

The thyroid hormone receptor functions as a ligand-operated developmental switch between proliferation and differentiation of erythroid progenitors

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The avian erythroblastosis virus (AEV) oncoprotein v-ErbA represents a mutated, oncogenic thyroid hormone receptor α (c-ErbA/TR α). v-ErbA cooperates with the stem cell factor-activated, endogenous receptor tyrosine kinase c-Kit to induce self-renewal and to arrest differentiation of primary avian erythroblasts, the AEV transformation target cells. In this cooperation, v-ErbA substitutes for endogenous steroid hormone receptor function required for sustained proliferation of non-transformed erythroid progenitors. In this paper, we propose a novel concept of how v-ErbA transforms erythroblasts. Using culture media strictly depleted from thyroid hormone (T3) and retinoids, the ligands for c-ErbA/TR α and its co-receptor RXR, we show that overexpressed, unliganded c-ErbA/TR α closely resembles v-ErbA in its activity on primary erythroblasts. In cooperation with ligand-activated c-Kit, c-ErbA/TR α causes steroid-independent, long-term proliferation and tightly blocks differentiation. Activation of c-ErbA/TR α by physiological T3 levels causes the loss of self-renewal capacity and induces synchronous, terminal differentiation under otherwise identical conditions. This T3-induced switch in erythroid progenitor development is correlated with a decrease of c-ErbA-associated histone deacetylase activity. Our results suggest that the crucial role of the mutations activating v-erbA as an oncogene is to 'freeze' c-ErbA/TR α in its non-liganded, repressive conformation and to facilitate its overexpression.

Keywords: differentiation/erythropoiesis/histone deacetylases/thyroid hormone receptor/v-ErbA

Introduction

The thyroid hormone receptor α (c-ErbA/TR α) is a typical member of the class II nuclear receptors (for reviews see Mangelsdorf and Evans, 1995; Mangelsdorf *et al.*, 1995; Tsai and O'Malley, 1994; see also Figure 1A). Its N-terminal domain (A/B region) contains a potential transactivation function (AF-1; Saatcioglu *et al.*, 1993b; Hadzic *et al.*, 1995) as well as phosphorylation sites important for receptor localization and activity (Glineur *et al.*, 1990; Andersson and Vennström, 1997). The A/B domain also

affects the DNA binding properties of the adjacent DNA binding domain (DBD, C-region; Judelson and Privalsky, 1996). The DBD consists of two conserved cysteine-rich zinc fingers, responsible for specific binding to thyroid hormone response elements (TREs) in TR target genes. The DBD is also involved in receptor dimerization (Rastinejad *et al.*, 1995; Perlmann *et al.*, 1996). In the absence of ligand, the hinge domain (D-region) of the receptor recruits transcriptional repressor complexes, consisting of receptor-bound nuclear co-repressors such as NCoR, SMRT and SUN-CoR (Chen and Evans, 1995; Hörlein *et al.*, 1995; Zamir *et al.*, 1996) which activate histone deacetylases (mRPD3/HDAC1) via a cascade of interacting proteins (Heinzel *et al.*, 1997; Nagy *et al.*, 1997). These activated co-repressor complexes were proposed to locally convert chromatin into an inactive state, which may restrict access of the basal transcriptional machinery to DNA promoter elements (for review see Pazin and Kadonaga, 1997).

The C-terminal, ligand-binding domain of TR α (LBD, E-region) contains the major ligand-inducible transactivation function 2 (AF-2; Baretino *et al.*, 1994; Wagner *et al.*, 1995). It also binds numerous proteins, among them the retinoid X receptors (RXR), the heterodimerization partners of class II receptors (for review see Mangelsdorf and Evans, 1995) and multiple co-activators, such as Trip1 (Lee *et al.*, 1995), TIF1 (LeDouarin *et al.*, 1995), TRAPs (Fondell *et al.*, 1996), CBP/p300 (Kamei *et al.*, 1996) and ACTR (Chen *et al.*, 1997). These co-activators interact with histone acetylases or display intrinsic histone acetylase activity (Ogryzko *et al.*, 1996), inducing chromatin to assume an active conformation facilitating transcriptional activation (Wong *et al.*, 1997).

These findings have led to a working model of TR action, suggesting that the TR remains bound to DNA but exists in two mutually exclusive conformations. In the absence of hormone, binding of the co-repressor complex leads to chromatin inactivation and gene repression, while binding of the ligand thyroid hormone (T3) causes dissociation of co-repressors, co-activator binding and transcriptional activation accompanied by local opening of chromatin structure (reviewed in Wolffe, 1997). In addition, the TR in its non-liganded state may activate certain genes via AF-1 or AF-2 (Saatcioglu *et al.*, 1993b; Hadzic *et al.*, 1995; Tomic-Canic *et al.*, 1996). Finally, the TR *trans*-represses genes in its liganded state via inhibiting other transcription factors such as AP-1 (Saatcioglu *et al.*, 1993a).

Despite this rather detailed knowledge about molecular events in TR function, the biological functions of the TR in relevant target cells are still poorly understood. In primary erythroblasts, the TR accelerates differentiation or induces apoptosis, while a role in proliferation induction and leukemic transformation was only assigned so far to its oncogenic counterpart, v-ErbA (for review see

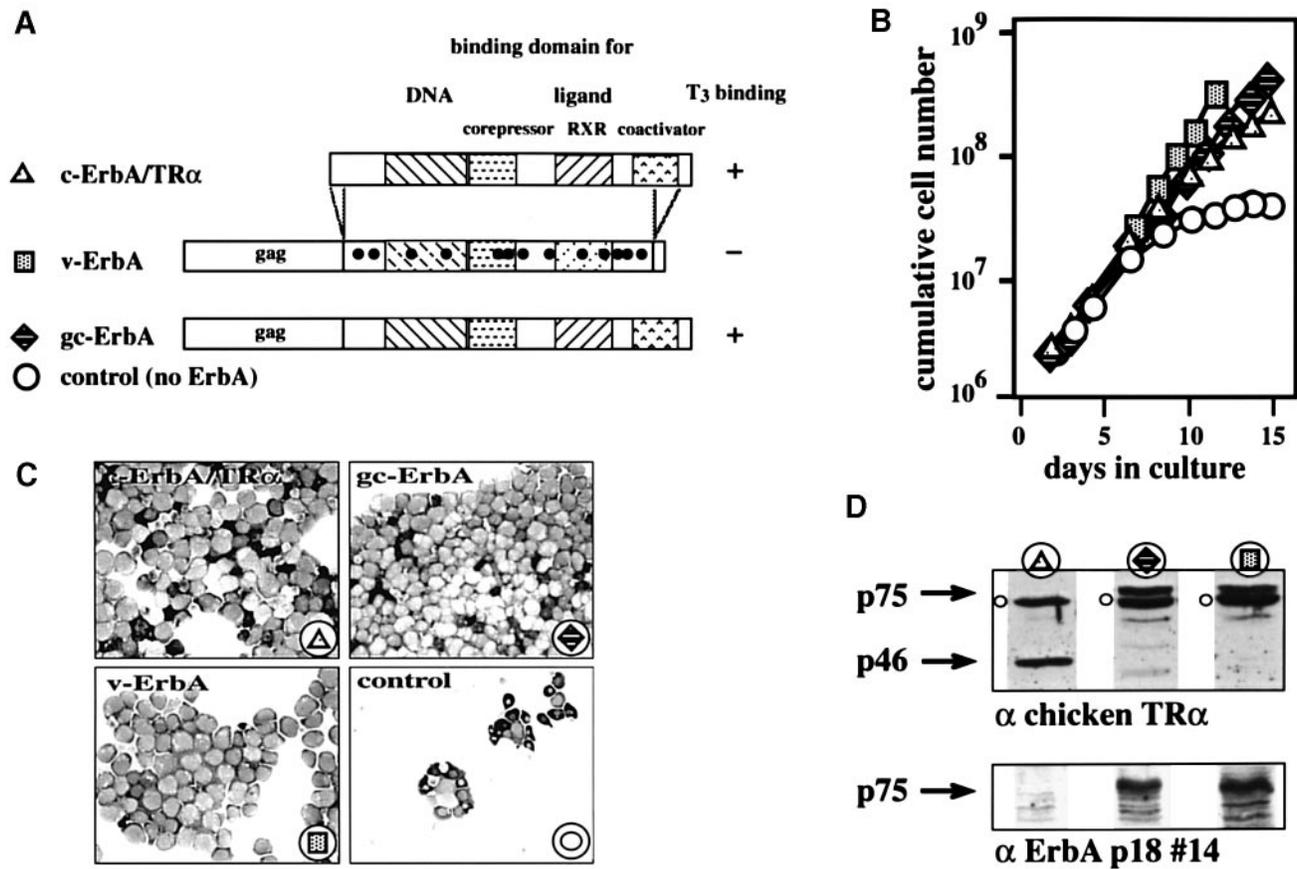


Fig. 1. Different forms of c-ErbA/TR α induce steroid-independent outgrowth of bone marrow erythroblasts in the absence of T₃/retinoids.

(A) Schematic representation of the domain structures of the thyroid hormone receptor c-ErbA/TR α , v-ErbA and gc-ErbA. Specific c-ErbA domains are indicated, including the DNA binding domain and domains specifically interacting with known proteins (co-receptor RXR, co-repressors like SMRT/NCOR/SUN-CoR and co-activators, e.g. p300/CBP). In v-ErbA, the virus-derived gag sequence, the N- and C-terminal deletions and the 13 v-ErbA specific point mutations (solid dots) are indicated. These mutations cause the loss of T₃-binding and abolish the binding of several interacting proteins (indicated by changed domain patterns). In gc-ErbA, all v-ErbA sequences are replaced by the corresponding c-ErbA sequences, restoring the function of the known, essential interaction domains. (B) Chicken bone marrow erythroblasts expressing c-ErbA/TR α (triangles), v-ErbA (squares) and gc-ErbA (diamonds) or infected with an empty control vector (circles) were seeded into CFU-E medium containing stripped sera (stripped medium; see Materials and methods) supplemented with the growth factors SCF and TGF α plus the steroid antagonists ICI and ZK. Cumulative cell numbers were determined as described (Fuerstenberg *et al.*, 1992). (C) Ten days after seeding, cytospin preparations from the cultures in (B) were stained with neutral benzidine plus histological dyes and images taken with a CCD camera (Photometrics), using a blue filter (480 nm) to reveal hemoglobin (dark staining; Bauer *et al.*, 1997). Images were processed with Adobe Photoshop software. Note darkly stained, small oval erythrocytes in the control culture (circle) whereas large, hemoglobin-negative erythroblasts prevail in the v- or c-ErbA-expressing cultures. (D) Expression of the ~75 kDa v-ErbA (square) and gc-ErbA (diamond), and the 46 kDa c-ErbA/TR α proteins (triangle) was analyzed in the respective primary erythroblasts 10 days after infection. Lysates were processed for Western blot analysis using two different antibodies (see Materials and methods). Arrows, positions of the v- and c-ErbA proteins; circles, a non-specific band detected by the c-ErbA antibody only.

Gandrillon *et al.*, 1995; Beug *et al.*, 1996). v-ErbA is one of the two oncoproteins encoded by the avian erythroblastosis virus (AEV-ES4; Vennström and Bishop, 1982) and causes acute erythroleukemia in cooperation with numerous receptor tyrosine kinase (RTK) oncoproteins or downstream signal transducers (v-ErbB, v-Sea, v-Src, v-Ras and v-Raf; Graf and Beug, 1983; Kahn *et al.*, 1986). Together with these oncoproteins, v-ErbA induces enhanced proliferation plus a tight differentiation arrest of erythroid progenitors in culture (Beug *et al.*, 1985; Kahn *et al.*, 1986). Recently, we demonstrated that v-ErbA substituted for the biological function of the activated estrogen and glucocorticoid receptors (ER and GR; Bauer *et al.*, 1997) which are essential for sustained self-renewal in normal erythroid progenitors (Wessely *et al.*, 1997). v-ErbA required cooperation with the stem cell factor

(SCF)-activated, endogenous RTK c-Kit, since v-ErbA overexpressing cells underwent normal terminal differentiation in the absence of SCF.

During the course of oncogene activation, v-ErbA was fused to viral gag sequences at its N-terminus and suffered N- and C-terminal deletions as well as numerous point mutations (Sap *et al.*, 1986; Weinberger *et al.*, 1986). This resulted in a loss of hormone binding activity and AF-2 function (Zenke *et al.*, 1990; Baretino *et al.*, 1994) as well as altered DNA binding, raising speculations that v-ErbA function may entail a gain of function mechanism (Chen *et al.*, 1993). However, v-ErbA retained the capacity to bind co-repressors, which are essential for its function (Damm and Evans, 1993; Chen and Evans, 1995; Hörlein *et al.*, 1995). In erythroblasts, v-ErbA represses several endogenous genes upregulated during terminal differenti-

ation such as carbonic anhydrase II (*CA II*), *band 3* and *ALA-S* (Zenke *et al.*, 1990; Disela *et al.*, 1991). Hence v-ErbA counteracts the effects of liganded c-ErbA/TR α , which induces those genes and accelerates erythroid differentiation (Schroeder *et al.*, 1992b; Gandrillon *et al.*, 1994). This led to the idea that v-ErbA would prevent access of liganded c-ErbA to TREs by constitutively binding to them, thus inhibiting c-ErbA function in a dominant-negative fashion (Damm *et al.*, 1989; Sap *et al.*, 1989).

However, this model is inconsistent with more recent observations. Both v-ErbA and unliganded c-ErbA/TR α bind co-repressor-histone deacetylase complexes and should therefore elicit similar molecular and biological functions. Accordingly, reversion of single, mutated amino acids in the N-terminal-, DNA binding- and D-domains of v-ErbA to the c-ErbA/TR α configuration failed to abolish its biological activity (Bauer *et al.*, 1997; A.Bauer, M.Andersson and H.Beug unpublished data). Furthermore, activating endogenous TR α /RXR by T3/9-*cis* retinoic acid (RA) induces differentiation and *CA II* mRNA upregulation in primary erythroid progenitors overexpressing v-ErbA (Schroeder *et al.*, 1992b; Bauer *et al.*, 1997). These and other findings (Damm and Evans, 1993) argued against a simple dominant negative function for v-ErbA.

Here we investigate an alternative model of oncogene function. We reasoned that v-ErbA may resemble the proto-oncogene c-ErbA/TR α in one of its functional states, i.e. TR expressed in the complete absence of ligand, and that the v-ErbA mutations would prevent c-ErbA/TR α from switching to the transcriptionally active conformation induced by T3 binding. Earlier results argued against this idea, since overexpressed gag-c-ErbA acting in the context of temperature-sensitive tyrosine kinase oncogenes inhibited rather than actively induced erythroblast proliferation and only partially arrested differentiation (Zenke *et al.*, 1990). Since the media used in these earlier studies still contained retinoids, this phenotype caused by the overexpressed gag-c-ErbA can be explained by partial ligand activation of the TR/RXR heterodimer.

In this study, we employed media strictly depleted of T3 and retinoids, as well as primary avian erythroblasts which represent the *in vivo* target cells for AEV transformation and express all interaction partners important for ErbA function. We demonstrate that overexpressed, ligand-deprived c-ErbA/TR α functions exactly like v-ErbA in these cells. In cooperation with liganded c-Kit, non-liganded c-ErbA/TR α actively promoted sustained proliferation in the absence of steroid hormone receptor function and tightly arrested differentiation. Physiological levels of T3 abolished this c-ErbA/TR α -induced differentiation arrest and caused accelerated terminal differentiation, rapid transcriptional activation of *CA-II*, and modest repression of *c-myb*. Finally, differentiation induction by T3 was accompanied by a decrease of histone deacetylase activity co-immunoprecipitated with TR α . Our results support the novel concept that c-ErbA/TR α represents a ligand-operated, molecular switch, regulating the balance between erythroblast self-renewal and differentiation. We also present a new model for v-ErbA function, simply mimicking the activity of c-ErbA/TR α in its non-liganded state.

Results

Exogenous c-ErbA supports steroid-independent outgrowth of chicken bone marrow cells in the absence of thyroid hormone and retinoids

Induction of sustained proliferation without differentiation (self-renewal) in primary avian erythroid progenitors requires the cooperation of activated RTKs with ligand-activated ER and GR (Wessely *et al.*, 1997). Recently, v-ErbA was shown to fully substitute for both these receptors (Bauer *et al.*, 1997). This biological function of v-ErbA was used as a tool to study whether the various mutations accumulated during the course of v-ErbA activation as an oncogene were important for its transforming ability.

First, we analyzed numerous v-ErbA variants in which amino acids in the N-terminal domain, the DNA-binding domain and hinge region had been changed back to the c-ErbA/TR α configuration. All these back-mutated v-ErbAs were still active in primary erythroblasts, thus failing to identify a crucial mutation responsible for leukemic transformation by v-ErbA (A.Bauer, unpublished). Therefore, we re-investigated the effect of the overexpressed proto-oncogene c-ErbA/TR α itself. In contrast to previous studies (Zenke *et al.*, 1990; Disela *et al.*, 1991; Schroeder *et al.*, 1992a), primary chicken bone marrow cells devoid of any oncogene were used to express c-ErbA/TR α . More importantly, we fully depleted the various sera required for erythroblast culture media of both T3 and retinoids (referred to as stripped medium; see Materials and methods) to prevent partial ligand activation of the TR α /RXR heterodimer, causing an aberrant phenotype in erythroblasts (Zenke *et al.*, 1990; see Introduction).

Using these stripped sera, we first tested whether overexpression of unliganded c-ErbA was now tolerated by the cells and whether it conferred steroid-independent self-renewal induction similar to v-ErbA. C-ErbA/TR α or a gag-fused c-ErbA, referred to as gc-ErbA (Figure 1A), were expressed in primary erythroblasts by retroviral infection. Empty vector- and v-ErbA-infected cells served as negative and positive controls, respectively. Gc-ErbA was employed since it resembles c-ErbA/TR α in all functions studied, including the ability to bind T3 (Munoz *et al.*, 1988; Zenke *et al.*, 1990) but is easily overexpressed and provides a 'tagged' c-ErbA version readily detected by the available, potent anti-gag antibodies (see Materials and methods). Expression of the different ErbA proteins was verified by Western blot analysis using antisera directed either against full-length chicken TR α or an 18 kDa fragment of v-ErbA, showing that the 75 kDa v-ErbA and gc-ErbA proteins as well as the 46 kDa c-ErbA/TR α protein were expressed at similar levels (Figure 1D).

These v- or c-ErbA-expressing erythroblasts were cultivated in stripped medium containing RTK ligands [SCF, the c-ErbB ligand transforming growth factor (TGF) α and insulin-like growth factor (IGF)]. To eliminate ER and GR function, we added the ER- and GR-antagonists ICI 182,780 (ICI) and ZK 112,993 (ZK), respectively (Wessely *et al.*, 1997). Outgrowth was monitored by determining cumulative cell numbers. All cells expressing either c- or v-ErbA exhibited exponential growth independ-

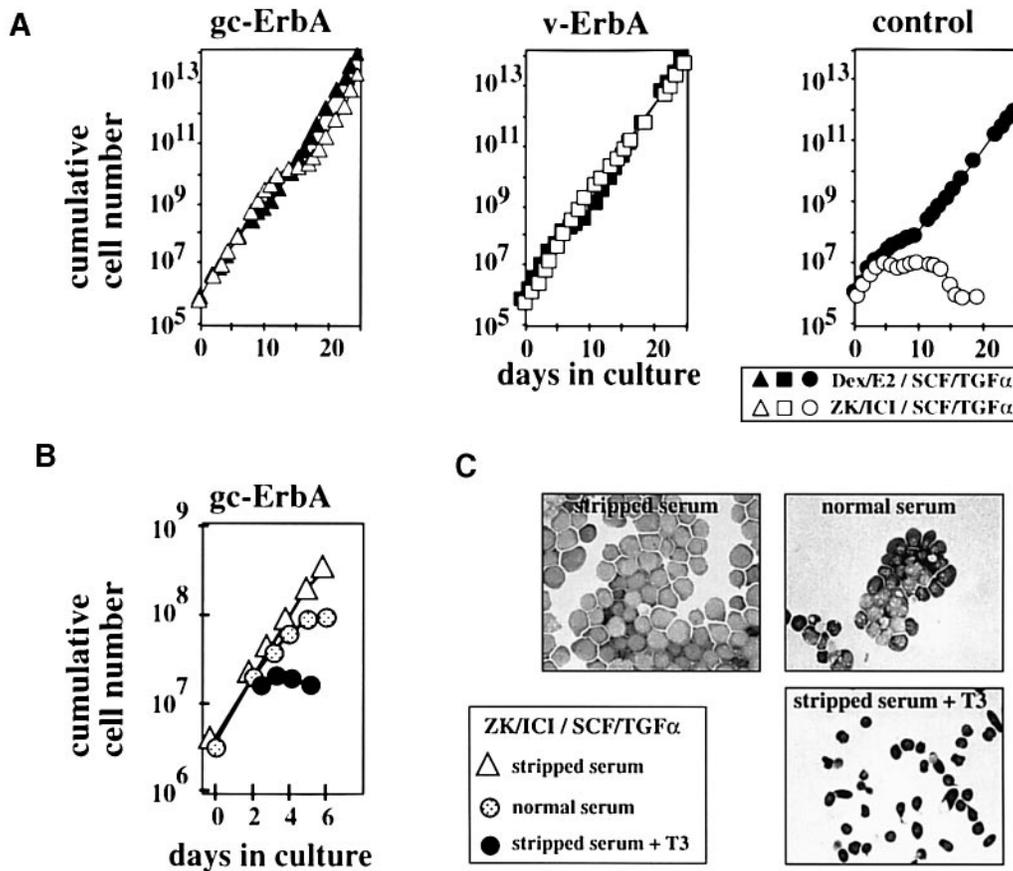


Fig. 2. c-ErbA/TR α -induced long-term self-renewal requires the absence of T3/retinoids. (A) Primary chicken erythroid progenitors expressing v-ErbA (squares), gc-ErbA (triangles) or empty vector (circles) were cultivated in media containing the RTK ligands SCF and TGF α in the presence of steroids (E2, Dex, closed symbols) or their antagonists (ICI, ZK, open symbols). Cells were counted daily and cumulative cell numbers were determined. Note that the empty vector control erythroblasts (circles) required E2 and Dex for continuous proliferation, while v-ErbA- (squares) and gc-ErbA-expressing (triangles) erythroblasts cultivated in the absence of T3/retinoids undergo long-term, steroid-independent proliferation with identical kinetics. (B) Aliquots from the gc-ErbA erythroblast culture shown in (A) were maintained in stripped medium (triangles) or switched to stripped medium containing 150 nM T3 (filled circles) and medium containing untreated instead of stripped serum (dotted circles). Cells were then analyzed for sustained proliferation as in (A). (C) Cells from the same cultures as in (B) were cytocentrifuged onto slides at day five, stained and processed for imaging as described in the legend to Figure 1. Note undifferentiated cells in stripped serum, partially and terminally differentiated cells in normal serum, and terminally differentiated plus apoptotic cells in stripped sera plus T3.

ent of GR and ER function until the experiment was terminated (15 days). In contrast, control cells lacking exogenous ErbA ceased to proliferate after 7 days under these conditions (Figure 1B).

Phenotypical analysis of these cells using stained cyto-spin preparations revealed large, hemoglobin-negative cells representing immature erythroblasts prevailed in the cultures expressing v-ErbA, c-ErbA/TR α or gc-ErbA. In contrast, the control culture mainly contained terminally differentiated, highly hemoglobinized erythrocytes (Figure 1C). Taken together these data indicate that both gag-fused and bona fide c-ErbA/TR α resemble v-ErbA in their ability to induce GR- and ER-independent proliferation and differentiation arrest in primary erythroid progenitors.

Non-liganded thyroid hormone receptor requires cooperation with endogenous, liganded c-Kit to induce steroid-independent, long-term self-renewal of erythroid progenitors

Does non-liganded c-ErbA/TR α also resemble v-ErbA in its ability to induce long-term erythroblast proliferation? In normal avian erythroid progenitors, this phenotype (proliferation without differentiation for ~30 generations,

the typical *in vitro* life span of chicken cells) is induced by the combined action of activated steroid hormone receptors and activated RTKs (Hayman *et al.*, 1993; Steinlein *et al.*, 1995; Wessely *et al.*, 1997). Erythroblast cultures expressing gc-ErbA, v-ErbA, c-ErbA/TR α and empty vector (generated as described above) were exposed to the c-Kit/c-ErbB ligands SCF and TGF α , and either treated with the steroids estradiol (E2) and dexamethasone (Dex), or with the respective antagonists ICI and ZK to inactivate steroid receptor function (Bauer *et al.*, 1997; Wessely *et al.*, 1997). Cells were serially passaged in stripped medium at optimal cell densities and proliferation kinetics determined by daily counting. As expected, v-ErbA (Figure 2A, squares) induced sustained self-renewal in erythroid progenitors, both in the presence of steroids and their antagonists, respectively. Control cells (Figure 2A, circles) proliferated only in the presence of steroids but ceased to grow after 6 days in the presence of steroid antagonists. To our surprise, gc-ErbA-expressing erythroblasts (Figure 2A, triangles) underwent long-term proliferation with identical kinetics as v-ErbA-expressing cells, again in a steroid-independent manner (Figure 2A). c-ErbA/TR α -expressing cells, as well as cells expressing

a gag-fused, full-length c-ErbA (g-flc-ErbA) behaved similarly (data not shown). Both v-ErbA and gc-ErbA cells increased $>10^8$ -fold within 25 days, equivalent to >27 population doublings. Thus, non-liganded c-ErbA and v-ErbA are similar, if not identical in their ability to promote steroid-independent, long-term self-renewal of erythroid progenitors.

The above results prompted the question whether or not the absence of T3 is crucial for long-term proliferation of c-ErbA-expressing progenitors. Aliquots of the above gc-ErbA erythroblast culture were switched to medium containing untreated sera or stripped medium supplemented with T3. Controls were maintained in stripped medium. Cumulative cell numbers were determined and the phenotype of cells analyzed using stained cytopsin preparations. gc-ErbA-expressing progenitors continued to proliferate as expected in stripped medium (Figure 2B, triangles), but ceased to proliferate 4 days after switching them to standard serum conditions (Figure 2B, dotted circles). Cells grown in untreated sera for 5 days represented partially mature or mature erythrocytes (Figure 2C, normal serum), whereas they retained a hemoglobin-negative erythroblast phenotype in stripped sera (Figure 2C). An even more striking result was obtained with the T3-treated cells. Proliferation ceased 1–2 days after T3 addition (Figure 2B, solid circles) and the cells differentiated into mature erythrocytes within 3 days, while a minority of cells underwent apoptosis. In conclusion, retrovirus-transduced, non-liganded c-ErbA resembles oncogenic v-ErbA in its ability to induce sustained proliferation, to block differentiation, and functionally replace activated steroid receptors in primary erythroid progenitors. Importantly, complete removal of T3 and retinoids is essential for this ability.

The biological activity of the v-ErbA oncoprotein in primary erythroblasts completely depends on the cooperation with endogenous c-Kit activated by SCF (Bauer *et al.*, 1997). In contrast, upregulation and activation of endogenous c-ErbB, required for long-term proliferation of non-transformed avian erythroid progenitors (Hayman *et al.*, 1993; Steinlein *et al.*, 1995; Wessely *et al.*, 1997), neither occurred nor was essential for self-renewal of cells expressing v-ErbA. We therefore asked which RTK was required for long-term outgrowth in cooperation with overexpressed c-ErbA. For this, gc-ErbA progenitors cultivated for 18 days with SCF/TGF α and ICI/ZK in stripped medium were compared with v-ErbA-expressing erythroblasts in a growth factor assay, measuring thymidine incorporation in response to SCF and TGF α (see Materials and methods). c- and v-ErbA-expressing progenitors were identical in that they showed a strong proliferative response to SCF, but did not detectably respond to TGF α (Figure 3A). In line with these findings, the c-ErbB protein was undetectable in both c- and v-ErbA-expressing cells by Western blot and phosphotyrosine blot analysis, while c-Kit was abundantly expressed (data not shown; Hayman *et al.*, 1993). Thus, non-liganded c-ErbA resembles v-ErbA in that it neither involves c-ErbB upregulation nor requires c-ErbB function for induction of sustained erythroblast proliferation.

Previous studies employing RTK oncoprotein-transformed erythroblasts that overexpressed c-ErbA suggested different biological activities of c-ErbA and v-

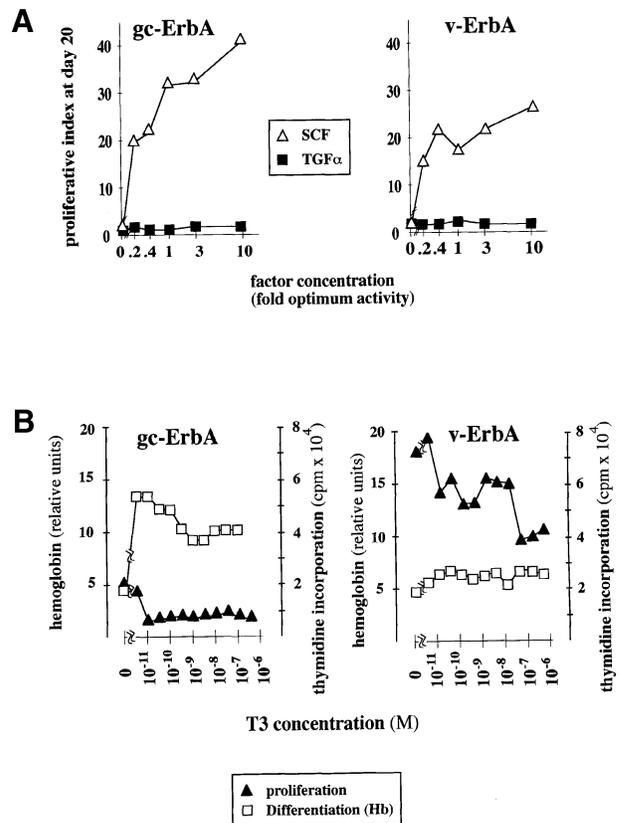


Fig. 3. Response of gc-ErbA-expressing erythroblasts to RTK ligands and T3. (A) Aliquots from 18-day-old gc-ErbA- (left panel) or v-ErbA-expressing erythroblasts (right panel), grown in the presence of SCF/TGF α and ICI/ZK, were tested in a growth-factor assay measuring [3 H]thymidine incorporation in response to SCF and TGF α stimulation. The proliferative index normalized to control samples receiving no factor is plotted against factor concentration (optimum factor concentrations: 100 ng/ml for SCF; 2 ng/ml for TGF α). The average of three independent determinations is shown. (B) Separate aliquots of the same gc-ErbA- or v-ErbA-expressing progenitor cultures were seeded in stripped media containing 100 ng/ml SCF plus the indicated amounts of T3. After incubation for 2 days, cultures were evaluated for their hemoglobin concentration using a photometric assay (left scale, open squares) and for [3 H]thymidine incorporation (right scale, filled triangles).

ErbA. c-ErbA conveyed a serious growth disadvantage rather than stimulating proliferation and only partially arrested erythroid differentiation (Zenke *et al.*, 1990). This can now be explained by the assumption that the media used in that study still contained ligands able to stimulate the TR α /RXR heterodimer (e.g. retinoids or residual T3). To verify that our stripped medium contained no detectable ligand activity for TR α /RXR and to analyze whether T3 acted at physiological concentrations in our system, we determined the concentration dependence of the T3 response in gc-ErbA erythroblasts. Two functional assays for the T3 response were employed: a thymidine incorporation assay to measure growth inhibition by T3; and a colorimetric hemoglobin assay to quantitate differentiation.

To our surprise, gc-ErbA progenitors already responded to picomolar amounts (3 pM) of T3 added to stripped medium (Figure 3B, left panel). Addition of 10 pM T3 reduced thymidine incorporation to basal levels (Figure 3B, solid triangles) and caused maximal levels of hemo-

globin accumulation (Figure 3B, open squares). These effects were not further enhanced at higher concentrations of T3. In contrast, erythroblasts expressing v-ErbA showed only a partial reduction of thymidine incorporation and hardly any hemoglobin accumulation in response to T3 (Figure 3B, right panels). Since these weak effects already occurred at low T3 levels (10^{-10} to 10^{-9} M), to which v-ErbA is clearly not responsive, they are probably due to endogenous c-ErbA/TR α present in the v-ErbA-expressing cells. These results indicate that physiological levels of T3 suffice to arrest proliferation and induce differentiation in c-ErbA-expressing erythroid progenitors, while the v-ErbA induced proliferation and differentiation arrest shows the expected insensitivity to T3.

Thyroid hormone is both necessary and sufficient to switch c-ErbA-expressing progenitors from self-renewal to terminal differentiation

v-ErbA causes a complete differentiation arrest in primary erythroblasts, when cooperating with the SCF-activated, endogenous c-Kit. In contrast, v-ErbA-expressing cells undergo normal terminal differentiation if c-Kit is inactivated by SCF withdrawal and replaced by insulin and anemic serum [AS, containing avian erythropoietin (Epo); Bauer *et al.*, 1997]. This prompted us to analyze whether the differentiation arrest caused by non-liganded c-ErbA plus activated c-Kit could be overcome by SCF withdrawal or T3 addition. However, stripped anemic serum may contain suboptimal concentrations of avian Epo as well as other factors influencing differentiation (O.Wessely, A.Bauer, C.Quang, E.Deiner, M.von Lindern, P.Steinlein, J.Ghysdael and H.Beug, submitted; H.Beug, unpublished). Earlier studies showed, that the murine Epo receptor (EpoR) substitutes for all known functions of the endogenous, chicken EpoR in avian erythroid progenitors (Steinlein *et al.*, 1994; Quang *et al.*, 1997). We therefore co-expressed c-ErbA and the murine EpoR in primary erythroblasts, thus generating a cell system solely dependent on recombinant factors. For this, a novel combination of retrovirus vectors allowing efficient co-infection was used (Quang *et al.*, 1997; see Materials and methods).

Avian erythroblasts co-expressing gc-ErbA and EpoR were induced to differentiate by addition of human recombinant Epo (hEpo) plus insulin, either alone or in combination with SCF, either and in the presence or absence of T3 (Figure 4A). In addition to phenotypical analysis using cytopspins, the cells were monitored for quantitative parameters such as proliferation and hemoglobin accumulation (Beug *et al.*, 1994). In the absence of SCF, i.e. in hEpo plus insulin alone, the gc-ErbA erythroblasts differentiated into partially mature or mature erythrocytes within 4–5 days, as indicated by loss of proliferative capacity (Figure 4C, filled circles), nuclear condensation, and strong staining for hemoglobin (Figure 4B, upper panel). Addition of SCF in the presence of hEpo/insulin caused a tight differentiation arrest of the EpoR-expressing gc-ErbA erythroblasts. The cells exhibited an immature erythroblast morphology (Figure 4B, middle panel), proliferated continuously (Figure 4C, open triangles) and did not accumulate hemoglobin (Figure 4D, open bars). Addition of T3 completely reversed the tight differentiation arrest observed in these cells after exposure to both SCF and hEpo/insulin. The T3-treated progenitors terminally

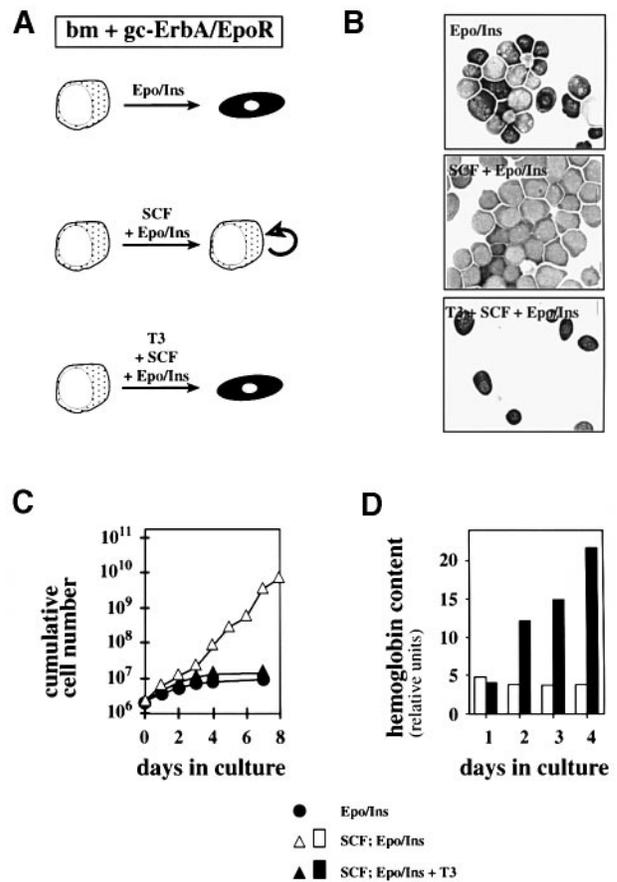


Fig. 4. T3-induced switch from self-renewal to terminal differentiation in erythroblasts expressing gc-ErbA plus the EpoR. (A) Scheme depicting the experimental approach to analyze effects of T3 on the differentiation of gc-ErbA progenitors expressing the mEpoR. Cells undergo self-renewal in the presence of SCF, Epo and insulin, but differentiate upon SCF withdrawal or T3 addition under otherwise identical conditions (see Material and methods). (B) gc-ErbA-expressing erythroblasts were treated as depicted in (A) and aliquots analyzed by cytoprecipitation, benzidine staining and imaging 4 days after differentiation induction, as described in the legend to Figure 1. Note differentiation in Epo/insulin, differentiation arrest in SCF + Epo/insulin and accelerated differentiation in T3 + SCF + Epo/insulin. (C) Aliquots of the same cultures as shown in (B) were counted daily and cumulative cell numbers plotted. (D) At the times indicated, aliquots were removed from cells grown in SCF, Epo and insulin in the presence (solid bars) or absence (open bars) of 150 nM T3 and hemoglobin content determined (normalized to cell number). Note growth arrest (C) and hemoglobin accumulation after T3 treatment.

differentiated within 4 days as indicated by loss of growth potential (Figure 4C, closed triangles), strong hemoglobin accumulation (Figure 4D, closed bars) and erythrocyte-like morphology plus strong staining for hemoglobin (Figure 4B, lower panel).

These data clearly show that T3 is both necessary and sufficient to trigger the switch from sustained proliferation to differentiation in gc-ErbA progenitors exposed to both SCF and differentiation factors. Thus, c-ErbA seems to function as a ligand-operated molecular switch between proliferation and differentiation of erythroblasts: repressing differentiation in the absence of ligand, but inducing it upon T3 addition.

The ErbA target gene *CA II* is rapidly induced after T3 stimulation of gc-ErbA progenitors

Having shown that c-ErbA functions as a ligand-operated switch between proliferation and differentiation, we sought to determine if this regulation also occurred at the level of target gene expression. v-ErbA represses the known target genes *CA II*, anion transporter *band 3* and erythroid δ -aminolevulinic synthase (*e-ALAS*), and maintains high expression of *c-myb* (Bauer *et al.*, 1997), a transcription factor essential for hematopoiesis (Mucenski *et al.*, 1991) and a potential target gene of the GR in avian erythroblasts (Wessely *et al.*, 1997). Thus, we analyzed if and how these putative ErbA target genes were regulated by c-ErbA upon switching EpoR/gc-ErbA-expressing erythroblasts proliferating in Epo/insulin plus SCF to the differentiating state by simple addition of T3. We also measured *GATA-1* expression because of its central role in erythropoiesis obvious from *GATA-1* $-/-$ mice (Pevny *et al.*, 1991). *GATA-1* has not yet been described as an ErbA target gene, but strongly accelerates erythroid differentiation in avian erythroblasts (Briegel *et al.*, 1996).

gc-ErbA progenitors expressing EpoR were stimulated to differentiate with T3 in the presence of SCF, Epo and insulin as above (Figure 4A). Control cultures were maintained in the proliferative state using the same growth factors but in the absence of T3. At the times indicated (Figure 5), aliquots were taken from the cultures and processed for Northern blot analysis. T3 addition caused a striking, rapid induction of *CA II* mRNA (Figure 5A). After 4 h, *CA II* mRNA was elevated 15-fold, and reached a maximum of 50-fold induction after 8 h (Figure 5B). A similarly rapid, but more modest (>2.5 -fold) downregulation of *c-myb* mRNA was also observed. In contrast, T3 only induced either minor or late upregulation of *e-ALAS* and *band 3* mRNA within 24 h. No changes in *GATA-1* mRNA levels were detected. In conclusion, T3-activated gc-ErbA exhibited striking, but quite selected effects on some, but not all putative v-ErbA target genes analyzed.

The T3-induced, gc-ErbA-mediated switch from proliferation to differentiation correlates with the loss of c-ErbA-associated histone deacetylase activity in primary erythroblasts

In their non-liganded state, class II NRs such as the thyroid hormone receptor activate histone deacetylases (HDs) via co-repressor complexes, a process thought to contribute to gene repression. Upon ligand induction, these repressor complexes dissociate from the receptor, being replaced by co-activator complexes which exhibit histone acetyltransferase activity (Heinzel *et al.*, 1997; Nagy *et al.*, 1997; Wolffe, 1997; Zamir *et al.*, 1997). If the rapid transcriptional induction of the potential c-ErbA target gene *CA II* was associated with respective changes in chromatin acetylation, we would expect that unliganded c-ErbA is associated with a HD complex in primary erythroblasts, and that T3 addition *in vivo* should release the HD-containing complex from the receptor.

To analyze this, EpoR-expressing gc-ErbA progenitors grown in SCF/hEpo/insulin were switched from proliferation to differentiation by T3 addition. At the times indicated, protein extracts were prepared and assayed for gc-ErbA-associated HD activity by co-immunoprecipitation (see Materials and methods). The results show

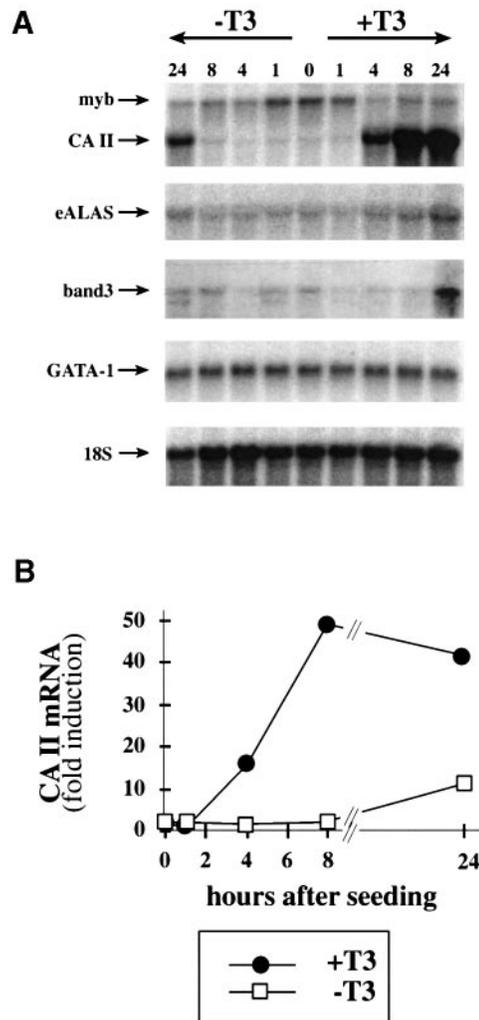


Fig. 5. *CA II* is a target gene of T3-activated gc-ErbA in primary erythroblasts. (A) Self-renewing gc-ErbA progenitors expressing the mEpoR were induced to differentiate in the presence or absence of T3 as described in the legend to Figure 4. Cells were harvested at the indicated time points (in hours) and 10 μ g of extracted total RNA from each sample was subjected to Northern blot analysis. Hybridization was performed sequentially with 32 P-labeled cDNA probes specific for chicken *c-myb*, *CA II*, *e-ALAS*, *GATA-1* and *band 3*. To confirm equal RNA loading, the signal obtained with the various probes was compared to that obtained with a probe for chicken 18S rRNA (18S). (B) *CA II* mRNA levels in cells treated or not treated with T3 as determined in (A) are shown after quantitative evaluation and normalization to the signal obtained with 18S rRNA. Blots were analyzed by phosphoimaging and quantitated using the Image Quant software.

that HD activity co-immunoprecipitating with gc-ErbA decreased 5-fold within 4 h after T3 addition and remained at low levels for at least 20 h (Figure 6, solid bars). In contrast, the total HD activity determined in whole-cell extracts did not decrease during the experiment and even increased slightly during the first 4 h (data not shown). To test whether T3 addition to extracts prepared from cells grown in the absence of T3 would influence gc-ErbA-associated HD activity *in vitro*, two aliquots from an extract of SCF/Epo/insulin grown cells were treated or not treated with T3, and then processed for co-immunoprecipitation analysis of gc-ErbA-HD association. Overnight incubation of the extract with T3 released about

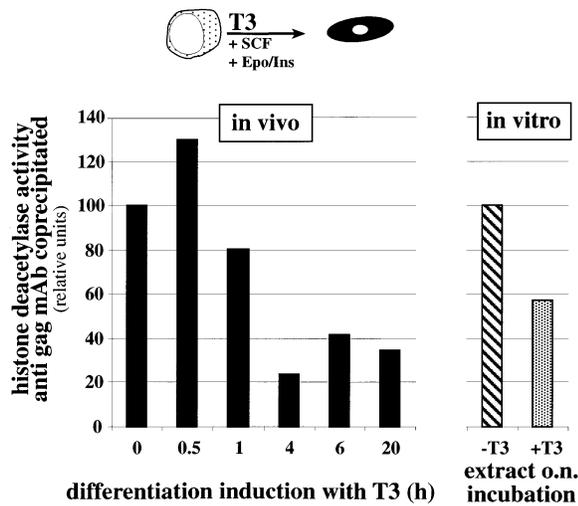


Fig. 6. T3-induced switch of gc-ErbA erythroblasts from proliferation to differentiation: Correlation with a decrease in gc-ErbA/histone deacetylase association. gc-ErbA progenitors expressing the EpoR and cultivated in SCF, Epo and insulin were induced to differentiate in the presence of T3 as described in the legend to Figure 4 (left panel, see scheme above panel). Cells were harvested at the times indicated (hours), and protein extracts were prepared and subjected to immunoprecipitation with the anti-gag monoclonal antibody 1G10 (anti gag mAb). In addition and as a control (right panel), two aliquots from a cell extract of gc-ErbA erythroblasts cultivated in the absence of T3 were incubated overnight in the presence (dotted bar) or absence of T3 (hatched bar) and then processed for immunoprecipitation with the 1G10 antibody as above. Histone deacetylase activity present in the immunoprecipitates was quantified by measuring the release of [³H]acetate from labeled chicken erythrocyte histones. Similar results were obtained in two further independent experiments.

half of the HD activity co-precipitated with gc-ErbA (Figure 6, gray bar) as compared with the untreated sample (Figure 6, hatched bar). Thus, the T3-induced differentiation induction mediated by gc-ErbA correlates with a disruption of a putative c-ErbA/co-repressor complex containing HD in primary avian erythroid cells.

Discussion

A recent model of c-ErbA/TR α and RAR α function suggests that these receptors act as ligand-operated molecular switches between transcriptional repression and activation, involving respective changes in chromatin structure (Wolffe, 1997; see Introduction). Here we show for the first time that a corresponding dual function of the thyroid hormone receptor α (c-ErbA/TR α) is important for determination of cell fate in hematopoietic progenitors. In the complete absence of its ligands, c-ErbA/TR α causes sustained proliferation accompanied by a tight differentiation arrest. After ligand (T3) addition, the same receptor readily promotes terminal red-cell differentiation. Thus, c-ErbA/TR α acts as a T3-driven molecular switch in regulating the balance between proliferation and differentiation of primary erythroblasts. In addition we propose a novel, surprisingly simple model for v-ErbA oncoprotein function, in which v-ErbA is constitutively 'frozen' in the ligand-free, repressing conformation of c-ErbA/TR α . Accordingly, the most likely role for the mutations occurring during oncogenic activation of v-ErbA is to stabilize or even enhance c-ErbA function in its non-liganded conformation.

The v-ErbA oncoprotein: function in erythroblast transformation

Initially, v-ErbA was thought to act as a dominant negative version of c-ErbA/TR α . v-ErbA would constitutively bind to TREs normally occupied by endogenous receptors and thus block their function (Damm *et al.*, 1989; Sap *et al.*, 1989). Later findings were inconsistent with this idea. T3 and retinoids activated their respective receptors despite the presence of excess v-ErbA and coexpressed c-ErbA was even dominant over v-ErbA (Disela *et al.*, 1991; Schroeder *et al.*, 1992a; Bauer *et al.*, 1997). Furthermore, the numerous mutations present in v-ErbA seemed to grossly alter its function as compared with c-ErbA/TR α . Heterodimerization with RXR is impaired (Baretino *et al.*, 1994), and multiple amino acid changes in the DNA binding- and N-terminal domains of v-ErbA caused a strongly altered DNA-binding specificity (Judelson and Privalsky, 1996 and references therein). v-ErbA is able to function as a transactivator despite its loss of the AF-2 domain, both in animal cells (Saaticioglu *et al.*, 1993b; Bigler and Eisenman, 1994; Tomic-Canic *et al.*, 1996) and in yeast (Sande and Privalsky, 1994). All these findings favored the assumption that v-ErbA transforms via mechanisms different from those employed by c-ErbA/TR α .

In vivo, v-ErbA cooperates with tyrosine kinase oncoproteins or endogenous c-Kit to induce erythroleukemia (Frykberg *et al.*, 1983 and references therein; Casini and Graf, 1995; see below). In our primary erythroblast cell system, where TR/RXR ligand concentrations can be manipulated, the functional alterations typical for v-ErbA are not required to induce proliferation and arrest differentiation, both essential features of the leukemic phenotype. Like v-ErbA, unliganded, overexpressed c-ErbA/TR α cooperates with SCF-activated endogenous c-Kit for proliferation induction and differentiation arrest in primary progenitors. Ligand-free c-ErbA/TR α also substitutes for steroid hormone receptor function (Wessely *et al.*, 1997) in normal erythroid progenitor self-renewal, a trait only ascribed to the oncogene so far (Bauer *et al.*, 1997). All these biological functions are carried out by different versions of c-ErbA, such as the bona fide 46 kDa c-ErbA/TR α and gag-fused versions lacking or containing the nuclear localization function (gc-ErbA, g-flc-ErbA).

To act like v-ErbA, c-ErbA proteins had to be expressed at levels comparable with v-ErbA. In addition, they had to be deprived of ligands able to activate the TR α /RXR heterodimer, e.g. T3 and retinoids such as 9-*cis* RA (Chen *et al.*, 1996). This probably explains the conflicting results obtained with overexpressed gc-ErbA in an earlier study (Zenke *et al.*, 1990). Since RXR and its complex formation with c-ErbA/TR α had not been identified, the sera used in that study had not been depleted from retinoids.

What is the function of the v-ErbA mutations in the context of this model? The deletion of the AF-2 transactivation domain and the inability to bind ligand are obviously required to 'lock' the mutated receptor in its ligand-free, non-hormone-bound conformation under physiological conditions occurring in chickens. A similar function may also apply to the mutations in the DNA-binding and N-terminal domains. These may stabilize a DBD conformation typical for ligand-free c-ErbA/TR α , which favors co-repressor binding (Zamir *et al.*, 1997) as

well as interaction with specific TREs utilized in gene repression (P.Ciana, G.Braliou, F.Demay, M.von Lindern, D.Barettino, H.Beug and H.Stunnenberg, submitted). Supporting this notion, a point mutation affecting co-repressor binding and biological activity of v-ErbA (Damm *et al.*, 1987; Chen and Evans, 1995) abolished target gene repression but not transactivation when introduced into c-ErbA/TR α (Damm and Evans, 1993). The striking similarity between the proposed, dual molecular function of c-ErbA/TR α in the regulation of chromatin structure (Wolffe, 1997) and its biological activity in primary erythroblasts is strengthened by our observation that T3-dependent differentiation induction correlates with loss of c-ErbA/TR α -associated HD activity in these cells.

It should be noted, however, that not all point mutations occurring in v-ErbA may be critical for its function *in vivo*, since only some of these mutations are conserved in the similarly transforming v-ErbA protein encoded by a different, independently isolated AEV retrovirus (AEV 193; A.Bauer and H.Beug, unpublished).

A second controversial aspect of v-ErbA function is its proposed ability to cause leukemic transformation on its own. Initially, transformation by v-ErbA required the cooperation with oncogenic RTKs or downstream signal transducers (Gandrillon *et al.*, 1995; for review see Beug *et al.*, 1996). Later, v-ErbA was proposed to transform erythroblasts in the absence of exogenous RTKs (Gandrillon *et al.*, 1989; Casini and Graf, 1995). However, both c-ErbA and v-ErbA cooperate with endogenous RTKs such as c-Kit. This is an event essential for ErbA activity since both v- and c-ErbA lack any biological activity when the endogenous RTKs are inactivated by ligand withdrawal or specific kinase inhibitors (Bauer *et al.*, 1997; H.Beug, unpublished; this study). RTK-independent transformation by v-ErbA is therefore possibly due to ligands for endogenous RTKs present in the chicken sera used (Gandrillon *et al.*, 1989; H.Beug, unpublished) or to c-Kit activation occurring during erythropoiesis *in vivo* (Casini and Graf, 1995).

c-ErbA, a ligand-operated molecular switch regulating proliferation versus differentiation?

A major finding of this paper was that overexpressed, ligand-deprived c-ErbA stimulated proliferation, arrested differentiation and replaced steroid receptor function under conditions triggering differentiation of normal erythroid progenitors (SCF, Epo, insulin; Beug *et al.*, 1996). Even the presence of an exogenous, ligand-activated EpoR did not alter this function of c-ErbA. However, T3 activation of the overexpressed c-ErbA/TR α completely switched the developmental program of the cells: they were arrested in proliferation and underwent accelerated terminal differentiation under otherwise identical conditions. Thus, T3 is both necessary and sufficient to switch the cells from a proliferative to a terminal differentiation program. This suggests that c-ErbA/TR α functions as a ligand-operated molecular switch that controls the balance between sustained proliferation and terminal differentiation in primary erythroid progenitors.

Our finding that T3 activation of c-ErbA/TR α in differentiation-arrested, primary erythroblasts also reduced c-ErbA-associated histone deacetylase activity (Figure 6) makes it attractive to speculate that proliferation induction

and/or differentiation arrest caused by non-liganded c-ErbA are at least in part due to repression of target genes via activation of histone deacetylases. Accordingly, T3 activation of c-ErbA would cause activation of the same or other target genes via loss of co-repressor complexes and recruitment of co-activator complexes that acetylate histones. While this is the first demonstration of a possible involvement of histone deacetylases in proliferation/differentiation of primary cells, direct evidence for the notion that c- and v-ErbA function require histone deacetylases is lacking. However, recent reports indicate that the RAR α -PML and RAR α -PLZF fusion proteins implicated in human acute promyelocytic leukemia arrest the differentiation of myeloid progenitor cell lines because of a constitutive activation of histone deacetylases via co-repressor complexes (Grignani *et al.*, 1998; Lin *et al.*, 1998).

Target genes of v-ErbA and c-ErbA

A main deficiency in our understanding of c-ErbA/TR α and v-ErbA function is that no target genes are known which could induce the biological effects of these receptors in erythroblasts. *CA II* mRNA is completely repressed by gc-ErbA erythroblasts in the absence of T3, but strongly upregulated after T3 addition (Figure 5). This and other, more direct evidence (P.Ciana, G.Braliou, F.Demay, M.von Lindern, D.Barettino, H.Beug and H.Stunnenberg, submitted) indicate that *CA II* is a v-ErbA target gene, induced by liganded c-ErbA, and repressed by the unliganded receptor. Although regulation of *CA II* is important for leukemogenesis (Fuerstenberg *et al.*, 1992), neither *CA II* nor band 3, another putative v-ErbA target gene (Zenke *et al.*, 1990), detectably contribute to v-ErbA-induced proliferation stimulation or differentiation arrest.

Another putative target gene for v-ErbA and c-ErbA is *c-myb*. v-ErbA maintains high *c-myb* mRNA levels in primary erythroblasts, a function retained in a v-ErbA mutant that induces proliferation but only partially arrests differentiation (Bauer *et al.*, 1997). In line with this, we observed a modest downregulation of *c-myb* mRNA in gc-ErbA cells after T3-induced switching from proliferation to differentiation (Figure 5). *c-myb* is a putative target gene of the GR. Activated oncogenic forms of c-Myb can substitute for the differentiation arrest caused by the ligand-activated GR (Wessely *et al.*, 1997). Interestingly, this Myb function also requires cooperation with c-Kit (H.Beug, unpublished). However, the observed c-ErbA effects on *c-myb* transcription alone are too minor to postulate *c-myb* as a c-ErbA target gene central to its biological functions, suggesting that c-Myb expression/function may also be controlled at the translational or post-translational level (e.g. the release from an inhibitory conformation of c-Myb prevalent in most cells tested; Ness, 1996). We are currently trying to identify new c- or v-ErbA target genes, using an approach allowing identification of genes that are regulated by ErbA at the transcriptional, post-transcriptional and/or translational level (Garcia-Sanz *et al.*, 1998).

Does the TR have a role in erythropoiesis?

Evidence for a role of the TR in erythropoiesis is indirect and fragmentary. Importantly, hypothyroidism is frequently associated with certain forms of anemia or hyper-

proliferation of immature erythroid progenitors (Horton *et al.*, 1976). In these anemias, the viability of erythrocytes is not affected (Das *et al.*, 1975; Means and Dessypris, 1993 and references therein). In contrast, decreased survival of red blood cells is always associated with anemias caused by lack of Epo due to renal failure (Cotes *et al.*, 1989). Studies in mice in which the genes encoding Epo or EpoR had been disrupted by homologous recombination confirmed that Epo and its receptor are crucially important for proliferation and survival of late-stage erythroid progenitor cells (Wu *et al.*, 1995). Thus, normal survival of red blood cells in anemia associated with hypothyroidism strongly argues against the simple explanation that anemia in these patients is due to insufficient Epo levels because of lower metabolic activity (Das *et al.*, 1975). Our results rather hint at a direct involvement of unliganded TR in inducing anemia by delaying maturation of erythroid progenitors *in vivo*. This is in agreement with the observation that thyroid replacement therapy corrects the pathological events typical for these anemias (Horton *et al.*, 1976).

In mice lacking TR α , TR β or both, standard hematopoiesis does not seem to be affected (Forrest *et al.*, 1996; Fraichard *et al.*, 1997; Wikstrom *et al.*, 1998; B.Vennström and D.Forrest, personal communication). However, this does not rule out a role of the TR in erythropoiesis under special physiological conditions. This becomes clear by comparison with the GR, another major regulator of the balance between proliferation and differentiation of erythroid progenitors (Wessely *et al.*, 1997). Similar to TR $^{-/-}$ mice, GR-deficient mice show no obvious defect in standard hematopoiesis (Cole *et al.*, 1995; Reichardt *et al.*, 1998). Nevertheless, the GR is essential for sustained proliferation of mouse fetal liver erythroblasts induced by a combination of Epo, SCF and Dexamethasone, since erythroid progenitors from GR deficient mice fail to proliferate under these conditions (Reichardt *et al.*, 1998; A.Bauer, F.Tronche, G.Schütz and H.Beug, unpublished).

What function could the GR have in erythropoiesis? It was noted earlier that hypophysectomized animals develop anemia, and that these animals have particular problems responding to stresses like hypoxia by increased erythropoiesis (Lindemann *et al.*, 1969). Combination treatment with several hormones, including androgens, corticosteroids and growth hormone, largely corrected this anemia, as did administration of pituitary extracts (Means and Dessypris, 1993). This points to a role of the ligand-activated GR in stress erythropoiesis, caused by blood loss, high altitudes, heavy injury or anemia. Under these conditions, both Epo and glucocorticoids are upregulated in the organism. Stress erythropoiesis requires the cooperation between Epo and c-Kit, and occurs almost exclusively in the spleen (Broudy *et al.*, 1996). Thus, it easily could have been overlooked in the GR-deficient mice.

Together with earlier findings, the results described here raise the possibility that the non-liganded TR might be similarly involved in stress erythropoiesis. On its own, the TR would not arrest differentiation *in vivo* since it would not be exposed to ligand-free conditions in the animal. Rather, we speculate that the TR functions downstream of the GR, perhaps being stabilized in a non-liganded conformation by unknown proteins induced by

the GR. Since the liganded GR transactivates certain reporter genes via the promoter of human TR α (Laudet *et al.*, 1993), even the level of TR expression might be directly influenced by the GR. In line with these ideas are our observations that v-ErbA and highly expressed, non-liganded TR completely substitute for the GR-effect in avian erythroid cells (Bauer *et al.*, 1997; Figure 2), and that liganded GR and the non-liganded TR cause identical phenotypes in primary erythroid progenitors. We are currently trying to approach several of these hypotheses in GR-deficient and TR knockout mice.

Materials and methods

Viruses and cells

The construction of the retroviral vectors pCRA (v-*erbA*-wt-neo), gag/c-*erbA*-neo and pNEO-CEA, the latter containing the perfect Kozak consensus sequence, has been described previously (Damm *et al.*, 1987; Disela *et al.*, 1991; Bauer *et al.*, 1997). The retroviral vector encoding gag/full-length c-*erbA* (g-flc-*erbA*-neo) was constructed by exchanging a *Bam*HI-*Eco*RV fragment of gag/c-*erbA*-neo with the respective *Bam*HI-*Eco*RV fragments of a plasmid containing part of the retroviral gag region of pSFCVneo (Fuerstenberg *et al.*, 1992), fused to full-length cDNA of c-*erbA*/TR α (pAX Δ G-CEAc-wt). The replication competent helper virus (RCAS) encoding the murine Epo receptor (RCAS/EpoR; described in Quang *et al.*, 1997) was a kind gift from J.Ghysdael.

Virus-producing chicken embryo fibroblasts (CEF) expressing the above replication-deficient retroviral constructs were generated by cotransfection with RCAN helper virus DNA or, when indicated, with RCAS/EpoR helper virus DNA, as described previously (Frykberg *et al.*, 1983), followed by G418 selection. The infected, virus-producing fibroblasts were expanded in standard growth medium modified as described previously (Beug *et al.*, 1995). Primary avian erythroid progenitors (SCF progenitors and SCF/TGF α progenitors) were grown from the bone marrow of 3–10 day old SPAFAS chicks as described previously (Hayman *et al.*, 1993; Beug *et al.*, 1995; Steinlein *et al.*, 1995; Wessely *et al.*, 1997).

Infection of primary erythroblasts with retroviruses

To infect primary erythroblasts, freshly prepared chicken bone marrow cells were co-cultivated with mitomycin-C-treated CEF expressing the respective retroviral constructs for 2 days (Fuerstenberg *et al.*, 1992; Beug *et al.*, 1995). During infection, the cells were grown in modified CFU-E medium supplemented with avian SCF (100 ng/ml) and the steroids estradiol and dexamethasone (5×10^{-7} M). Unless stated otherwise, progenitors expressing c-ErbA, gc-ErbA and g-flc-ErbA were cultivated in CFU-E medium, in which both chicken and fetal calf sera were replaced by the respective stripped, T3 and retinoid-free serum batches. In addition, these media were supplemented with recombinant human IGF-1 (40 ng/ml; Sigma).

Preparation of thyroid hormone/retinoic acid depleted (stripped) serum

Two grams of anion exchange resin AG 1-X8 (Bio-Rad) and 0.8 g activated charcoal (Norit A, Serva) were mixed and washed twice with sterile distilled water. The wet mixture was added to 45 ml of serum batches to be treated and shaken for 6 h at room temperature. The resin was centrifuged and the procedure repeated with the same amount of fresh resin/charcoal mixture by incubating overnight with continuous shaking. Thereafter, the resin/charcoal mixture was removed by centrifugation for 30 min at ~ 3000 g, the supernatant cleared by filtration through fluted paper filters, sterilized by filtration through a 0.45 μ m membrane filter and stored frozen in aliquots. The efficiency of the stripping procedure was monitored by measuring the proliferation kinetics of gc-ErbA-expressing erythroblasts over 5–7 days, using tested serum batches as controls.

Proliferation kinetics

Cells infected with the various *erbA* constructs as well as control cells were propagated as mass cultures (2–10 ml cultures) in modified CFU-E medium, containing combinations of the following factors (as indicated in the text): SCF (100 ng/ml; Beug *et al.*, 1995), TGF α (2 ng/ml; Promega), the steroids estradiol (1 μ M) and dexamethasone (1 μ M), or

the steroid antagonists ICI 182,780 (5×10^{-7} M; Schroeder *et al.*, 1993) and ZK 112,993 (1.5×10^{-6} M; a kind gift from Dr E.Müllner, Vienna Biocenter; Wessely *et al.*, 1997). Cells were kept at densities of $2-4 \times 10^6$ /ml by subjecting them to daily, appropriate dilution with fresh factor-containing medium or to partial medium changes plus re-addition of fresh factors. Aliquots were counted daily in an electronic cell counter (CASY-1, Schärfe-System, FRG). Cumulative cell numbers were calculated from the cell counts plus dilution factors as described previously (Fuerstenberg *et al.*, 1992).

Growth factor assay

To titrate SCF, TGF α and T3, a modification of the assay described in Steinlein *et al.* (1995) was used. Briefly, serial dilutions of SCF, TGF α or T3 were prepared in 96-well plates. For testing the growth factors, progenitors were washed twice with phosphate-buffered saline (PBS) and 25 000 cells added to 100 μ l of factor dilutions made up in stripped modified CFU-E medium without chicken serum added. For testing T3 effects, the same number of washed cells were seeded in 100 μ l of modified differentiation medium containing the T3 dilutions (Zenke *et al.*, 1990) and supplemented with stripped FCS, stripped anemic serum (2%), SCF (100 ng/ml), recombinant human IGF-1 (40 ng/ml) and insulin (23 mU/ml). After incubation at 37°C for 2 days, thymidine incorporation was determined by labeling the cells for 3 h with 0.8 mCi [3 H]thymidine per well ([3 H]TdR, ~30 Ci/mmol; Amersham). Labeled cells were harvested onto glass fiber filters using an automated cell harvester (TOMTEC), the cell-bound radioactivity was determined in a 96-well scintillation counter (Wallac, Microbeta 1450).

Differentiation induction

Cells co-expressing gc-ErbA and murine EpoR were washed twice in serum-free medium and seeded at 2×10^6 cells/ml into 35 mm dishes containing 2 ml of modified stripped BFU-E medium supplemented with human recombinant Epo (2 U/ml), recombinant human IGF-1 (40 ng/ml) and insulin (23 mU/ml, corresponding to 1.4 nM). When indicated, SCF (100 ng/ml) and triiodothyronine (T3, 150 nM) were added. Cells were counted daily to follow proliferation kinetics and cell volume (Bauer *et al.*, 1997) and maintained at densities of $2-4 \times 10^6$ cells/ml by daily medium addition or partial medium changes plus re-addition of factors.

Analysis of differentiation by cell morphology and staining

Cells were cytocentrifuged onto slides and subsequently stained with histological dyes and neutral benzidine for hemoglobin as described previously (Bauer *et al.*, 1997). Images were taken using a CCD camera (Photometrics) and a blue filter (480 nm), resulting in dark staining of mature, hemoglobin-containing cells. Images were processed using the Adobe Photoshop software.

Photometric hemoglobin assay

Three 50 μ l aliquots of the cultures to be tested were removed and processed for photometric determination of hemoglobin as described previously (Beug *et al.*, 1995).

Northern blot analysis

Cells were harvested by centrifugation and total RNA prepared by lysis in guanidinium-isothiocyanate buffer and subsequent extraction in acidic phenol as described previously (Dolznig *et al.*, 1995). The resulting preparations were treated with 200 μ g/ml Proteinase K for 30 min at 55°C in a buffer containing 0.5% SDS, followed by an additional extraction with phenol/chloroform and re-precipitated. Ten micrograms of RNA from each sample were separated on 1% formaldehyde-agarose gels and transferred to nylon membranes (Gene Screen, DuPont-NEN). After UV-fixation, filters were sequentially hybridized with random primer 32 P-labeled probes specific for chicken *c-myb*, *CA II*, *e-ALAS*, *GATA-1*, *band 3* and 18S rRNA (Zenke *et al.*, 1990; Schroeder *et al.*, 1993). Hybridized filters were washed and exposed in phosphorimager cassettes as well as using X-ray films at -80°C using intensifying screens. Specific hybridization signals were quantified by the Image Quant PhosphorImager (Molecular Dynamics).

Immunoblot analysis

Avian erythroblasts expressing the various retroviral vectors were washed in PBS, harvested by centrifugation, lysed in RIPA buffer (Hayman *et al.*, 1993) and subjected to Western blot analysis. ErbA expression was analyzed using a rabbit antiserum to an 18 kDa polypeptide derived from the DNA binding domain of v-ErbA (Goldberg *et al.*, 1988; a kind gift from J.Ghysdael, Orsay, France) and a rabbit serum raised against full-length chicken TR α (Santa Cruz). The enhanced chemiluminescence

(ECL) detection system (Amersham) was used for visualization of the antibody-labeled proteins according to the manufacturer's instructions.

Immunoprecipitation and histone deacetylase assay

Equal numbers of cells ($60-80 \times 10^6$) were washed twice in cold PBS and once in washing buffer (10 mM Tris pH 8.1, 10% glycerol, 35 mM NaCl). Cells were then lysed in lysis buffer (washing buffer supplemented with 1% NP-40) for 20 min under gently rocking at 4°C. After high-speed centrifugation, the supernatants were directly used for immunoprecipitations. Extracts were incubated overnight with 2 μ l of 1G10 serum and 20 μ l of protein G-Sepharose bead suspension (10% v/v; Pharmacia). After three washes, the beads were resuspended in 50 μ l of lysis buffer. For histone deacetylase assays, 20 μ l aliquots of the beads containing the immunoprecipitated proteins were incubated at 30°C for 1 h with 10 μ l of [3 H]acetate-labeled chicken erythrocyte histones and the reaction stopped by the addition of 35 μ l of 1 N HCl plus 0.4 M Na-acetate. For extraction, 0.8 ml of ethyl acetate was added and the mix was vortexed for 15 s. After centrifugation at 8400 g for 5 min, a 600 μ l aliquot of the organic phase was counted in 3 ml of liquid scintillation cocktail. Histone deacetylase activity in whole-cell extracts was determined as described elsewhere (Lechner *et al.*, 1996).

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References

- Andersson, M.L. and Vennström, B. (1997) Chicken thyroid hormone receptor alpha requires the N-terminal amino acids for exclusive nuclear localization. *FEBS Lett.*, **416**, 291–296.
- Baretino, D., Vivanco, R.M. and Stunnenberg, H.G. (1994) Characterization of the ligand-dependent transactivation domain of thyroid hormone receptor. *EMBO J.*, **13**, 3039–3049.
- Bauer, A., Ulrich, E., Andersson, M., Beug, H. and von Lindern, M. (1997) Mechanism of transformation by v-ErbA: Replacement of steroid hormone receptor function in self renewal induction. *Oncogene*, **15**, 701–715.
- Beug, H., Kahn, P., Doederlein, G., Hayman, M.J. and Graf, T. (1985) In Neth, R., Gallo, R., Greaves, M. and Janka, K. (eds), *Characterization of Hematopoietic Cells Transformed In Vitro by AEV-H, A v-erbB-Containing Avian Erythroblastosis Virus*. Vol. 29. Springer Verlag, Berlin, Heidelberg, Germany, pp. 284–289.
- Beug, H., Mullner, E.W. and Hayman, M.J. (1994) Insights into erythroid differentiation obtained from studies on avian erythroblastosis virus. *Curr. Opin. Cell Biol.*, **6**, 816–824.
- Beug, H., Bartunek, P., Steinlein, P. and Hayman, M.J. (1995) In Vogt, P.K. and Verma, I.M. (eds), *Avian Hematopoietic Cell Culture: In Vitro Model Systems To Study The Oncogenic Transformation of Hematopoietic Cells*. Vol. 254. Academic Press Inc., New York and London, pp. 41–76.
- Beug, H., Bauer, A., Dolznig, H., von Lindern, M., Lobmayer, L., Mellitzer, G., Steinlein, P., Wessely, O. and Mullner, E. (1996) Avian erythropoiesis and erythroleukemia: towards understanding the role of the biomolecules involved. *Biochim. Biophys. Acta*, **1288**, M35–M47.
- Bigler, J. and Eisenman, R.N. (1994) Isolation of a thyroid hormone-responsive gene by immunoprecipitation of thyroid hormone receptor-DNA complexes. *Mol. Cell. Biol.*, **14**, 7621–7632.
- Briegleb, K., Bartunek, P., Stengl, G., Lim, K.C., Beug, H., Engel, J.D. and Zenke, M. (1996) Regulation and function of transcription factor GATA-1 during red blood cell differentiation. *Development*, **122**, 3839–3850.
- Broudy, V.C., Lin, N.L., Priestley, G.V., Nocka, K. and Wolf, N.S. (1996) Interaction of stem cell factor and its receptor c-kit mediates lodgment and acute expansion of hematopoietic cells in the murine spleen. *Blood*, **88**, 75–81.
- Casini, T. and Graf, T. (1995) Bicistronic retroviral vector reveals capacity of v-erbA to induce erythroleukemia and to co-operate with v-myb. *Oncogene*, **11**, 1019–1026.

- Chen, J.D. and Evans, R.M. (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature*, **377**, 454–457.
- Chen, H., Smit-McBride, Z., Lewis, S., Sharif, M. and Privalsky, M.L. (1993) Nuclear hormone receptors involved in neoplasia: erbA exhibits a novel DNA sequence specificity determined by amino acids outside of the zinc-finger domain. *Mol. Cell. Biol.*, **13**, 2366–2376.
- Chen, J.Y., Clifford, J., Zusi, C., Starrett, J., Tortolani, D., Ostrowski, J., Reczek, P.R., Chambon, P. and Gronemeyer, H. (1996) Two distinct actions of retinoid-receptor ligands. *Nature*, **382**, 819–822.
- Chen, H., Lin, R.J., Schiltz, R.L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M.L., Nakatani, Y. and Evans, R.M. (1997) Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell*, **90**, 569–580.
- Cole, P.J. et al. (1995) Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. *Genes Dev.*, **9**, 1608–1621.
- Cotes, P.M., Pippard, M.J., Reid, C.D., Winearls, C.G., Oliver, D.O. and Royston, J.P. (1989) Characterization of the anaemia of chronic renal failure and the mode of its correction by a preparation of human erythropoietin (r-HuEPO). An investigation of the pharmacokinetics of intravenous erythropoietin and its effects on erythrokinetics. *Q. J. Med.*, **70**, 113–137.
- Damm, K. and Evans, R.M. (1993) Identification of a domain required for oncogenic activity and transcriptional suppression by v-erbA and thyroid-hormone receptor alpha. *Proc. Natl Acad. Sci. USA*, **90**, 10668–10672.
- Damm, K., Beug, H., Graf, T. and Vennström, B. (1987) A single point mutation in erbA restores the erythroid transforming potential of a mutant avian erythroblastosis virus (AEV) defective in both *erbA* and *erbB* oncogenes. *EMBO J.*, **6**, 375–382.
- Damm, K., Thompson, C.C. and Evans, R.M. (1989) Protein encoded by v-erbA functions as a thyroid-hormone receptor antagonist. *Nature*, **339**, 593–597.
- Das, K.C., Mukherjee, M., Sarkar, T.K., Dash, R.J. and Rastogi, G.K. (1975) Erythropoiesis and erythropoietin in hypo- and hyperthyroidism. *J. Clin. Endocrinol. Metab.*, **40**, 211–220.
- Disela, C., Glineur, C., Bugge, T., Sap, J., Stengl, G., Dodgson, J., Stunnenberg, H., Beug, H. and Zenke, M. (1991) v-erbA overexpression is required to extinguish c-erbA function in erythroid cell differentiation and regulation of the erbA target gene CA II. *Genes Dev.*, **5**, 2033–2047.
- Dolznic, H., Bartunek, P., Nasmyth, K., Müllner, E. and Beug, H. (1995) Terminal differentiation of normal chicken erythroid progenitors: Shortening of G1 correlates with loss of D cyclin/CDK-4 expression and altered cell size control. *Cell Growth Differ.*, **6**, 1341–1352.
- Fondell, J.D., Ge, H. and Roeder, R.G. (1996) Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. *Proc. Natl Acad. Sci. USA*, **93**, 8329–8333.
- Forrest, D., Erway, L.C., Ng, L., Altschuler, R. and Curran, T. (1996) Thyroid hormone receptor beta is essential for development of auditory function. *Nature Genet.*, **13**, 354–357.
- Fraichard, A. et al. (1997) The T3R α gene encoding a thyroid hormone receptor is essential for post-natal development and thyroid hormone production. *EMBO J.*, **16**, 4412–4420.
- Frykberg, L., Palmieri, S., Beug, H., Graf, T., Hayman, M.J. and Vennström, B. (1983) Transforming capacities of avian erythroblastosis virus mutants deleted in the *erbA* or *erbB* oncogenes. *Cell*, **32**, 227–238.
- Fuerstenberg, S., Leitner, I., Schroeder, C., Schwarz, H., Vennström, B. and Beug, H. (1992) Transcriptional repression of band 3 and CA II in v-erbA transformed erythroblasts accounts for an important part of the leukemic phenotype. *EMBO J.*, **11**, 3355–3366.
- Gandrillon, O., Jurdic, P., Pain, B., Desbois, C., Madjar, J.J., Moscovici, M.G., Moscovici, C. and Samarut, J. (1989) Expression of the v-erbA product, an altered nuclear hormone receptor, is sufficient to transform erythrocytic cells *in vitro*. *Cell*, **58**, 115–121.
- Gandrillon, O., Ferrand, N., Michaille, J.J., Roze, L., Zile, M.H. and Samarut, J. (1994) c-erbA alpha/T3R and RARs control commitment of hematopoietic self-renewing progenitor cells to apoptosis or differentiation and are antagonized by the v-erbA oncogene. *Oncogene*, **9**, 749–758.
- Gandrillon, O., Rasclé, A. and Samarut, J. (1995) The v-erbA oncogene: a superb tool for dissecting the involvement of nuclear hormone receptors in differentiation and neoplasia. *Int. J. Oncol.*, **6**, 215–231.
- Garcia-Sanz, J.A., Mikulits, W., Livingstone, A., Lefkovits, I. and Müllner, E.W. (1998) Translational control: a general mechanism of gene regulation during T cell activation. *FASEB J.*, **12**, 299–306.
- Glineur, C., Zenke, M., Beug, H. and Ghysdael, J. (1990) Phosphorylation of the v-erbA protein is required for its function as an oncogene. *Genes Dev.*, **4**, 1663–1676.
- Goldberg, Y., Glineur, C., Gesquiere, J.C., Ricouart, A., Sap, J., Vennström, B. and Ghysdael, J. (1988) Activation of protein kinase C or cAMP-dependent protein kinase increases phosphorylation of the c-erbA-encoded thyroid hormone receptor and of the v-erbA-encoded protein. *EMBO J.*, **7**, 2425–2433.
- Graf, T. and Beug, H. (1983) Role of the v-erbA and v-erbB oncogenes of avian erythroblastosis virus in erythroid cell transformation. *Cell*, **34**, 7–9.
- Grignani, F. et al. (1998) Fusion protein of the retinoid acid receptor- α recruit histone deacetylase in promyelocytic leukemia. *Nature*, **391**, 815–818.
- Hadzic, E., Desai Yajnik, V., Helmer, E., Guo, S., Wu, S., Koudinova, N., Casanova, J., Raaka, B.M. and Samuels, H.H. (1995) A 10-amino-acid sequence in the N-terminal A/B domain of thyroid hormone receptor alpha is essential for transcriptional activation and interaction with the general transcription factor TFIIB. *Mol. Cell. Biol.*, **15**, 4507–4517.
- Hayman, M.J., Meyer, S., Martin, F., Steinlein, P. and Beug, H. (1993) Self renewal and differentiation of normal avian erythroid progenitor cells: Regulatory roles of the c-erbB/TGF α receptor and the c-kit/SCF receptor. *Cell*, **74**, 157–169.
- Heinzel, T. et al. (1997) A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature*, **387**, 43–48.
- Hörlein, A.J. et al. (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature*, **377**, 397–404.
- Horton, L., Coburn, R.J., England, J.M. and Himsworth, R.L. (1976) The haematology of hypothyroidism. *Q. J. Med.*, **45**, 101–123.
- Judelson, C. and Privalsky, M.L. (1996) DNA recognition by normal and oncogenic thyroid hormone receptors. Unexpected diversity in half-site specificity controlled by non-zinc-finger determinants. *J. Biol. Chem.*, **271**, 10800–10805.
- Kahn, P., Frykberg, L., Brady, C., Stanley, I., Beug, H., Vennström, B. and Graf, T. (1986) v-erbA cooperates with sarcoma oncogenes in leukemic cell transformation. *Cell*, **45**, 349–356.
- Kamei, Y. et al. (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell*, **85**, 403–414.
- Laudet, V., Vanacker, J.M., Adelmant, G., Begue, A. and Stehelin, D. (1993) Characterization of a functional promoter for the human thyroid hormone receptor alpha (c-erbA-1) gene. *Oncogene*, **8**, 975–982.
- Lechner, T., Lusser, A., Brosch, G., Eberharter, A., Goralik-Schramel, M. and Loidl, P. (1996) A comparative study of histone deacetylases of plant, fungal and vertebrate cells. *Biochim. Biophys. Acta*, **1296**, 181–188.
- LeDouarin, B. et al. (1995) The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. *EMBO J.*, **14**, 2020–2033.
- Lee, J.W., Ryan, F., Swaffield, J.C., Johnston, S.A. and Moore, D.D. (1995) Interaction of thyroid-hormone receptor with a conserved transcriptional mediator. *Nature*, **374**, 91–94.
- Lin, R.J., Nagy, L., Inoue, S., Shao, W., Miller, W. and Evans, R.M. (1998) Role of the histone deacetylase complex in acute promyelocytic leukaemia. *Nature*, **391**, 811–814.
- Lindemann, R., Trygstad, O. and Halvorsen, S. (1969) Pituitary control of erythropoiesis. *Scand. J. Haematol.*, **6**, 77–86.
- Mangelsdorf, D.J. and Evans, R.M. (1995) The RXR heterodimers and orphan receptors. *Cell*, **83**, 841–850.
- Mangelsdorf, D.J. et al. (1995) The nuclear receptor superfamily: the second decade. *Cell*, **83**, 835–839.
- Means, R.T.J. and Dessypris, E.N. (1993) In Bick, R.L. (ed.), *The Anemias of Chronic Disease, Renal Failure and Endocrine Disorders*. Vol. 1. Mosby-Year Book Inc., St Louis, MO, USA pp. 419–429.
- Mucenski, M.L., McLain, K., Kier, A.B., Swerdlow, S.H., Schreiner, C.M., Miller, T.A., Pietryga, D.W., Scott, W.J., Jr and Potter, S.S. (1991) A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. *Cell*, **65**, 677–689.
- Munoz, A., Zenke, M., Gehring, U., Sap, J., Beug, H. and Vennström, B. (1988) Characterization of the hormone-binding domain of the chicken c-erbA/thyroid hormone receptor protein. *EMBO J.*, **7**, 155–159.

- Nagy,L., Kao,H.Y., Chakravarti,D., Lin,R.J., Hassig,C.A., Ayer,D.E., Schreiber,S.L. and Evans,R.M. (1997) Nuclear receptor repression mediated by a complex containing SMRT, mSin3A and histone deacetylase. *Cell*, **89**, 373–380.
- Ness,S.A. (1996) The Myb oncoprotein: regulating a regulator. *Biochim. Biophys. Acta*, **1288**, F123–F139.
- Ogryzko,V.V., Schiltz,R.L., Russanova,V., Howard,B.H. and Nakatani,Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell*, **87**, 953–959.
- Pazin,M.J. and Kadonaga,J.T. (1997) What's up and down with histone deacetylation and transcription? *Cell*, **89**, 325–328.
- Perlmann,T., Umesono,K., Rangarajan,P.N., Forman,B.M. and Evans,R.M. (1996) Two distinct dimerization interfaces differentially modulate target gene specificity of nuclear hormone receptors. *Mol. Endocrinol.*, **10**, 958–966.
- Pevny,L., Simon,M.C., Robertson,E., Klein,W.H., Tsai,S.F., D'Agati,V., Orkin,S.H. and Costantini,F. (1991) Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature*, **349**, 257–260.
- Quang,C.T., Wessely,O., Pironin,M., Beug,H. and Ghysdael,J. (1997) Cooperation of Spi-1/PU.1 with an activated erythropoietin receptor inhibits apoptosis and Epo-dependent differentiation in primary erythroblasts and induces their Kit ligand-dependent proliferation. *EMBO J.*, **16**, 5639–5653.
- Rastinejad,F., Perlmann,T., Evans,R.M. and Sigler,P.B. (1995) Structural determinants of nuclear receptor assembly on DNA direct repeats. *Nature*, **375**, 203–211.
- Reichardt,H.M. *et al.* (1998) DNA binding of the glucocorticoid receptor is not essential for survival. *Cell*, **93**, 531–541.
- Saatcioglu,F., Bartunek,P., Deng,T., Zenke,M. and Karin,M. (1993a) A conserved C-terminal sequence that is deleted in v-ErbA is essential for the biological activities of c-ErbA (the thyroid hormone receptor). *Mol. Cell. Biol.*, **13**, 3675–3685.
- Saatcioglu,F., Deng,T. and Karin,M. (1993b) A novel *cis* element mediating ligand-independent activation by c-ErbA: implications for hormonal regulation. *Cell*, **75**, 1095–1105.
- Sande,S. and Privalsky,M.L. (1994) Reconstitution of thyroid hormone receptor and retinoic acid receptor function in the fission yeast *Schizosaccharomyces pombe*. *Mol. Endocrinol.*, **8**, 1455–1464.
- Sap,J., Munoz,A., Damm,K., Goldberg,Y., Ghysdael,J., Leutz,A., Beug,H. and Vennström,B. (1986) The c-erb-A protein is a high-affinity receptor for thyroid hormone. *Nature*, **324**, 635–640.
- Sap,J., Munoz,A., Schmitt,J., Stunnenberg,H. and Vennström,B. (1989) Repression of transcription mediated at a thyroid hormone response element by the v-erb-A oncogene product. *Nature*, **340**, 242–244.
- Schroeder,C., Gibson,L. and Beug,H. (1992a) The v-erbA oncogene requires cooperation with tyrosine kinases to arrest erythroid differentiation induced by ligand-activated endogenous c-erbA and retinoic acid receptor. *Oncogene*, **7**, 203–216.
- Schroeder,C., Gibson,L., Zenke,M. and Beug,H. (1992b) Modulation of normal erythroid differentiation by the endogenous thyroid hormone and retinoic acid receptors: a possible target for v-erbA oncogene action. *Oncogene*, **7**, 217–227.
- Schroeder,C., Gibson,L., Nordström,C. and Beug,H. (1993) The estrogen receptor cooperates with the TGF α receptor (c-erbB) in regulation of chicken erythroid progenitor self renewal. *EMBO J.*, **12**, 951–960.
- Steinlein,P., Deiner,E.M., Leutz,A. and Beug,H. (1994) Recombinant murine erythropoietin receptor expressed in avian progenitors mediates terminal erythroid differentiation *in vitro*. *Growth Factors*, **10**, 1–16.
- Steinlein,P., Wessely,O., Meyer,S., Deiner,E.M., Hayman,M.J. and Beug,H. (1995) Primary, self-renewing erythroid progenitors develop from BFU-E through activation of both tyrosine kinase and steroid hormone receptors. *Curr. Biol.*, **5**, 191–204.
- Tomic-Canic,M., Day,D., Samuels,H.H., Freedberg,I.M. and Blumenberg,M. (1996) Novel regulation of keratin gene expression by thyroid hormone- and retinoid receptors. *J. Biol. Chem.*, **271**, 1416–1423.
- Tsai,M.J. and O'Malley,B.W. (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem.*, **63**, 451–486.
- Vennström,B. and Bishop,J.M. (1982) Isolation and characterization of chicken DNA homologous to the two putative oncogenes of avian erythroblastosis virus. *Cell*, **28**, 135–143.
- Wagner,R.L., Apriletti,J.W., McGrath,M.E., West,B.L., Baxter,J.D. and Fletterick,R.J. (1995) A structural role for hormone in the thyroid hormone receptor. *Nature*, **378**, 690–697.
- Weinberger,C., Thompson,C., Ong,E., Gruol,D. and Evans,R. (1986) The c-erbA gene encodes a thyroid hormone receptor. *Nature*, **324**, 641–646.
- Wessely,O., Deiner,E.M., Beug,H. and von Lindern,M. (1997) The glucocorticoid receptor is a key regulator of the decision between self renewal and differentiation in erythroid progenitors. *EMBO J.*, **16**, 267–280.
- Wikstrom,L., Johansson,C., Salto,C., Barlow,C., Campos Barros,A., Baas,F., Forrest,D., Thoren,P. and Vennström,B. (1998) Abnormal heart rate and body temperature in mice lacking thyroid hormone receptor alpha1. *EMBO J.*, **17**, 455–461.
- Wolffe,A.P. (1997) Sinful repression. *Nature*, **387**, 16–17.
- Wong,J., Shi,Y.B. and Wolffe,A.P. (1997) Determinants of chromatin disruption and transcriptional regulation instigated by the thyroid hormone receptor: hormone-regulated chromatin disruption is not sufficient for transcriptional activation. *EMBO J.*, **16**, 3158–3171.
- Wu,H., Liu,X., Jaenisch,R. and Lodish,H.F. (1995) Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell*, **83**, 59–67.
- Zamir,I., Harding,H.P., Atkins,G.B., Hörlein,A., Glass,C.K., Rosenfeld,M.G. and Lazar,M.A. (1996) A nuclear hormone receptor corepressor mediates transcriptional silencing by receptors with distinct repression domains. *Mol. Cell. Biol.*, **16**, 5458–5465.
- Zamir,I., Zhang,J. and Lazar,M.A. (1997) Stoichiometric and steric principles governing repression by nuclear hormone receptors. *Genes Dev.*, **11**, 835–846.
- Zenke,M., Munoz,A., Sap,J., Vennström,B. and Beug,H. (1990) v-erbA oncogene activation entails the loss of hormone-dependent regulator activity of c-erbA. *Cell*, **61**, 1035–1049.

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