-C have been associated with lethal respiratory failure in newborn children (reviewed in 68). Moreover, targeted disruption of the SP-B gene leads to respiratory failure in newborn mice, evidencing its crucial role in surfactant homeostasis after birth (69). STAT3 may well play a role in surfactant proteins expression since the alpha isoform was shown to stimulate the SP-B gene promoter in a human respiratory epithelial cell line (70). However, the mRNA levels for all four surfactants were comparable between

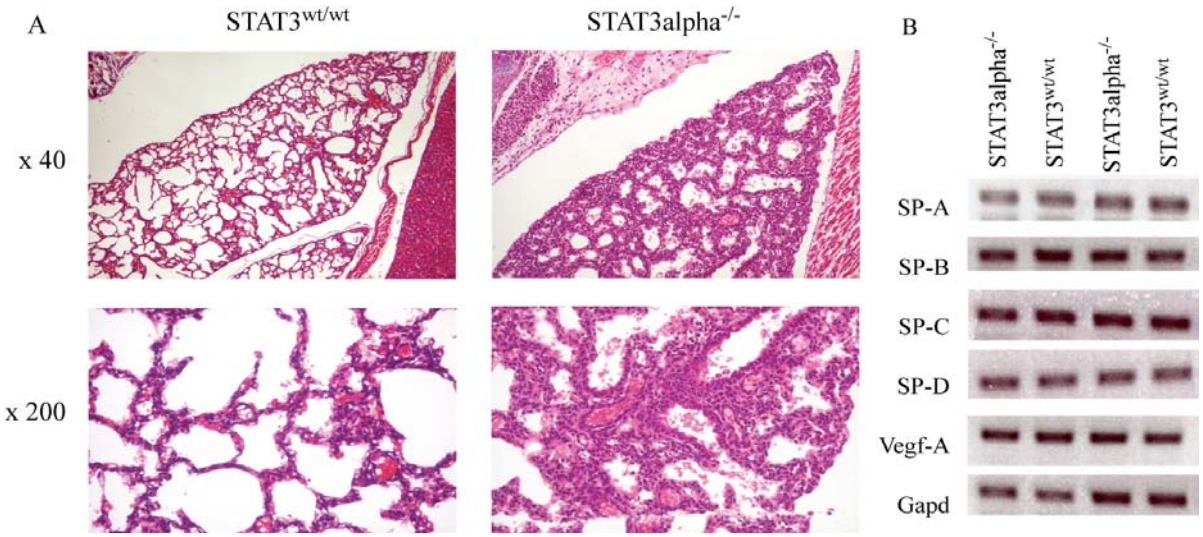

Figure 3. Lung atelectasis and levels of surfactant proteins in STAT3alpha$^{-/-}$ newborns. A. Lungs from STAT3$^{wt/wt}$ and STAT3alpha$^{-/-}$ newborns were dissected, formalin-fixed, and 8 micrometer-thick sections were stained with hematoxylin and eosin. B. Transcription levels of pulmonary surfactant proteins and Vegf-A in STAT3alpha$^{-/-}$ and control newborns. Lungs were collected from two STAT3alpha$^{-/-}$ and two wild type littermates, at the time of death (lane 1) or sacrificed 8 hours after birth (lanes 2, 3, 4). Total RNA was extracted and subjected to semiquantitative retrotranscriptase PCR analysis for SP-A, SP-B, SP-C, SP-D and Vegf-A. Gapd levels were measured for sample normalization.

STAT3alpha$^{-/-}$ and wild type control mice both at the time of death and when sacrificed 9 hours after birth (Figure 3B). Surfactant mRNAs expression, at least in newborns, is therefore not dependent on STAT3alpha, and the defective airways expansion observed in the mutant pups is likely not dependent on defective surfactant production. This is in agreement with the observation that mice specifically lacking STAT3 in lung epithelial cells developed normally and showed impaired SP-B production only in adulthood under hyperoxic conditions (71).

Whatever the cause, defective airways expansion may cause hypoxia and ultimately death, particularly if lung vascularization or responses to hypoxia are altered. However, the number and size of lung vessels and the distribution of the endothelial and smooth muscle cell layers, analyzed by immunohistochemistry, were normal in the STAT3alpha$^{-/-}$ pups (data not shown). VEGF-A is a known STAT3 target (72) and is induced during hypoxia. We therefore measured VEGF-A lung mRNA levels but found them to be comparable between the STAT3alpha$^{-/-}$ pups and their control littermates (Figure 3B). Normal were likewise the expression levels of the mRNA for SOCS3 (data not shown), a canonical STAT3 target that specifically requires the STAT3alpha isoform in MEF cells (29). All together, these observations do not support a direct role for defective lung structure/function or for impaired responses to hypoxia in the lung atelectasis and/or in the lethality caused by specific ablation of the STAT3alpha isoform. It is worth noting that the expression of recognized STAT3alpha targets such as SP-B, VEGF-A or SOCS3 is unaffected by the absence of STAT3alpha. This may suggest that in newborn lung epithelial cells STAT3beta is able to compensate for the lack of STAT3alpha in the transcriptional induction of the these genes. Alternatively, other pathways bypassing STAT3 may be activated in these cells.

4.4. Normal nodose neuron numbers

Both the respiratory problems and the failure to feed observed in the STAT3alpha$^{-/-}$ newborns could be secondary to neurological defects. Indeed, STAT3 is a central mediator of the action of neurotrophic cytokines belonging to the gp130 family (CNTF, LIF, OSM), and mice lacking LIF or CNTF receptor alpha were unable to breath at birth and displayed perinatal lethality which was linked to impaired survival of neurons in the dorsal root ganglia (73, 74). In contrast to STAT3alpha$^{-/-}$ mice, however, both LIFR$^{-/-}$ and CNTFRalpha$^{-/-}$ newborns also displayed evident motor defects such as impaired righting reflex and response to tail pinch. Impaired righting reflex and perinatal lethality were also described in Bal-Cre mice, where STAT3 was efficiently deleted in the CNS (63). Lethality correlated with defective survival of the neurons in the nodose-petrosal ganglion, a neuronal complex providing sensory innervation to visceral tissues and thus mediating critical cardiorespiratory and gastrointestinal functions. Therefore, the STAT3alpha isoform may be specifically involved in mediating nodose neuron survival and/or function. In order to test this hypothesis, neurons in the nodose-petrosal ganglia were counted in serial sections from STAT3alpha$^{-/-}$ and control wild type newborns sacrificed 10 hours after birth. However, nodose neuron numbers appeared to be equivalent in the ganglia from STAT3alpha$^{-/-}$ and wild type pups (Figure 4). This observation suggests that STAT3alpha$^{-/-}$ nodose-petrosal neurons do not present any major survival defect, although functional alterations cannot be ruled out. Therefore, the expression of STAT3beta in the CNS and in the nodose neurons appears to be able to rescue at least some of the neurological defects triggered by ablating both isoforms

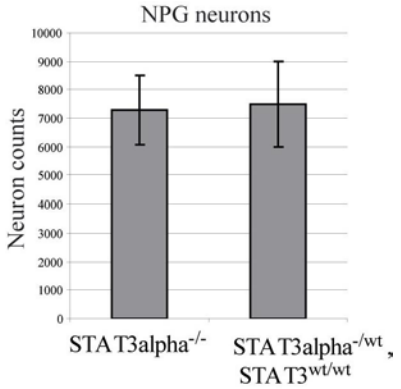

Figure 4. Nodose-petrosal neuron counts of STAT3alpha$^{-/-}$ newborns. Two STAT3alpha$^{-/-}$ newborns and two control littermates were perfused with paraformaldehyde and the dissected heads fixed in paraformaldehyde, decalcified, dehydrated and included in paraffin. 8 micrometer-thick serial sections were stained with cresylviolet. The left and right nodose-petrosal ganglia were identified (n=4). The total number of neurons per ganglion was calculated by adding all neurons of all sections containing the nodose-petrosal ganglion.

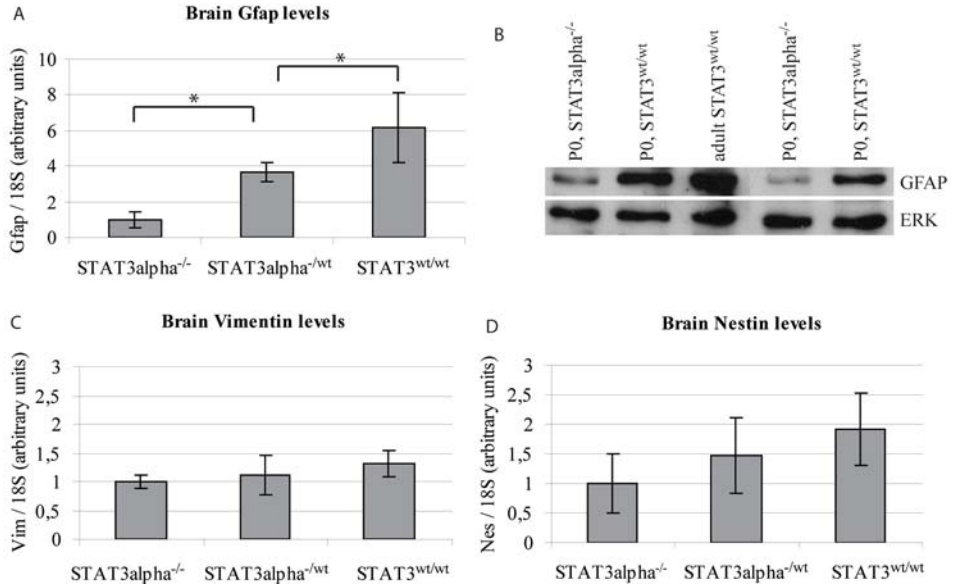

Figure 5. Levels of GFAP, Vimentin and Nestin in STAT3alpha$^{-/-}$ brains. Brains were dissected from STAT3alpha$^{-/-}$ (n=4), STAT3$^{alpha-/wt}$ (n=4) and STAT3$^{wt/wt}$ (n=4) littermates sacrificed between 3 and 20 hours after birth. A. GFAP mRNA levels in the brain were measured by retrotranscription of total RNA and Real-Time PCR analysis. 18S RNA levels were used for sample normalization. *p<0.05 B. GFAP protein levels were measured by Western Blot analysis. Proteins were extracted from brain tissue, fractionated on SDS-PAGE gel and transferred to nitrocellulose membrane for immunoblotting. ERK is used as a loading control. A wild type adult brain was included as positive control. C. and D. Brain Vimentin and Nestin mRNA levels were measured by Real-Time PCR analysis (n=4). 18S RNA levels were used for sample normalization.

in the Bal1-Cre,STAT3$^{fl/fl}$ mice, including nodose neurons survival and impaired righting reflexes. However, this rescue may be functionally incomplete, thus explaining why also STAT3alpha$^{-/-}$ pups die, although a few hours later.

4.5. Defective GFAP expression and *in vitro* astrocyte differentiation

STAT3 is thought to play an important role in astrocytic differentiation downstream of the gp130 cytokines IL-6, LIF and CNTF (75, 76), and to be required for the expression of the gene encoding glial fibrillary acidic protein (GFAP), a cytoskeletal intermediate filament which is an exquisite marker of astrocytic lineage. GFAP transcript levels were indeed 6-fold lower in the brains of STAT3alpha$^{-/-}$ newborns than in the wild type control, with heterozygous mice showing intermediate levels (Figure 5A). Western blot analysis confirmed that also GFAP protein levels were significantly decreased (Figure 5B). These data suggest that the STAT3alpha isoform is

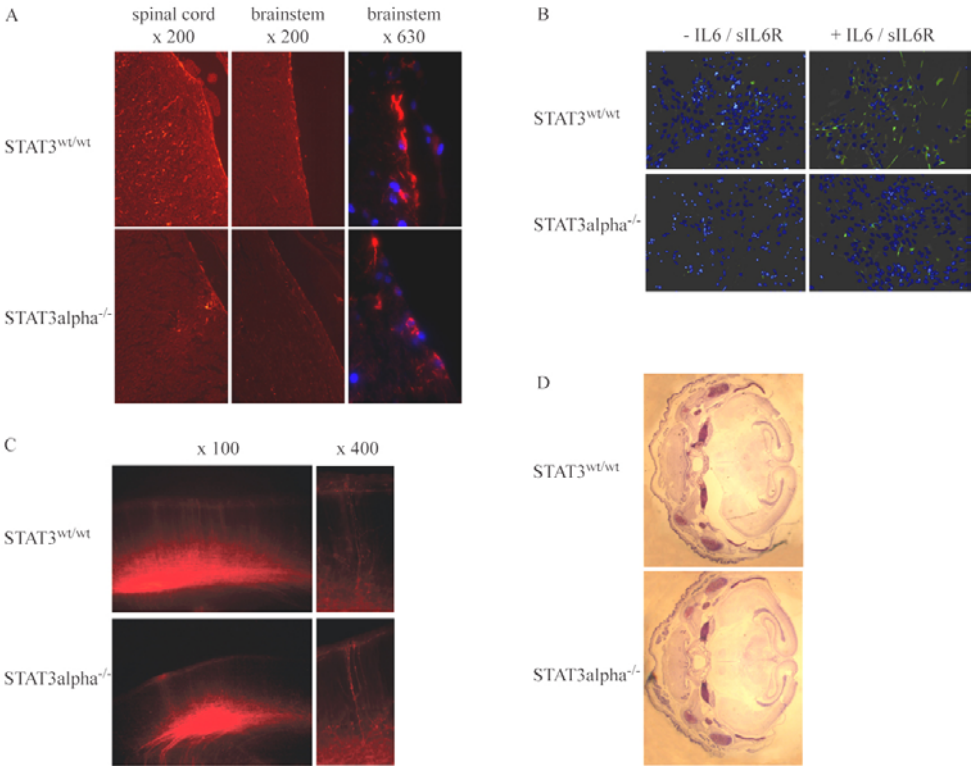

Figure 6. GFAP expression, *in vitro* astrocyte differentiation, radial glia formation and brain structure of STAT3alpha$^{-/-}$ newborns. A. GFAP immunofluorescence. STAT3alpha$^{-/-}$ (n=2) and wild type littermates (n=2) were sacrificed 10 hours after birth, brains were dissected and sectioned. Sections comprising the spinal cord and brain stem area were immunostained for GFAP and analyzed by fluorescence microscopy. B. *In vitro* astrocyte differentiation from P0 newborns. Neuroepithelial cells derived from the brain of STAT3alpha$^{-/-}$ or wildtype newborns were cultured with or without IL-6 and sIL-6R. After three days, cells were fixed and stained for GFAP by specific antibody and for nuclei with Hoechst, followed by fluorescent microscopy. GFAP-positive cells and nuclei are shown in green and blue, respectively. Results are representative of three independent experiments. C. DiI labeled radial glia. DiI crystals were deposited into the corpus callosum of coronal brain sections of PFA-perfused STAT3alpha$^{-/-}$ (n=1) and control (n=3) newborns. After diffusion of DiI for one week at room temperature, brains were included in agar and 100 micrometer-thick sections analyzed by fluorescence microscopy with a Nikon Eclipse 800 microscope. Original magnification as reported. D. Brain structure of STAT3alpha$^{-/-}$ and control newborns. STAT3$^{wt/wt}$ (n=2) and STAT3alpha$^{-/-}$ (n=2) mice were sacrificed 10 hours after birth, brains were dissected, sectioned, and stained with cresylviolet.

specifically required for efficient GFAP expression in astrocytes *in vivo*, also supported by the normal GFAP levels detected in the brains of STAT3beta$^{-/-}$ mice (data not shown). There was no apparent compensation by other intermediate filaments, as the mRNA levels for vimentin were normal, and those for nestin were even slightly reduced in the STAT3alpha$^{-/-}$ brains (Figure 5C and D).

In agreement with the residual GFAP expression detected by Western blot, a few GFAP positive cells could still be detected by immunohistochemistry in the STAT3alpha$^{-/-}$ spinal cord and brain stem (Figure 6A), although reduced in number and displaying decreased staining intensity. Therefore, despite the significant reduction in GFAP levels, a certain degree of astrocyte differentiation can still take place *in vivo* in the absence of STAT3alpha. To assess *in vitro* differentiating potential, neuroepithelial cells were isolated from P0 STAT3alpha$^{-/-}$ mice brains and cultured for 3 days in the presence of IL-6 and its soluble receptor, known to activate gp130-mediated astrocyte differentiation, and then analyzed for GFAP expression by immunofluorescence. Similar to what observed *in vivo*, some GFAP-positive cells were detected in the absence of STAT3alpha, although significantly less than in the wild type controls (Figure 6B). In addition, a few of these showed the typical elongated processes of differentiated astrocytes, although both staining intensity and length of the cellular processes were reduced as compared to wild type control cells. These results suggest that STAT3alpha is an important factor for GFAP expression and astrocyte differentiation both *in vitro* and *in vivo*, but that other pathways are also involved and can partially compensate for STAT3alpha function. Interestingly, *in vitro* astrocytic differentiation of neuroepithelial cells was completely impaired in gp130$^{-/-}$ mice and in mice where the gp130 docking site for STAT3 was eliminated, and GFAP-staining of histological brain sections was almost completely absent in gp130$^{-/-}$ mice but only slightly decreased in mice with mutated gp130 STAT3 docking sites (76). It is tempting to speculate that

STAT3beta may be partially able to compensate for STAT3alpha in transducing the signal from gp130 cytokines to induce astrocytic differentiation, including partial activation of GFAP transcription.

Finally, the development of radial glia, which is essential for the correct development of neuroepithelial structures in the brain and is connected with astrocyte differentiation (77) was analyzed by DiI staining in STAT3alpha$^{-/-}$ and wild type newborns. Morphology, direction and branching potential of radial glia in the STAT3alpha$^{-/-}$ samples was similar to that of the wild type samples, suggesting that the astrocytic defect observed is cell autonomous and does not correlate with overall radial glia defects (Figure 6C). In keeping with the normal radial glia structure, STAT3alpha$^{-/-}$ brains did not display any gross structural abnormalities in the organization of the cortex layers or in the structure of the hippocampus, as shown by coronal brain sections stained with cresylviolet (Figure 6D).

4.6. Concluding remarks

Complete germline deletion of STAT3 triggers early embryonal lethality, and exactly for which function(s) is STAT3 crucially required for embryonal development past gastrulation still remains unknown. Whatever these functions, they are clearly shared by both STAT3 isoforms, as suggested by the ability of STAT3alpha$^{-/-}$ embryos, still expressing STAT3beta, to reach birth. In keeping with the idea that STAT3beta possesses indeed specific functions that contribute to the overall biological role of STAT3, we were further able to demonstrate that this isoform can indeed perform some of the activities attributed to STAT3alpha, such as regulating IGF1 expression and body weight at birth and allowing nodose neurons survival. In addition, we have shown that STAT3beta may be able to partially compensate for STAT3alpha in supporting astrocyte differentiation, which was only partially defective in the absence of STAT3alpha. On the other hand, we were able to add to the previously demonstrated unique functions of the STAT3alpha isoform also the capacity to confer crucial feeding properties to the thymic epithelial stroma, as judged by increased thymocytes apoptotic death *in vivo* but not *in vitro*.

STAT3beta is however unable to warrant postnatal survival, and the STAT3alpha$^{-/-}$ pups die within 24 hours of birth. Despite our efforts, we were unable to understand the causes of death. Extensive use of conditional mutagenesis has allowed in the past the analysis of STAT3 (alpha plus beta) functions in many different tissues and cell systems, including myeloid cells precursors, B and T lymphocytes, liver and epithelial cells (6). So far, the only deletion causing perinatal lethality has been that targeting the whole CNS (63). Even though survival of nodose neurons is normal in the STAT3alpha$^{-/-}$ newborns, it is likely that other, still undetected neuronal functions uniquely dependent on the alpha isoform are impaired, impacting among others on breathing and feeding abilities. Inducible isoform-specific deletion in different neuronal populations will be required in order to elucidate these functions.

5. ACKNOWLEDGEMENTS

We wish to thank Drs. S. Rose-John for the gift of the soluble IL-6 receptor encoding plasmid, D. Maritano for helpful discussion, L. Minichiello, C. Sciarretta and P. Muzzi for help with the neuron counts and the brain immunofluorescence. This work was supported by the Italian Ministry of Research (MIUR). Sarah Dewilde, Present address: BioIndustryPark del Canavese, Via Ribes 5, 10010 Colleretto Giacosa, Italy.

6. REFERENCES

1. Wegenka, U. M., J. Buschmann, C. Lutticken, P. C. Heinrich and F. Horn: Acute-phase response factor, a nuclear factor binding to acute-phase response elements, is rapidly activated by interleukin-6 at the posttranslational level. *Mol Cell Biol* 13, 276-88 (1993)

2. Akira, S., Y. Nishio, M. Inoue, X. J. Wang, S. Wei, T. Matsusaka, K. Yoshida, T. Sudo, M. Naruto and T. Kishimoto: Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. *Cell* 77, 63-71 (1994)

3. Zhong, Z., Z. Wen and J. E. Darnell, Jr.: Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* 264, 95-8 (1994)

4. Wegenka, U. M., C. Lutticken, J. Buschmann, J. Yuan, F. Lottspeich, W. Muller-Esterl, C. Schindler, E. Roeb, P. C. Heinrich and F. Horn: The interleukin-6-activated acute-phase response factor is antigenically and functionally related to members of the signal transducer and activator of transcription (STAT) family. *Mol Cell Biol* 14, 3186-96 (1994)

5. Takeda, K., K. Noguchi, W. Shi, T. Tanaka, M. Matsumoto, N. Yoshida, T. Kishimoto and S. Akira: Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci U S A* 94, 3801-4 (1997)

6. Poli, V.: STAT3 function in vivo. *Signal Transducers and Activators of Transcription (STATs)*, D. Sehgal, Ed., Kluwer Academic Publishers, 493-512 (2003).

7. Bowman, T., R. Garcia, J. Turkson and R. Jove: STATs in oncogenesis. *Oncogene* 19, 2474-88 (2000)

8. Lee, C. K., R. Raz, R. Gimeno, R. Gertner, B. Wistinghausen, K. Takeshita, R. A. DePinho and D. E. Levy: STAT3 is a negative regulator of granulopoiesis but is not required for G-CSF-dependent differentiation. *Immunity* 17, 63-72 (2002)

9. Cuesta, N., Q. M. Nhu, E. Zudaire, S. Polumuri, F. Cuttitta and S. N. Vogel: IFN regulatory factor-2 regulates macrophage apoptosis through a STAT1/3- and caspase-1-dependent mechanism. *J Immunol* 178, 3602-11 (2007)

10. Gamero, A. M., R. Potla, J. Wegrzyn, M. Szelag, A. E. Edling, K. Shimoda, D. C. Link, J. Dulak, D. P. Baker, Y. Tanabe, J. M. Grayson and A. C. Larner: Activation of Tyk2 and Stat3 is required for the apoptotic actions of interferon-beta in primary pro-B cells. *J Biol Chem* 281, 16238-44 (2006)

11. Chapman, R. S., P. Lourenco, E. Tonner, D. Flint, S. Selbert, K. Takeda, S. Akira, A. R. Clarke and C. J. Watson: The role of Stat3 in apoptosis and mammary gland involution. Conditional deletion of Stat3. *Adv Exp Med Biol* 480, 129-38 (2000)

12. Alonzi, T., D. Maritano, B. Gorgoni, G. Rizzuto, C. Libert and V. Poli: Essential role of STAT3 in the control of the acute-phase response as revealed by inducible gene inactivation [correction of activation] in the liver. *Mol Cell Biol* 21, 1621-32 (2001)

13. Alonzi, T., I. P. Newton, P. J. Bryce, E. Di Carlo, G. Lattanzio, M. Tripodi, P. Musiani and V. Poli: Induced somatic inactivation of STAT3 in mice triggers the development of a fulminant form of enterocolitis. *Cytokine* 26, 45-56 (2004)

14. Kobayashi, M., M. N. Kweon, H. Kuwata, R. D. Schreiber, H. Kiyono, K. Takeda and S. Akira: Toll-like receptor-dependent production of IL-12p40 causes chronic enterocolitis in myeloid cell-specific Stat3-deficient mice. *J Clin Invest* 111, 1297-308 (2003)

15. Takeda, K., B. E. Clausen, T. Kaisho, T. Tsujimura, N. Terada, I. Forster and S. Akira: Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity* 10, 39-49 (1999)

16. Caldenhoven, E., T. B. van Dijk, R. Solari, J. Armstrong, J. A. Raaijmakers, J. W. Lammers, L. Koenderman and R. P. de Groot: STAT3beta, a splice variant of transcription factor STAT3, is a dominant negative regulator of transcription. *J Biol Chem* 271, 13221-7 (1996)

17. Schaefer, T. S., L. K. Sanders and D. Nathans: Cooperative transcriptional activity of Jun and Stat3 beta, a short form of Stat3. *Proc Natl Acad Sci U S A* 92, 9097-101 (1995)

18. Shao, H., A. J. Quintero and D. J. Tweardy: Identification and characterization of cis elements in the STAT3 gene regulating STAT3 alpha and STAT3 beta messenger RNA splicing. *Blood* 98, 3853-6 (2001)

19. Wen, Z., Z. Zhong and J. E. Darnell, Jr.: Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell* 82, 241-50 (1995)

20. Biethahn, S., F. Alves, S. Wilde, W. Hiddemann and K. Spiekermann: Expression of granulocyte colony-stimulating factor- and granulocyte-macrophage colony-stimulating factor-associated signal transduction proteins of the JAK/STAT pathway in normal granulopoiesis and in blast cells of acute myelogenous leukemia. *Exp Hematol* 27, 885-94 (1999)

21. Hevehan, D. L., W. M. Miller and E. T. Papoutsakis: Differential expression and phosphorylation of distinct STAT3 proteins during granulocytic differentiation. *Blood* 99, 1627-37 (2002)

22. Schaefer, T. S., L. K. Sanders, O. K. Park and D. Nathans: Functional differences between Stat3alpha and Stat3beta. *Mol Cell Biol* 17, 5307-16 (1997)

23. Park, O. K., L. K. Schaefer, W. Wang and T. S. Schaefer: Dimer stability as a determinant of differential DNA binding activity of Stat3 isoforms. *J Biol Chem* 275, 32244-9 (2000)

24. Huang, Y., J. Qiu, S. Dong, M. S. Redell, V. Poli, M. A. Mancini and D. J. Tweardy: STAT3 isoforms, alpha and beta , demonstrate distinct intracellular dynamics with prolonged nuclear retention of STAT3beta mapping to its unique C-terminal end. *J Biol Chem* (2007)

25. Yoo, J. Y., D. L. Huso, D. Nathans and S. Desiderio: Specific ablation of Stat3beta distorts the pattern of Stat3-responsive gene expression and impairs recovery from endotoxic shock. *Cell* 108, 331-44 (2002)

26. Schuringa, J. J., H. Schepers, E. Vellenga and W. Kruijer: Ser727-dependent transcriptional activation by association of p300 with STAT3 upon IL-6 stimulation. *FEBS Lett* 495, 71-6 (2001)

27. Karni, R., R. Jove and A. Levitzki: Inhibition of pp60c-Src reduces Bcl-XL expression and reverses the transformed phenotype of cells overexpressing EGF and HER-2 receptors. *Oncogene* 18, 4654-62 (1999)

28. Sinibaldi, D., W. Wharton, J. Turkson, T. Bowman, W. J. Pledger and R. Jove: Induction of p21WAF1/CIP1 and cyclin D1 expression by the Src oncoprotein in mouse fibroblasts: role of activated STAT3 signaling. *Oncogene* 19, 5419-27 (2000)

29. Maritano, D., M. L. Sugrue, S. Tininini, S. Dewilde, B. Strobl, X. Fu, V. Murray-Tait, R. Chiarle and V. Poli: The STAT3 isoforms alpha and beta have unique and specific functions. *Nat Immunol* 5, 401-9 (2004)

30. Yang, X. P., K. Irani, S. Mattagajasingh, A. Dipaula, F. Khanday, M. Ozaki, K. Fox-Talbot, W. M. Baldwin, 3rd and L. C. Becker: Signal transducer and activator of transcription 3alpha and specificity protein 1 interact to upregulate intercellular adhesion molecule-1 in ischemic-reperfused myocardium and vascular endothelium. *Arterioscler Thromb Vasc Biol* 25, 1395-400 (2005)

31. Yeh, M., N. M. Gharavi, J. Choi, X. Hsieh, E. Reed, K. P. Mouillesseaux, A. L. Cole, S. T. Reddy and J. A. Berliner: Oxidized phospholipids increase interleukin 8 (IL-8) synthesis by activation of the c-src/signal

transducers and activators of transcription (STAT)3 pathway. *J Biol Chem* 279, 30175-81 (2004)

32. de Koning, J. P., A. C. Ward, E. Caldenhoven, R. P. de Groot, B. Lowenberg and I. P. Touw: STAT3beta does not interfere with granulocyte colony-stimulating factor-induced neutrophilic differentiation. *Hematol J* 1, 220-5 (2000)

33. Sasse, J., U. Hemmann, C. Schwartz, U. Schniertshauer, B. Heesel, C. Landgraf, J. Schneider-Mergener, P. C. Heinrich and F. Horn: Mutational analysis of acute-phase response factor/Stat3 activation and dimerization. *Mol Cell Biol* 17, 4677-86 (1997)

34. Zhang, X., M. H. Wrzeszczynska, C. M. Horvath and J. E. Darnell, Jr.: Interacting regions in Stat3 and c-Jun that participate in cooperative transcriptional activation. *Mol Cell Biol* 19, 7138-46 (1999)

35. Yoo, J. Y., W. Wang, S. Desiderio and D. Nathans: Synergistic activity of STAT3 and c-Jun at a specific array of DNA elements in the alpha 2-macroglobulin promoter. *J Biol Chem* 276, 26421-9 (2001)

36. Ivanov, V. N., M. Krasilnikov and Z. Ronai: Regulation of Fas expression by STAT3 and c-Jun is mediated by phosphatidylinositol 3-kinase-AKT signaling. *J Biol Chem* 277, 4932-44 (2002)

37. Nakashima, K., M. Yanagisawa, H. Arakawa, N. Kimura, T. Hisatsune, M. Kawabata, K. Miyazono and T. Taga: Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. *Science* 284, 479-82 (1999)

38. Giraud, S., F. Bienvenu, S. Avril, H. Gascan, D. M. Heery and O. Coqueret: Functional interaction of STAT3 transcription factor with the coactivator NcoA/SRC1a. *J Biol Chem* 277, 8004-11 (2002)

39. Schaefer, L. K., S. Wang and T. S. Schaefer: c-Src activates the DNA binding and transcriptional activity of Stat3 molecules: serine 727 is not required for transcriptional activation under certain circumstances. *Biochem Biophys Res Commun* 266, 481-7 (1999)

40. Lutticken, C., P. Coffer, J. Yuan, C. Schwartz, E. Caldenhoven, C. Schindler, W. Kruijer, P. C. Heinrich and F. Horn: Interleukin-6-induced serine phosphorylation of transcription factor APRF: evidence for a role in interleukin-6 target gene induction. *FEBS Lett* 360, 137-43 (1995)

41. Wen, Z. and J. E. Darnell, Jr.: Mapping of Stat3 serine phosphorylation to a single residue (727) and evidence that serine phosphorylation has no influence on DNA binding of Stat1 and Stat3. *Nucleic Acids Res* 25, 2062-7 (1997)

42. Chung, J., E. Uchida, T. C. Grammer and J. Blenis: STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. *Mol Cell Biol* 17, 6508-16 (1997)

43. Frank, D. A.: STAT3 as a central mediator of neoplastic cellular transformation. *Cancer Lett* 251, 199-210 (2007)

44. Turkson, J. and R. Jove: STAT proteins: novel molecular targets for cancer drug discovery. *Oncogene* 19, 6613-26 (2000)

45. Turkson, J., T. Bowman, R. Garcia, E. Caldenhoven, R. P. De Groot and R. Jove: Stat3 activation by Src induces specific gene regulation and is required for cell transformation. *Mol Cell Biol* 18, 2545-52 (1998)

46. Bromberg, J. F., C. M. Horvath, D. Besser, W. W. Lathem and J. E. Darnell, Jr.: Stat3 activation is required for cellular transformation by v-src. *Mol Cell Biol* 18, 2553-8 (1998)

47. Catlett-Falcone, R., T. H. Landowski, M. M. Oshiro, J. Turkson, A. Levitzki, R. Savino, G. Ciliberto, L. Moscinski, J. L. Fernandez-Luna, G. Nunez, W. S. Dalton and R. Jove: Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity* 10, 105-15 (1999)

48. Zhang, Y. W., L. M. Wang, R. Jove and G. F. Vande Woude: Requirement of Stat3 signaling for HGF/SF-Met mediated tumorigenesis. *Oncogene* 21, 217-26 (2002)

49. Burke, W. M., X. Jin, H. J. Lin, M. Huang, R. Liu, R. K. Reynolds and J. Lin: Inhibition of constitutively active Stat3 suppresses growth of human ovarian and breast cancer cells. *Oncogene* 20, 7925-34 (2001)

50. Cheng, L., S. Wang, Y. J. Ye, J. Q. Zhang, X. M. Qiao, D. Q. Yang and Z. R. Cui: [The effect of transfecting Stat3beta cDNA on human breast cancer]. *Zhonghua Yi Xue Za Zhi* 85, 23-7 (2005)

51. Rivat, C., S. Rodrigues, E. Bruyneel, G. Pietu, A. Robert, G. Redeuilh, M. Bracke, C. Gespach and S. Attoub: Implication of STAT3 signaling in human colonic cancer cells during intestinal trefoil factor 3 (TFF3) -- and vascular endothelial growth factor-mediated cellular invasion and tumor growth. *Cancer Res* 65, 195-202 (2005)

52. Niu, G., R. Heller, R. Catlett-Falcone, D. Coppola, M. Jaroszeski, W. Dalton, R. Jove and H. Yu: Gene therapy with dominant-negative Stat3 suppresses growth of the murine melanoma B16 tumor in vivo. *Cancer Res* 59, 5059-63 (1999)

53. Niu, G., K. H. Shain, M. Huang, R. Ravi, A. Bedi, W. S. Dalton, R. Jove and H. Yu: Overexpression of a dominant-negative signal transducer and activator of transcription 3 variant in tumor cells leads to production of soluble factors that induce apoptosis and cell cycle arrest. *Cancer Res* 61, 3276-80 (2001)

54. Wang, T., G. Niu, M. Kortylewski, L. Burdelya, K. Shain, S. Zhang, R. Bhattacharya, D. Gabrilovich, R. Heller, D. Coppola, W. Dalton, R. Jove, D. Pardoll and H.

Yu: Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. *Nat Med* 10, 48-54 (2004)

55. Chakraborty, A., K. F. Dyer, M. Cascio, T. A. Mietzner and D. J. Tweardy: Identification of a novel Stat3 recruitment and activation motif within the granulocyte colony-stimulating factor receptor. *Blood* 93, 15-24 (1999)

56. Panopoulos, A. D., L. Zhang, J. W. Snow, D. M. Jones, A. M. Smith, K. C. El Kasmi, F. Liu, M. A. Goldsmith, D. C. Link, P. J. Murray and S. S. Watowich: STAT3 governs distinct pathways in emergency granulopoiesis and mature neutrophils. *Blood* 108, 3682-90 (2006)

57. McLemore, M. L., S. Grewal, F. Liu, A. Archambault, J. Poursine-Laurent, J. Haug and D. C. Link: STAT-3 activation is required for normal G-CSF-dependent proliferation and granulocytic differentiation. *Immunity* 14, 193-204 (2001)

58. Croker, B. A., D. Metcalf, L. Robb, W. Wei, S. Mifsud, L. DiRago, L. A. Cluse, K. D. Sutherland, L. Hartley, E. Williams, J. G. Zhang, D. J. Hilton, N. A. Nicola, W. S. Alexander and A. W. Roberts: SOCS3 is a critical physiological negative regulator of G-CSF signaling and emergency granulopoiesis. *Immunity* 20, 153-65 (2004)

59. Kamezaki, K., K. Shimoda, A. Numata, T. Haro, H. Kakumitsu, M. Yoshie, M. Yamamoto, K. Takeda, T. Matsuda, S. Akira, K. Ogawa and M. Harada: Roles of Stat3 and ERK in G-CSF signaling. *Stem Cells* 23, 252-63 (2005)

60. Chakraborty, A., S. M. White, T. S. Schaefer, E. D. Ball, K. F. Dyer and D. J. Tweardy: Granulocyte colony-stimulating factor activation of Stat3 alpha and Stat3 beta in immature normal and leukemic human myeloid cells. *Blood* 88, 2442-9 (1996)

61. Redell, M. S., A. Tsimelzon, S. G. Hilsenbeck and D. J. Tweardy: Conditional overexpression of Stat3alpha in differentiating myeloid cells results in neutrophil expansion and induces a distinct, antiapoptotic and pro-oncogenic gene expression pattern. *J Leukoc Biol* 82, 975-85 (2007)

62. Mackiewicz, A., M. Wiznerowicz, E. Roeb, A. Karczewska, J. Nowak, P. C. Heinrich and S. Rose-John: Soluble interleukin 6 receptor is biologically active in vivo. *Cytokine* 7, 142-9 (1995)

63. Alonzi, T., G. Middleton, S. Wyatt, V. Buchman, U. A. Betz, W. Muller, P. Musiani, V. Poli and A. M. Davies: Role of STAT3 and PI 3-kinase/Akt in mediating the survival actions of cytokines on sensory neurons. *Mol Cell Neurosci* 18, 270-82 (2001)

64. Vercelli, A., M. Repici, D. Garbossa and A. Grimaldi: Recent techniques for tracing pathways in the central nervous system of developing and adult mammals. *Brain Res Bull* 51, 11-28 (2000)

65. Shen, Y., K. Schlessinger, X. Zhu, E. Meffre, F. Quimby, D. E. Levy and J. E. Darnell, Jr.: Essential role of STAT3 in postnatal survival and growth revealed by mice lacking STAT3 serine 727 phosphorylation. *Mol Cell Biol* 24, 407-19 (2004)

66. Sano, S., Y. Takahama, T. Sugawara, H. Kosaka, S. Itami, K. Yoshikawa, J. Miyazaki, W. van Ewijk and J. Takeda: Stat3 in thymic epithelial cells is essential for postnatal maintenance of thymic architecture and thymocyte survival. *Immunity* 15, 261-73 (2001)

67. Bridges, J. P. and T. E. Weaver: Use of transgenic mice to study lung morphogenesis and function. *Ilar J* 47, 22-31 (2006)

68. Hamvas, A.: Inherited surfactant protein-B deficiency and surfactant protein-C associated disease: clinical features and evaluation. *Semin Perinatol* 30, 316-26 (2006)

69. Clark, J. C., S. E. Wert, C. J. Bachurski, M. T. Stahlman, B. R. Stripp, T. E. Weaver and J. A. Whitsett: Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. *Proc Natl Acad Sci U S A* 92, 7794-8 (1995)

70. Yan, C., A. Naltner, M. Martin, M. Naltner, J. M. Fangman and O. Gurel: Transcriptional stimulation of the surfactant protein B gene by STAT3 in respiratory epithelial cells. *J Biol Chem* 277, 10967-72 (2002)

71. Hokuto, I., M. Ikegami, M. Yoshida, K. Takeda, S. Akira, A. K. Perl, W. M. Hull, S. E. Wert and J. A. Whitsett: Stat-3 is required for pulmonary homeostasis during hyperoxia. *J Clin Invest* 113, 28-37 (2004)

72. Niu, G., K. L. Wright, M. Huang, L. Song, E. Haura, J. Turkson, S. Zhang, T. Wang, D. Sinibaldi, D. Coppola, R. Heller, L. M. Ellis, J. Karras, J. Bromberg, D. Pardoll, R. Jove and H. Yu: Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis. *Oncogene* 21, 2000-8 (2002)

73. DeChiara, T. M., R. Vejsada, W. T. Poueymirou, A. Acheson, C. Suri, J. C. Conover, B. Friedman, J. McClain, L. Pan, N. Stahl, N. Y. Ip and G. D. Yancopoulos: Mice lacking the CNTF receptor, unlike mice lacking CNTF, exhibit profound motor neuron deficits at birth. *Cell* 83, 313-22 (1995)

74. Li, M., M. Sendtner and A. Smith: Essential function of LIF receptor in motor neurons. *Nature* 378, 724-7 (1995)

75. Bonni, A., Y. Sun, M. Nadal-Vicens, A. Bhatt, D. A. Frank, I. Rozovsky, N. Stahl, G. D. Yancopoulos and M. E. Greenberg: Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. *Science* 278, 477-83 (1997)

76. Nakashima, K., S. Wiese, M. Yanagisawa, H. Arakawa, N. Kimura, T. Hisatsune, K. Yoshida, T. Kishimoto, M.

Sendtner and T. Taga: Developmental requirement of gp130 signaling in neuronal survival and astrocyte differentiation. *J Neurosci* 19, 5429-34 (1999)

77. Tramontin, A. D., J. M. Garcia-Verdugo, D. A. Lim and A. Alvarez-Buylla: Postnatal development of radial glia and the ventricular zone (VZ): a continuum of the neural stem cell compartment. *Cereb Cortex* 13, 580-7 (2003)

Abbreviations: basic fibroblast growth factor (bFGF), central nervous system (CNS), epidermal growth factor (EGF), granulocyte colony-stimulating factor (G-CSF), granulocyte colony-stimulating factor receptor (G-CSFR), glial fibrillary acidic protein (GFAP), hepatocyte growth factor (HGF), interleukin (IL), mouse embryonal fibroblast (MEF), surfactant protein (SP), suppressor of cytokine signaling (SOCS), signal transducer and activator of transcription (STAT), transcription activation domain (TAD), terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), vascular endothelial growth factor (VEGF)

Key Words: STAT3, STAT3alpha, STAT3beta, STAT, Acute Phase Response factor, Review, GFAP, Astrocyte, Nodose, Cytokine Signaling

Send correspondence to: Valeria Poli, Molecular Biotechnology Center, University of Turin, Via Nizza 52, 10126 Turin, Italy, Tel: 39-011-6706428, Fax: 39-011-6706432, E-mail: valeria.poli@unito.it

http://www.bioscience.org/current/vol13.htm