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Identifizierung von LEF-1 Zielgenen

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Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Professor Dr. R. Grosschedl betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

München, den

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Surface is an illusion, but so is depth.

David Hockney

For my parents.

Identification of LEF-1 Target Genes

Abbreviations

μM	micromolar
APS	ammoniumperoxodisulfate
BSA	bovine serum albumin
cDNA	copy DNA
cpm	counts per minute
C-terminal	carboxyterminal
dATP	desoxyadenosintriphosphate
dCTP	desoxycytosintriphosphate
DEPC	diethylpyrocarbonate
DNA	desoxyribonucleicacid
dNTP	desoxynucleosidtriphosphate
DTT	dithiothreitol
EDTA	ethylendiamintetraacetate
FCS	fetal calf serum
HEPES	N-[2-hydroxyethyl]-piperazin-N-[2-ethansulforicacid]
HMG	high mobility group
kDa	kilodalton
M	molar
mM	millimolar
mRNA	messenger RNA
N-terminal	aminoterminal
PAGE	polyacrylamide-gel electrophoresis
PBS	phoshate buffered saline
PCR	polymerase chain reaction
PEG	polyethylenglycol
PMSF	phenylmethylsulfonylfluoride
primer	starting oligo-nucleotides for the DNA polymerase
rpm	rounds per minute
SDS	sodiumdodecylsulfate
TEMED	N,N,N',N'-tetraethylethylen-diamin
TRIS	Tris-(hydroxylmethyl)-aminomethan
U	unit

1. Introduction

1.1. Cell signaling

The ability of one cell to influence the behavior of another cell is achieved through cell communication, termed cell signaling. These communication mechanisms depend heavily on extracellular signal molecules that are produced from the cells to signal to their neighbors or cells further away. Mostly the communication does not only consist of one signal, but whole networks of signaling were developed in multicellular organisms. Over the time several different signaling pathways have been developed, as for example the Notch-, the Hedgehog-, the BMP-, and the Wnt signaling pathway. At the end of each intracellular signaling pathway are target proteins, which are altered when the pathway is active and change the behavior of the cell. Moreover, a crosstalk between the different pathways occurs, what leads to a big network of signals and a tight regulation within a cell. Disturbance of the balanced systems often leads to diseases, as for example cancer. In the following I will concentrate on the Wnt signaling pathway.

1.2. Overview of the different Wnt signaling pathways

The Wnt signaling consists of three different pathways. The classical Wnt/β-catenin pathway, termed canonical Wnt pathway, the frizzled regulated planar cell polarity pathway (PCP), and the Wnt/Ca^{2+} pathway (Kuhl 2000; Wang 2003). The PCP pathway involves the small GTPases rho and cdc42 as well as the Jun-N-terminal kinase (JNK) (Weber 2000) and regulates *Drosophila* development independently of β-catenin (David 2002). The mechanism is not completely understood, but it seems that it is not a linear signaling pathway from the receptor frizzled (Fz) through a downstream cytoplasmic protein Dishevelled (Dsh), to

tissue specific proteins, but that the signaling involves asymmetric distribution of Fz and Dsh and is functioning through a feedback loop (David 2002).
In the Wnt/Ca^{2+} pathway, Fz appears to act through heterotrimeric guanine nucleotide-binding proteins (G proteins) (Slusarski 1997) and seems to activate phospholipase C (PLC) and phosphodiesterase (PDE) (Ahumada 2002), which lead to increased concentrations of free intracellular calcium and to decreased intracellular concentrations of cyclic guanosine monophosphate (cGMP).
The canonical Wnt cascade plays a critical role in many developmental processes. It has been implicated in the development of B and T cells (Okamura 1998; Reya 2000) and in the self-renewal of haematopoietic stem cells (HSC) (Reya 2003). The transcription factors LEF/TCF mediate a nuclear response to Wnt signals by interacting with β-catenin. After a Wnt signal, β-catenin is stabilized and transported to the nucleus, and is binding to the LEF/TCF proteins bnto turn on target genes. The following overview delineates the function of the canonical Wnt pathway, respectively of LEF/TCF.

1.3. The canonical Wnt Cascade

In unstimulated cells, β-catenin is in a big cytoplasmic complex together with the tumor suppressor adenomatous polyposes coli (APC), the constitutively active kinase Glycogen synthase kinase 3β (GSK-3β), and Axin. In this complex, β-catenin is captured and subjected to phosphorylation by GSK-3β at four N-terminal serine and threonine residues (Ikeda 1998). The phosphorylation of β-catenin is recognized by different proteins like Slimb/TrcP and getting conveyed to ubiquitin conjugating enzymes, which mark β-catenin for degradation (Jiang 1998; Marikawa 1998). β-catenin is rapidly degraded via the ubiquitin-proteasome pathway (Aberle 1997). The Wnt family members are ligands of the Frizzled (Fz) family of serpentine receptors (Bhanot 1996). Wnt proteins comprise a large family of so far 19 identified family members that have been found in round worms, insects, and vertebrates (Sidow 1992). Wnt proteins are secreted glycoproteins

that have been shown to be associated with the cell surface or extracellular matrix of secreting cells, making a local activity through its biochemical properties most likely (Bradley 1990; Papkoff 1990). They are involved in a number of developmental and physiologic processes. The Low-density lipoprotein receptor-related proteins (LRP) can bind together with Fz to the Wnt proteins, thus activating the Wnt cascade (Pinson 2000; Tamai 2000). As a consequence of Wnt signal, GSK-3β is inhibited by the cytoplasmic protein Dishevelled (Dsh) (Noordermeer 1994; Kishida 1999; Smalley 1999; Itoh 2000), preventing the phosphorylation of β-catenin and its degradation. The so stabilized β-catenin is transferred into the nucleus where it can interact with the nuclear mediators of Wnt signaling, the LEF/TCF proteins and turn on Wnt target genes by interaction with the mediators (Hsu 1998). Another interaction that was shown to occur in the nucleus is the binding of β-catenin to the BCL9/ Pygopus (Pyg) complex what might help to stabilize β-catenin.

In the absence of a Wnt signal, LEF/TCF proteins cannot activate target genes of the Wnt pathway, moreover they can interact with Groucho, a co-repressor and actively repress the transcription of genes (Figure1).

1.4. LEF/TCF protein family

The first members of the LEF/TCF family to be identified were T cell factor 1 (*Tcf1*) (Oosterwegel 1991; van de Wetering 1991) and Lymphoid enhancer factor 1 (*Lef1*) (Travis 1991; Carlsson 1993). Proteins of the LEF/TCF family share an 80-amino-acid high mobility group (HMG) box. It was shown that the HMG box can bind to DNA as a monomer in a sequence specific manner (Giese 1991; Travis 1991).

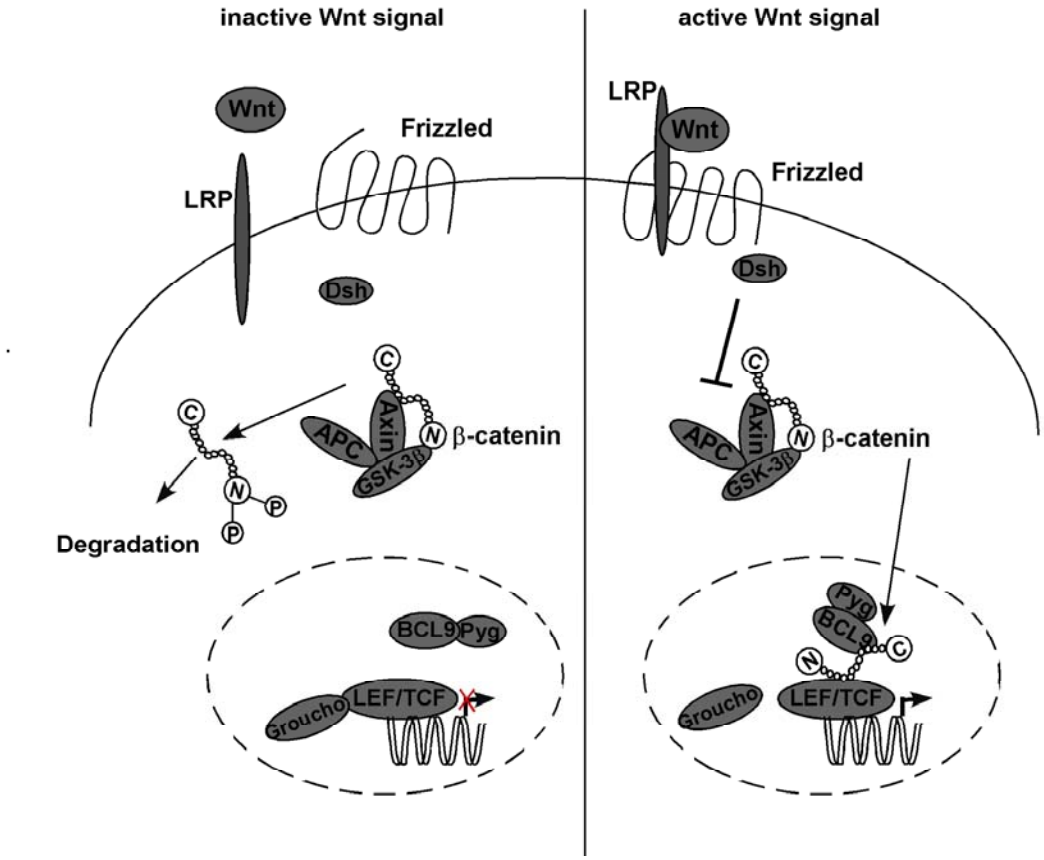


Figure 1. Schematic view of the canonical Wnt pathway.

Other features of LEF-1 are the β-catenin binding domain (βBD), through which the interaction with β-catenin is achieved, and the context dependent activation domain (CAD) that can interact for example with the Ally of AML-1 and LEF-1 (ALY), an ubiquitously expressed nuclear protein that was shown to be necessary for the T cell receptor α (TCRα) enhancer function (Bruhn 1997) (Figure2).

The LEF/TCF family members are expressed in a great variety of tissues such as immature T and B cells of adult mice and in the neural crest, mesencephalon, tooth germs, whisker follicles, and other sites during embryogenesis. It was shown that LEF-1 has an architectural function and can interact with different proteins what results in either activation or repression of target genes. For the activating effect, the LEF/TCF family members mostly interact with β-catenin to turn on Wnt target genes what makes them for this regulation a member of the Wnt signaling pathway. In a distinct number of cases LEF-1 can also positively regulate target genes without the help of β-catenin, thus acting independently of the Wnt pathway, as it was shown for example for the regulation of TCRα by LEF-1 (Travis 1991).

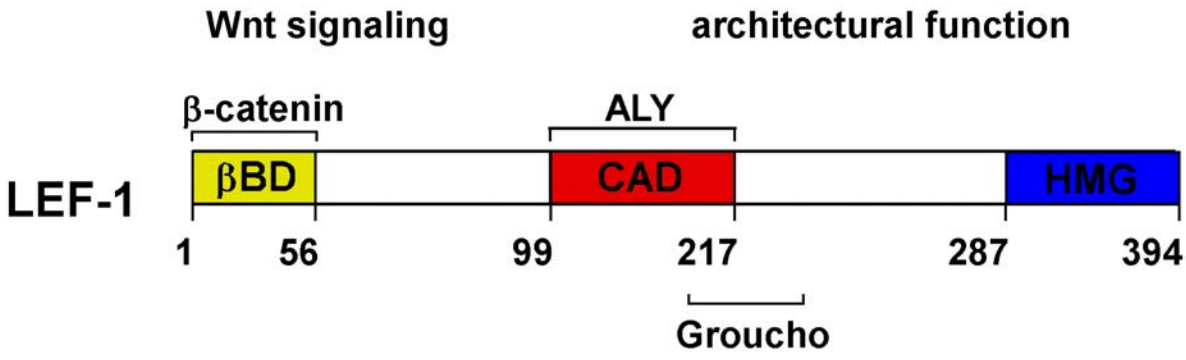


Figure 2: Schematic diagram of LEF-1.
The most important elements of LEF-1 are indicated. The β-catenin interaction domain (βBD), the context dependent activation domain (CAD), and the high mobility group (HMG). ALY can bind to LEF-1 via the CAD domain, β-catenin, a coactivator of LEF-1 interacts with the βBD domain. Groucho, a corepressor binds to part of the CAD domain.

But LEF/TCF proteins can also actively repress transcription. This was first observed with experiments in *Drosophila* and *Xenopus*, showing that in the absence of a Wnt/Wg signal the repression of Ultrabithorax (Ubx) and Siamois is released by mutating the LEF/TCF consensus sites in their transcriptional control elements (Brannon 1997; Riese 1997; Bienz 1998). There are some co-repressors known to directly interact with LEF/TCF proteins that help to repress target genes. One of them is Groucho that also interacts with several other DNA-binding proteins such as Hairy, Engrailed, and Dorsal (Cavallo 1998; Fisher 1998; Levanon 1998; Roose 1998). Groucho binds to part of the CAD domain of LEF-1, making it possible that its binding can occur at the same time as β-catenin binding leading to a repressive effect in the context of a Wnt signal. Repression through LEF-1 and β-catenin interaction was shown for E-cadherin without the help of any co-repressors (Jamora 2003). Nevertheless it seems to be more likely, that the main mechanism for repression is mediated without the help of β-catenin. The contribution of β-catenin to the downregulation might be sometimes necessary for the induction of Groucho or other co-repressors, but then β-catenin is not conducive to the repressive effect itself as it was shown recently in the analysis of They could provide evidence, that Groucho is localized to the same areas as β-catenin before the formation of the bud. At later stages of bud maturation, the expression pattern of Groucho and β-catenin are not overlapping any more. In this

stage Groucho is expressed only in areas where no β-catenin is expressed and vice versa.
Taking together, the reports about the mechanism of repression through LEF-1 are contradictionary, and no main pathway was discovered yet. Thus, for the repressive effect of LEF/TCF proteins there are still a lot of questions to be answered.

1.5. Haematopoiesis

All of the mature blood cells in the body are generated from a relatively small number of haematopoietic stem cells (HSCs) and progenitors (Weissman 2000). In the mouse, a single HSC can reconstitute the entire haematopoietic system for the natural lifespan of the animal (Osawa 1996). HSCs generate the multiple haematopoietic lineages through a series of intermediate progenitors. Those are the common lymphoid progenitors (CLPs) that give rise to natural killer cells (NK), T cells, and B cells, and the common myeloid progenitors (CMPs), which can generate monocytes, granulocytes, megacaryothytes, and erythrocytes (Kondo 1997; Akashi 2000). Out of the CMPs develop more specialized progenitors, that are further restricted to a number and type of cell lineages that they can generate. These are the granulocyte/monocyte progenitors (GMP), which give raise to the granulocytes and monocytes, and the megacarythrocyte/erythrocyte progenitors (MEP), which can develop to megacarythrocytes and erythrocytes (Akashi 2000). Further downstream of the CLPs, the NK cells and the pro T and pro B cells develop. Terminally differentiated cells are produced that cannot divide any longer and undergo apoptosis after days to decades depending on their cell type (Figure 3)

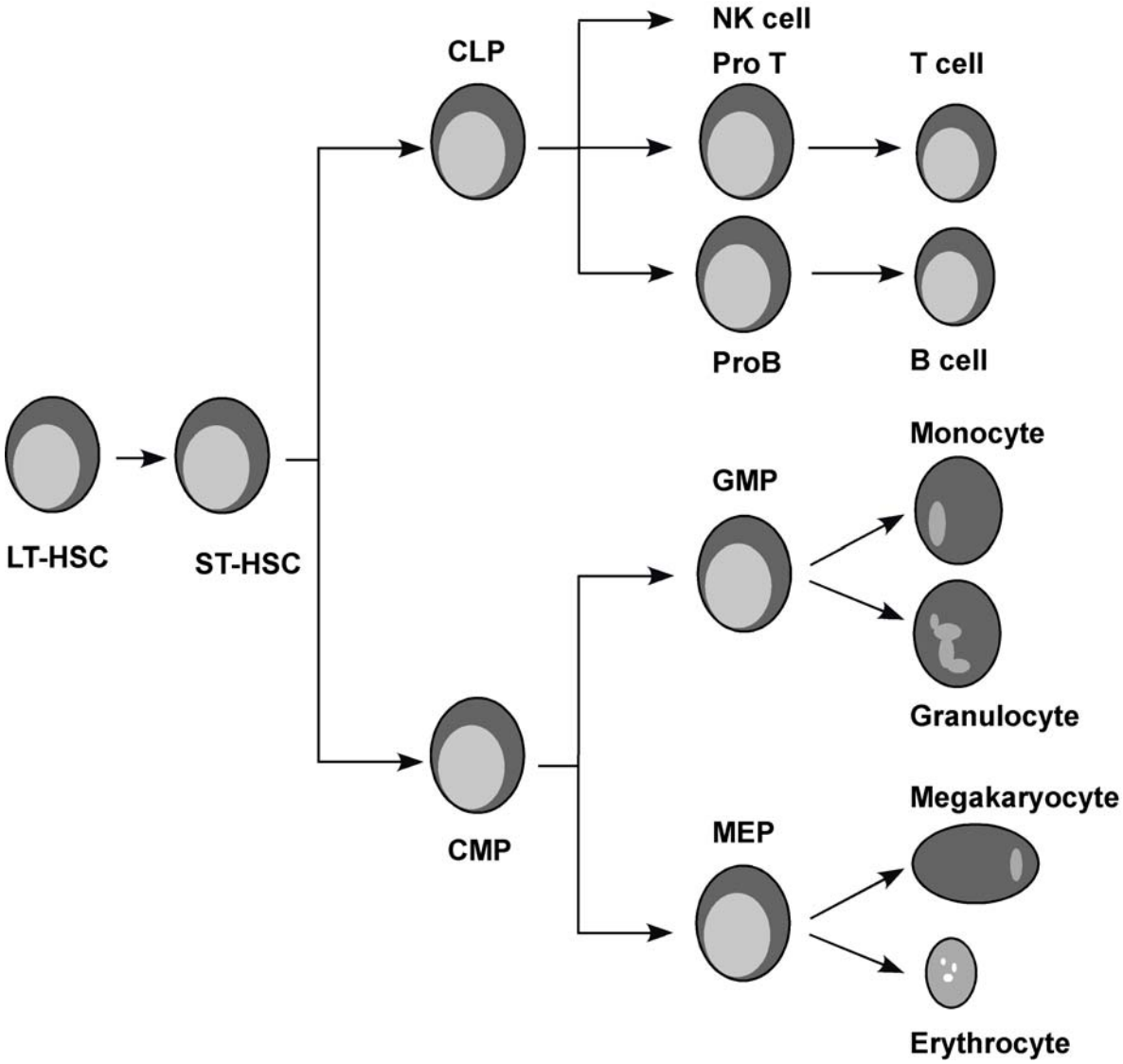


Figure 3. Haematopoiesis.
Long term haematopoietic stem cells (LT-HSC) give rise to short term (ST) HSCs. Due to different stimuli they either become common lymphoid progenitors (CLP) or common myeloid progenitors (CMP). Downstream of CLPs the cells either develop to natural killer cells (NK), to B or T cells. The CMPs give rise to more specialized progenitors, granulocyte/monocyte progenitors (GMP) and megakaryocyte/erythrocyte progenitors (MEP). Those finally differentiate to granulocytes, monocytes, megakaryocytes, and erythrocytes.

1.6. Role of the Wnt pathway in haematopoietic stem cells

Haematopoietic stem cells (HSCs) are pluripotent cells with the capacity to produce cells of all blood lineages over the whole life span. For that, the cells need a balance between their plasticity that is the production of progenitor cells, which generate specific blood lineages, and their own self-renewal. Until today not much is known about the mechanisms underlying both processes. It was shown that Wnt5a and Wnt10b are expressed in the murine yolk sac and other microenvironments of haematopoietic stem cells, such as the microenvironment of the fetal liver (Austin 1997). Furthermore Wnt5a is also expressed in fetal liver stromal cells. These findings together with the fact that Wnt expression of HSCs stimulates their proliferation (Austin 1997; Van den Berg 1998), suggested that the Wnt signaling is involved either in the cell fate decision or in the process of self-renewal. Recently more evidence could be provided, showing that the Wnt signaling is most likely involved in the self-renewal process of the HSCs (Reya 2003). HSC cells were sorted using cells of H2K-BCL-2 transgenic mice, to reduce the effects of pro-differentiation stimuli necessary for the infection prior to the experiment readout. The sorted HSC cells were infected with a retrovirus carrying either β-catenin-IRES-GFP or IRES-GFP alone for control, and then again sorted, this time for GFP expression. It was shown that almost twice as many cells expressing β-catenin are in the active cell cycle than cells infected with the control. The complete withdrawal of growth factors showed, that Wnt signaling increases the long-term growth, as the β-catenin expressing cells could still proliferate for more than four weeks, whereas the control cells showed only minimal growth for less than two weeks. At the same time the cells were not starting to upregulate lineage specific markers, showing that the majority of the cells retained the phenotype of HSCs. To control if the cells were still able to give rise to the different lymphoid lineages, retrovirally transduced cells were transplanted into lethally irradiated mice in limiting numbers and the mice were analyzed after 11 weeks. Clear reconstitution of the myeloid, T and B lineages could be achieved using the cells infected with a β-catenin expressing virus, whereas the control cells failed to reconstitute all lineages. They also tested if LEF/TCF proteins are activated in

HSC cells, by infection of the cells with destabilized GFP driven by the LEF/TCF reporter (TOP-dGFP) or by the mutated form of the reporter (FOP-dGFP) as a control. The infected HSC cells were transplanted into lethally irradiated mice and the bone marrow was studied for GFP expression 14 days post transplantation. The bone marrow transplanted with β-catenin expressing cells showed a large number of GFP positive cells derived from the donor whereas host cells and transplanted control cells were only low in GFP expression. This showed that HSCs in vivo normally signal through LEF/TCF elements. The requirement of an intact Wnt signal for HSCs was tested with two different setups. First the soluble form of the frizzled cysteine-rich domain (CRD) that inhibits binding of Wnt proteins to the frizzled receptor was added as an IgG fusion (CRD-IgG) to wildtype HSCs in culture or IgG alone as a control. The CRD domain inhibited growth by four fold whereas the control did not change the proliferation, indicating that indeed the Wnt signaling is necessary for proliferation. As a second evidence for this, the independent inhibitor axin was ectopically expressed in HSCs. Axin increases β-catenin degradation and leads to a reduced Wnt signal. Cells infected with an axin expressing retrovirus could be shown to have a seven fold reduced growth potential, and a decreased cell survival potential compared to control cells. In addition to those findings, genes known to be involved in self-renewal as HoxB4 and Notch1 were tested for regulation after infection of HSCs with β-catenin. HoxB4 and Notch1 were found to be upregulated in HSCs overexpressing β–catenin compared to control cells. Those findings indicate strongly that a Wnt signal is needed for cell survival and proliferation of HSCs, but it does not give signals for differentiation.

1.7. Role of the Wnt signaling in B cell development

The B cell differentiation is characterized by the rearrangement of the immunoglobin genes and by the expression of different molecules in the cell and on its surface. The first B cells develop in the fetal liver, whereas after birth the

process is taking place in the bone marrow. The differentiation is a tightly regulated process that is strongly depending on the expression of certain genes. The earliest B cell precursors are derived from pluripotent stem cells. Those cells express AA4.1, B220, and CD43 at their surface and belong to the fraction A of pre-B cells, also called preBI cells (Hardy 1991; Rolink 1993; Li 1996). They go on in differentiation to fraction B cells, so called pro-B cells, which express B220, CD43, and the heat stable antigen (HAS) on the surface. In this phase the rearrangement of the immunoglobin D-J_H occurs. In the next stage (fraction C), cells express BP-1 on their surface and V_H-DJ_H gene recombination takes place. Cells of fraction B and C express the immunoglobulin surrogate light chain genes, λ5 and VpreB. After rearrangement of the light chain gene, the heavy and light chains are expressed on the surface together with the signal transduction elements Igα (mb-1) and Igβ (B-29), forming together the pre-B cell complex (pre BCR). Now the cell is in the pre-B cell stage where the last H elements have been rearranged, and the L chains are starting to get rearranged what results in the expression of IgM on the surface. As soon as the cells express IgM, they are called immature B cell. Those cells migrate now to the periphery, express IgD and become fully capable of responding to antigens (mature B cells). Many genes are involved in the process of B cell development, whereby the exact function of them has been subject to intensive studies. LEF-1 is known to be expressed in transformed pre-B cell lines but not in mature B cell lines (Travis 1991). The other family members of the LEF/TCF family are not found to be expressed in any stage of the B cell development. Only little is known about the influence of Wnt signaling on B cell development. The first evidence that Wnt might play a critical role came from the finding, that some leukemic B cell lines overexpress a novel Wnt protein, Wnt 16 (McWhirter 1999). The exact expression pattern of the Wnt proteins and LEF-1 in B cells and the role of Wnt signaling and LEF-1 in the development remained unclear. Recently the effects of LEF-1 on B cell development were subject to an intensive analysis (Reya 2000). First the precise pattern of LEF-1 in developing B cells was studied. It could be shown by visualizing the lacZ gene that was inserted into one allele of the *Lef1* locus by homologous recombination (Galceran 2000), that LEF-1 is expressed during early B cell development in the

fetal liver and adult bone marrow. The upregulation occurs in fraction B pro-B cells and LEF-1 can also be detected in fraction C cells. There is no LEF-1 expression found in IgM-positive B cells from the adult spleen or adult bone marrow. To test for a correlation between the expression pattern and the function, fetal liver of *Lef1*$^{-/-}$ embryos and perinatal bone marrow was analyzed, as an analysis of older mice is not possible due to the early death of *Lef1*$^{-/-}$ mice. The number of B220^{+} cells was reduced by more than two fold and was even more obvious after excluding the dying and dead cells. To specify the stage of the cells, B220^{+} positive cells were tested for other surface markers and it could be shown, that the majority of the B220^{+} cells were also CD43^{+}, placing them in the pro-B cell compartment. To test if LEF-1 deficiency results in a differentiation defect, they tested bone marrow of mice at postnatal day 13 (P13) for the ratio of IgM^{-} to IgM^{+} B lymphocytes. Although the total number of the cells was reduced as shown before, the ratio remained still the same and there were no defects in rearrangement of the immunoglobulin heavy and light chains occurring. Those findings were also confirmed with adoptive transfer experiments where the mutant B cells behaved like wildtype cells in a wildtype environment. They went on to analyze if the reduced number of B220^{+} cells is due to reduced cell survival. With TUNNEL assay and Annexin V staining it could be shown that indeed the B220^{+} cells of Lef1$^{-/-}$ mice die at an up to 20-fold higher frequency. As a cause for the reduced survival, Reya and coworkers were analyzing the expression level of several genes known to be involved in apoptosis. Whereas the levels of Bcl-2, Bcl-x, and p53 remained unchanged in sorted pro-B cells (fraction B) of *Lef1*$^{-/-}$ mice compared to wildtype, the expression of Fas and c-myc was elevated. A second defect that can contribute to the reduced size of the B cell compartment is the diminished proliferation of the B cells. With a thymidine incorporation assay it could be shown, that in addition to the increased apoptosis the proliferation is decreased, arguing that LEF-1 has an important function for the proliferation of B cells. As LEF-1 and β-catenin together are members of the Wnt signaling pathway, they went on testing the responsiveness of B cells to Wnt stimuli. Whereas Wnt10B, Wnt3A, and Wnt5A were found to be expressed in bone marrow, only Wnt5A was expressed in the stromal cells of the bone marrow, indicating that the

other family members are produced by the haematopoietic cells themselves. They could show, that proliferation of wildtype pro-B cells is increased after LiCl stimulation, and that the soluble Wnt3A could stabilize β-catenin in the cells. Furthermore comparing the responsiveness of wildtype and *Lef1*$^{-/-}$ cells to Wnt3A stimulation revealed the LEF-1 dependence. Only a small proportion of cells deficient for LEF-1 started to proliferate after Wnt3A addition whereas the majority of the wildtype cells were found to start dividing. Those findings taken together strongly indicate an essential role of Wnt signaling and LEF-1 expression for B cell development.

1.8. Role of Wnt signaling in T cell development

Differentiation of T cells in the thymus is a well-defined process that can be characterized by the expression of specific surface markers. The T cell precursors that migrate to the thymus express almost no surface markers typical for T cells, only low levels of CD4 (Wu 1991). When the precursors start to differentiate, they reach first a stage termed double negative (DN) stage, expressing neither CD4 nor CD8. This stage can be divided into four distinct differentiation stages, defined by the surface markers CD44 and CD25, starting up as $CD44^+CD25^-$, followed by $CD44^+CD25^+$. In the third phase they downregulate CD44 again ($CD44^-CD25^+$). During this step the *Rag1* and *Rag2* genes are upregulated and the T cell receptor (TCR) β, δ, and γ chains are rearranged (Godfrey 1994). Only if they succeed in rearranging the β chain they can go to the next step, downregulating CD25 again ($CD44^-CD25^-$) and can associate with the pre-TCRα (pTα), generating a pre-T cell receptor complex (Saint-Ruf 1994). The formation of the pTα complex is essential for the generation of α/β T cells (Mombaerts 1992). If the cells fail to rearrange the β chain and build the pTα complex but successfully rearrange the γ and δ chain, they can develop to γ/δ T cells. The differentiation of α/β cells continues with the upregulation of CD8, generating immature single positive (ISP) cells, followed by the upregulation of CD4 to double positive (DP) $CD4^+CD8^+$ cells. This stage is

accompanied by the second upregulation of *Rag1* and *Rag2* and rearrangement of the TCRα locus. The majority of the cells in this differentiation stage fails to rearrange the TCRα locus successfully and over 90% of the cells undergo apoptosis. Cells that succeeded in rearranging, downregulate either the CD4 or the CD8 to get mature single positive (SP) $CD4^+CD8^-$ or $CD4^-CD8^+$ cells (Figure 4).

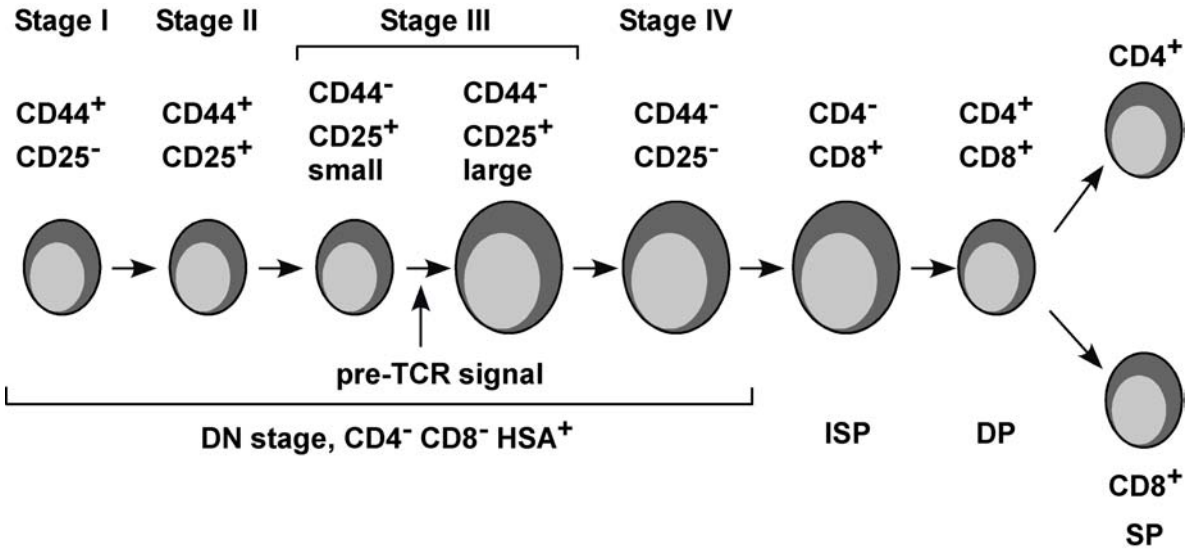


Figure 4. Schematic overview of T cell development.

To investigate the role of Wnt signaling in T cell development, several different approaches have been conducted. Many different knockout mice were created with adjactant rescue and overexpression studies.

One approach to study effects of the LEF/TCF family members and the connected Wnt pathway on T cell development was to investigate their role by gene knockout experiments in mice. As the first gene of the LEF/TCF family members, LEF-1 was targeted with a knockout construct, leading to mice carrying a homozygous germ-line mutation in the *Lef1* gene that eliminates its protein expression and causes postnatal lethality (van Genderen 1994). For TCF-1 two different knockout mice have been made, targeting either exon V (*Tcf1(V)*$^{-/-}$) or exon VII (*Tcf1(VII)*$^{-/-}$) of the *Tcf1* gene (Verbeek 1995). The *Tcf1(V)*$^{-/-}$ knockout allows the production of a truncated but still functional TCF-1 protein, whereas the *Tcf1(VII)*$^{-/-}$ knockout is seen as real knockout, where the production of a functional TCF-1 protein is

completely abolished. Studies of the T cell development in all three knockouts revealed, that there are only marginal defects visible due to the redundancy of LEF-1 and TCF-1 (Okamura 1998). Whereas in *Lef1*$^{-/-}$ knockout mice the T cell development is comparable with the one in wildtype mice, a very weak block of T cell development is visible in the two *Tcf1*$^{-/-}$ knockout mice. But only the crossing of the *Lef1*$^{-/-}$ and *Tcf1(V)*$^{-/-}$ knockout resulted in an almost complete block of the T cell development after the DN stage due to the redundancy of LEF-1 and TCF-1 (Figure 5).

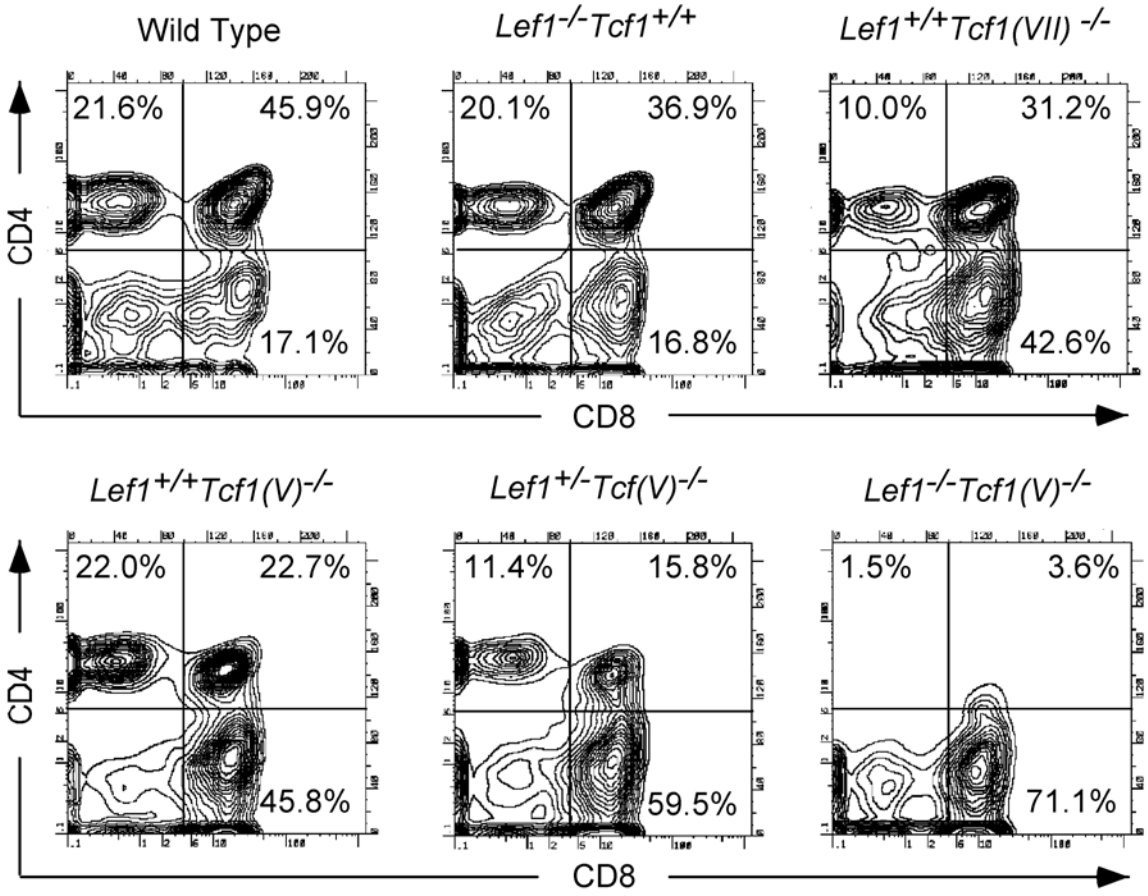


Figure 5 adopted from Okamura 1998. Defects in T cell development in *Lef1*$^{-/-}$/*Tcf1*$^{-/-}$ knockout mice.
Flow cytometry analysis of E17.5 fetal thymic organ cultures, after seven days of culture. A strong block after the ISP CD8^{+} stage can be detected for *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ double null cells, whereas only minor defects are seen in single knockout cells.

A more precise analysis of the T cells in the DN stage with fetal thymic organ cultures revealed, that the double null mice have a 3-fold increase of this

proportion of cells. Anti-CD3ε antibody was used to test for a defect in pre-TCR signaling, but the treatment of fetal organic cultures revealed that even cells of the double null mice were able to develop to double positive $CD4^+CD8^+$ cells after treatment, indicating that the pre T cell receptor is not abrogated in double-deficient animals. To test if the developmental defect is derived by the stroma or by the haematopoietic cells themselves, adoptive transfer experiment into SCID (severe combined immunodeficiency) mice were carried out. Those revealed that cells derived from double null mice were not capable to develop into the different lineages and the T cell development was also blocked compared to normal development of wildtype cells. Those findings indicated, that the haematopoietic cells themselves and not the stroma cells are due to the defects. To get an idea why the block in T cell development occurs, Okamura and coworkers were testing whether the TCRα levels or the V(D)J rearrangement are changed. The fact that the V(D)J recombination of other genes like TCRγ is detectable in double null mice, but not the one of TCRα, suggested that the expression levels of TCRα had to be changed. PCR reaction for TCRα out of sorted ISP $CD8^+$ cells revealed, that indeed no TCRα was detectable in double null cells, leading to an explanation for the defects observed.

Another knockout mouse created, was targeting the Wnt3a protein (Takada 1994). Interestingly, the phenotypic abnormalities seen in the *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ double knockout resemble most of the changes detected in the Wnt3a targeted mutant mice, indicating that most of the defects are due to the absence of an active Wnt signaling.

Overexpression of the extracellular Wnt binding domain of the Fz receptor in FTOC cultures using retroviral constructs producing the soluble form of the extracellular inhibitor of Wnt signaling was another approach studied (Staal 2001). It could be shown, that addition of Fz inhibited the development of T cells and resulted in an almost complete block of early thymocyte development, similar to the one seen in *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ mice.

In a subsequent study the *Tcf1(VII)*$^{-/-}$ knockout was subjected to a rescue experiment (Ioannidis 2001). Two different isoforms of *Tcf1* were tested for rescuing, a full length *Tcf1*, including also the β-catenin interaction domain, and a

shorter isoform, missing the β-catenin interaction domain. Only the full length *Tcf1* could overcome the defects in T cell development, whereas the shorter form failed to rescue the knockout. This strongly indicates that the defects examined in the different LEF/TCF family member knockouts are due to the disruption of the Wnt signaling pathway.

Another knockout that was created is the double knockout of *Wnt1*$^{-/-}$*Wnt4*$^{-/-}$ (Mulroy 2002). The phenotype of this knockout is very mild compared to the one of *Lef1*$^{-/-}$ *Tcf1(V)*$^{-/-}$. This is probably due to the redundancy of the Wnt proteins. It was shown, that at least three other family members of the Wnt proteins that could compensate for the two targeted ones are expressed in thymocytes.

The studies discussed so far give proof that the proliferation of T cells is connected to the Wnt signaling, but it could not be shown yet, that Wnt signaling is also essential for the differentiation of T cells. Boehmer and coworkers (Gounari 2001) have been given the first hint, that this might be the case. They were examining the effects of overexpression of a shortened, stabilized form of β-catenin in a heterozygous floxed transgenic mouse (β-cat$^{Thy\Delta ex3}$). They could show that expression of this shortened form of β-catenin in the thymus lowered on one hand the number of thymocytes and altered their contribution of the different stages, on the other hand DP $CD4^+CD8^+$ cells were produced that were lacking the expression of intracellular TCR-β chains. These findings indicated that a large proportion of the thymocytes with stabilized β-catenin proceeded to the DP stage in the absence of pre-TCR and TCRαβ signaling. They were also testing the effects of the stabilized β-catenin in RAG-2$^{-/-}$ mice that normally fail to develop B and T cells as a result of their lack of TCR chains. Thymocytes of RAG-2$^{-/-}$ mice crossed to the β-cat$^{Thy\Delta ex3}$ mice were able to overcome the block at the DN3 stage and could develop to DP and even SP cells. Those findings suggest that the effects of the constitutive active β-catenin have to occur either in parallel and most likely independent of the pre-TCR signaling, as development is achieved in the absence of TCR chains.

Furthermore could be shown, that in β-cat$^{Thy\Delta ex3}$ more cells of the DN3 stage were in cell cycle as compared to wildtype mice. But the proportion of cells in cell cycle

in the DN4 stage was higher in wildtype mice than in β-cat$^{Thy\Delta ex3}$. It seems that the β-catenin stabilization induced a greater initial activation of DN3 cells followed by diminished cycling. This and the finding that thymocytes of β-cat$^{Thy\Delta ex3}$ mice undergo increased apoptosis can count for the reduced cellularity found in β-cat$^{Thy\Delta ex3}$ compared to wildtype mice.

The studies outlined give strong indications that Wnt signaling and the LEF/TCF proteins play an essential role in T cell development. A Wnt signal is probably necessary for two events. In the first place is the Wnt stimulus essential for the proliferation of T cells, secondly helps a Wnt signal T cells to differentiate.

1.9. Phenotype of the Lef1$^{-/-}$ knockout

As described before, there was a knockout mouse generated, carrying a homozygous germ-line mutation in the LEF-1 gene (van Genderen 1994). The mice displayed a severe phenotype in the B and T cells, characterized in the previous paragraphs. Furthermore could defects also be detected in other tissues. The most obvious phenotype of the LEF-1 deficient mice was the lack of body hair and whiskers. The normal onset of hair follicle development is between E13 and E14. The development starts with the formation of small focal epidermal thickenings, so called placodes, in association with small dense aggregates of mesenchyme, called dermal papilla (Sengel 1976). The epidermal placodes grow into the underlying dermis and are getting characteristics of mature follicles by E18. In the LEF-1 deficient mice the onset of hair follicle development started normal, but only in reduced number, and was blocked around E17, resulting in no mature follicles. The skin of the mutant mice also lacked melanin and dermal fat. Moreover the deficient mice showed a pointed snout and were smaller in size compared to heterozygous and wildtype mice. Besides, no mammary glands could be detected in *Lef1*$^{-/-}$ mice. It could be shown that the development of mammary glands started in reduced number, and a developmental block occurred before they could mature. TMN (trigerminal nerves) neurons are located in the midbrain

and are derived from the neural crest (Narayanan 1978). Because a high concentration of LEF-1 was detected in this area, in situ analysis on TMNs was performed. In deficient mice the TMN was absent, indicating that LEF-1 plays also an essential role in the development of TMN.

Taking the effects of LEF-1 together, it becomes clear, that LEF-1 is an essential gene, and mutations can lead to severe defects and even death.

2. Aim of the study

The function of LEF-1 has been already subject of intensive studies as described before. Although the LEF/TCF family members have been characterized extensively, only little is known about events and genes downstream of LEF-1. Until now just few target genes of LEF-1 and its family members were identified and characterized. So far, most of the identified target genes are positively regulated by LEF-1 through its interaction with β-catenin. But it was shown before, that LEF-1 can also activate genes without the help of β-catenin (Travis 1991) and can also have repressive effects on them. In order to understand the function of LEF-1 and the defects following a deregulation it is important to identify further downstream target genes.

Our interest is to identify LEF-1 target genes and to gain a deeper insight in the regulatory processes mediated through LEF-1. For that purpose we will use different approaches.

In our first project, the main goal is to identify Wnt dependent LEF-1 target genes by controlled overexpression of a fusion of LEF-1 to the C-terminal part of β-catenin. This is a constitutively active form of LEF-1 (CatCLEF) that we introduced in NIH3T3 cells. This method allows us to search predominantly for primary target genes as we are inducing CatCLEF for only 8 hours and to identify β-catenin dependent and independent target genes.

With the second and third approach we want to explore LEF-1 dependent target genes in *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ double knockout mice and in *Lef1*$^{m5/m5}$ knockout mice. The *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ double null mice have a developmental block of T cells at the ISP CD8^{+} stage. We will compare a very well defined population of T cells, ISP CD8^{+} cells, from wildtype and *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ mice. This stage in T cell development was chosen as it is the last stage that occurs in both mice lines, in the wildtype and in the *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ mice. This finding argues that important regulatory, LEF-1 dependent processes are going on in this specific cell population, which we hope to uncover. Another advantage of this approach is, that

we can minimize the unspecific effects that are due to different contributions of the cell compartments as the cells were sorted before the analysis.

In a third attempt we want to take a closer look at the differences between Wnt dependent and Wnt independent LEF-1 target genes. The published data imply, that mainly the repressive function of LEF-1 is Wnt independent whereas the majority of activating events acts through the Wnt pathway. In our lab a knockout mouse line was generated, carrying a mutation in the β-catenin interaction domain of LEF-1, leading to a mutant LEF-1 (LEF-$1^{m5/m5}$) protein that is expressed at normal levels but cannot interact with β-catenin any more (W. Roth, unpublished data). With *in situ* analysis a clear difference could be detected in skin between the *Lef1*$^{-/-}$ and *Lef1*$^{m5/m5}$ mutant mice, indicating that in this tissue regulatory processes are going on that depend on LEF-1 but not on β-catenin. To analyze the target genes depending only on LEF-1 and those that need the interaction between LEF-1 and β-catenin, we will compare whole skin of E16.5 embryos from wildtype, *Lef1*$^{-/-}$, and *Lef1*$^{m5/m5}$ mice.

Those three approaches will provide us with a better understanding of the complex regulatory processes in which LEF-1 is involved.

3. Materials and methods

3.1. Common buffers

0.5 M EDTA, pH 8.0 (ethylenediamine tetraacetic acid)	Dissolve 186.1 g $Na_2EDTA{\cdot}2H_2O$ in 700 ml H_2O Adjust pH to 8.0 with 10 M NaOH (~50 ml) Add H_2O to 1 liter
50 x TAE (Tris/acetate/EDTA) electrophoresis buffer	242 g Tris base 57.1 ml glacial acetic acid 37.2 g $Na_2EDTA{\cdot}2H_2O$ H_2O to 1 liter
10 x TBE (Tris/borate/EDTA) electrophoresis buffer	108 g Tris base (890 mM) 55 g boric acid (890 mM) 40 ml 0.5 M EDTA, pH 8.0
TE (Tris/EDTA) buffer	10 mM Tris–Cl, pH 8.0 1 mM EDTA, pH 8.0

3.2. Cell lines

293T (DuBridge et al., 1987)	Adenovirus 5-transformed human embryonic kidney cell line
NIH 3T3 (Jainchill er al., 1991)	Fibroblastic cell line from mouse embryo
EL4 (Gorer, 1950)	Murine T cell lymphoma
Jurkat (Gillis et al., 1980)	Human acute T cell leukemia lymphoma

Hut78 (O'Conell et al., 1995)	Human cutaneous T cell lymphoma
J558 L (Gehring et al., 1969)	Murine plasmacytoma cell line
HeLa (Gey et al., 1952)	Aneuploid, human epithelial cell line originating from a cervical carcinoma
10T1/2 (Reznikoff et al., 1973)	Murine mesenchymal stem cell line
Neuro-2a (Olmsted et al., 1970)	Neuronal and amoeboid stem cells

3.3. Cell culture

3.3.1. Culture conditions

All suspension cells were cultured in filter-capped culture flasks with RPMI 1620 media supplemented with 10% heat-inactivated fetal calf serum (FCS) and 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.3 μg/ml L-glutamine (referred to as RPMI complete) at 37°C in a 5% CO_2-gassed atmosphere.
Adherent cells were grown on culture tissue plates in DMEM, 1000 mg/l glucose media supplemented with 10% heat-inactivated FCS and 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.3 μg/ml L-glutamine (referred to as DMEM complete) at 37°C in a 5% CO_2-gassed atmosphere.

3.3.2. Freezing, thawing, and storage of the cells

The cells were frozen in their relevant media containing 10% Dimethylsulfoxide (DMSO) to prevent crystal formation. Cooling the cells to -80°C and lower temperatures had to occur slowly. Therefore they were stored in an isopropanol containing box at -80°C, resulting in a temperature drop of one degree per minute. For long term storage the cells were stored in liquid nitrogen. Thawing of cells was

done by submerging the frozen vial in a 37°C water bath and resuspending the cell pellet in pre-warmed media before transferring the cells to culture plates or flasks.

3.4. Isolation of peripheral blood monolayer cells (PBMC)

PBMCs were isolated from human whole blood. Between 100 and 200 ml of donor blood were taken and anticoagulant-treated with Heparin. The blood was diluted 1:2 with Dulbecco's phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}. 12.5 ml of Ficoll-Paque™ Plus (Amersham) were added to a 50 ml Falcon tube. 25 ml of the diluted blood were carefully layered on the Ficoll-Paque. The samples were centrifuged at 400 x g for 30 minutes at room temperature without brake. The lymphocyte layer was transferred to a clean 50 ml Falcon tube. Three volumes of PBS were added and cells were resuspended by gently drawing them in and out of a Pasteur pipette. They were centrifuged at 100 x g for 10 minutes at room temperature with brake. The supernatant was removed and the lymphocytes were resuspended in 6 ml PBS, followed by another centrifugation step at 100 x g for 10 minutes with brake. The supernatant was removed again and the lymphocytes were resuspended in RPMI complete complemented with non essential amino acids (Gibco) and additional 2 mM glutamine and left over night in a culture plate to allow macrophages to attach. The following day the lymphocytes were counted and plated onto new plates. For stimulation Phytohemaglutamin-P (PHA-P) was added to a final concentration of 5 μg/ml.

3.5. Transfections

3.5.1. Adherent cells

Adherent cells were transfected using calcium phosphate.
Cells were trypsinised and counted. Between 1.75 x 10^5 (for NIH 3T3) and 3.25 x 10^5 (for 293T) cells were plated per 6 cm plate, containing 5 ml of media. The cells were allowed to settle for 4 to 12 hours before addition of the transfection mix. Therefore the appropriate DNA's and 10 μg salmon sperm DNA as carrier were mixed together for each sample. 250 μl of a 250 mM $CaCl_2$ solution were added and samples were mixed. Slowly 250 μl of either 2 x HBS pH 7.05 (280 mM NaCl; 10 mM KCl; 1.5 mM Na_2HPO_4; 12 mM dextrose; 50 mM HEPES) or 2 x BES pH 7.1 (50 mM N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid; 280 mM NaCl; 1.5 mM Na_2HPO_4) were added drop by drop and the mixture was incubated for 30 minutes at room temperature. The solution was added to the prepared plates and incubated between 8 and 20 hours depending on the cell type. Cells with low transfection efficiency were incubated using 2 x BES and the incubation was done at least over night in an incubator with 3% CO_2. Following the incubation the cells were once washed with media, then 5 ml of fresh media were added. 36 to 48 hours after the addition of the transfection mix the cells were harvested.

3.5.2. Non-adherent cells

Non adherent cells, mostly lymphoid cells, were transfected using electroporation. Per transfection 5 x 10^6 cells were diluted either into 500 μl medium for easier transfectable cells as Jurkat cells or into 250 μl medium for harder transfectable cells as Hut78 or primary cells. DNA was added to the cell suspension and the whole mixture was transferred into a Bio-Rad Gene Pulser Cuvette (0.4 cm

electrode). The cell suspension was shocked with 250V and 975F. Folowing the electro shock the cells were immediately transferred into a Falcon tube containing 5ml of media and incubated one to two hours at 37°C. After this period of recreation the cells were centrifuged and resuspended in 10 ml fresh media. After 36 to 48 hours the cells were harvested and analyzed.

3.6. Luciferase assay

Transfected cells were harvested after 36 to 48 hours and washed once with PBS without Ca^{2+} and Mg^{2+}. The cell pellets were resuspended in 100 - 200 µl of 1 x Reporter Lysis buffer (proprietary formulation of bicine buffer and Tween® detergents, Promega) or 1 x Passive Lysis buffer (Promega) for Dual Luciferase measurements. The cell suspensions were frozen in ethanol/dry ice and thawed at room temperature. The luciferase reagent (Luciferase Assay Substrate, Promega) and also the second component needed for Dual Luciferase measurements, Stop & Glo® Reagent (Promega) were thawed at room temperature and 20 µl of the lysates were measured in the “Luminat LB 9507”.
The values were normalized for transfection efficiency either using β-galactosidase, chloramphenicol acetyltransferase (CAT), or Renilla Luciferase measured in parallel with the firefly Luciferase.

3.7. β-gal assay

The co-transfected β-galactosidase was measured using chlorphenolred- β-D galactopyranosid (CPRG). Therefore 20 µl of the cell lysates were mixed with 180 µl of a mixture of 10 ml Z buffer (60 mM Na_2HPO_4; 40 mM NaH_2PO_4; 10 mM KCl; 10 mM $MgSO_4$), 100 µl 50 mM CPRG, and 10 µl β-mercaptoethanol. The release

of chlorphenolred by the β–galactosidase was monitored in a 96-well plate using the Spectra Max 250 of Molecular Devices®.

3.8. CAT assay

The chloramphenicol acetyltransferase (CAT) assay was carried out with ^{14}C labeled acetyl coenzym A (acetyl CoA). 20 µl of the CAT reaction mixture

Reagent	**Volume**
^{14}C acetyl CoA	3 µl
20 mM acetyl CoA	0.1 µl
0.25 M Tris-Cl, pH 7.4	5 µl
8 mM Chloramphenicol	10 µl
H_2O	1.9 µl
Final Volume	**20 µl**

were added to 30 µl of transfection lysates. The reaction was incubated for 4 hours at 37°C, then 210 µl of ethylacetate were added and the aqueous phase extracted by mixing. The samples were centrifuged at room temperature at maximum speed for 4 minutes. 180 µl of the upper phase were transferred into 3 ml of scintillation fluid and counted for ^{14}C activity.

3.9. Flow cytometry

3.9.1. FACS sorting

Cells were transfected with the appropriate constructs to look for endogenous gene regulations. GFP was either co-expressed from a bicistronic vector or co-transfected with the other plasmids to detect which cells were transfected. 24 to 36 hours after transfection, the cells were harvested and resuspended in cell dissociation solution (SIGMA). GFP positive cells were sorted either with a BD Facs sorter Advantage using the Cell Quest software or with a Cytomation MoFlo sorter using the Summit software.

3.9.2. FACS analysis

To identify different cell populations the cells were stained with antibodies conjugated to different dyes specific for the surface markers of interest. The colors used were fluorescein isothiocyanat (FITC), phycoerythrin (PE), allophycecyanin (APC), and propidium iodide to exclude dead cells. First the cells were harvested as described before, then washed twice with FACS buffer (3% FCS; 0.1% Sodium Azide; in PBS), and finally resuspended in an appropriate volume of this buffer. The cells were counted and $1x10^7$ cells per sample were transferred to small FACS tubes. For blocking of unspecific binding the cells were incubated on ice for 15 minutes in a 1:200 dilution of F_cR (αCD16/CD32) block in FACS buffer to target the F_c receptors on lymphoid cells, which in non-blocked state unspecifically bind the antibodies employed for FACS analysis. Following the cells were incubated with the desired antibody in FACS buffer for 30 minutes on ice. Next the cells were washed three times with FACS buffer and finally they were resuspended in 250 μl of FACS buffer and ready for the analysis.

3.10. RNA preparation

Total RNA was isolated form adherent or non-adherent cells. For adherent cells, the media of the culture plate was aspirated off, the plates were washed with PBS without Ca^{2+} and Mg^{2+} and 1 ml of TRIZOL (Invitrogen) was added. The TRIZOL/cell mixture was transferred to an Eppendorf tube. Non-adherent cells were centrifuged, the supernatant was aspirated off, and the cell pellet was washed with PBS without Ca^{2+} and Mg^{2+}. TRIZOL was added and the cells were transferred to an Eppendorf tube. The tubes were frozen at -80°C for storage or for further preparation of the RNA. After thawing the TRIZOL/cell mixture the genomic DNA was destroyed by shearing it through a syringe ten times. 200 µl of chloroform were added and vortexed for mixing. After 5 minutes at room temperature phase separation was achieved by centrifugation for 20 minutes at 4°C at maximal speed. The upper phase was transferred to a new tube, avoiding touching the interphase. 500 µl of isopropanol were added followed by a 10 minutes incubation phase at room temperature. Then the samples were centrifuged at maximal speed for 10 minutes at 4°C. The supernatant was aspirated off and the pellet was washed with 70% ethanol. Finally the pellet was resuspended in an adequate volume of
diethylpyrocarbonate **(**DEPC) treated water.

3.11. Reverse transcription

RNA was transcribed to cDNA serving as a template for PCR reactions. Therefore the following reaction was set up:

Reagent	Volume
Total RNA (5μg)	x μl
H_2O	y μl
Oligo d(t15) primer	2 μl
Final volume	10.6 μl

The samples were heated up to 70°C for 10 minutes, then kept on ice for two minutes and supplemented with:

Reagents	Volume
First strand buffer	4 μl
Dithiothreitol (DTT)	2 μl
dNTP (25 mM each)	0.4 μl
RNAsin (Sigma)	1 μl
Superscript II, Reverse Transcriptase (Invitrogen 200U/μ)	2 μl
Final Volume	20 μl

As negative control, one reaction was prepared without Superscript II. The reactions were incubated at 37°C for one hour. The volume was adjusted to 40 μl with water and 0.5 μl were used for one PCR reaction.

3.12. Northern blot hybridization

3.12.1. Formaldehyde agarose gel electrophoresis

For visualization of RNA in agarose gels, ethidium bromide was added directly to the samples to avoid high background fluorescence. A volume corresponding to 3

μg poly-A^+ RNA or 15 μg total RNA was withdrawn and DEPC treated H_2O was added to 20 μl final volume. Then 20 μl of a formaldehyde/formamide mix were added to the RNA:

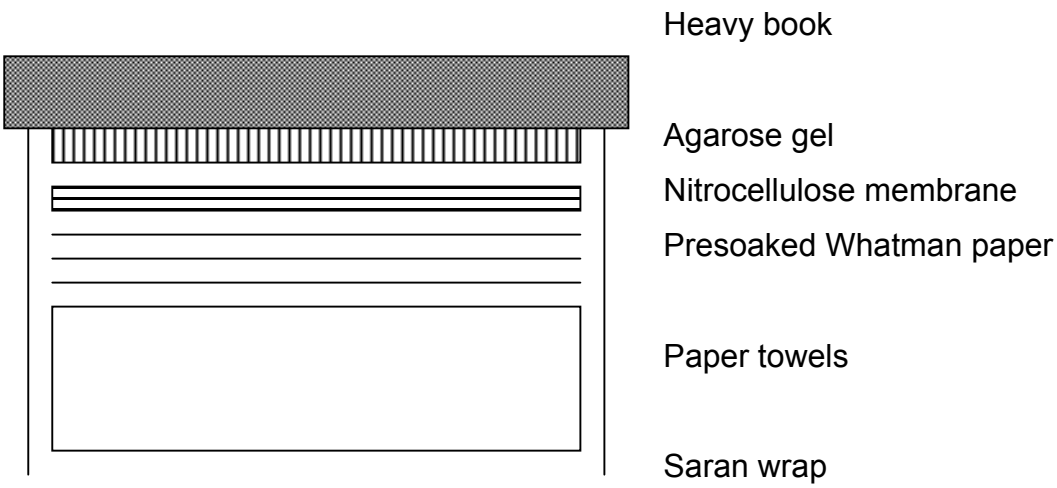
Reagents	**Volume**
Formamide	100 μl
Formaldehyde	20 μl
10x MOPS (0.2 M MOPS, pH 7.0; 5 mM EDTA, pH 8.0; 40 mM Sodium Acetate, pH 7.0)	20 μl
Fthidium bromide (10 mg/ml)	1 μl
H_2O	9 μl
Final Volume	**150 μl**

The samples were incubated at 65°C for 10 minutes, then cooled on ice for 10 minutes. The samples were separated on a 1 x MOPS gel containing 1% agarose and the gel was run at 140 V. The RNA was visualized and the gel photographed with a gel documentation system (IS-100 from Alpha Innotech Corporation). The location of the 28S (corresponding to 4718 nucleotides) and 18S (corresponding to 1874 nucleotides) rRNA molecules were marked in the gel with a Pasteur pipette.

3.12.2. Transfer of RNA to nitrocellulose membrane

3 pieces of Whatman 3MM paper and one piece of Hybond-N^+ nitrocellulose membrane (Amersham) were cut in the size of the gel. The Whatman papers and the gel were presoaked in 20 x NaCl/sodium citrate (SSC) (3 M NaCl; 0.3 M sodium citrate; adjusted to pH 7.0). A stack of paper towels was arranged as a sponge on the bench, on top three Whatman papers, the membrane, and last the gel topside facing up, were added. A saran wrap enclosed the whole transfer to

avoid evaporation of the buffer. On top of the stack a heavy book was placed for compression.



The RNA was transferred to the nitrocellulose membrane over night. To immobilize the RNA, the membrane was UV-crosslinked in a transilluminator (Spectronics Corporation)

3.12.3. Preparation of the probe

The probes were prepared with the Rediprime II random labeling system kit (Amersham), which takes advantage of random sequence hexanucleotides to prime DNA synthesis at numerous sites along the denatured template DNA. 25 ng of linearized template in 45 µl of TE-buffer were denatured by boiling for 5 minutes at 100°C. The solution was cooled on ice, collected by centrifugation, and added to the Rediprime reaction tube. 5 µl of Redivue [α-^{32}P]-dCTP (3000 mCi) were added to the reaction mix and incubated for 30 minutes at 37°C. 50 µl H_2O was added to increase the volume. To remove free nucleotides, the probe mixture was loaded on a pre-spun (1100 x g for 2 minutes) Quick Spin Column (Roche) and centrifuged at 1100 x g for 4 minutes to collect the purified probe in a fresh

Eppendorf tube. 1 μl of the probe was used for counting the activity in a scintillation counter.

3.12.4. Hybridization

The nitrocellulose membrane was placed in a hybridization tube with the RNA-side facing the center of the tube. For hybridization a mix without formamide was used (50 mM Na_xPO_4, pH 7.0; 1% (w/v) SDS; 1 x Denhardt's; 5 x SSC; 1 mg/ml yeast t-RNA). The membrane was first pre-hybridized for 1-2 hours in 20 ml of the hybridization mix at 68°C. The probe was added to the tube in a volume corresponding to an activity of 750000 cpm/ml. The hybridization was carried out at 68°C over night.

The next morning the hybridization mix was poured off and the nitrocellulose membrane was washed in the tube once with 5 x SSC for 10 minutes at 68°C, twice with 2 x SSC; 0.5% (w/v) SDS for 15 minutes at 68°C.

The wetted nitrocellulose membrane was wrapped in foil and autoradiography films (Kodak) were exposed to it in a cassette at -80°C.

For reprobing, the membrane was stripped by boiling in hot 40 mM Tris-Cl, pH 7.5; 1% (w/v) SDS) for 10 minutes.

3.13. Affymetrix chip

3.13.1. Preparation and labeling of the RNA

3.13.1.1. Standard procedure

Total RNA was prepared as described previously. For a standard procedure between 30 and 50 μg of total RNA were used as starting material. RNA was first transcribed to single stranded (ss) cDNA.

Reagents	Volume
Total RNA (5 – 50 μg)	x μl
DEPC- treated H_2O	y μl
T_7-$(dT)_{24}$ primer (100 pmol/μl)	1 μl
Final volume	**11 μl**

The reactions were incubated at 70°C for 10 minutes. Then 5 x First strand buffer (250 mM Tris-Cl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$), 0.1 M DTT, and 10 mM dNTP mix were added.

Reagents	Volume
5x First strand buffer	4 μl
DTT (0.1 M)	2 μl
dNTP mix (10 mM)	1 μl
Final Volume	**18 μl**

The samples were heated to 37°C for two minutes. Finally the reverse transcriptase Superscript II was added.

Reagents	Volume
Superscript II (200 U/μl)	2 μl
Final Volume	**20 μl**

The reaction was incubated at 42°C for one hour. The First Strand reaction was placed on ice and the reagents listed below in the Second Strand final reaction composition table were added.

Reagents	Volume
DEPC treated H_2O	91 µl
5 x Second strand buffer (Invitrogen)	30 µl
DNA Polymerase I (Invitrogen, 40 U/µl)	4 µl
dNTP mix (10 mM each)	3 µl
DNA ligase (Invitrogen, 10 U/µl)	1 µl
RNAse H (Invitrogen, 2 U/µl)	1 µl
Final Volume	**150 µl**

The samples were incubated at 16c for two hours. The reaction were purified by performing one phenol/chloroform and one chloroform extraction, followed by an ethanol precipitation. Finally the pellet was washed with 80% ethanol and air dried before resuspending in 24 µl DEPC treated H_2O.

The last reaction step was an in vitro transcription of the ds cDNA into RNA, thereby using biotinylated dNTP's to incorporate biotin into the produced RNA. For this purpose the labeling kit RNA Transcript Labeling Kit supplied by Enzo BioArray was used.

Reagents	Volume
Template ds DNA	24 µl
10 x HY reaction buffer	4 µl
10 x Biotin Labeled Ribonucleotides	4 µl
10 x DTT	4 µl
10 x RNAse Inhibitor mix	4µl
20 x T7 RNA Polymerase	2 µl
Total Volume	**40 µl**

The tube was immediately incubated for 4 hours at 37v, mixing the contents every 30 - 45 minutes. The reaction was cleaned by using the RNeasy Mini Kit supplied by Quiagen. The amount of labeled RNA was quantitated by spectrophotometry.

3.13.1.2. Preparation for low RNA amounts

When the starting material was limited, the RNA had to be amplified. Therefore the procedure was accomplished as described, preparing ds cDNA. Then the in vitro transcription (IVT) was carried out with normal not biotinylated nucleotides, using the Ambion™ T_7 Megascript Kit.

Reagents	**Volume**
DEPC treated H_2O	y µl
dATP	4 µl
dCTP	4 µl
dGTP	4 µl
dUTTP	4 µl
10 x buffer (Ambion®, T_7 Megascript)	4 µl
Enzyme Mix	4 µl
DNA	x µl
Total Volume	**40 µl**

The IVT was followed by a second and third round of ds cDNA amplification. The third IVT was carried out using the Enzo™ Kit with its biotinylated nucleotides as described before.

3.13.2. Fragmentation of the RNA

16 µg of labeled RNA were fragmented to obtain pieces mainly between 100 and 500 basepairs. This was achieved by adding a 5 x Fragmentation buffer (200 mM Tris-acetate, pH 8.1; 500 mM potassiumacetate, 150 mM magnesiumacetate) to a final concentration of 1 x and incubation of the sample at 94°C for 35 minutes.

3.13.3. Hybridization of the microarray

The components for the hybridization were added to the fragmented RNA.

Reagents	Volume
RNA	30 μl
Control oligo B2 (oligo for the antisense probe array)	5 μl
20 x Eukaryotic Mix (eukaryotic control oligos for hybridization quality check)	15 μl
Hering sperm DNA	3 μl
Acetylated Bovine Serum Albumin (BSA)	3 μl
2 x Hybridization buffer (200 mM MES; 2 M $[Na^+]$; 40 mM EDTA; 0.01% Tween 20)	150 μl
H_2O	94 μl
Final volume	**300 μl**

The contents were heated at 95°C for 5 minutes and then centrifuged at maximal speed at room temperature for 5 minutes. The Affymetrix Chip was calibrated by filling it with 1 x MES buffer (0.1 M MES; 0.07 M $[Na^+]$) through one of the septa and incubated at 45°C for 10 minutes under rotation. Then the buffer solution was removed and the array was filled with the clarified hybridization cocktail avoiding any insoluble material at the bottom of the tube. Finally the probe array was placed in a rotisserie box in a 45°C oven and hybridized for 16 hours.

3.13.4. Washing, Staining, and Scanning of the microarrays

After 16 hours of hybridization, the hybridization cocktail was removed from the probe array and set aside in a microcentrifuge tube. The probe array was

completely filled with non-stringent wash buffer (0.9 M NaCl; 6 mM EDTA; 0.01% Tween 20; 0.005% Antifoam), called buffer A. The Affymetrix Fluidics Station was prepared and the protocol for the chip chosen. The Streptavidin Phycoerythrin (SAPE) staining solution was prepared:

Reagents	**Volume**
2 x Stain buffer (200 mM MES; 2 M [Na^+]; 0.05% Tween 20; 0.005% Antifoam)	600 µl
Acetylated BSA (50 mg/ml)	48 µl
Streptavidin Phycoerythrin (SAPE) (1 mg/ml)	12 µl
H_2O	540 µl
Final Volume	**600 µl**

At the same time the antibody solution was prepared.

Reagents	**Volume**
2 x Stain buffer	300 µl
Acetylated BSA (50 mg/ml)	24 µl
Normal goat IgG (10 mg/ml)	6.0 µl
Biotinylated antibody (0.5 mg/ml)	3.6 µl
H_2O	266,4 µl
Final Volume	**600 µl**

The microarray was inserted into the Fluidics Station and the program started that controls the staining and washing procedure independently. The procedure comprised several washings with buffer A and the more stringent buffer B (100 mM MES; 0.1 M [Na^+]; 0.01% Tween 20). Next, the chip was incubated with the SAPE solution for several minutes. The probe array was washed again followed by the addition of the antibody solution. Before adding the SAPE solution for the

second time, the array was washed the same way. Following the final washing cycle, the chip was put for scanning.

3.14. Preparation of total protein extracts

Plates of adherent cells were washed twice with PBS without Ca^{2+} and Mg^{2+} and kept on ice during further preparation steps. The cells were lysed in an appropriate volume of RIPA buffer (10mM Na_xPO_4, pH 7.2; 150 mM NaCl; 1% (v/v) Triton X-100; 1% (w/v) Sodium Deoxycholate; 0.1% (w/v) SDS; 1 mM DTT) with 1 x protease inhibitor mix (pim) (5 μg/ml Soybean Trypsin/Chymotrypsin inhibitor; 5 μg/ml Antipain; 5 μg/ml Aprotinin; 5 μg/ml Leupeptin; 0.5 μg/ml Pepstatin A; 5 μg/ml Bestain in 20 mM Tris-Cl, pH 7.9), and 1 mM phenylmethylsulfonyl fluoride (PMSF) added just before harvesting. After 5 minutes of incubation the cells were scraped off with a rubber policeman and transferred to a new Eppendorf tube.
Suspension cells were shaken from the walls of the culture flasks and the culture suspension was transferred to a Falcon tube. The cells were spun down, washed twice in PBS without Ca^{2+} and Mg^{2+}, and transferred to Eppendorf tubes, which were kept on ice during subsequent preparation. Following a short centrifugation step, the supernatant was aspirated off and the pellet was resuspended in 5 times the volume of the pellet of RIPA buffer including 1 x pim and 1 mM PMSF. If necessary the sample was sonicated to destroy the genomic DNA. After centrifugation for 2 minutes at maximal speed at 4°C, the supernatant containing the total protein extract was transferred to a new Eppendorf tube and the concentration of protein was determined by Bradford assay. The total protein extract was stored at –80°C.

3.15. Western blot analysis

3.15.1. Gel electrophoresis and transfer to nitrocellulose filter

A protein gel was made up of a stacking gel (3.9% acrylamide; 0.104% bisacrylamide; 0.125 M Tris-Cl, pH 6.8; 0.1% (w/v) SDS; 0.15% (w/v) ammonium persulfate; 0.2% TEMED) and a separating gel with the appropriate acrylamide/bisacrylamide content. For analysis of LEF-1, a protein running at approximately 60 kDa, a 10% acrylamide gel was used (10% acrylamide; 0.24% bisacrylamide; 0.375 M Tris-Cl, pH 8.8; 0.1% (w/v) ammonium persulfate; 0.13% TEMED).

The protein samples were filled up with H_2O to 40 µl and 10 µl of sample buffer (100 mM Tris-Cl, pH 6.8; 200 mM DTT; 4% SDS; 2% bromphenolblue; 20% glycerol) were added. The samples were boiled at 95°C for 4 minutes and centrifuged shortly to bring contents down to the bottom. Then they were loaded on the gel together with a broad range prestained protein marker (BioRad). The gel was run in 1 x Tris-glycine buffer (1.25 M glycine, 125 mM Tris-Cl; 0.5% (w/v) SDS) at 20 mA through the stacking gel and at 40 mA through the separating gel.

The transfer of separated proteins from the gel to a piece of nitrocellulose transfer membrane (Schleicher and Schuell) was carefully set up under transfer buffer (20% methanol in 1x Tris-glycine buffer) to avoid air pockets. The transfer was run in a cold room at 60 V for two hours or at 20 V overnight.

3.15.2. Immunodetection

To block non-specific binding, the nitrocellulose filter was incubated in 5% (w/v) non- fat dry milk in 1 x PBS-T (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.4 mM KH_2PO_4, pH 7.4 with 0.1% Tween 20) or in 5% (w/v) BSA in 1 x PBS-T for one hour at room temperature or overnight in the cold room on a rocking plate. The blot

was washed once for 15 minutes and twice for 10 minutes in PBS-T. A primary antibody recognizing the protein of interest was diluted in PBS-T to the optimized concentration. The blot was incubated in the antibody solution for one hour at room temperature with agitation and washed once for 15 minutes and twice for 10 minutes in PBS-T. A horseradish peroxides (HRP)-labeled secondary antibody specific for the utilized primary antibody was diluted in PBS-T and the blot was incubated in the solution for one hour at room temperature with agitation. For immunoblotting the following antibody concentrations were used:

Primary antibody	**Dilution**	**Sec. antibody**	**Dilution**
Rabbit αmouse LEF-1	1:4000	αrabbit-HRP	1:10000
Rabbit αmouse SATB1	1:2500	αrabbit-HRP	1:10000
Goat αmouse LaminB	1:1000	αgoat-HRP	1:3000

The nitrocellulose filter was washed once for 15 minutes and twice for 10 minutes in PBS-T and the liquid was drained off on a piece of Kim Wipes.

Immunodetection was performed with ECL Western blotting analysis system (Amersham). 3 ml of reagent 1 and 3 ml of reagent 2 were added to the protein side of the membrane and incubated for one minute at room temperature. The filter was wicked dry on a piece of Kim Wipes and it was carefully wrapped in Saran Wrap carefully avoiding air bubbles. Auto radiography film were exposed to the membrane for appropriate time periods.

If the membrane was to be reprobed, primary and secondary antibodies were stripped off by incubating it in stripping buffer (100 mM β-mercaptoethanol; 2% (w/v) SDS; 62.5 mM Tris-Cl, pH 6.7) at 65°C for 30 minutes. The membrane was washed twice in PBS-T for 10 minutes and blocked for one hour at room temperature in 5% non-fat dry milk before performing immunodetection.

3.16. Bacteria cultures

Escherichia coli (E. coli) strains were grown up by culturing them in liquid Luria Bertani (LB) media (1% tryptone; 0.5% yeast extract; 1% NaCl) at 37°C with agitation or plating them out on LB plates (LB media with 1.5% agar), which where incubated at 37°C.

For storage, bacteria cultures were quick-frozen in liquid nitrogen and stored at -80°C in LB media containing 25% glycerol. To recover the bacteria, the top of the frozen sample was scraped with an inoculation loop and the bacteria were plated onto a media plate, which was incubated overnight at 37°C.

3.17. Mini preps

To check if a cloning step worked, small amounts of DNA were prepared, so called Mini preps. DNA was prepared using the boil-lysis method. 1.5 ml of an over night culture were centrifuged at full speed for 5 minutes. The supernatant was discarded and the pellet resuspended in 200 µl of STET buffer (0.08% (w/v) sucrose; 0.05% (v/v) Triton X-100; 5 mM Tris-Cl, pH 8.0; 5 mM EDTA, pH 8.0) and 10 µl Lysozym/Rnase mix (Lysozym 10 mg/ml; RNase 1 mg/ml; 5 mM EDTA, pH 8.0). The samples were boiled at 95°C for 10 minutes. The suspension was centrifuged at maximal speed and the supernatant was transferred into a new Eppendorf tube. For precipitation 200 µl 5 M ammoniumacetat and 400 µl isopropanol were added and the samples were centrifuged at full speed for 15 minutes in a cold centrifuge. The DNA pellet was washed with 70% ethanol, dried at 37°C, and resuspended in 30 µl of H_2O. 5 µl were used for restriction digest analysis.

3.18. Maxi preps

If larger amounts of DNA were needed, DNA was prepared in a large scale, so called Maxi preps. An 800 ml culture was inoculated and grown over night. The saturated culture was transferred to centrifuge bottles and centrifuged at 4000 rpm at 4°C for 15 minutes. The supernatant was discarded and the pellet dissolved in 6 ml of solution I (5 mM glucose; 2.5 mM Tris-Cl, pH 8.0; 1 mM EDTA, pH 8.0). Then 12 ml of solution II (20 mM NaOH; 1% SDS) were added and the samples incubated for 5 minutes on ice. Then 10 ml of solution III (3 M potassium acetate; 11.5% glacial acetic acid) were added and placed on ice again for 5 minutes. The tubes were centrifuged in a Sorvall SS34 rotor for 15 minutes at 12000 rpm and the supernatant was filtered into a 50 ml Falcon tube. Isopropanol was added to the 50 ml mark, the tube was inverted several times and was incubated for 30 minutes at room temperature. The precipitate was collected by centrifugation in a Rotanta Hettich swinging bucket centrifuge at 4000 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet dissolved in 3 ml of H_2O. 5.0 g cesium chloride were dissolved and 100 µl ethidium bromide (10 mg/ml) added and the solution transferred into an ultracentrifuge quick seal tube. The tube was sealed and put into a VTI 65.2 rotor where it was run in an ultracentirfuge at 65000 rpm for at least 3.5 hours at roomtemperature. The DNA band was pulled out with a syringe and the volume adjusted to 6 ml with H_2O. N-butanol was added to the 12 ml level and ethidium bromide extracted by shaiking. After phaseseparation the upper phase was aspirated off and the procedure was repeated until the pink color was completely removed, paying attention that the H_2O level stayed above 5 ml. After the last extraction cold ethanol was added to the 15 ml mark. The samples were stored at -20 °C for 30 minutes to allow precipitation. The tubes were centrifuged in a Rotanta Hettich centrifuge at 4000 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet washed with 70% ethanol, dried, and finally resuspended in an appropriate volume of H_2O.

3.19. Mutagenenesis

3.19.1. Production of single stranded DNA

The DNA of interest was transformed into the E.coli strain CJ236. The bacterial suspension was plated on a LB plate containing ampicillin (100 μg/ml). The plate was incubated over night at 37°C. The next day a single colony was picked and a 50 ml culture of LB containing 70 μg/ml ampicillin inoculated. After inoculation 2 μl of VCSM13 helper phage (1 ml ≅ 2 x 10^7 pfu/ml) were added. The culture was grown for about 2-3 hours, then 70 μg/ml kanamycin were added, and the culture was vigourously agitated over night. 40 ml of the infected cultures were centrifuged at 10000 rpm in a Sorvall SS34 fixed angel rotor. The supernatant was transferred to another centrifuge tube and spun again for another 10 minutes at 10000 rpm to ensure that no bacterial cells were present. The supernatant was transferred to a 50 ml Falcon tube and 7 ml of 20% polyethylenglycol 8000 in 2.5 M NaCl were added and mixed by inverting the tube several times. The reaction was left at room temperature for 30 minutes. The precipitated bacteriophage particles were recovered by centrifugation at 4000 rpm for 20 minutes at 4°C in a Beckmann swinging bucket centrifuge. The supernatant was discarded and the pellet resuspended in 300 μl of TE (pH 8.0) and transferred to an Eppendorf tube. An equal volume of phenol was added and mixed vigorously. The sample was centrifuged at maximal speed for 2 minutes. The phenol extraction was repeated, followed by one phenol/chloroform (1:1 v/v) and one chloroform extraction. After the final extraction the supernatant was transferred to a new Eppendorf tube and 2.5 volumes of 100% ethanol and 1/10^{th} volume of 3 M sodium acetate, pH 5.0 were added. The ssDNA was precipitated for 20 minutes at -80°C and centrifuged at 4°C for 20 minutes at full speed. The pellet was washed with 70% ethanol and the supernatant was discarded. The pellet was dried and resuspended in 20 μl of TE.

3.19.2. Oligo-nucleotide mediated site directed mutagenesis

For the mutagenesis a primer was designed, that encoded the desired mutation in an antisense orientation to the origin of replication in the plasmid used. As a first step, the mutagenic primer was kinased.

Reagent	**Volume**
Mutagenic primer (100 pmol/μl)	4 μl
Kinase buffer (70 mM Tris-Cl, pH 7.6; 10 mM $MgCl_2$; 5 mM DTT)	2 μl
ATP (10 mM)	1 μl
T4 Polynucleotide Kinase (NEB)	1 μl
H_2O	9.5 μl
Final Volume	**16.5 μl**

The reaction was placed at 37°C for one hour, then stopped by heating to 65°C for 15 minutes. The kinased primer were annealed with the single stranded DNA.

Reagent	**Volume**
Single stranded DNA, 1 μg	x μl
Kinased mutagenic primer (10 pmol)	1 μl
Reverse primer (1 pmol)	0.5 μl
10 x PE1 buffer (200 mM Tris-Cl, pH 7.5; 100 mM $MgCl_2$; 500 mM NaCl; 10 mM DTT)	1 μl
H_2O	y μl
Final Volume	**10 μl**

For better transcription of the DNA template a second perfect match primer was added, also antisense to the origin of replication, referred to as reverse primer. If

two or more sites had to be mutated, this primer was exchanged with a second kinased mutagenic primer. Up to three kinased mutagenic primers were used successfully in one reaction to obtain a three point mutant. The reaction was put into a 95°C water bath and cooled slowly to room temperature. While the annealing reaction was cooling, the following mix was made up.

Reagent	**Volume**
PE2 buffer (200 mM Tris-Cl, 7.5; 100 mM $MgCl_2$; 100 mM DTT)	1 µl
dNTP mix (10 mM each)	1 µl
T4 DNA ligase (NEB)	1 µl
Klenow (Roche, 2U/µl)	1 µl
H_2O	5 µl
Final Volume	**10 µl**

Once the annealing mixture had cooled to room temperature, the mix was added and the sample was incubated at 16°C over night. The next day 10 µl of the sample were transformed into DH5α. Between 10 and 20 colonies were analyzed for mutations.

3.20. Electro mobility shift assay (EMSA)

The DNA-binding assay using nondenaturing polyacrylamide gel electrophoresis (PAGE) provides a simple, rapid, and extremely sensitive method for detecting sequence-specific DNA-binding proteins. Proteins that bind specifically to an end-labeled DNA fragment retard the mobility of the fragment during electrophoresis, resulting in discrete bands corresponding to the individual protein-DNA complexes. The assay can be used to test binding of purified proteins or of proteins found in crude extracts.

3.20.1. Probe preparation

To generate the probe for an electro mobility shift assay (EMSA), oligos with the desired sequence were designed in sense and antisense direction. One of the oligos, or for stronger probes both oligos were endlabeled with γ-^{32}P ATP.

Reagents	**Volume**
Oligo (100 pmol/µl)	1 µl
Polynucleotide Kinase buffer (NEB)	5 µl
γ-^{32}P ATP (6000 Ci/mmol)	5 µl
T4 Polynucleotide Kinase (NEB)	2 µl
H_2O	37 µl
Final Volume	**50 µl**

The labeling reaction was precipitated by adding 2 µl of tRNA as carrier, 1/10 3 M Sodium acetate, pH 5.2, and 2.5 volumes of ethanol. The precipitate was resuspended in 4 µl of TE buffer. Next the annealing reaction was set up.

Reagent	**Volume**
Labeled oligo sense (100pmol)	4 µl
Labeled/unlabeled oligo antisense (100pmol)	4 µl
NEB buffer 3	2 µl
H_2O	10 µl
Final Volume	**20 µl**

The sample was annealed in a thermo cycler: 85°C for 3 minutes; 65°C for 10 minutes; 42°C for 15 minutes; 37°C for 10 minutes; 20°C for 5 minutes. Meanwhile a 20% gel, 2 mm thick, 25 cm long, was prepared with 1xTBE as buffer. It was pre run for 30 minutes at 500 V. The annealed probe was loaded on the gel and separated for 4-5 hours at 500 V. An autoradiography film was exposed to

visualize the labeled and annealed probe and the double stranded oligo was cut out. The slice of gel was crushed into small pieces and eluted over night with 450 μl of elution buffer (0.5 M ammonium acetate; 10 mM magnesium acetate; 1 mM EDTA, pH 8.0; 0.1% SDS). The supernatant was transferred to a column to hold back small acrylamide pieces. At the end the sample was precipitated, washed with 70% ethanol, and resuspended in 20 μl of TE buffer.

3.20.2. Gel preparation

For the assay a 2 mm thick, 4% native acrylamide gel was prepared. The buffer was either 0.25 x TBE or 1 x Tris glycine (25 mM Tris-Cl, pH 8.3; 190 mM glycine; 1 mM EDTA). The gel was prerun for 30 minutes at 150V before the samples were loaded.

3.20.3. Band shift

While the gel was prerunning, the binding reaction was assembled by combining the protein (10-500 ng recombinant protein, 0.5-2 μl) and 18 μl band shift buffer (75 mM NaCl; 20 mM Hepes pH 7.6; 10% glycerol; 2 mM DTT; 0.1 mg/ml BSA; 2 μg/ml dI/dC). The total protein concentration was kept constant with additional BSA to obtain the same protein concentration in each sample. For supershifts a second protein or an antibody was added and the reaction incubated for 15 minutes. The probe was diluted to 10000-20000 cpm/μl and 1 μl of it was added to the protein/buffer mix. The samples were incubated for 30 minutes and then loaded on the gel. After one hour the gel was put for drying and was exposed to an autoradiography film (Kodak).

4. Results

4.1. NIH3T3 Fibroblasts

4.1.1. Stable transfection of the inducible β-catenin-LEF1 fusion (CatCLEF) in NIH3T3 cells

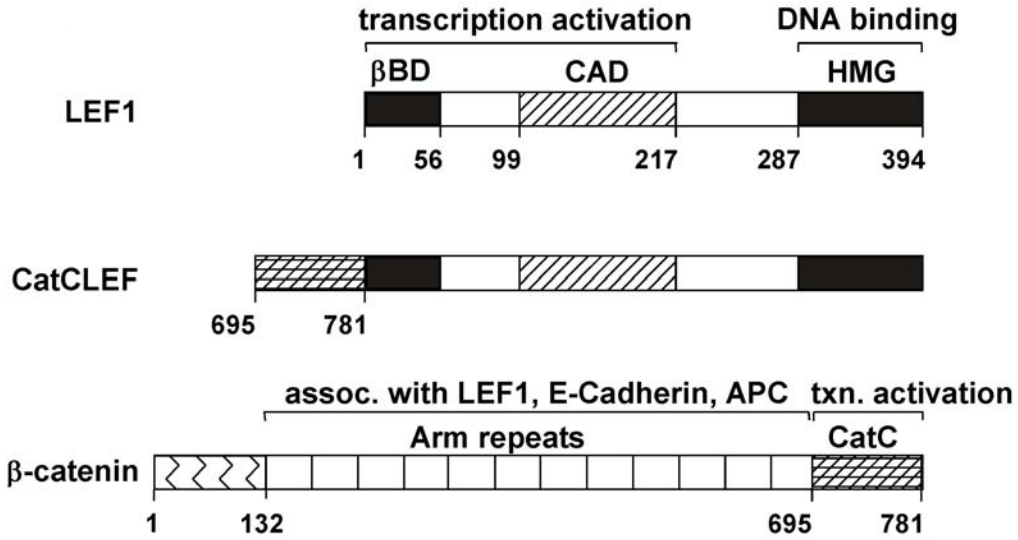
In a first approach to identify LEF-1 target genes in different regulatory processes, we were investigating the role of LEF-1 in a cell culture system. Therefore we decided to search for LEF-1 target genes in NIH3T3 cells, a murine fibroblastic cell line without endogenous LEF-1 expression, what gave us the possibility to express LEF-1 through transfection under controlled conditions. We mainly looked for genes regulated within the canonical Wnt pathway, thus regulated through the interaction of LEF-1 and β-catenin, so it was not enough to express LEF-1 alone. Overexpression of LEF-1 without β-catenin would most likely result in regulatory processes occurring without the help of β-catenin. Therefore, we were expressing a fusion of the C terminal part of β-catenin to LEF-1 (CatCLEF), which is a constitutive active form of LEF-1 (Figure 6A). This fusion ensures sufficient transcriptional activation as it was shown before (Hsu 1998) and will result in β-catenin dependent and β-catenin independent target genes. To control the expression of CatCLEF and to look mainly at primary targets, the transfection was carried out with a two-step inducible system that allowed us to express CatCLEF only for a short period of time. We used the ecdysone-inducible mammalian expression system that consists of the two vectors pVgRXR and the pIND-based inducible expression vector. PVgRXR constitutively expresses the RXR and VgEcR receptor subunits that assemble to the ecdysone receptor. The pIND vector carried CatCLEF or was empty as control and could be selected with neomycin, while pVgRXR could be selected with zeocin. First one plasmid was

transfected and a stable clone selected, then the second plasmid was stably integrated. A clone stable for control (RXR) and CatCLEF (CatCLEF#29) was selected for further analysis. The stable cell lines were kindly provided by S.-C. Hsu. The clones were induced for 8 hours with 10 mM Ponasterone A and then harvested. An immunoblot analysis confirmed the induction of the CatCLEF#29 clone but not of the RXR clone, showing that the system is completely shut off without estrogen addition (Figure 6B).

4.1.2. Microarray analysis of induced NIH3T3 cells, carrying either CatCLEF or the empty plasmid

4.1.2.1. Probe array hybridization

CatCLEF#29 and RXR cells were compared using Affymetrix chip technology to find differentially regulated genes. Both cell lines were induced for 8 hours with Ponasterone A and then harvested. Total RNA was prepared and a standard labeling procedure was carried out according to Affymetrix protocols. The labeled cRNA was fragmented, mixed with the blocking reagents and standards and hybridized to the probe arrays Mu 6500 A, B, and C by S-C. Hsu. Mu 6500 arrays are mouse arrays, each spotted with 6500 oligo-nucleotides, so that the total analysis covered 19500 sequences. One third of them are known genes two thirds are sequences derived from EST's. The chip was scanned, controlled, and subjected to analysis. We repeated that experiment using this time only one microarray MGU74Avs2 that contained 12000 spotted oligo-nucleotides on one array, some of them not spotted on the other type of chip. The microarrays are not completely comparable as for some genes the spotted sequence was changed what can result in a different strength of hybridization.



B

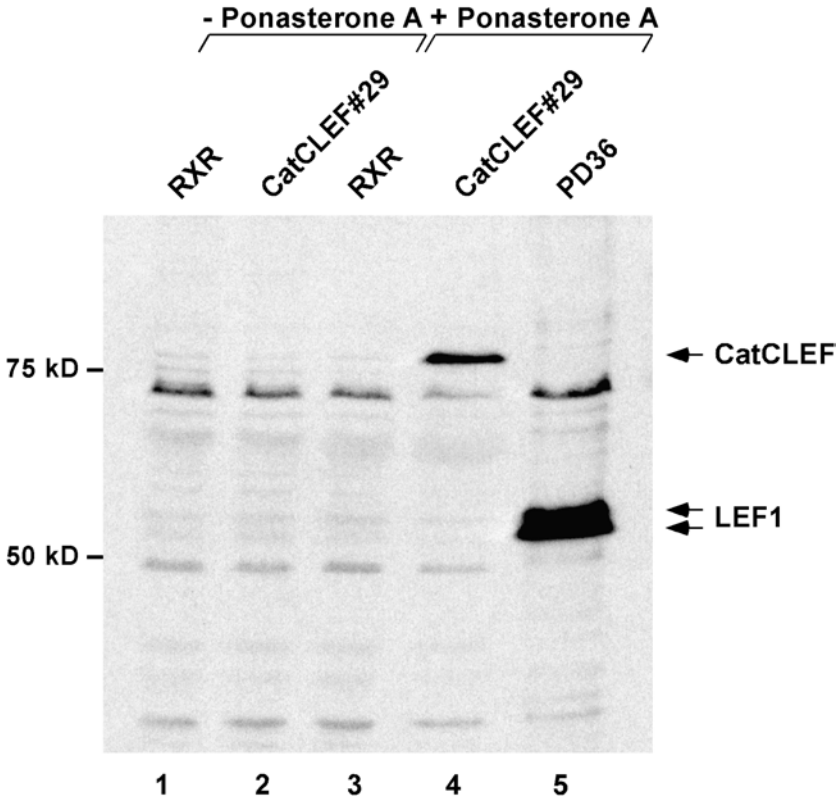
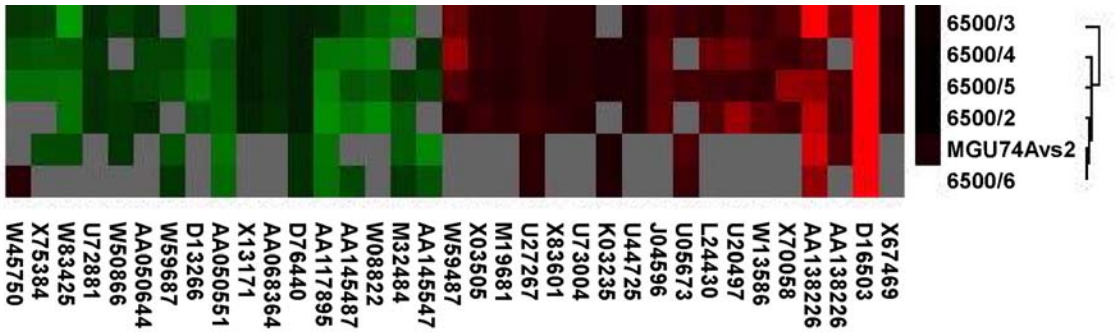


Figure 6. Induction control of the stable NIH3T3 cell lines.
(A) Schematic diagram of LEF-1 and β-catenin and their fusion CatCLEF in which the carboxyterminal (CatC) domain of β-catenin was linked to full length LEF-1. The β-catenin binding domain (βBD), context-dependent activation domain (CAD), high-mobility-group domain (HMG), and the armadillo repeats (Arm repeats) are indicated.
(B) The control cell line (RXR) and the CatCLEF expressing cell line (CatCLEF#29) were tested for their CatCLEF induction ability. Cells untreated and treated with Ponasterone A (8 hours, 10 mM) were harvested and whole cell extract was prepared. The expression of LEF-1 was tested by immunoblot analysis against LEF-1. Whole cell extract of PD36 cells was used as positive control. Only induced CatCLEF#29 cells showed expression of CatCLEF without any leakiness in the uninduced sample.

4.1.2.2. Analysis of the probe arrays

Of each cell line three Mu 6500 A, B, and C probe arrays and one MGU74Avs2 were hybridized. First the Mu 6500 chips were compared to each other, counting a gene as potential target if it was regulated at least in four out of the six comparisons (Figure 7B). Then we expanded the results with some new targets identified within the MGU74Avs2 screen and performed a cluster analysis for better visualization of the targets (Figure 7A).

A



B

Acc. #	FC	FC	FC	FC	FC	FC	count	av FC	
D16503	26.9	45.6	24.9	33	30.8	45.6	6	34.4	Lymphoid enhancer binding factor 1
AA138226	13.5	19.1	6.3	10	10.6	15	6	12.5	Homologous to CLATHRIN LIGHT CHAIN B
U20497	11.1	5.5	8.3	4.1			4	7.3	Cdk4 and Cdk6 inhibitor p19 protein
W59487	2.6	7.6	10.9	5.8			4	6.7	Homologous to ZINC FINGER PROTEIN 40
X70058	5.1	6.2	4.1	11			4	6.5	Cytokine (fic)
J04596	6.5	5.6	6.9	6			4	6.3	Platelet-derived growth factor-inducible KC protein
AA071802	4.6	8		5.3		5.7	4	5.9	Homologous to APOPAIN PRECURSOR
L24430	5.7	3.4	7.4	4.5			4	5.3	Osteocalcin precursor. bone gla protein
W13586	6.6	4.1	5	3.7			4	4.9	Atrial/fetal isoform myosin alkali light chain (MALC)
U05673		4.1		4.7	4.3	5.5	4	4.7	Adenosine receptor subtype
U27267	4.4	4.1	3.7	3.5	3.2	3.1	6	3.7	LPS-induced C-X-C chemokine LIX

								precursor
X67469	3.3	4.1	3.2	4			4	3.7	AM2 receptor
X03505	4.1	3.5	3.8	3.1			4	3.6	Serum amyloid A (SAA) 3 protein
Msa.26.0		5.4	3.3	7.4	-4.2		4	3.0	Homologous to PHOSPHATIDYLINOSITOL-4-PHOSPHATE 5-KINASE FAB1
X83601	3.4	3	2.7	2.6			4	2.9	PTX3 mRNA
M19681	3.1	2.9	3	2.7			4	2.9	Platelet-derived growth factor-inducible protein (JE) gene,
U73004	2.7	2.7	2.9	2.9			4	2.8	Secretory leukocyte protease inhibitor
U44725	2.4	2.3	2.4	2.4			4	2.4	Sl-d mutant allele kit ligand (KL)
K03235			2.2	2.2	2.4	2.3	4	2.3	Mrp/plf3 gene for mitogen regulated protein/proliferin (MRP/PLF), exon 1
X13171	-2.7	-2.4	-2.4	-2.2			4	-2.4	Histone H1(0) gene
D76440	-2.4	-2.5	-2.2	-2.4	-3.4	-3.6	6	-2.8	Necdin
AA068364	-3.4	-3.1	-3.2	-3.1			4	-3.2	Homologous to POSSIBLE DNA-REPAIR PROTEIN XP-E
U72881	-3.4	-3.2	-3.9	-3.6			4	-3.5	RGS-r protein
W45750		-4.2	-6.1	-8.2	2.9		4	-3.9	Homologous to GUANINE NUCLEOTIDE-BINDING PROTEIN G(T)
AA050644	-3.5	-3.6	-5	-5.1			4	-4.3	Homologous to MELANOMA-ASSOCIATED ANTIGEN XP
W50866	-4.1	-4.8		-4.5		-3.9	4	-4.3	Homologous to CALCINEURIN B SUBUNIT ISOFORM 1
W59687			-5	-6.1	-4	-6.1	4	-5.3	Homologous to ADENOSINE A2A RECEPTOR.
M32484	-6.6	-8.6		-4.2	-4.2	-6.5	5	-6.0	Placenta and embryonic expression gene (pem)
AA145547			-3.6	-5.1	-6.2	-9.9	4	-6.2	Homologous to LARGE PROLINE-RICH PROTEIN BAT2
X75384		-4.9	-6.6	-8		-5.5	4	-6.3	Sax-1 gene encoded protein
AA145487	-8.6	-5.5	-8.6	-6.3	-5.4		5	-6.9	Homologous to REPLICATION PROTEIN A 70 KD DNA-BINDING SUBUNIT
AA050551	-7.2	-8.3	-6.2	-6.8	-7.7	-8.5	6	-7.5	Homologous to CELL SURFACE GLYCOPROTEIN A15 (TALLA-1).
D13266	-6.2	-7.7	-7.5	-8.7			4	-7.5	Glutamate receptor channel delta 2 subunit
W83425	-8.3	-11	-6.3	-8.3		-5.5	5	-7.9	Homologous to Y BOX BINDING PROTEIN-

AA117895	-10.5	-3	-8.5	-9.8	-8.1	-8.5	6	-8.1	1 (Y-BOX TRANSCRIPTIO Homologous to PROBABLE ATP-DEPENDENT TRANSPORTER
W08822	-10.1	-7.3	-9.4	-7.1			4	-8.5	Homologous to ZINC FINGER PROTEIN GLO3.

Figure 7. Analysis of the chip screen of NIH3T3 cells.
(A) Cluster analysis of the chip results; red for positive, green for negative regulation.
(B) List of differentially regulated genes found on Mu 6500 probe arrays. Accession number (Accession#), fold changes of the six comparisons (FC), regulation seen in how many comparisons (count), average fold change (av FC), gene name or homology are indicated.

4.1.3. Validation of targets with northern blot analysis

For validation of the differentially regulated genes, the number of genes was cut down by choosing only the strongest regulated genes. Northern blot analysis was performed using RNA of induced CatCLEF#29 and RXR cells. For targets that were most likely to be expressed in pre-B cells, two additional cell lines were included. Pre-B cells isolated from fetal liver of wildtype mice (fl1) and of *Lef1*$^{-/-}$ mice (fl3) that have been infected with the Abelson virus for immortalization were added as a comparison for more natural conditions. The probes for the targets were synthesized by PCR, end-labeled with α ^{32}P-dCTP and hybridized over night. Clathrin light chain, MAGE D2, pem, Glycoprotein A15, IGF-IIR, spi2, MCP-1 and two EST sequences could be validated (Figure 8).

In table 1 the results of the chip screen were compared to the results of northern blot analysis. The fold changes detected with the microarrays are good reproducible with the northern blot analyses.

Clathrin light chains are components of clathrin coated vesicles, structural constituents involved in endocytosis and membrane recycling (Broadsky 1988). In mammalian cells one form of clathrin heavy chain is known, but two classes of light chains, light chain A (LCA) and light chain B (LCB), in contrast to

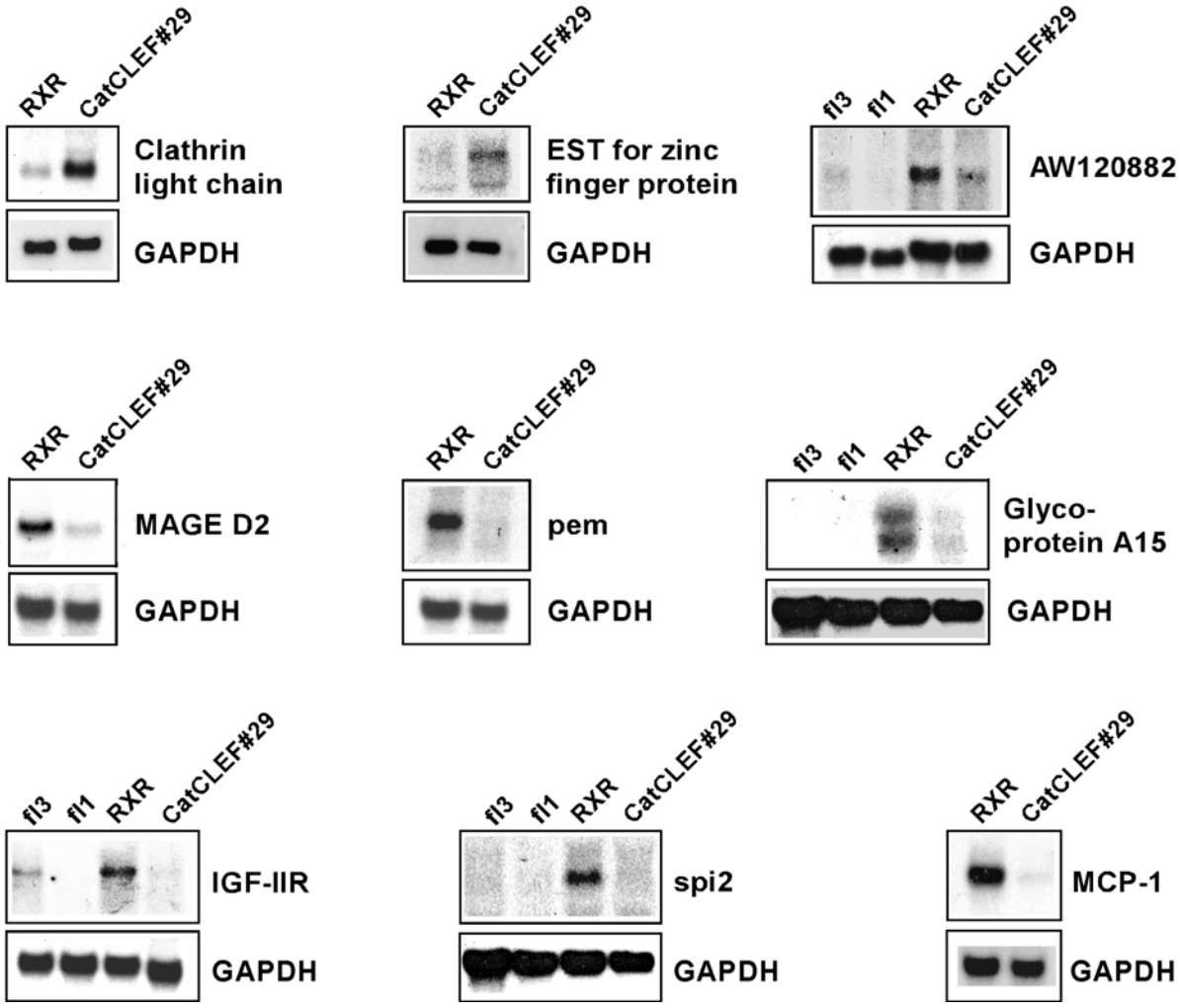


Figure 8. Northern blot analysis for validation of target genes of the NIH3T3 screen.
Northern blot analysis was carried out with 15 μg of total RNA or 5 μg of mRNA of CatCLEF#29 and RXR cell lines induced for 8 hours with 10mM Ponasterone A, or of fetal liver derived, Abelson infected pre-B cells of wildtype (fl1) and *Lef1*$^{-/-}$ (fl3) cells. PCR amplified and ^{32}P-labeled probes of the validated genes were hybridized to the RNA. The pre-B cell lines fl1 and fl3 are included for some genes as controls. We could validate clathrin light chain, MAGE D2, pem, Glycoprotein A15, IGF-IIR, spi2, MCP-1, and two ESTs without close homologies.

Gene name	FC Mu 6500	FC MG U 74A vs2	FC Northern blot
Clathrin light chain	12.5	1.6	5
EST for zinc finger protein	6.7	n.d.	2.1
Mage D2	-4.3	-2.2	-3.4
EST	-5.7	n.d.	-5.7
Glycoprotein A15	-7.5	-2.7	-7.5

Insulin like growth factor receptor (IGF-IIR)	n.d.	-7.9	-7.7
Small inducible cytokine (MCP-1)	n.d.	-9.3	-5.7
Pem	-6.0	-20	-15.7
Spi2 proteinase inhibitor	n.d.	-15.6	-19.4

Table 1. Comparison of the results detected with the microarray analysis and the northern blot analysis.

the single light chain of yeast (Payne 1985; Jackson 1987; Kirchhausen 1987; Jackson 1988; Silveira 1990). The EST found
to be regulated was homologue to the human LCB form. The precise role of the Clathrin light chains is uncertain. In vitro evidence that they bind calmodulin (Pley 1995) and are essential for the activity of an uncoating ATPase (DeLuca-Flaherty 1990) points to them being regulatory elements in Clathrin function. Neither is the purpose of the light chain polymorphism established. LCB is specifically phosphorylated both *in vitro* (Bar-Zvi 1986) and *in vivo* (Cantournet 1987) by a casein-kinase II-like activity.

Glycoprotein A15 is a member of the transmembrane 4 superfamily (TM4SF), also known as the tetraspanin that is characterized by four transmembrane domains. The TM4SF family comprises more than 20 known genes (Hotta 1988; Classon 1989; Amiot 1990). A15 is most abundantly expressed in brain but can also be detected in heart, lung, kidney, colon, and muscle (Hosokawa 1999). The function of A15 and its family members is largely unknown, but it is likely that they are involved in diverse processes such as cell activation and proliferation, adhesion and motility, differentiation, and cancer.

The serine proteinase inhibitor 2 (**spi2**) is a protein subfamily that belongs to a superfamily including active serine proteinase inhibitors, as well as proteins with other biological roles but no known inhibitory activity. The proteins are involved in the control of proteinases central to the coagulation cascade, fibrinolysis, the complement cascade, and the acute phase response (Travis 1983; Carell 1987).

The melanoma antigen-encoding gene (**MAGE**) D2 belongs to the MAGE family. Those family members are expressed in a variety of tumors but not in normal cells,

with the exception of the male germ cell, placenta, and, possibly cells of the developing embryo (Clotman 2000; Osterlund 2000). MAGE-D genes are particularly well conserved between man and mouse, suggesting that they exert an important function (Chomez 2001), although the cellular function of this protein family remains still unknown.

The small inducible cytokine A2 encodes the monocyte specific chemotactic factor **MCP-1**. MCP-1 is thought to play an important role in monocyte infiltration in the immune response (Yoshimura 1989; Leonard 1990) and is a monocyte specific chemotactic factor that belongs to the newly identified cytokine superfamily, intercrine/chemokine (Oppenheim 1991; Schall 1991). It is produced by a variety of cell types, including monocytes, fibroblasts, vascular endothelial cells, and smooth muscle cells in response to various stimuli such as lipopolysaccherid (LPS), interleukin-1 (IL-1), tumor necrosis factor (TNF), plateled-derived growth factor, Interferon-γ (IFN-γ), and TPA (Rollins 1991; Colotta 1992).

The placenta and embryonic expression early gene (**pem**) is an oncofetal gene expressed in a stage-specific manner during murine embryonic development. Pem is highly expressed in T-lymphoma cell clones and not detectable in normal lymphoid tissue (Mac Leod 1990). It is abundantly expressed in immortalized and malignant cells from different cell lineages, including epithelial cell lines, but is not detectable in differentiated adult tissues (Mac Leod 1990; Wilkinson 1990). Pem encodes a protein containing a homeodomain (Sasaki 1991). The homeobox motif is a 60 amino acid segment that encodes a helix-turn-helix (HTH) domain, which binds DNA and has structural similarities with prokaryotic HTH transcriptions factors (reviewed by (Scott 1989)). Pem has greatest homologies with the paired (prd) family, also encoding a homeodomain. In mammals, the known members are the murine S8 (Kongsuwan 1988), and the murine Pax 3, 6, and 7 genes (Kessel 1990). We found pem to be expressed in the stable cell line RXR as it is described for immortalized cell lines. Expression of LEF-1 resulted in a drastic down regulation within 8 hours to undetectable levels, making it a potent candidate for direct regulation.

The insulin-like growth factor II receptor (**IGF-IIR**) is a multifunctional membrane-spanning glycoprotein that interacts with diverse proteins bearing a mannose 6-

phosphate (M6P) recognition signal and with the nonglycosylated growth factor IGF-II (Kornfeld 1992). Biochemical studies have demonstrated that the receptor is responsible for targeting lyosomal enzymes from their sites of synthesis in the Golgi to an acidic prelysosomal compartment. About the regulation of IGF-IIR not much is known so far, but it could be shown that the promoter has two E-boxes, potential binding sites for helix-loop-helix proteins (Faisst 1992), and a binding site for the transcriptional activator SP1 (Liu 1995). As a helix-loop-helix leucine-zipper protein, c-myc can bind the identified E-box sequence. C-myc is broadly expressed during embryogenesis and in tissue compartments of the adult that possess high proliferative capacity such as skin. As The IGF-IIR receptor was shown to have the potential to be bound by c-myc and c-myc is a known target of the Wnt pathway and was identified to be regulated through its LEF/TCF binding sites (He 1998), we decided to further analyze the IGF-IIR gene.

4.1.4. IGF-IIR promoter

4.1.4.1. Cloning of the IGF-IIR promoter

To study whether the IGF-IIR is a direct target of LEF-1, we analyzed its promoter for LEF-1 responsiveness. The promoter has already been identified (Liu 1995) and so we were searching within the sequence for potential LEF-1 binding sites. One strong consensus and two weaker binding sites compared to the perfect TCRα site could be identified. The whole promoter was cloned upstream of the firefly luciferase together with two truncated promoter constructs (Figure 9). The truncations had either only two LEF-1 binding sites (IGF-IIR-2), or only one residual LEF-1 consensus motif (IGF-IIR-1).

```
-716  ccgccttat gtaatatgga tgctgcatat tatctttaca gaataataaa
-667 actttgtaac agaggtggtg gtacacgctt ctaaacctag aactcggtag
-617 gcttaagtag agggatctga actccagtat agcctgagct acatagtaag
                                                        •
-567 caatctcacg ctaaaagttt ttttttgttt gtcatttgct tgttacattt
-517 tggtttctct gcacactgca tattagtcca ccagtcacct aacttgctgt
                              *
-467 agaaagccct gcttgggaag tcacatttcc acagagctgc tgtgaggctc
-417 tccggggcat gagtcggaaa ctcccacgcg ggattctaga aagactgacc
-367 tcttaaccct gcatccactt gcaacactaa acatcaacct gggcttttcc
-317 acctaactcc atctcggcca ccgtactggt ctcggttgaa gaagagagag
-267 ttaggaagcg ctcaagcgca gacgcaacct gggtgctgga cggggaaact
-217 gaggtctggc tctgaggcgt caccctcgc cgcgcgtgag caaccctggg
-167 gttgtcaggc ctcgagtagg tacctggcgc tcgtgcccgg cccgcaacac
-117 ttcctgtccc gcgcgcgtgc gatgctcatg tgacccggga ctgggcggag
 -67 agcacctgaa cgaggacgtc acgtgagcag gaggcggggc gggggcgggc
 -17 cgactcaggt cacgtgaCGC TCCGGGGACG GCCACGGAGC GCCTCCTCGT
  34 CGCACTCCCC CCTGGCTCCA GTTCTCTCTC CTCTTTCTCC CTCCAGCTCC
  84 CGTTGCAGCT
```

B

Figure 9. IGF-IIR promoter.
(A) Promoter sequence of the IGF-IIR promoter
Letters in bold indicate exon 1; counting starts at exon 1; underlined, bold sequences are potential LEF-1 binding sites; dot marks start of the truncated form IGF-IIR-2, asterisk marks start of the truncated form IGF-IIR-1.
(B) Schematic diagram of the cloned IGF-IIR promoter fragments.

4.1.4.2. Transfection assays of the IGF-IIR promoter to test LEF-1 responsiveness

For reporter analysis the IGF-IIR promoter (Figure 10B) and the IGF-IIR-1 construct (Figure 10A) were chosen and the luciferase activity monitored after

Lef1, *Lef1*/β-catenin, *CatCLef*, and VP16*Lef1* transfection. VP16*Lef1* is a construct where a viral promoter is fused to LEF-1 and therefore LEF-1 is constitutive active. No specific effects could be detected, as the weak repression detected after LEF-1/β-catenin or CatCLEF expression was only occurring with the shortened promoter construct, which should be less responsive than the full length IGF-IIR promoter. A titration for LEF-1 dependent repression was carried out using increasing amounts of LEF-1 together with the full-length construct (Figure 10C). The upregulating effects occurring are most likely due to unspecific activation of the promoter and do not display a specific effect, as the same effect was also observed for the shortened construct (data not shown). In summary no effect on the IGF-IIR promoter could be detected after *Lef1* transfection.

4.1.4.3. Stable integration of the IGF-IIR promoter in NIH3T3 cells

The fact that we could not detect any regulation of the IGF-IIR promoter after LEF-1 expression could be due to the necessity of a stable integration of the promoter into the genome, as chromatin has also an influence on regulatory effects. Therefore, the full length promoter construct carrying the three LEF-1 binding sites driving the firefly luciferase was stably integrated into NIH3T3 cells. The promoter construct was linearized and together with a linearized plasmid carrying the resistance for neomycin co-transfected into NIH3T3 cells. One day post transfection, the culture was supplemented with neomycin and stable pools were created. The different pools were tested for luciferase activity to ensure that the IGF-IIR promoter was inserted. Three different pools with high luciferase activity could be detected. To test the stable cell pools for LEF-1 dependent repression, the cells were transfected with different plasmids carrying either a CatCLEF-IRES-eGFP, a Δ56LEF-IRES-eGFP, an HMG-IRES-eGFP, only containing the HMG domain of LEF-1, or the empty plasmid IRES-eGFP for control. After 48 hours GFP positive cells were sorted to obtain only cells that were transfected with the different plasmids. CMV-βgal was always co-transfected for normalization. The experiment was carried out five times and two representable

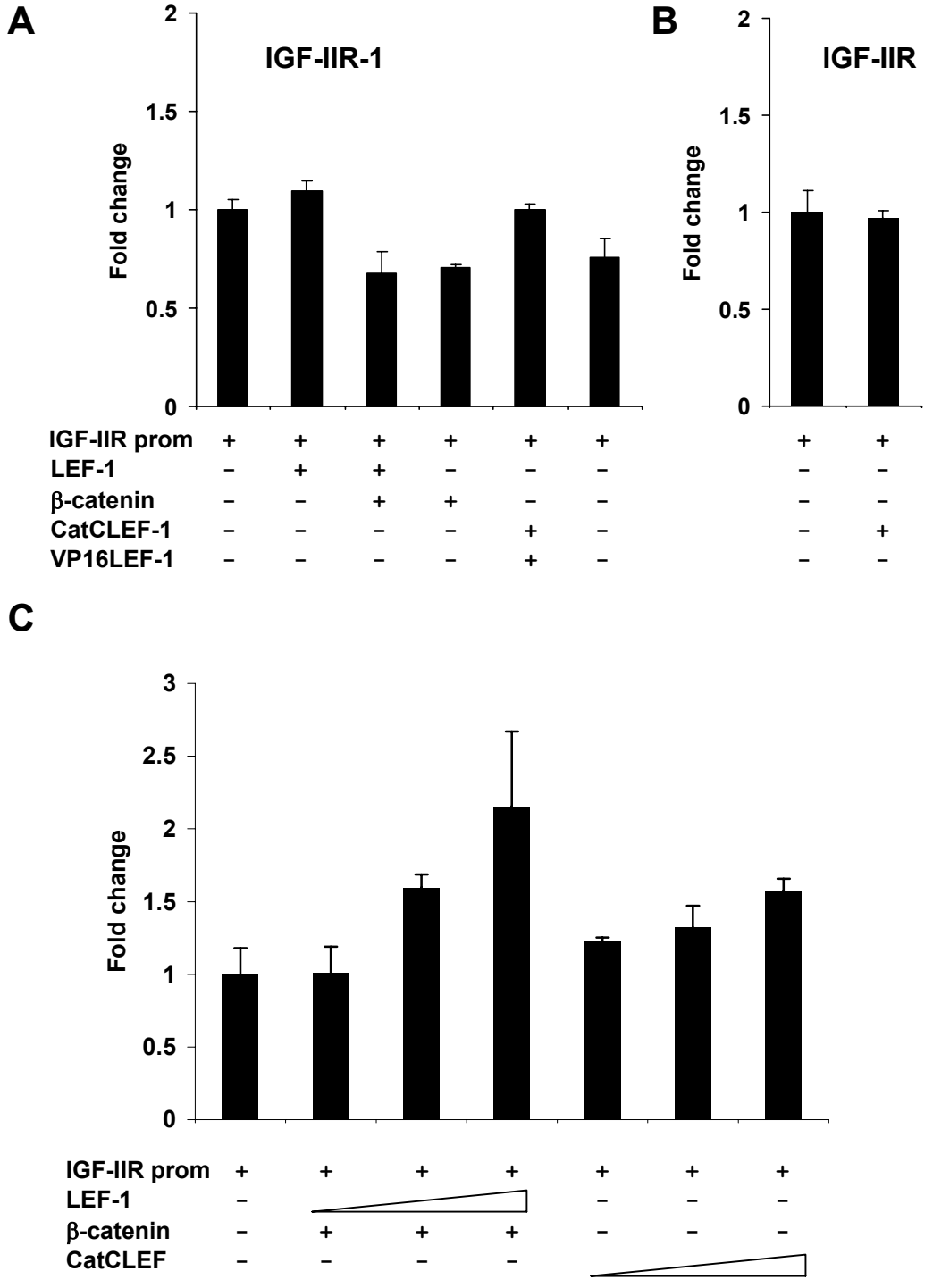


Figure 10. Promoter analysis of the IGF-IIR promoter.
NIH3T3 cells were transfected with calcium-phosphate with 50 ng of the IGF-IIR-1 promoter (A) or the full length IGF-IIR promoter (B) together with plasmids expressing LEF-1 (30 ng), β-catenin (1 μg), CatCLEF (30 ng), and VP16LEF-1 (30 ng). To test whether higher LEF-1 expression levels are necessary for repression of the promoter, increasing amounts of LEF-1 (30, 100, 300 ng) together with β-catenin (1 μ g) or CatCLEF (30, 100, 300 ng) were transfected using the full-length promoter. When using the highest amounts of LEF-1/CatCLEF some upregulation could be detected, but this is most likely an unspecific activation.

measurements are shown (Figure 11). No repression could be detected. As the weak upregulation sometimes observed after CatCLEF transfection only occurred randomly and was not reproducible (only in experiment B, not in A), it is most likely that this effect is due to an unspecific effect. Neither Δ56LEF nor the HMG domain changed the measured luciferase levels. The stable integration of the IGF-IIR promoter into NIH3T3 cells did not help to observe the repressive effect we wanted to examine.

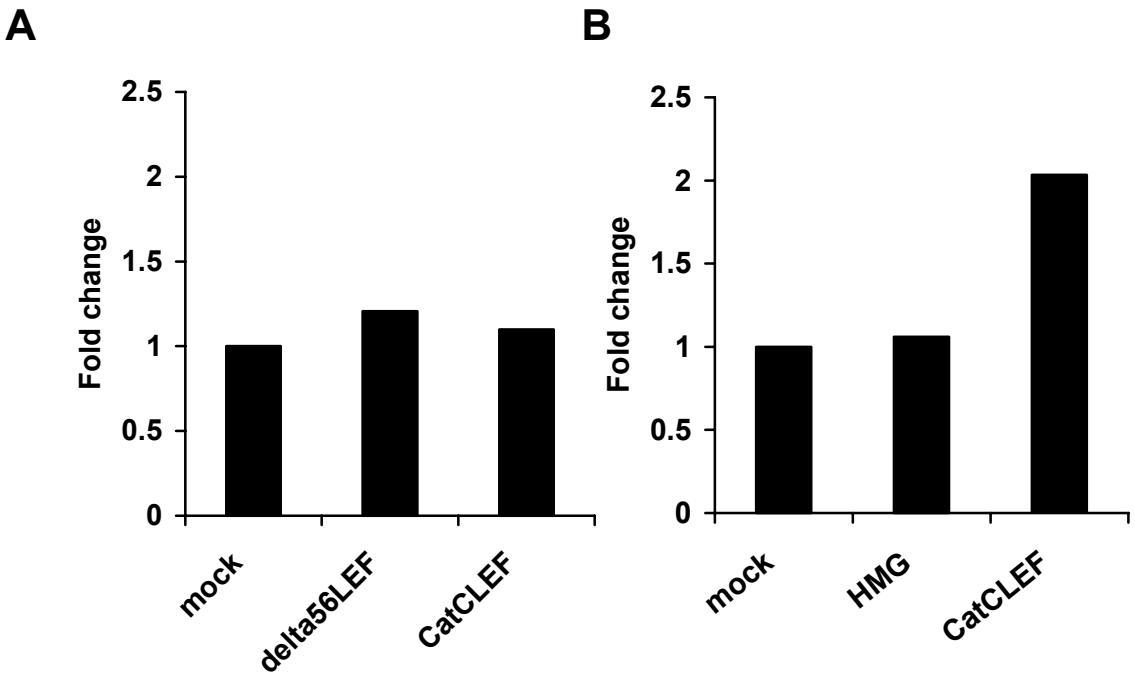


Figure 11. Effects of LEF-1 expression on NIH3T3 cells stably transfected with the IGF-IIR promoter.
A stable pool of NIH3T3 cells expressing IGF-IIR driving the luciferase was transfected with Δ56LEF-IRES-eGFP, CatCLEF-IRES-eGFP, HMG-IRES-eGFP, or the empty plasmid IRES-eGFP for control. 48 hours after transfection the cells were sorted for GFP expressing cells and luciferase activity was measured. The results were normalized to the co-transfected βgal and the values expressed as fold change relative to the empty control plasmid. A and B are two different experiments. No reproducible effect could be detected following the transfection.

4.1.4.4. Infection of stable NIH3T3 IGF-IIR pools with CatCLEF-ER

Since regulatory effects are better to study if protein expression is inducible, we cloned CatCLEF and Δ56LEF as a fusion to the human estrogen receptor (ER). This method affects protein localization but has no influence on its translation. The

translated protein is kept inactive in the cytoplasm due to the ER signal. After addition of estrogen to the cells, the protein translocates into the nucleus where it becomes active and regulates target genes. To obtain higher cell numbers for further analysis we decided to use the estrogen system together with a retroviral system, thus infecting the IGF-IIR stable NIH3T3 cells with the different LEF-1 constructs. For this purpose two NIH3T3 IGF-IIR cell pools (I+II) were infected with retroviral vectors carrying either CatCLEF-ER or ∆56LEF-ER. The infected pools were selected with neomycin for IGF-IIR and zeocin for the retroviral vectors. We obtained homogenous cell pools expressing equal levels of the IGF-IIR promoter driving the luciferase as well as equal levels of the inducible CatCLEF-ER/∆56LEF-ER. To ensure that LEF-1 is inactive without the addition of estrogen, a special estrogen free serum was used that was stripped with activated charcoal which binds estrogen. The inducibility of the established system was tested by preparing nuclear and cytoplasmic extracts of induced and uninduced cells. The extracts were used in an electro mobility shift experiments with a LEF-1 probe containing a LEF-1 consensus site. By incubation of the extracts with the LEF-1 probe we could also test if the system was leaky (Figure 12A). To control if the extracts were properly prepared, the electro mobility shift experiment was carried out in parallel with a second probe, encoding a SP1 consensus site, binding the nuclear transcription factor SP1 that should only give a signal for the nuclear fraction. It could be shown that the fractions were prepared without any contamination between nuclear and cytoplasmic fraction and that the system is inducible and no leakiness is detectable taking into account that the faster migrating band is unspecific.

The four different cell pools were induced for 40 hours with estrogen, the uninduced cells served as a control for regulation. The cells were harvested and normalized to total protein concentration and cell number. It could be shown that the different infected pools display different basal levels of luciferase activity, but in none of the infected pools a downregulation of the luciferase activity following induction could be observed (Figure 12B).

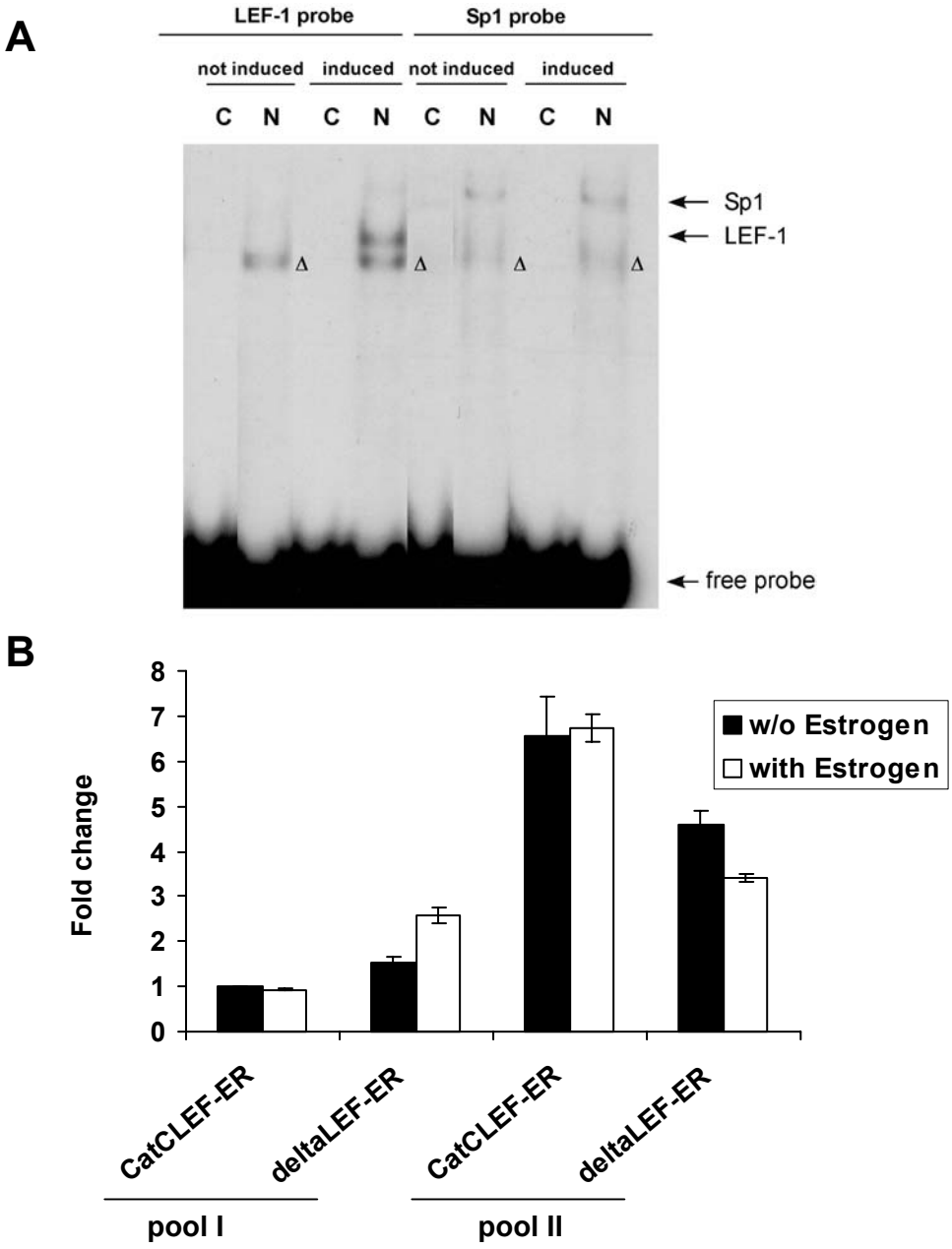


Figure 12. Responsiveness of LEF-1 infected NIH3T3 stable cell pools expressing the IGF-IIR promoter driven luciferase.
(A) Test of estrogen inducible LEF-1 expression. NIH3T3 cells infected with LEF-1-ER were grown in media supplemented with dextran charcoal stripped serum to reduce the estrogen concentration in the serum. Cytosolic and nuclear fractions were prepared and tested in an EMSA: As a control for proper preparation of the extracts a probe was used that carried the consensus sequence for SP1 that is only expressed in the nucleus. The shift only occurs in the nuclear fraction (N) and not in the cytosolic fraction (C): For LEF-1 a shift can only be detected with the induced nuclear fraction. Δ indicates unspecific binding to the probes.
(B) Analysis of two different NIH3T3 cell pools with the IGF-IIR promoter driving the luciferase stably integrated (pool I+II) that were infected with CatCLEF-ER or Δ56LEF-1-ER (deltaLEF-ER). CatCLEF and Δ56LEF-1 were activated following the addition of estrogen and induced and uninduced cells were harvested 40 hours later. Luciferase activities were measured and normalized to the cell number and the total protein concentration and are expressed as fold changes relative to the uninduced CatCLEF-ER infected cell pool I. Besides the different base lines of the infected pools no reproducible regulatory effects could be detected after activation of the proteins.

Because we could not detect any significant regulation of the IGF-IIR promoter following LEF-1 expression we concluded that either the regulatory effect of the IGF-IIR gene detected with the microarray screen cannot directly be ascribed to LEF-1 or the element through which the direct regulation occurs is an enhancer in a region different from the promoter we cloned.

4.1.5. Pem endogenous regulation

The oncofetal gene pem was another subject of interest. It was shown before that pem expression can be elevated by more than 50 fold in $10T^1/_2$ cells by treating them with 5-Aza-cytidine (5AzaC) (Saski 1991). $10T^1/_2$ cells are a mesenchymal stem cell line that can become committed to specific mesenchymal cell lineages, but they do not terminally differentiate unless the proliferative stimulus to the cells is removed. A large portion of 5AzaC treated $10T^1/_2$ cells (25-50%) becomes myoblasts committed to the muscle cell lineage (Konieczyny 1984). Without stimulus $10T^1/_2$ cells express a low but constitutive pem level. We decided to investigate if the change of pem expression levels in 5AzaC treated $10T^1/_2$ cells also resulted in a change of LEF-1 levels. This would indicate whether the regulation of pem is tightly bound to LEF-1. On the other hand it is not clear why pem gets upregulated after 5AzaC treatment. There is still the possibility of a potential positive stimulus that gets activated through 5AzaC that can override the LEF-1 inhibiting mechanism.

We were seeding out $10T^1/_2$ cells very sparse and induced half of the samples with 3 μM 5AzaC, the other half stayed untreated for control. Samples were harvested after 3, 5, and 7 days of induction. RNA was prepared, transcribed to cDNA, and a PCR for *pem*, *Lef1*, and GAPDH was performed (Figure 13). The experiment was repeated three times to rule out secondary effects. *Pem* was highly upregulated after 5AzaC treatment, but *Lef1* levels stayed unchanged.

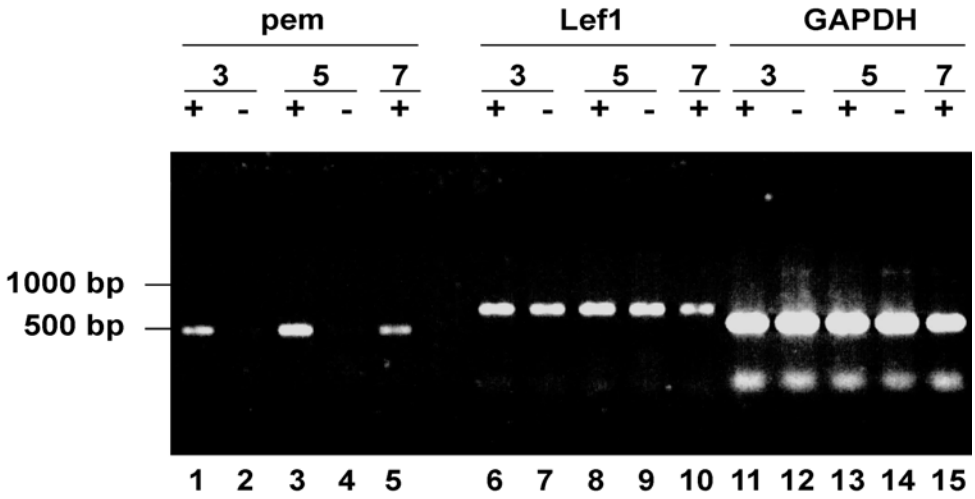


Figure 13. RT-PCR analysis of 10T½ cells treated with 5-Aza-cytidine (5AzaC).
10T½ cells were treated with 3 μM 5AzaC for 3, 5, and 7 days. Untreated cells were used as control. Cells were harvested after the indicated time points (3,5,7 days) and total RNA was prepared. The RNA was transcribed to cDNA and a PCR with 25 cycles was performed for pem, Lef1 and GAPDH as control. The experiment was repeated three times to rule out unspecific effects. Pem was upregulated already 3 days after treatment. The *Lef1* levels stayed unchanged during the treatment phase.

This analysis did not show a closer connection between LEF-1 and pem. Nevertheless, this does not rule out the possibility, that the regulation observed in the chip screen is direct. To further address that question, we would have to perform a promoter analysis.

4.2. T cells

4.2.1. Arrest of T cell growth

As a second approach we were analyzing LEF-1 target genes in T cells, thereby comparing the expression pattern in thymic cells of wildtype mice with those of *Lef1*$^{-/-}$ mice. It was shown before that T cell development in *Lef1*$^{-/-}$ mice is almost not altered (Okamura 1998). This is due to a redundancy of LEF-1 with other members of the LEF/TCF family like TCF-1. Only mice mutant for *Lef1* and *Tcf1* have an almost complete block in T cell development, occurring after the immature single positive CD8^{+} (ISP CD8^{+}) state.

4.2.2. Microarray analysis of ISP CD8^{+} cells of wildtype and *Lef1*$^{-/-}$ *Tcf1(V)*$^{-/-}$ mice

4.2.2.1. Sorting of ISP CD8^{+} cells

For studies of genes regulated by LEF-1 in T cells, we were choosing *Lef1*$^{-/-}$ *Tcf1(V)*$^{-/-}$ mice, as the redundancy of LEF-1 with TCF-1 does not allow direct investigation of expression level changes in the *Lef1*$^{-/-}$ knockouts. To observe changes in gene regulations due to the lack of LEF-1/TCF-1 we worked with a specific cell population that is present in both wildtype and mutant mice, in order to rule out the possibility, that changed expression levels are a result of changed proportions of the cell pools. Because in *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ mice the T cell development stops at the ISP CD8^{+} stage, is this stage the last stage occurring in both mice lines. This makes it most likely that important regulatory processes are going on in this cell type. We sorted cells of the ISP CD8^{+} stage from wildtype and from *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ E17.5 embryos. The analysis of those cells should unveil

important target genes. Fetal thymic organ cultures of wildtype and *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ mice were first sorted for the lack of surface expression of CD4 and CD3. Cells that met these criteria were then further sorted for expression of CD8. The sort was carried out by R. Okamura.

4.2.2.2. Linear Amplification of RNA

For Affymetrix microarray analysis the amount of total RNA to start with was between 5 and 50 μg. The sort of the ISP CD8$^+$ cells only resulted in very few cells. We could obtain 10^5 cells for wildtype and double null mice each. Therefore it was necessary to increase the RNA amount. To amplify the genes in a linear and therefore proportional range, a PCR amplification was not suitable as this results in an exponentially amplification. For this reason a linear amplification strategy was developed (Figure 14). The procedure was started as described by Coleman and coworkers (Eberwine 1992), using a poly-dT primer with a fused T7 promoter site to prime for the first strand cDNA synthesis. Following the first strand synthesis, the second cDNA strand was produced, resulting in an intact T7 promoter. For the *in vitro* translation (IVT) back to RNA, the T7 polymerase was used that could prime within the T7 promoter region. As the RNA produced after one round was still not enough for the microarray hybridization, we had to develop a method to further amplify the RNA. For that we were choosing to do another round of cDNA and *in vitro* transcription. As the poly-A tail of the total RNA is absent after one round of amplification, we could not use the poly-dT primer again. Therefore, we were starting the second round of amplification with random hexamer primers, followed by the poly-dT primer with a fused T7 promoter site for the second strand cDNA synthesis. This resulted again in cDNA that could be transcribed by the T7 polymerase. This procedure was repeated once more, but this time biotinylated nucleotides were used for the last step, the IVT reaction, to obtain RNA with incorporated biotin. This biotin was detected by Streptavidin Phycoerythrin within the following staining process. Affymetrix later offered a similar protocol for the amplification of low RNA amounts.

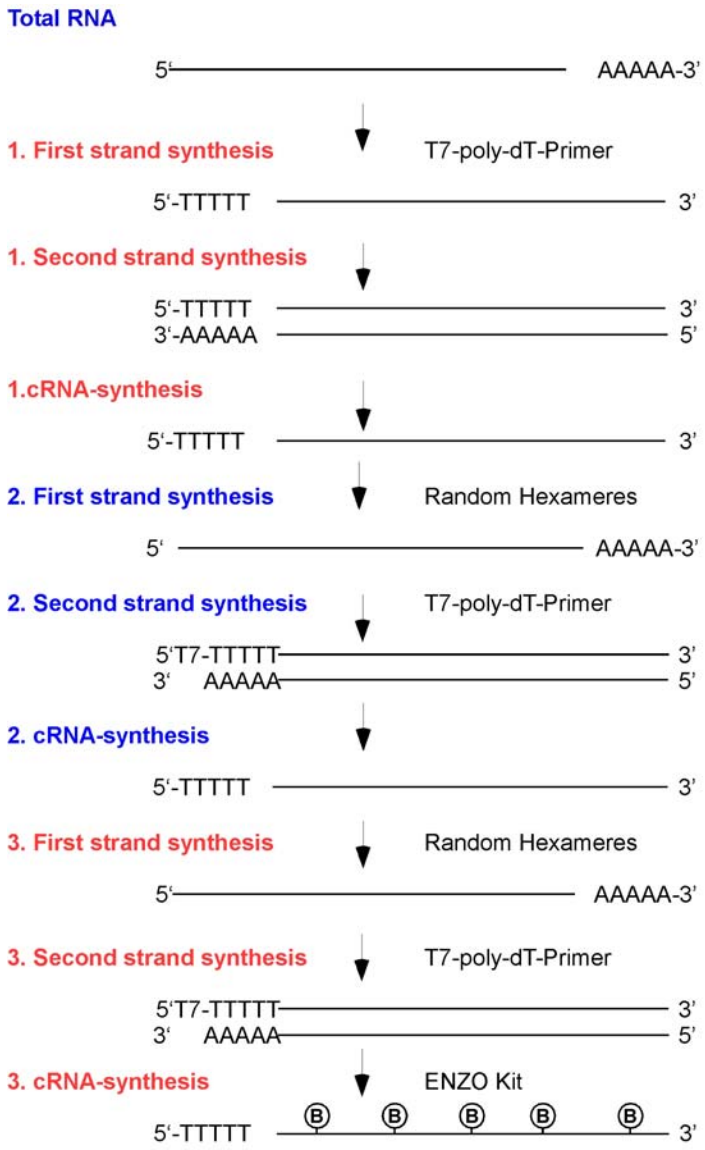


Figure 14. Flow diagram for the procedure of linear RNA amplification.
Total RNA of 10000 cells was transcribed to double stranded cDNA using a poly-dT primer with the T7 promoter site fused. The in vitro transcription (IVT) reaction was carried out with the T7 polymerase. This procedure was repeated twice, taking random primer for the second cDNA synthesis and biotinylated nucleotides for the third IVT reaction.

4.2.2.3. Microarray Hybridization and Scan

The labeled cRNA was subjected to a fragmentation reaction to obtain small pieces with sizes between 100 to 200 basepairs. The cRNA was mixed with blocking reagents and standards for quality control and the reaction mixture was incubated for 16 hours on the microarray MGU74Avs2. Following hybridization the microarray was washed several times, stained and finally scanned. The whole procedure was carried out in an Affymetrix Fluidics Station. One gene is represented by 21 oligo-nucleotides. Specificity is controlled taking the ratio of the perfect match oligo-nucleotides to the mismatch oligo-nucleotide, which has one base mutated in the 20 basepairs spotted. The scanned image was controlled for proper alignment of the grid and intensities of the control genes (Figure 15).

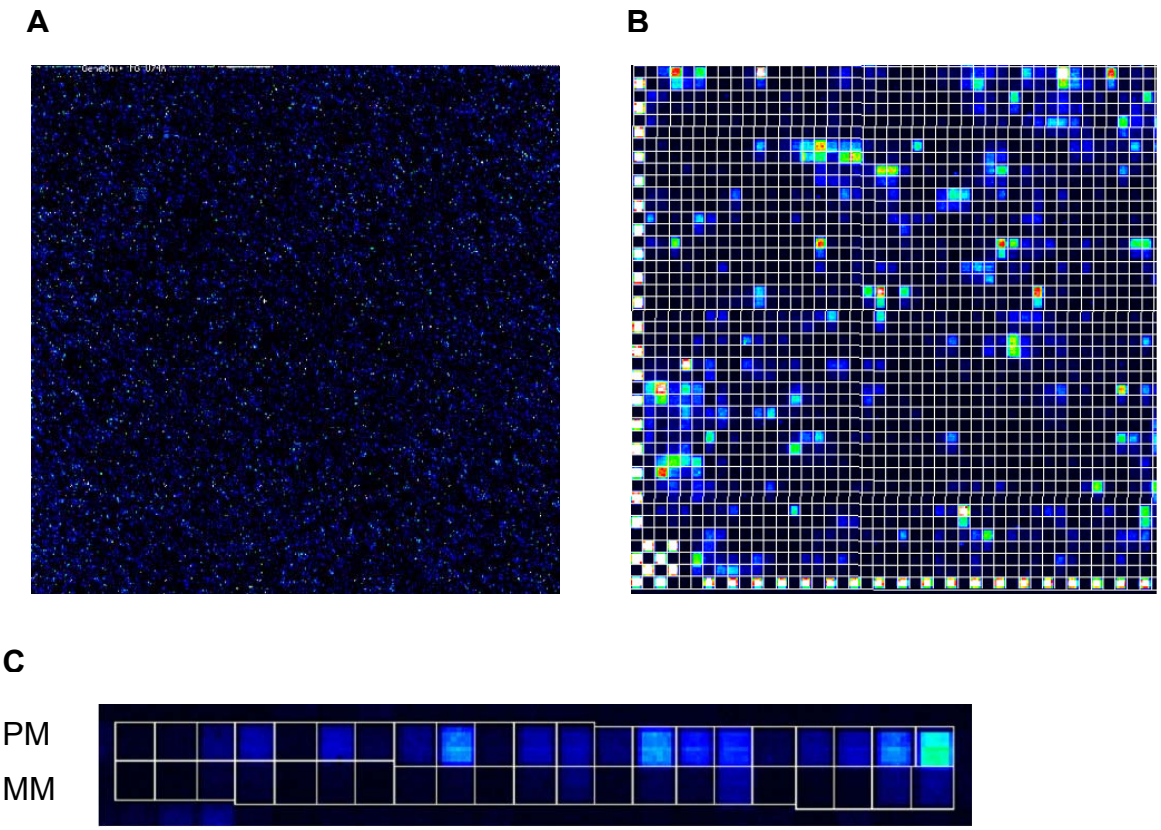


Figure 15. Images of the scanned microarray.
(A) Total view of the microarray MGU74Avs2 after scanning.
(B) Control of the grid alignment in the left corner.
(C) 21 spotted oligo-nucleotides representing one gene; upper row perfect match (PM), lower row one mismatch (MM) in 20 bases of the oligo-nucleotide for specificity control.

4.2.2.4. Regulated genes

The expression profiles of the two arrays, either probed with wildtype or *Lef1*$^{-/-}$ *Tcf1(V)*$^{-/-}$ cells, were analyzed. The two chips were compared using the wildtype as the baseline. The experiment was repeated twice. 28 genes were detected to be upregulated at least three fold in *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ double null cells in respect to wildtype cells and 63 downregulated at least three fold in *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ double null cells (Table 2). Sequences of the ESTs were searched for homologies with known genes.

Affymetrix ID number	Fold Change	Sort Score	EST's homology	Gene name
98406_at	49	189.26		RANTES/small inducible cytokine A5
93038_f_at	27	108.68		Lipocortin 1
100468_g_at	16	79.37		LYL gene
102185_f_at	13	46.74	Mouse lysosyme M gene	
102744_at	12	67.34		T-cell gamma gene
95611_at	12	43.22	LP1 lipoprotein lipase	
95673_s_at	11	45.07	No homology	
97113_at	11	36.02		Fas antigen ligand
102272_at	10	50.61		Natural killer cell BY55 precursor
102695_at	9	30.09		T cell receptor gamma locus
93013_at	8	35.44		Inhibitor of DNA binding 2
104217_at	8	23.81	FLJ22603 fis (86%)	
93604_f_at	8	24.91		Immunosuperfamily protein BI2
103571_at	7	21.88		Lymphocyte specific transcript (LST)
96047_at	7	27.2		Retinol binding protein (RBP)
93534_at	7	18.26		Decorin
100611_at	6	16.33		Lysozyme M gene
102877_at	6	13.28		Granzyme B
99214_at	6	12.66	C-C chemokine receptor 5	
93714_f_at	6	17.41	MHC class I	
95339_r_at	5	12.07		Macrophage metalloelastase
96372_f_at	5	13.05	Acrogranin percursor	

100944_at	5	24.32	No homology	
92328_at	5	32.05		T cell receptor delta chain. C region
94887_at	5	35.93	No homology	
96172_at	4	22.46	No homology	
103451_at	3	7.84	No homology	
104079_at	-3	-8.17	No homology	
95702_at	-3	-7.69	No homology	
100212_f_at	-3	-5.57	Cytokine inducible SH2-containing protein	
100958_at	-3	-5.73	No homology	
96791_at	-3	-7.85	No homology	
98893_at	-3	-5.52	No homology	
94367_at	-3	-10.9	Uridine kinase	
96083_s_at	-3	-16.21	Ribonucleoprotein D-like	
97411_at	-3	-5.72		Ect2 oncogene
96073_at	-3	-5.13		Requiem
101065_at	-3	-11.94		Proliferating cell nuclear antigen
94348_f_at	-3	-6.11	No homology	
96135_at	-3	-6.06	Homo sapiens PRO2013	
97250_at	-3	-15.12	Homo sapiens Nop10p	
99607_at	-3	-7.27		Transcription elongation factor B
103267_i_at	-4	-5.14	ZT2 gene zinc finger	
93254_at	-4	-5.14	BCRL2	
93274_at	-4	-7.07		CDC-like kinase
97181_f_at	-4	-9.32		IgE-binding factor
103359_at	-4	-5.51	No homology	
100720_at	-4	-10.26		Poly(A) binding protein
101081_at	-4	-15.21		CtBP1 protein
103667_at	-4	-5.21	Homo sapiens polyA site DNA (85%)	
102838_at	-4	-5.21		Lymph node homing receptor
93255_at	-4	-5.73		Ral-interacting protein 1
94433_at	-4	-5.03	Rattus norvegicus amino acid transporter system A (87%)+J85	
97312_at	-4	-6.23		MGC-24v

93538_at	-4	-5.78	Mus musculus Traf and Tnf receptor associated protein	
98981_s_at	-4	-8.53		Transcription factor 12
101958_f_at	-4	-7.26		Transcription factor Dp 1
97812_at	-4	-7.5		B cell antigen receptor Ig beta associated protein 1 (IBAP-1)
92513_at	-4	-6.47		Nuclear protein SA2
94192_at	-4	-6.97		Ganglioside-induced differentiation associated protein 10
102040_at	-4	-7.8		G-protein coupled receptor kinase 6-B
104578_f_at	-4	-22.72	Human chromosome 14 DNA sequence	
102821_s_at	-4	-10.93		Mouse (clone M2) GTPase (Ran)
103634_at	-4	-6.89		Interferon dependent positive acting Transcription factor 3 gamma
103081_at	-5	-7.49	No homology	
95994_at	-5	-7.17	Mus Musculus Chromosome 2 Clone RP23-291P1	
93511_at	-5	-8.24		Integral membrane protein 2
94788_f_at	-5	-9.12		Beta-tubulin
99086_g_at	-5	-7.26	Homo sapiens ubiquitin specific protease 3(80%)	
104152_at	-5	-7.51	No homology	
103562_f_at	-5	-14.3		Endogenous retrovirus truncated gag protein
92596_at	-5	-11.28		Calcyclin binding protein (CACYBP)
94027_at	-5	-10.07	Mouse DNA for t-haplotype-specific elements	
103753_at	-5	-13.22	Homo sapiens cDNA FLJ10362 fis (89%)	
94830_at	-5	-10.23	No homology	
92614_at	-5	-6.67		Inhibitor of DNA binding 3
95204_f_at	-5	-12.11	Mouse beta D galactosidasae fusion	
96891_at	-5	-13.69	Proliferation protein	
104343_f_at	-6	-25.58	No homology	

100900_at	-6	-16.15	Transcription factor C1	
97556_at	-6	-15.42	Homo sapiens FLJ21971 fis	
97944_f_at	-6	-25.73		T cell receptor alpha chain variable region
99632_at	-7	-18.07		Mitotic checkpoint component Mad2
99665_at	-7	-31.44		Nuclear matrix attachment DNA-binding protein SATB1
94835_f_at	-8	-23.03		Tubulin, beta 2
93637_at	-9	-28.66		CD5 antigen
102539_at	-9	-31.07	T cell differentiation antigen (Ly3)	
96302_at	-10	-32.87	Homo sapiens splicing factor	
98756_at	-13	-46.66	No homology	
96609_at	-16	-62.08	Rat casein kinase II	

Table 2. List of genes regulated more than 3-fold in both directions found in the T cell screen.

4.2.3. Validation of target genes by real-time PCR

The list of regulated genes was further reduced. The genes strongest regulated in both directions were examined. We decided to concentrate on the full-length genes and keep the ESTs for later analysis. For the repressed genes we wanted to analyze potential targets that were at least 11-fold regulated. Therefore we were selecting RANTES, Lipocortin, LYL, and Fas antigen ligand (FasL) for validation. T-cell gamma we did not include in our analysis, as the sequences for the spotted oligo-nucleotides were not published up to this date, and we could not distinguish between the different variable forms existing. As the genes activated through LEF-1 were only to lower levels regulated as compared to the repressed genes, we were choosing potential target genes that were at least 7-fold regulated. The positively regulated genes included in our validation were CD5, SATB1, and Mad2. Tubulin-β was not included in the validation due to similar reasons as the T cell-γ. For tubulin-β many isoforms were identified. As we did not have the spotted

sequence, we could not rule out, which of the isoforms was regulated, or whether all isoforms showed this effect.

Real-time PCR was performed out of 10^5 ISP CD8$^+$ cells of wildtype and *Lef1*$^{-/-}$ *Tcf1(V)*$^{-/-}$ mice for the selected genes. Total RNA was transcribed to cDNA and was used unamplified for the quantitative PCR reaction. TCRα, an already published target gene (Travis 1991), was selected as a positive control. Lipocortin, LYL, and Mad2 could not be detected before 40 cycles, neither in the cDNA out of wildtype cells nor in the cDNA out of *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ double null cells, because of the low cDNA amount used (data not shown). This is probably due to lower abundance of these genes in the ISP CD8$^+$ cells as compared to the other genes selected for validation. RANTES, FasL, SATB1, and CD5 could be detected and were found to be regulated as seen with the microarray analysis (Figure 16).

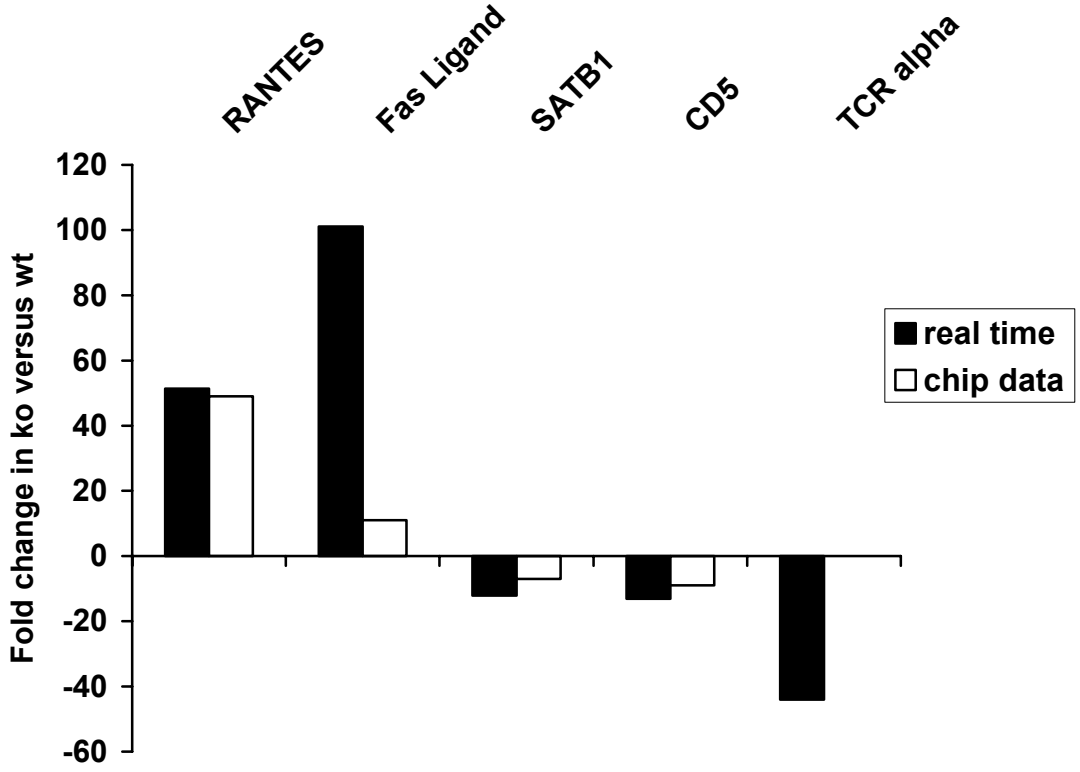


Figure 16. Validation of the T cell screen results by real-time PCR.
Total RNA of sorted, unamplified ISP CD8$^+$ cells of wildtype and *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ double null mice was prepared, transcribed to cDNA and endogenous levels of the target genes were evaluated by real-time PCR. RANTES, Fas Ligand, SATB1, and CD5 could be shown to be regulated. TCRα served as positive control, as it was already shown to be regulated by LEF-1.

RANTES, the small inducible cytokine 5, is expressed only late in T cell development. The induction occurs about five days after activation of peripheral blood monolayer cells (PBMCs) (Schall 1988). RANTES Factor of Late Activated T Lymphocytes-1 (RFLAT-1) was shown to be necessary for activation of RANTES in T cells (Song 1999) but it is still unclear why the onset of this cytokine occurs so late compared to other cytokines. There is the possibility that an active suppressor for RANTES exists. Since RANTES belongs to the same cytokine family as MCP-1, a target identified with the NIH3T3 screen, and both are found to be deregulated independently, the question arises if the whole family of this type of cytokines is regulated through LEF-1.

Fas antigen ligand (FasL) is a tumor necrosis factor (TNF) related type II membrane protein and binds to Fas. It is expressed in highly activated T-lymphocytes (Nagata 1999; Pinkoski 1999). Activated T-lymphocytes undergo apoptosis following homotypic interaction of FasL and its receptor, Fas (Brunner 1995; Dhein 1995; Ju 1995). Thus, the elimination of highly activated T-cells by the Fas/FasL system is critical for the downregulation of immune responses, the homeostasis of lymphocytes, and the maintenance of peripheral tolerance. FasL, the second gene negatively regulated by LEF-1 that we validated is also highly expressed in activated T cells, making it possible that the regulation is direct, as LEF-1 is only highly expressed in the developing T cell and is almost not detectable in PBMCs, with or without activation.

CD5 (Ly-1) is a pan-T marker present at higher levels on helper T cells than on suppressor or cytotoxic T cells. Little is known about the role of CD5 in lymphocyte function and/or differentiation. Antibodies against CD5 can augment alloantigen- or mitogen-induced lymphocyte proliferation, suggesting a possible role for CD5 in regulating T-cell proliferation (Hollander 1981; Loydberg 1985). As LEF-1 is also clearly connected to the proliferation of T cells it seems to be likely that those two genes are interdependent.

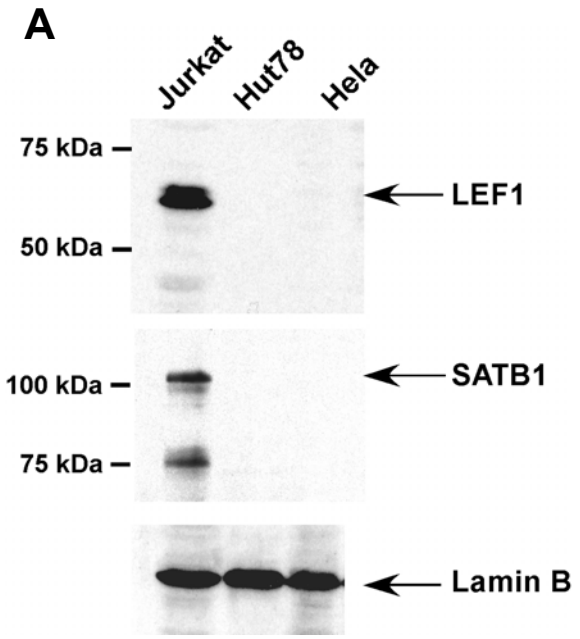
SATB1, a MAR-binding protein is known to regulate expression of multiple genes during T-cell development. It was also found to be downregulated in *Lef1*$^{-/-}$ *Tcf1(V)*$^{-/-}$ T-cells, indicating that it is normally upregulated by LEF-1. SATB1 knockout mice develop a thymic phenotype similar to that found in *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$

mice. The thymus is highly reduced in size and there is a block in T-cell development (Alvarez 2000). Nevertheless the block occurs at a later stage than it was seen in *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ mice. SATB1 null mice can still develop double positive cells but further development is greatly reduced. The later onset of the T cell developmental block in SATB1 mice could be a hint that SATB1 is indeed lying downstream of LEF-1, making it an interesting target for further analysis.

4.2.4. Correlation of LEF-1 expression with the expression of the putative target genes

The endogenous levels of the target genes were analyzed in different cell lines to examine relative expression levels of the target genes and LEF-1. This can already be an indication whether the regulation detected on the microarray and validated by real-time PCR is also occurring naturally in transformed cell lines. We were expecting to find high expression levels of SATB1 and CD5 in cell lines expressing high LEF-1 levels and low in those cell lines where LEF-1 is almost not detectable. RANTES and FasL were supposed to behave the opposite way. For the analysis two different T cell lines were chosen. On the one hand we were examining Jurkat cells, a mature T cell line without activated features, on the other hand Hut78 cells, also a mature T cell line, but this cell line is already displaying features of activated cells such as the presentation of the IL2 receptor on the surface. The levels of LEF-1 in those cell lines were determined by immunoblot analysis, using Hela cells as a negative control. It could be shown that LEF-1 is expressed at high levels in Jurkat cells and cannot be detected in Hut78 cells (Figure 17A). The expression levels of the targets were examined by real-time PCR. SATB1 and CD5 are expressed highly in Jurkat cells and at lower levels in Hut78 cells, whereas FasL and RANTES behave vice versa (Figure 17B). Expression of RANTES and FasL seemed to be almost completely blocked in Jurkat cells as their levels are only few cycles above the background, indicating an active repression. The strong regulatory effects found for RANTES and FasL

expressing the levels as fold change relative to the cell line expressing the genes only at low levels, the Jurkat cells, are contradictionary to the modest regulation observed for SATB1 and CD5. This might be due to the fact that the expression level of SATB1 and CD5 are lower in Hut78 cells, but are still clearly detectable above background, indicating that an active repressor might be missing in Hut78 cells to completely abolish the expression of SATB1 and CD5 or that they can also get activated by other LEF/TCF family members. We also examined the protein levels for SATB1. The analysis revealed the same as seen at RNA levels and is overlapping with the expression profile of LEF-1 (Figure 17A).
RANTES is highly expressed in other tissues besides T cells, but they are always low on LEF-1 expression (data not shown).



B

	Jurkat	Hut78
RANTES	1	6700
FasL	1	361842
SATB1	59	1
CD5	5	1

Figure 17. Endogenous expression levels of target genes compared to endogenous LEF-1 levels.
(A) Protein levels of LEF-1, SATB1, and Lamin B as loading control in Jurkat, Hut78, and Hela cells. LEF-1 and SATB1 are present in Jurkat cells, but there is no expression in Hut78 and Hela cells detectable.
(B) RNA levels of RANTES, FasL, SATB1, and CD5 in Jurkat and Hut78 cells measured by real-time PCR. RANTES and FasL are not detectable in Jurkat cells, but expressed at high levels in Hut78 cells. SATB1 and CD5 are behaving vice versa, showing high levels in Jurkat and lower levels in Hut78 cells. The values are denoted as fold changes relative to the cell line where lower expression levels are detected.

4.2.5. Endogenous upregulation of CD5 and SATB1 after transfection of *Lef1* and β-catenin

To study the regulation of CD5 and SATB1 in more detail, *Lef1* and β-catenin were transfected into Hut78 cells. As shown before, this cell line is expressing only low levels of LEF-1, SATB1 and CD5. Hut78 cells were electroporated either with an empty vector carrying IRES-eGFP as control or with LEF-1-IRES-eGFP together with a plasmid encoding β-catenin. The cells were sorted after 24 hours for GFP expression and RNA was prepared. After reverse transcription the cDNA was analyzed by real-time amplification. LEF-1 protein levels were analyzed to verify the LEF-1 expression in LEF-1-IRES-eGFP transfected cells (Figure 18A). It could be shown that after 24 hours the CD5 level was upregulated 8-fold and the SATB1 level 12-fold (Figure 18C). To analyze how fast the upregulation occurs, this experiment was repeated but this time the cells were already sorted 12 hours post transfection. CD5 and SATB1 were still found to be upregulated (Figure 18B), leading to the conclusion that they are most likely directly regulated by LEF-1. Nevertheless it cannot be ruled out that the regulations are due to secondary effects.

4.2.6. Endogenous levels of LEF-1 and RANTES in peripheral blood monolayer cells (PBMCs)

It was shown before that RANTES could be induced with the mitogen phytohemaglutamine (PHA-P) (5 μg/ml) in peripheral blood monolayer cells (PBMCs) (Schall 1988). RANTES reaches the maximum level 5 days after the induction. RFLAT, a positive regulator of RANTES was shown to get posttranscriptionally modified shortly before the upregulation occurs (Song 1999), indicating that the activation is driven by RFLAT.

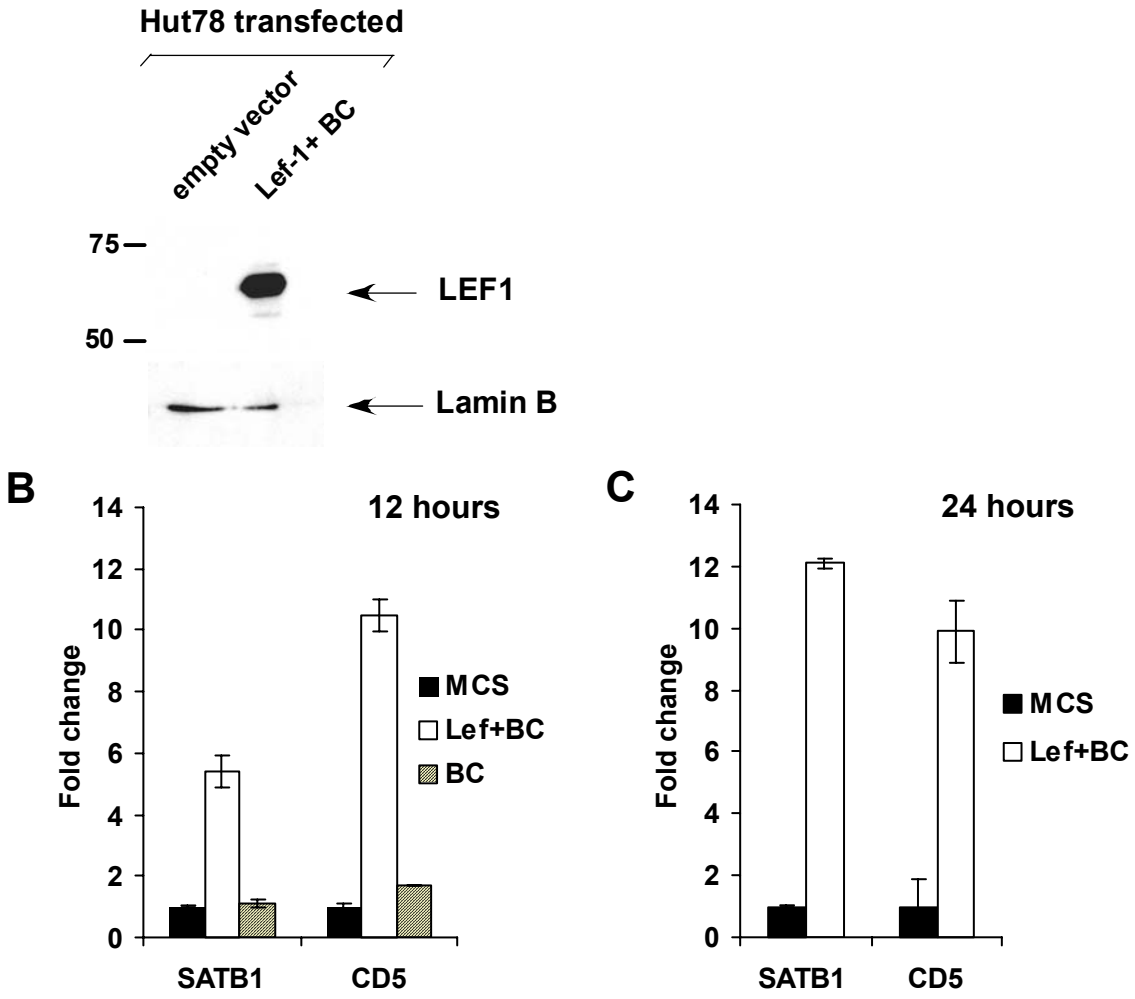


Figure 18. Regulatory effects after transfection of plasmids expressing LEF-1 and β-catenin into Hut78 cells measured by real-time PCR.
Hut78 cells were transfected with plasmids expressing LEF-1-IRES-eGFP and β-catenin or the empty plasmid IRES-eGFP (MCS) as control.
(A) The expression of LEF-1 in transfected cells was controlled by an immunoblot.
(B), (C) Endogenous SATB1 and CD5 levels were examined by real-time PCR sorting the cells 12 (B) or 24 hours (C) after transfection for GFP positive cells. CD5 and SATB1 were found activated in both cases. The levels are expressed as fold change relative to the mock transfected sample.

We examined whether LEF-1 also plays a role in the induction and whether the upregulation of RANTES is accompanied by LEF-1 downregulation. We isolated PBMCs out of 150 ml human blood and seeded them onto culture dishes. Half of the cells were induced with PHA-P (5 μg/ml), the other half was kept untreated as control group. The cells were checked for activation after 3 days by FACS measurement, staining the surface marker of the T cell marker CD3 and the activation marker CD25 (Figure 19A). The majority of the uninduced cells was

found to be not activated, whereas 81.5% of the cells were found active 3 days after induction. Cells of the induced and control group were harvested at 0, 1, 3, 5, and 7 days after the induction. The RNA levels of RANTES and LEF-1 were examined by real-time PCR in uninduced and induced cells (Figure 19B). An upregulation of RANTES could be shown starting at day 5, *Lef1* levels were very low and almost not higher than the background over the whole period, but they also showed a weak upregulation.

We ruled out the possibility that LEF-1 is regulated posttranscriptionally and is influencing RANTES levels by modifications at the protein level, as RFALT is only posttranscriptionally regulated and its RNA levels are staying constant after activation of PBMCs (Song 1999). We were performing an immunoblot against LEF-1 to determine the LEF-1 protein levels (Figure 19C). LEF-1 was hardly detectable because there is almost no protein translated in PBMCs and no obvious change in the protein levels during the activation occurred. This makes it unlikely that LEF-1 is involved in the process of the late upregulation of RANTES in PBMCs. Nevertheless, this finding is not a proof that there is no direct repression of RANTES by LEF-1, it just shows that there is no automatic upregulation of RANTES if LEF-1 is not expressed at high levels. The question of a direct effect of LEF-1 expression on RANTES repression had to be addressed in a promoter analysis.

4.2.7. RANTES promoter

LEF-1 was shown before to positively regulate target genes. Mostly LEF-1 has to interact with β-catenin to turn on genes. So far not much is known how LEF-1 can repress genes. A recent study showed that LEF-1 together with β-catenin could repress E-cadherin (Jamora 2003). But as described before, it is still unclear whether β-catenin is really necessary for active repression or just for the activation of the co-repressor. The effects reported in the E-cadherin analysis are only 2-fold,

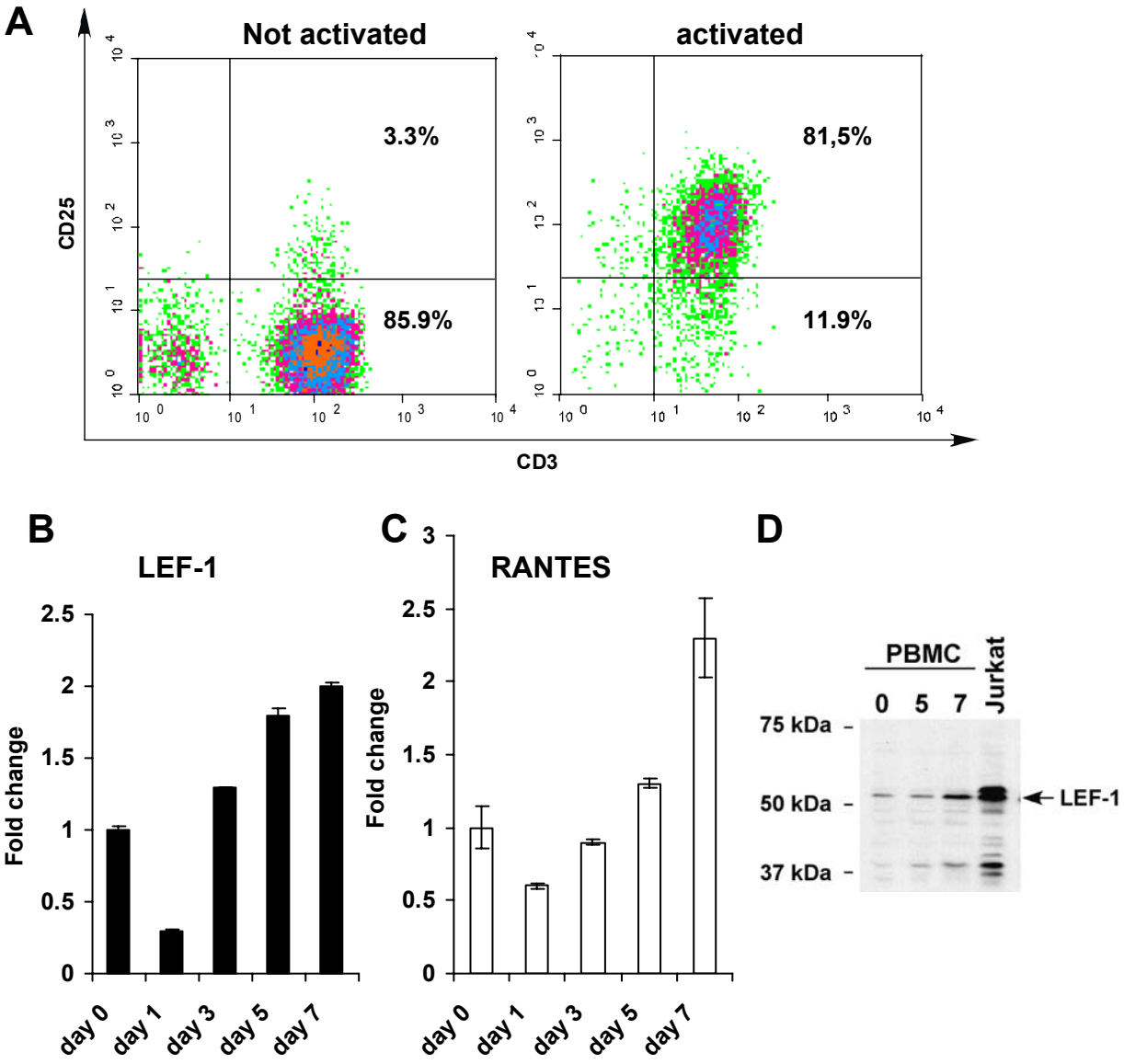


Figure 19. Control of endogenous LEF-1 and RANTES levels after stimulation of PBMCs.
PBMCs were isolated out of whole blood and stimulated with the mitogen PHA-P (5 μg/ml) for 0, 1, 3, 5, and 7 days.
(A) Control for activation of PBMCs. Three days after stimulation of the PBMCs the cells were stained for the T cell marker CD3 and the activation marker CD25. An unstimulated group was taken as control. Without stimulation only very few cells are in the activated stage, but 3 days after activation 81.5% of the cells are expressing CD25.
(B/C) Endogenous RNA levels of *Lef1* and RANTES at day 0, 1, 3, 5, 7 after activation measured by real-time PCR. LEF-1 is activated weakly after stimulation, but the RNA levels are only slightly above background. RANTES is found to be expressed at high levels and could be further activated over the time.
(C) Endogenous protein levels of LEF-1 at day 0, 5, 7 after activation detected by immunoblot. LEF-1 levels are low compared to the positive control (whole protein extract of Jurkat cells) and there is no change of the expression levels visible following activation.

therefore it is possible that a co-repressor is missing in order to obtain greater regulatory effects.

As one third of the genes found to be regulated in our T cell screen appeared to be negatively regulated by LEF-1, we examined whether the repression is directly mediated by LEF-1. To analyze this, we choose the *Rantes* gene as this is involved in many severe diseases like asthma, HIV and others and has a well characterized promoter.

The RANTES promoter was already cloned out of the mouse and out of the human genome (Nelson 1993; Danoff 1994). Human and mouse RANTES are ortholog genes that show significant sequence similarity within their coding region. However, alignment of the two promoters showed that the promoter regions are not conserved. In mouse, RANTES does not bind to the same receptors as in human (Gao 1995; Gao 1997). Facing this problem we decided to clone the human and the mouse promoter, as we did not know if the regulation only works by combining mouse cells and mouse promoter and vice versa.

4.2.7.1. Cloning of the mouse RANTES promoter

The mouse RANTES promoter (Danoff 1994) has two strong and one weak LEF-1 binding site. It was shown before that the minimal active promoter is only 175 basepairs long, but to be able to detect repression we cloned a long promoter piece consisting of 1041 basepairs including all three LEF-1 consensus motifs in front of the firefly luciferase (Figure 20A). To be able to distinguish between unspecific effects and LEF-1 depending effects on the promoter we mutated the two strong LEF-1 binding sites by site directed mutagenesis and also cloned a truncated form lacking all three LEF-1 binding sites (Figure 20B).

```
-1041 aagcttgact ctggactagg acctcaacaa agaataaggc ggagcatgat
 -991 tgaatgctaa gcatcctcct gtgcccacca ttcacacact gattgaatgt
 -941 taggcatcct cctgtgccca ccattcacac acatgcgcac atacacatgc
 -891 acacacatgc acacacacat gtacacacat gcacacacat gcacacacat
 -841 atgtacacac acatgtacac acacacatgc acacacacat gcacacacac
 -791 acacacacac acacacacac acacacacac ttttttaaa tctttatttc
 -741 cacagagatt ctttgtagcc ctatcagatc ccttgactcc tagacaaaca
 -691 gacagacaga cagacaataa atagatatta cagatagata atagatggac
 -641 atagaggaca caactctttt gttcccatct tagttactaa tgttaactct
 -591 cagatcacat gtcacacact aagtgtaagt atgcctatat ctacctagtt
 -541 atctgggaat caggattacc tggcaaattc cttacaacaa atctcctata
 -491 ccttgttaac ttattgttat caatgacaca agtgtggtct gtttctgata
 -441 tggtatgctc atgacaaata ctcttccatg gagaaaacag aaagaccaaa
 -391 atcaagtctg ggctacaact tgggaatttg ccaagtgaag accaatggct
       •
 -341 tgaccttaac tgacccctac ttgccttaag acaacagctc cctgctacct
 -291 ggagaggccc tgagtgggac ggcagatctg agggggaggg ggagggggag
 -241 gaagaaattt tcccctactg tatttggcca gagagggagt catcctggac
 -191 tggagggcag ttagaggcag agtcatactt ccaagggtga tttcagtttt
 -141 cttttccatt ttgtgttttc attttatgac agcaacaagt gtttggtgtc
  -91 ttttgtggaa actccccaag tcctggggct gggggggggc acttcctctg
  -41 ctaccctggc tcccTATAAa aggtctgcct gagctgcaga gCACCCCTTG
   10 CAGAGGACTCT GAGACAGCAC ATGCATCTC CCACAGCCTC TGCCGCGGGT
        ↓
   50 ACCATGAAGA TCTC
```

B

Wildtype –1100	gacct**caacaaag**aataaggcg
Mutant –1100	gacctcaacgaattctaaggcg
Wildtype –725	agatt**ctttgtag**ccctatcag
Mutant –725	agattccttaaggccctatcag

C

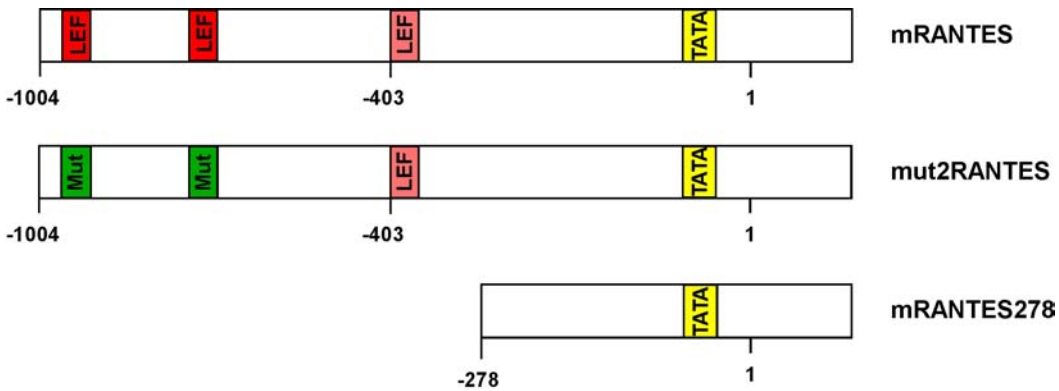


Figure 20. DNA sequence and consensus elements of the mouse RANTES promoter.
(A) Cloned mouse RANTES DNA sequence including 991 basepairs of the promoter region. Exon in capital letters; the transcription start site has been designated +1. Arrow denotes the translation start site. The TATA box at –27 has been double underlined. Bold underlined sequences represent the LEF-1 binding sites. Dot represents start site of truncated form mRANTES278.
(B) Strong LEF-1 consensus sites with the mutated sequence.
(C) Schematic diagramm of the mRANTES promoter constructs. In mut2RANTES were the two strong LEF-1 binding sites mutated (Mut), mRANTES278 is the truncated form, missing all LEF-1 binding sites.

4.2.7.2. Cloning of the human RANTES promoter

The human RANTES promoter (Nelson 1993) has even more perfect LEF-1 binding sites. Two are identical to the site found in TCRα, a gene that was shown to interact with LEF-1, and two are less perfect. We cloned the promoter piece including all four LEF-1 binding sites in front of the firefly luciferase (Figure 21A) and performed site directed mutagenesis with all four sites resulting in a four point mutated promoter (Figure 21B).

4.2.7.3. Analysis of the LEF-1 binding sites by Electro Mobility Shift Assay (EMSA)

The two major LEF-1 binding sites of the mouse promoter were analyzed for LEF-1 binding capacity using an electro mobility shift assay (EMSA). Recombinant LEF-1 was tested with the two wildtype LEF-1 binding sites and their mutants for activity (Figure 20B; 22A). It could be shown that LEF-1 binds to the wildtype sites whereas for the mutated sites no binding could be detected. LEF-1 binds to site –725 with almost comparable strength as to the TCRα positive control, whereas the binding to site –1100 is detectable but not as strong as seen for the other site or the positive control.

We also tested the binding capacity of LEF-1 to the sites of the human promoter. As site –942 and –376 are identical and sites –667 and –209 are almost the same, we tested sites –942 and –676 as representatives (Figure 21B; 22B). LEF-1 was shown to bind with at least the same strength to site –942 as to TCRα, whereas the binding to site –667 was only weakly detectable. The mutated site –942 was bound poorly by LEF-1, approximately to the same extent as site –667. The remaining binding activity can be explained with another less perfect LEF-1 binding site (-934) next to the mutated perfect match consensus site.

A direct regulation of the RANTES promoter through LEF-1 seems possible, because LEF-1 can bind in the promoter region.

```
-961 gtcgaggatc cctaaagtcc tttgaagctt tcatattctg taacttttgt
-911 gccaagaagg ccttacagtg agatgggatc ccagtattta ttgagtttcc
-861 tcattcataa aatggggata ataatagtaa atgagttgac acgcgctaag
-811 acagtggaat agtggctggc acagataagc cctcggtaaa tggtagccaa
-761 taatgataga gtatgctgta agatatcttt ctctccctct gcttctcaac
-711 aagtctctaa tcaattattc cactttataa acaaggaaat agaactcaaa
-661 gacattaagc acttttccca aaggtcgctt agcaagtaaa tgggagagac
-611 cctatgacca ggatgaaagc aagaaattcc cacaagagga ctcattccaa
-561 ctcatatctt gtgaaaaggt tcccaatgcc cagctcagat caactgcctc
-511 aatttacagt gtgagtgtgc tcacctcctt tggggactgt atatccagag
-461 gaccctcctc aataaaacac tttataaata acatccttcc atggatgagg
-411 gaaaggagat aagatctgta atgaataagc aggaactttg aagactcagt
-361 gactcagtga gtaataaaga ctcagtgact tctgatcctg tcctaactgc
-311 cactccttgt tgtccccaag aaagcggctt cctgctctct gaggaggacc
-261 ccttccctgg aaggtaaaac taaggatgtc agcagagaaa tttttccacc
-211 attggtgctt ggtcaaagag gaaactgatg agctcactct agatgagaga
-161 gcagtgaggg agagacagag actcgaattt ccggaggcta tttcagtttt
-111 cttttccgtt ttgtgcaatt tcacttatga taccggccaa tgcttggttg
 -61 ctattttgga aactcccctt aggggatgcc cctcaactgg ccctataaag
 -11 ggccagcctg aGCTGCAGAG CGATTCCTGC ACGAGGATCA AGCACAGCA
```

B

Wildtype -942	5′ tccctaaagtcctttgaagctttcatattc 3′
Mutant -942	5′ tccctaaagtgaattcaagctttcatattc 3′
Wildtype -667	5′ aggaaatagaactcaaagacattaagcac 3′
Mutant -667	5′ aggaaatagaactgaattccattaagcac 3′
Wildtype -376	5′ aataagcaggaactttgaagactcagtgactca 3′
Mutant -376	5′ aataagcaggagaattcaagactcagtgactca 3′
Wildtype -210	5′ attggtgcttggtcaaaggaaactgat 3′
Mutant -210	5′ attggtgcttggtgaattcggaaactgat 3′

C

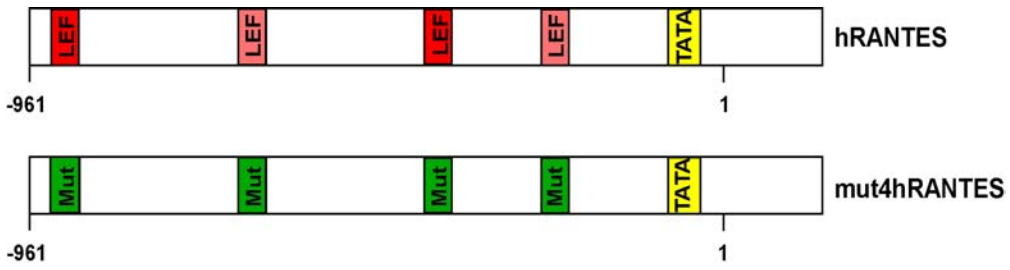


Figure 21. DNA sequence and consensus elements of the human RANTES promoter.
(A) Part of the human RANTES gene including 961 basepairs of the promoter. Exon in capital letters. The transcription start site has been designated +1. Bold, underlined sequences represent the LEF-1 binding sites.
(B) LEF-1 consensus sites with the mutated sequence.
(C) Schematic diagram of the hRANTES promoter constructs. In mut4hRANTES were all four LEF-1 binding sites mutated (Mut).

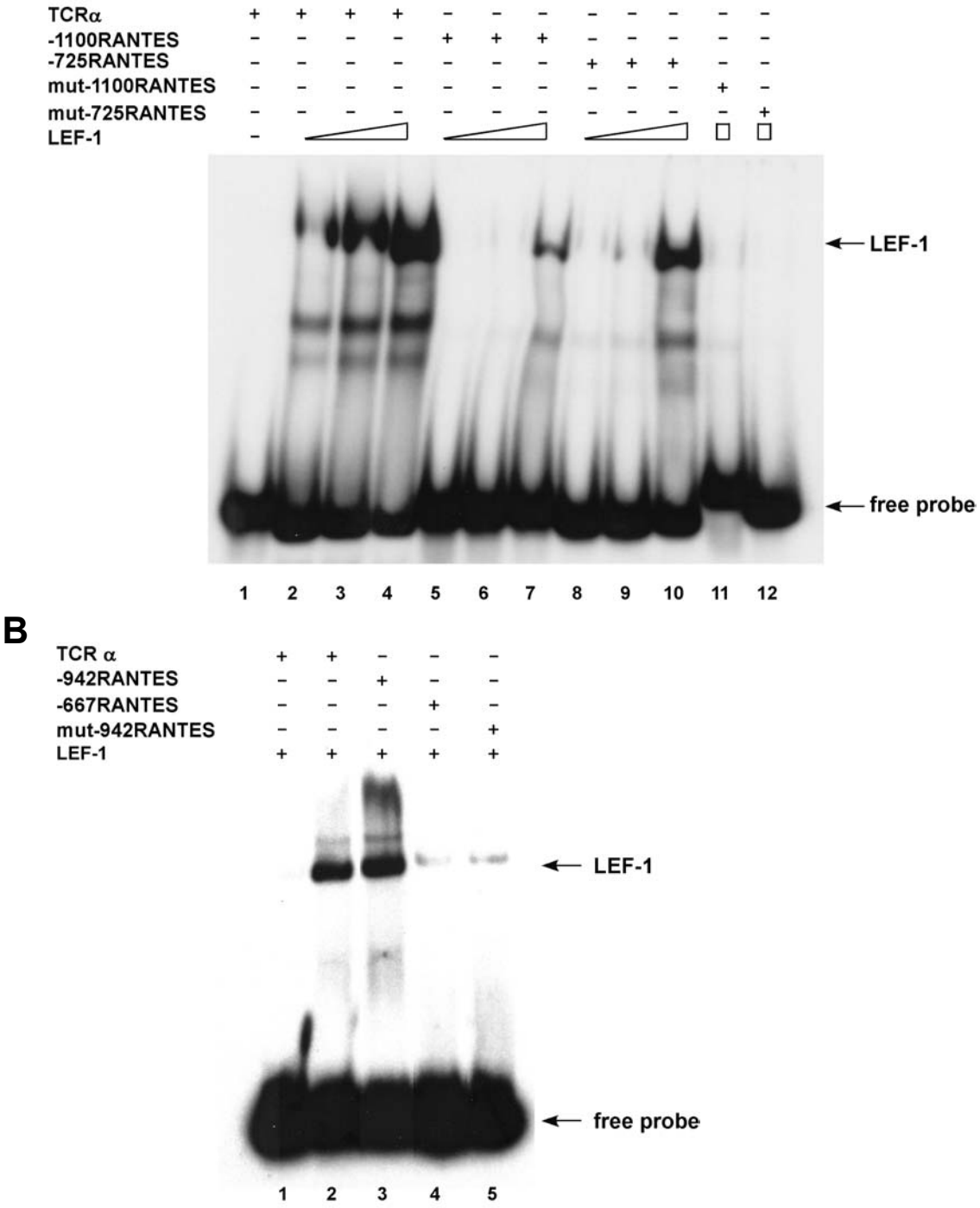


Figure 22. EMSA to determine binding capacity of LEF-1 to the binding sites in the mouse and human RANTES promoter.

(A) Oligo-nucleotides encompassing a perfect LEF-1 consensus site (TCRα) and the two good LEF-1 binding sites (-1100, -725) of the mouse RANTES promoter were labeled with ^{32}P. 30, 50, and 100 ng of recombinant LEF-1 were used together with 10000 cpm of the probe to test for binding capacity of the two binding sites in comparison to the control. LEF-1 binds to the –725 consensus motif as good as to the control, –1100 is found to be bound with lower capacity. No binding could be detected for the mutated sites.

(B) The binding of LEF-1 to two representative sites of the human RANTES promoter (site –942, site –667) was tested. The probes were labeled with ^{32}P and 30 ng of recombinant LEF-1 were used together with 10000 cpm of the probe for the band shift. With site –942 LEF-1 shows at least equal binding strength as with TCRα, but the site –667 was only weakly bound. LEF-1 is still able to shift the mutated site –942, probably due to another minor LEF-1 consensus site next to the mutated one.

4.2.7.4. Transfection assays to test LEF-1 responsiveness of the RANTES promoter

The cloned mouse and human promoter driving the firefly luciferase were tested for activity in Jurkat cells. Both promoters were found to be active (data not shown). Therefore, we conducted the next experiments concentrating on the mouse promoter. As shown before, LEF-1 is expressed at high levels in Jurkat cells. We first compared the basal levels of the wildtype mouse promoter and the mut2RANTES mouse promoter (Figure 23A). If LEF-1 alone is responsible for the repression, we could expect higher levels for the mutated promoter. 1 μg of the mouse wildtype and the mouse mutated RANTES promoter were transfected into Jurkat cells and the luciferase activity was measured. The experiment was conducted in duplicate and repeated several times. The mutated promoter behaved the opposite as we expected and it was found to have a lower basal level than the wildtype one. This could be due to the absence of interaction partners, necessary for the repression. Next we tested the effect of *Lef1*, β-catenin or both on the mouse wildtype RANTES promoter in Jurkat cells (Figure 23B). After β-catenin expression we could detect an upregulation, less than 1.5-fold that was pronounced if we coexpressed LEF-1. As this regulation was not strengthened when we were transfecting more β-catenin and never exceeded 1.7-fold, we were accounting this effect as not significant. After transfection of higher amounts of LEF-1 together with β-catenin, a repression could be detected, getting stronger when we were expressing more of β-catenin but not exceeding 1.8-fold. As in this effect the increasing amounts used are reflected in the repression pattern, we concluded that the weak repression might be real and went on with the analysis of the RANTES promoter.

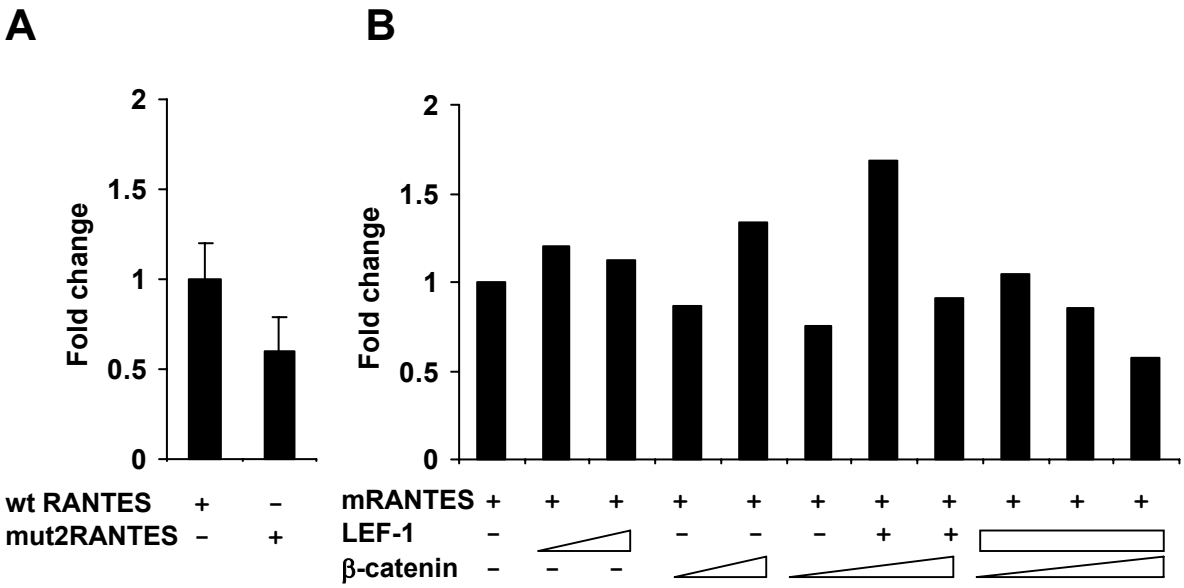


Figure 23. Mouse RANTES promoter analysis in Jurkat cells.
(A) Promoter activity of the wildtype and the double mutant mouse RANTES promoter (mut2 RANTES). Electroporation was used to transfect Jurkat cells with two different RANTES promoter-luciferase constructs in the pGL-3 vector backbone. Cells were transfected with 1 μg of each plasmid and luciferase activity was determined 36 hours after transfections. Luciferase activity is expresses as fold change relative to the activity of the wildtype promoter. The mutated promoter showed a lower basal level compared to the wildtype promoter.
(B) LEF-1 can repress RANTES promoter activity by 1.8-fold. 1 μg mRANTES reporter construct were transfected together with increasing amounts of expression constructs encoding for LEF-1 (1, 3 μg) and β-catenin (1, 3, 10 μg). The luciferase activity is expressed as fold change relative to the level of luciferase activity from cells transfected with the promoter alone. No significant effect could be detected with LEF-1 and β-catenin expression alone. Co-expression showed a weak repression of the promoter activity.

Since Jurkat cells have high levels of endogenous LEF-1 and are also expressing β-catenin, we were choosing Hut78 cells for further analysis of the RANTES promoter, because those cells express almost no LEF-1 as shown previously. The human and the mouse RANTES promoter were also active in this cell line, even at a 2- to 3-fold higher level than in Jurkat cells (data not shown). We co-transfected the cells with *Lef1*, β-catenin, and both of them, using the mouse and the human RANTES wildtype promoter to rule out the possibility that the mouse promoter is not getting regulated in a human environment. With the mouse RANTES promoter no significant changes could be detected (Figure 24A), whereas with the human promoter a weak (less than two fold) but reproducible downregulation could be seen when LEF-1 and β-catenin were both expressed (Figure 24B).

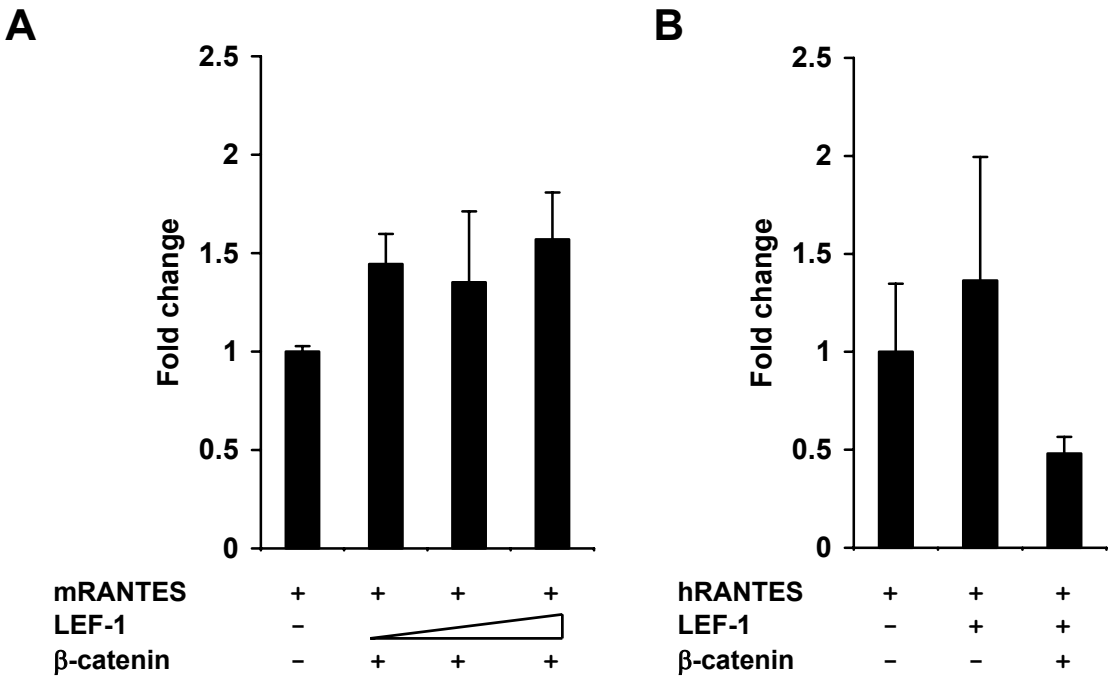


Figure 24. RANTES promoter analysis in Hut78 cells.
Luciferase activities are expressed as fold changes relative to the promoter alone.
(A) Mouse RANTES promoter activity is not affected by LEF-1 and β-catenin expression. Hut78 cells were transfected by electroporation with 1 μg of the reporter construct together with LEF-1 (1, 3, 10 μg) and β-catenin (10 μg).
(B) Human RANTES promoter activity is weakly repressed by LEF-1 and β-catenin. Hut78 cells were transfected by electroporation together with LEF-1 (1 μg) and β-catenin (5 μg).

We also wanted to test the activity of the promoter in non-lymphoid cells. Therefore, we performed a reporter assay in NIH3T3 cells that do not express LEF-1 but are positive for RANTES. As these are mouse cells we were using the mouse promoter and also the truncated form to rule out any LEF-1 dependent effects. Transfection of the RANTES promoter together with only *Lef1* and in combination with β-catenin resulted in an upregulation of the full length promoter, whereas the effect was pronounced if only *Lef1* was used. No changes were detectable following the transfection of β-catenin alone. For the truncated form we could also detect upregulation following *Lef1* transfection, showing that the upregulating event is not mediated directly by the LEF-1 consensus sites. LEF-1 seemed to act on another gene, specific or not, that could after activation turn on the RANTES promoter. This finding was not contradictionary to the fact that LEF-1

normally represses RANTES, as the upregulation will probably never occur *in vivo* simply due to the fact, that LEF-1 is normally never expressed in fibroblasts.

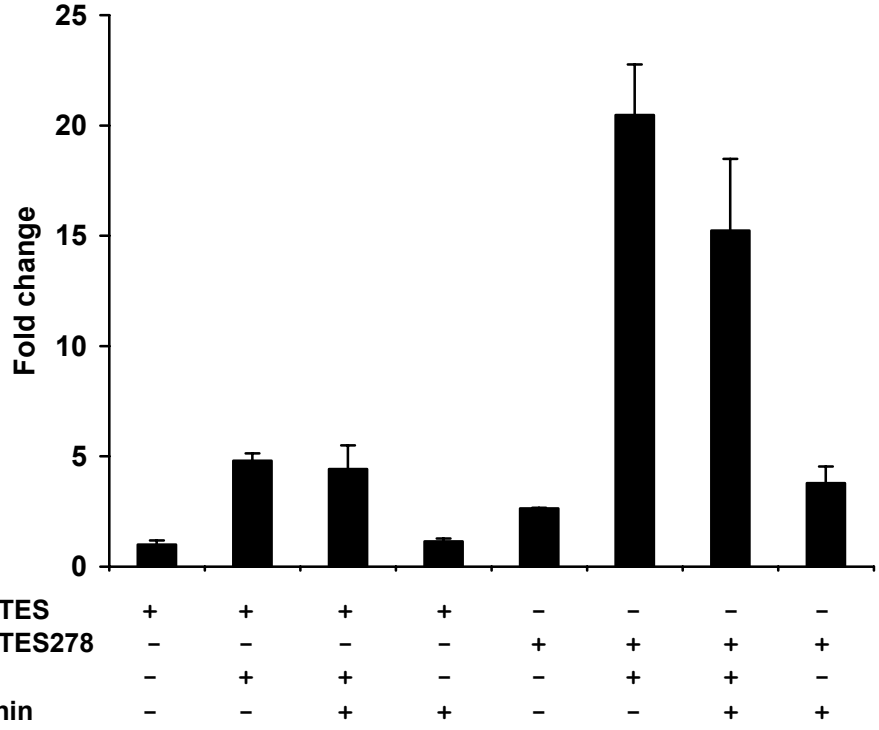


Figure 25. Mouse RANTES promoter analysis in NIH3T3 cells.
NIH3T3 fibroblasts were transfected by $CaCl_2$ with 100 ng of the mRANTES promoter or the truncated form (mRANTES278) together with plasmids expressing LEF-1 (100 ng) and β-catenin (1 μg) in combination or alone. Luciferase activity is expressed as fold changes relative to the promoter alone. For both promoters we could detect activation, pronounced after cotransfection of *Lef1* alone.

Although we could not detect any changes in the LEF-1 levels after activation of PBMCs, we wanted to test the human RANTES promoter in primary T cells, as sometimes effects are more obvious in untransformed cells. The PBMCs were purified out of whole blood and split into two groups. One group was kept as control group and stayed untreated, the other group we activated with the mitogen PHA-P (5 μg/ml). After 24 hours of activation the cells were transfected with the hRANTES promoter. The control and induced group were harvested 36 hours post transfection and luciferase activity was measured (Figure 26A). In the control cells the promoter was not active, corresponding to the fact, that at this stage RANTES is not expressed. In activated cells the promoter showed activity. We further analyzed the regulatory processes in activated PBMCs by investigating the effects

of LEF-1 and β-catenin on the wildtype and the mutated human RANTES promoter. To distinguish between regulatory processes conducted by LEF-1 alone and regulations caused by the interaction of LEF-1 and β-catenin we were transfecting the m5-mutant of LEF-1, missing the β-catenin interaction domain, and LEF-1 together with β-catenin. The analysis revealed that m5LEF-1 could repress the wildtype promoter reproducible almost 2-fold, whereas activation is seen for the mut4hRANTES promoter (Figure 26B). This can be explained by the fact that there are indirect activating effects following *Lef1* transfection as seen in the NIH3T3 reporter assay. Nevertheless, the lymphoid cells probably express the needed co-repressor, in contrast to NIH3T3 cells, so that the positive secondary upregulation effecting the wildtype RANTES promoter in PBMCs can be overruled with LEF-1 expression. Mutation of the LEF-1 consensus sites resulted in the loss of the repression and the indirect activating effect was visible. Transfection of LEF-1 and β-catenin resulted in a 5-fold decrease of the wildtype promoter and a 2-fold of the mutated. The downregulation of the mutated promoter can be explained with two factors. First is one weak LEF-1 binding site still intact in the promoter as shown with the EMSA, where the mutated site was still bound by LEF-1. Secondly caused *Lef1*/β-catenin coexpression also a downregulation of the β-galactosidase readout, arguing that the effect is probably due to lower proliferation rates or onset of apoptosis.

Taken together we can say that the regulation of RANTES is directly regulated by LEF-1 as we could detect clear differences between the wildtype and the mutated human RANTES promoter in PBMCs following LEF-1 expression.

4.2.7.5. Transfection assays to test the effect of the co-repressor Groucho-1 together with LEF-1 on the RANTES promoter

Groucho is a co-repressor that is known to interact with LEF/TCF proteins and helps to repress genes. It can also interact with other DNA binding proteins, such

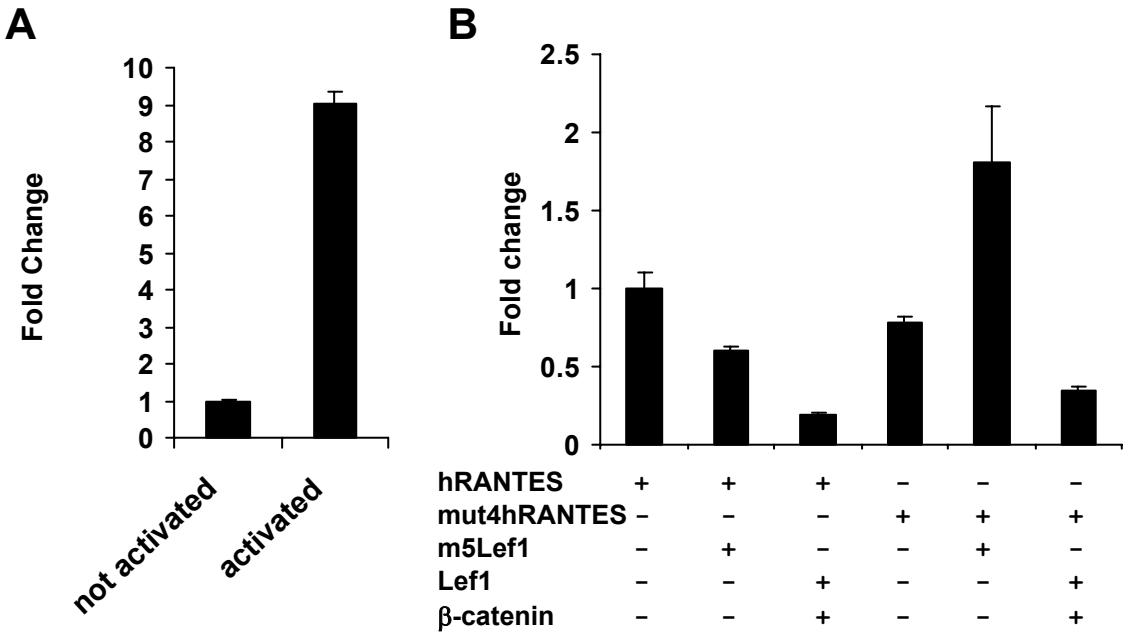


Figure 26. Activity of the RANTES promoter in PBMCs.
(A) PBMCs were isolated out of whole blood and seeded onto culture plates. One half was activated with the mitogen PHA-P (5 μg/ml), the other half was kept untreated as control. 24 hours after activation the cells were transfected with 5 μg hRANTES promoter by electroporation. 36 hours after the transfection the cells were harvested and luciferase activity was measured. The levels are expressed as fold change relative to the level of luciferase from untreated cells. No activity was detected for the promoter transfected into untreated cells, but the promoter was active in induced cells.
(B) Activated PBMCs were transfected 5 days after induction by electroporation with 2 μg of the wildtype (hRANTES) or mutated (mut4hRANTES) hRANTES promoter together with plasmids encoding m5LEF-1 (2 μg), LEF-1 (6 μg), and b-catenin (10 μg). The levels are expressed as fold change relative to the level of luciferase from promoter alone. Repression of the wildtype promoter could be detected after transfection of *m5Lef1* or *Lef1*/β-catenin. The mutated promoter was shown to get activated by m5LEF-1 and a modest downregulation is seen after LEF-1 and β-catenin expression.

as Hairy, Engrailed, and Dorsal (Cavallo 1998). To test whether the downregulation of RANTES can be enforced by the interaction of LEF-1 with Groucho-1, we performed a reporter assay using the mouse RANTES promoter in M12 cells, mature B cells that do not express LEF-1, by adding LEF-1 and β-catenin and increasing amounts of Groucho-1. Again we could show a minor downregulation of the promoter after LEF-1 expression, but an up to 10-fold downregulation is detectable if Groucho is coexpressed. But the repression is also observed using Groucho-1 alone (Figure 27). Mouse RANTES promoter reacts on Groucho-1 alone and LEF-1 is not necessary to mediate the repression, so the effect is not mediated through LEF-1. The same assay was conducted in activated

PBMCs but Groucho-1 showed no specific effect (data not shown), implicating that the effect observed in M12 cells is not occurring in general, but only appearing in special cell types. Therefore, we can say that RANTES is repressed directly by LEF-1, probably through an interaction with a specific lymphoid co-repressor, but Groucho was not found to participate.

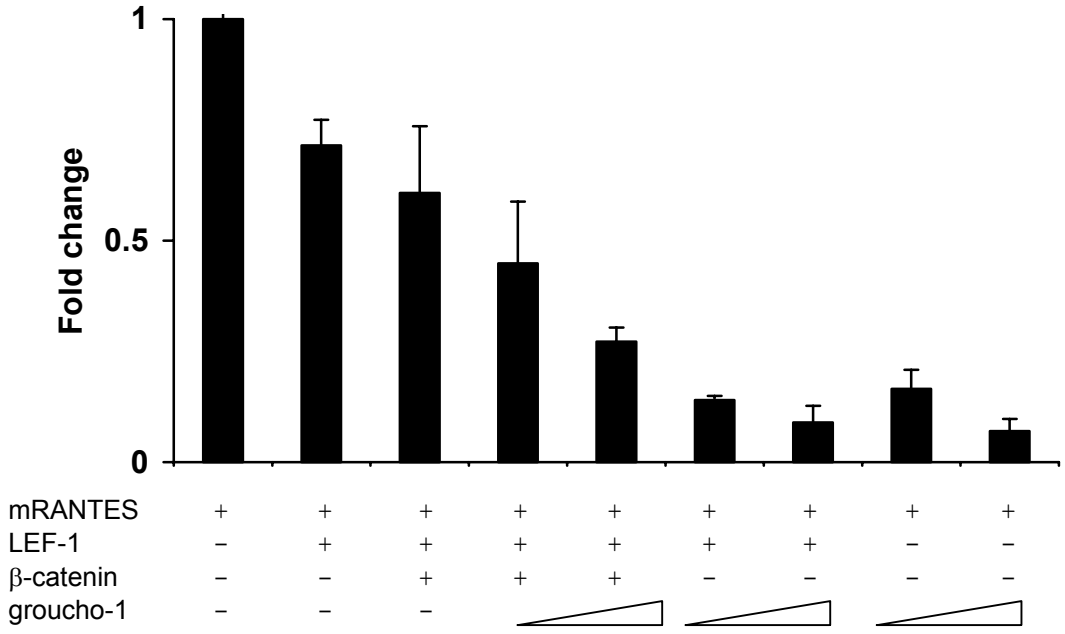


Figure 27. Groucho-1 represses the RANTES promoter. M12 cells were transfected with 1 μg mouse RANTES promoter by electroporation together with expression plasmids encoding for LEF-1 (2 μg), β-catenin (5 μg), and increasing amounts of groucho-1 (1, 3 μg). Luciferase activities are expressed as fold change relative to the luciferase activity of the promoter alone. LEF-1 alone and in combination with β-catenin gives only minor effects on the RANTES promoter, whereas addition of groucho-1 results in a drastic downregulation. But the regulatory effect does not seem to be mediated by LEF-1 as Groucho-1 can repress the RANTES promoter on its own.

4.3. Skin

4.3.1. Microarray analysis of skin of E 16.5 wildtype, *Lef1*$^{-/-}$, and *Lef1*$^{m5/m5}$ embryos

4.3.1.1. Probe array hybridization

In our attempt to determine the effects of LEF-1 and search for LEF-1 target genes, we wanted to be able to distinguish between effects of LEF-1 arising from the canonical Wnt pathway, and regulations apart of the canonical Wnt signaling, where the interaction with β-catenin is not important. For this purpose a mouse carrying a mutation in the β-catenin interaction domain of LEF-1 (*Lef1*$^{m5/m5}$) was created in our lab (W. Roth, unpublished data). It was shown by an electro mobility shift analysis, that the *Lef1*$^{m5/m5}$ mouse can still produce a form of LEF-1 that can bind to its consensus sites, but no interaction of LEF-1 with β-catenin is possible. The analysis of the *Lef1*$^{m5/m5}$ mice revealed that the phenotype is similar to the one observed in *Lef1*$^{-/-}$ mice. The *Lef1*$^{m5/m5}$ embryos show also a pointed snout and the same lethality as observed for *Lef1*$^{-/-}$ mice. Nevertheless, the phenotype of the skin of *Lef1*$^{m5/m5}$ mice is altered as in comparison to *Lef1*$^{-/-}$ mice and reveals more the phenotype of wildtype mice. *Lef1*$^{-/-}$ mice show, as described before, no mature hair follicle. This is due to a developmental block at embryonic stage E17. In contrast to the *Lef1*$^{-/-}$ mice, the *Lef1*$^{m5/m5}$ mice show almost normal hair follicle development, just in slightly reduced numbers. To further examine the differences between the *Lef1*$^{m5/m5}$, *Lef1*$^{-/-}$, and wildtype mice, and to uncover effects mediated by LEF-1 without the interaction with β-catenin, we were performing an Affymetrix microarray analysis of skin from wildtype, *Lef1*$^{-/-}$, and *Lef1*$^{m5/m5}$ E16.5 embryos in order to get more insight into possible target genes. With this analysis we also hoped to encircle the question of repressive effects of LEF-1 and the mechanisms underlying the whole process.

Whole skin of E16.5 embryos of wildtype, *Lef1*$^{-/-}$, and *Lef1*$^{m5/m5}$ was prepared and total RNA isolated. The RNA was used for microarray analysis with MGU74Avs2 probe arrays of Affymetrix. The screen of wildtype and *Lef1*$^{-/-}$ was repeated three times with total RNA from different embryos and the labeled RNA was hybridized to the probearrays, whereas the *Lef1*$^{m5/m5}$ analysis was repeated four times with total RNA from different embryos. The microarrays were stained and scanned in the Affymetrix fluidics station and scanner.

4.3.1.2. Analysis of the probe arrays

To analyze differences between wildtype and *Lef1*$^{-/-}$ skin, and between wildtype and *Lef1*$^{m5/m5}$ skin, comparisons for each pair of microarrays were performed, using the wildtype as baseline. For the differences between *Lef1*$^{-/-}$ and *Lef1*$^{m5/m5}$ skin we used *Lef1*$^{m5/m5}$ as the baseline, leading us to genes, which are regulated by LEF-1 but independently of β-catenin. Setting the criteria to an average of at least 3-fold differences in 6 out of 9 comparisons, we could detect 47 genes differentially regulated within the *Lef1*$^{-/-}$ wildtype comparisons, 6 of them being repressed, and 39 activated by LEF-1 (Figure 28A, D). For the *Lef1*$^{m5/m5}$/wildtype comparison, where we analyzed β-catenin dependent activation and repression, we were choosing genes that were in average at least 3-fold regulated in 7 out of the 12 comparisons. We obtained 86 genes regulated, 8 of them displaying a β-catenin dependent repression and 78 showing β-catenin dependent activation (Figure 28B, D). For the comparison between *Lef1*$^{-/-}$/*Lef1*$^{m5/m5}$ we could identify 52 genes, in average at least 3-fold regulated in 6 out of 12 comparisons. 7 of the genes displayed β-catenin independent activation, 45 β-catenin independent repression (Figure 28C, D). As at least some of the targets found in the two screens mutant against wildtype should be overlapping, we were searching for genes identified in both comparisons, the comparison *Lef1*$^{-/-}$/wildtype and the comparison *Lef1*$^{m5/m5}$/wildtype. 28 genes were identified in both analyses,

indicating that the results we obtained are not random regulations but real LEF-1 targets.

A

***Lef1*$^{-/-}$ vs wildtype**

ID#	FC	Count	Description
1	8.3	7	XXX
2	4.5	7	XXX
3	4.5	7	XXX
4	3.6	8	XXX
5	3.1	6	XXX
6	3.1	9	XXX
7	-2.8	6	XXX
8	-2.9	6	XXX
9	-2.9	9	XXX
10	-2.9	8	XXX
11	-3.1	9	XXX
12	-3.4	9	XXX
13	-3.4	9	XXX
14	-3.6	9	XXX
15	-3.6	6	XXX
16	-3.6	7	XXX
17	-3.6	6	XXX
18	-3.7	9	XXX
19	-3.7	6	XXX
20	-3.7	7	XXX
21	-3.7	6	XXX
22	-3.9	6	XXX
23	-3.9	6	XXX
24	-4.0	6	XXX
25	-4.0	9	XXX
26	-4.0	6	XXX
27	-4.1	9	XXX
28	-4.2	9	XXX

29	-4.2	6	XXX
30	-4.3	6	XXX
31	-4.3	7	XXX
32	-4.4	9	XXX
33	-4.9	9	XXX
34	-5.0	9	XXX
35	-5.0	6	XXX
36	-5.4	8	XXX
37	-5.8	6	XXX
38	-5.9	9	XXX
39	-6.5	7	XXX
40	-7.0	8	XXX
41	-7.2	7	XXX
42	-7.4	9	XXX
43	-7.5	7	XXX
44	-7.7	6	XXX
45	-15.0	9	XXX
46	-15.5	9	XXX
47	-17.1	9	XXX

B

Lef1$^{m5/m5}$ vs wildtype:

ID#	FC	Count	Descriptions
1	10.3	7	XXX
48	9.8	7	XXX
49	5.0	11	XXX
50	4.8	7	XXX
6	4.3	7	XXX
51	3.7	7	XXX
52	3.5	8	XXX
53	3.1	9	XXX
54	-2.8	8	XXX
55	-2.8	10	XXX

56	-2.8	8	XXX
57	-2.8	8	XXX
58	-2.8	8	XXX
43	-2.8	8	XXX
59	-2.8	7	XXX
60	-2.9	8	XXX
61	-2.9	8	XXX
62	-2.9	7	XXX
63	-2.9	8	XXX
64	-3.0	7	XXX
65	-3.1	7	XXX
66	-3.2	8	XXX
67	-3.2	7	XXX
68	-3.2	8	XXX
69	-3.2	8	XXX
70	-3.3	7	XXX
42	-3.3	11	XXX
71	-3.3	8	XXX
72	-3.3	9	XXX
28	-3.4	12	XXX
19	-3.4	10	XXX
73	-3.4	8	XXX
13	-3.5	9	XXX
74	-3.5	7	XXX
18	-3.6	12	XXX
75	-3.6	8	XXX
21	-3.6	7	XXX
76	-3.6	7	XXX
77	-3.6	8	XXX
78	-3.7	8	XXX
79	-3.7	7	XXX
80	-3.7	8	XXX
81	-4.0	8	XXX
82	-4.0	8	XXX
7	-4.1	10	XXX
83	-4.1	7	XXX

84	-4.3	9	XXX
85	-4.4	8	XXX
86	-4.4	8	XXX
87	-4.5	7	XXX
40	-4.6	9	XXX
88	-4.7	7	XXX
36	-4.7	11	XXX
27	-4.7	7	XXX
89	-4.9	8	XXX
90	-4.9	7	XXX
91	-5.0	7	XXX
92	-5.1	7	XXX
93	-5.2	9	XXX
94	-5.4	10	XXX
95	-5.8	8	XXX
37	-6.0	8	XXX
96	-6.0	7	XXX
38	-6.2	10	XXX
97	-6.5	7	XXX
15	-6.5	8	XXX
98	-6.6	7	XXX
99	-6.6	7	XXX
25	-7.2	7	XXX
100	-7.3	9	XXX
101	-7.3	7	XXX
29	-7.3	8	XXX
41	-7.5	10	XXX
35	-7.9	8	XXX
11	-8.6	9	XXX
102	-8.8	7	XXX
103	-8.8	7	XXX
34	-9.1	8	XXX
104	-9.1	7	XXX
105	-10.3	7	XXX
106	-11.0	7	XXX
47	-13.4	12	XXX

46	-14.6	12	XXX
107	-15.2	7	XXX
45	-24.3	11	XXX
108	-46.2	7	XXX

C

Lef1$^{-/-}$ vs *Lef1*$^{m5/m5}$

ID	FC	Count	Description
109	13.1	6	XXX
110	9.6	6	XXX
111	8.3	6	XXX
112	8.1	6	XXX
113	7.7	6	XXX
114	7.5	6	XXX
115	7.4	6	XXX
116	6.7	6	XXX
117	6.5	6	XXX
118	6.5	6	XXX
119	4.3	7	XXX
120	4.3	6	XXX
121	4.2	6	XXX
122	4.2	7	XXX
123	4.2	6	XXX
124	4.0	6	XXX
125	4.0	8	XXX
126	3.9	6	XXX
127	3.9	6	XXX
128	3.9	6	XXX
129	3.8	6	XXX
130	3.8	6	XXX
131	3.7	6	XXX
132	3.7	6	XXX
133	3.7	10	XXX

134	3.6	6	XXX
135	3.6	6	XXX
136	3.6	6	XXX
137	3.6	6	XXX
138	3.6	6	XXX
139	3.4	6	XXX
140	3.3	6	XXX
141	3.3	6	XXX
142	3.3	6	XXX
143	3.2	7	XXX
144	3.2	6	XXX
145	3.2	6	XXX
146	3.1	6	XXX
147	3.0	6	XXX
148	3.0	6	XXX
149	2.9	6	XXX
150	2.9	6	XXX
151	2.9	6	XXX
152	2.8	6	XXX
153	2.8	6	XXX
154	-3.2	6	XXX
155	-3.5	6	XXX
156	-4.0	6	XXX
157	-4.3	11	XXX
158	-8.8	7	XXX
159	-8.8	8	XXX
160	-20.0	12	XXX

D

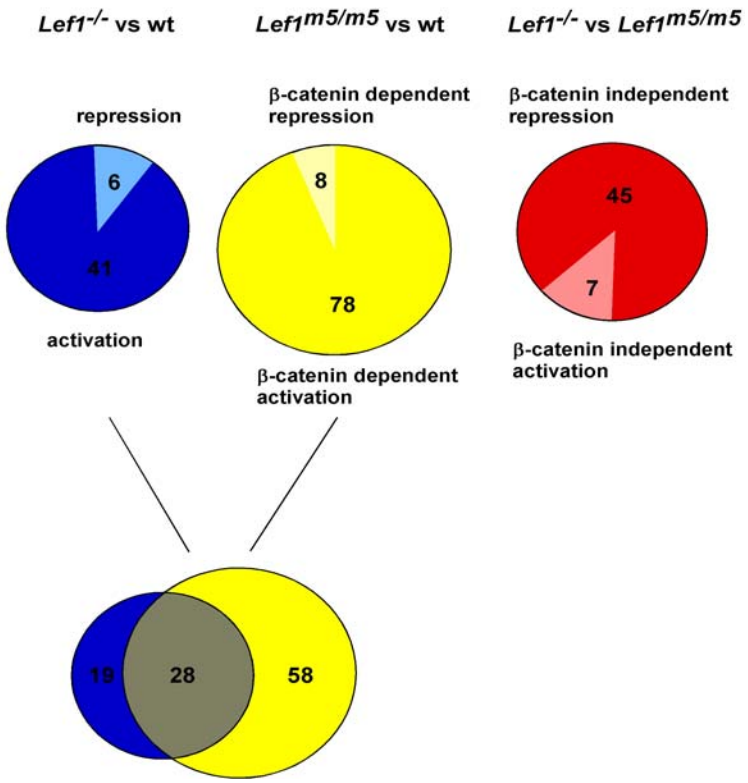


Figure 28. Identified target genes of the skin microarray screen.
Accession number (Acc. #) of the identified genes, fold change (FC) as level of regulation, count, displays in how many comparisons the gene was found to be regulated; name of the gene (description) are indicated
(A) Comparison of *Lef1*$^{-/-}$ versus (vs) wildtype. 41 genes were found to be activated through LEF-1 (negative FC), 6 repressed (positive FC).
(B) Comparison of *Lef1*$^{m5/m5}$ vs wildtype. 76 genes were found to be β-catenin dependent activated (negative FC), 8 β-catenin dependent repressed (positive FC).
(C) Comparison of *Lef1*$^{-/-}$ vs *Lef1*$^{m5/m5}$. 45 genes were identified to be β-catenin independent repressed (positive FC), 7 β-catenin independent activated.
(D) Schematic overview of the distribution of the different comparisons.

The microarray analyses of the three different mouse lines revealed, that many genes are regulated by LEF-1 without the interaction of β-catenin. Those genes are mainly genes that get repressed by LEF-1, whereas the majority of genes activated by LEF-1 are regulated through the canonical Wnt pathway.

5. Discussion

In this analysis we demonstrate that a large number of genes is dependent on LEF-1 expression. We were able to identify genes that are positively and negatively regulated by LEF-1.

5.1. Comparison of the different approaches, used for our LEF-1 target gene screens

We could provide evidence that a great variety of genes is regulated by LEF-1 using three different approaches in our search for LEF-1 target genes. This can be explained on the one hand with differences in the expression patterns of the identified target genes that vary throughout the tissues, so that a number of genes expressed in skin, is for example not expressed in lymphocytes and vice versa. On the other hand it may also be a result of different expression patterns of required co-activators or co-repressors. But by searching for LEF-1 target genes in T cells and skin, the analysis already covered very different tissues and their profiles.
The T cell screen was carried out with sorted ISP $CD8^+$ cells, helping to minimize unspecific effects that are only due to different proportions of the compartments what was a great advantage of this approach.
In our skin analysis we could not reduce the screen to a distinct cell population as the skin of E16.5 embryos is too small to be sectioned and analyzed separately. But as the skin of the *Lef1*$^{m5/m5}$ mutants displays a phenotype closely related to the wildtype phenotype, we could neglect those minor effects. For the *Lef1*$^{-/-}$ skin however we had to keep in mind, that some of the genes we wanted to look at are probably not expressed in this mutant skin, as the hair follicles are almost completely absent. The great advantage of the skin screen was clearly, that we could distinguish between β-catenin dependent and β-catenin independent targets, as we were using a mouse cell line mutant for the β-catenin interaction domain.

Those two loss-of-function experiments were complemented with a gain-of-function experiment, where a constitutive active form of LEF-1, a fusion of LEF-1 to the C-terminal part of β-catenin, was overexpressed in NIH3T3 fibroblasts under controlled conditions. Although the target genes identified in the NIH3T3 screen depend to some extend on co-activators/repressors that are present in NIH3T3 cells, this system displays definitely the most cell type independent analysis of the three chosen approaches. Another advantage of the cell culture system was, that we were able to limit the expression of LEF-1 to a short period of time (eight hours), what helped to minimize the number of secondary targets.

5.2. Criteria for selection of potential targets

The Affymetrix chip technology is very reproducible and has an intern control for unspecific hybridization, consisting of mismatch oligo-nucleotides, where the intensity of the perfect match oligo-nucleotide is compared to the mismatch oligo-nucleotides, having one base of the 21 bases exchanged against another. This method ensures reproducible results. The analyses are based on differentially expressed genes that differ by a factor of three or more. This changes should be significant because a number of genes shows already a functional significant change if they are expressed only mono- instead of bi-allelic. Furthermore, the experiments were done in multiplex and the regulation had to occur in more than half of the comparisons.

5.3. Model for repression mediated through LEF-1

The mechanism underlying the repressive effects of LEF-1 is not completely understood until today. One recent study showed that LEF-1 together with β-catenin represses E-cadherin (Jamora 2003). Fuchs and coworkers could provide evidence that E-cadherin could not only be downregulated by LEF-1 in the

presence of a constitutively stable β-catenin, but that the downregulation of the promoter, examined with a promoter analysis, occurred only after LEF-1 and β-catenin coexpression. However, they could not exclude, that *in vivo*, the ability of LEF-1 and β-catenin to downregulate *E-cadherin* mRNA expression may be indirect. The model of an indirect effect is further supported by findings in the chicken feather bud development (Houghton 2003). In this case β-catenin is only needed for the onset of Groucho, an identified co-repressor of LEF-1, but not for the active process of repression. This could be shown with co-localization studies of β-catenin and Groucho. They co-localize only before the repression occurs, but the expression pattern during and after the repression is altered and Groucho and β-catenin are not found to be expressed in the same compartments.

To further analyze the difference between the β-catenin dependent and the β-catenin independent function of LEF-1, we analyzed the skin of *Lef1*$^{m5/m5}$, *Lef1*$^{-/-}$ and wildtype mice. The *Lef1*$^{m5/m5}$ mouse line was created in our lab, carrying a mutation in the β-catenin interaction domain. This results in a shortened LEF-1, which cannot interact with β-catenin (W. Roth, unpublished data). As described previously, we could observe differences in the hair follicle development of *Lef1*$^{m5/m5}$ mice and of *Lef1*$^{-/-}$ mice. Whereas the development of hair follicles is blocked in *Lef1*$^{-/-}$ mice, we could observe hair follicle development of *Lef1*$^{m5/m5}$ mice at almost normal levels as compared to the wildtype mice. The comparison of skin from *Lef1*$^{m5/m5}$ to skin from *Lef1*$^{-/-}$ mice provided us with a number of genes, regulated by LEF-1 independently of β-catenin, thus genes that are not regulated through the canonical Wnt pathway. Only 7 of the 52 identified target genes were found to be genes that get upregulated by LEF-1. Those genes are probably regulated in a similar way like TCRα, which is getting induced without the help of β-catenin (Travis 1991). Nevertheless, the majority of genes regulated without LEF-1/β-catenin interaction, are genes that are repressed by LEF-1, supporting the idea, that co-repressors like Groucho are necessary for the repressive effects instead of β-catenin and maybe in very few cases the repression is mediated through LEF-1 and β-catenin. This theory was also supported by the comparison of wildtype skin with either *Lef1*$^{-/-}$ skin, or *Lef1*$^{m5/m5}$ skin. In both analyses we mainly

detected genes that get activated by LEF-1. Although we cannot distinguish between β-catenin dependent and independent targets in the wildtype/*Lef1*$^{-/-}$ screen, as LEF-1 is completely abolished, the wildtype/*Lef1*$^{m5/m5}$ comparison revealed only genes that are regulated with the help of β-catenin, as there is only the β-catenin interaction domain missing. For the *Lef1*$^{m5/m5}$ comparison we could identify more targets (86) as for the *Lef1*$^{-/-}$ (47), what might be puzzling at the first sight, as one expects to see stronger effects after complete withdrawal of LEF-1. But there are several explanations for this finding. It is possible that the *Lef1*$^{m5/m5}$ mutant acts on some proteins as dominant negative form of LEF-1 by preventing the binding of other LEF/TCF family members. This would lead to more severe changes as compared to the *Lef1*$^{-/-}$ mutant. Nevertheless the phenotype of the *Lef1*$^{m5/m5}$ is even milder as the one observed for *Lef1*$^{-/-}$ mice, indicating that the dominant negative effect cannot be very pronounced or is acting mainly on genes whose deregulation is not resulting in visible phenotypes. The second explanation for the discrepancy between those two analyses is that there are some genes detected in the wildtype/*Lef1*$^{m5/m5}$ comparison that are due to the altered genetic background. As the knockout constructs were stably integrated into 129 SV/J embryonic stem cells (ES cells) and then injected into C57 BL/6J mice, the genetic background is mixed for the first generations. The *Lef1*$^{-/-}$ knockout is already crossed back to C57 BL/6J for many generations, but the *Lef1*$^{m5/m5}$ knockout mouse is only in the third generation after back cross, so that it is most likely that there are some genetic background differences compared to the C57 BL/6J wildtype mice. Taken together we can predict that activation processes are most likely predominantly mediated through LEF-1 and β-catenin, whereas the majority of repressive events seems to be regulated without the help of β-catenin (Figure 29).

5.4. Analysis of validated target genes

The target genes identified are of a great variety in function. The role of some of the genes has not been revealed yet, and the knowledge that they are regulated by

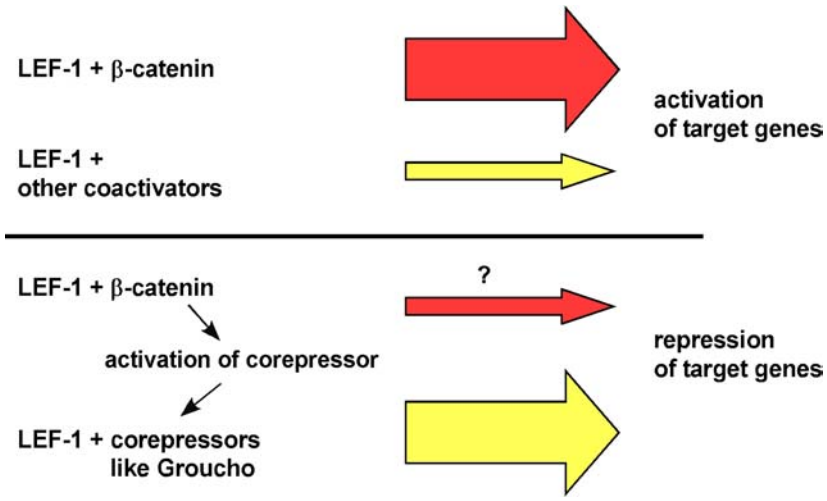


Figure 29: Model of activation and repression mediated through LEF-1.
We predict that activation of target genes is mainly achieved by interaction of LEF-1 with β-catenin within the canonical Wnt pathway, whereas repression occurs mostly without the help of β-catenin but with the interplay of LEF-1 and co-repressors like Groucho.

LEF-1 might help to define their role. We were validating identified genes either with northern blot analysis or with real time PCR. The validation showed that the genes are to great percentage real targets and the fold change regulations found on the microarray are in the right range. For the T cell screen, where we were using amplified RNA, the validation was conducted with unamplified RNA, still giving the same results as seen on the array. This was also a necessary control whether the amplification method we developed was working and the RNA was amplified in a linear range.
To address the question if the connection of the identified targets to LEF-1 is meaningful and a direct regulation seems likely, we were studying the genes, their expression profiles and promoters, if characterized, to get a sense of the possible interplay.

5.4.1. Targets genes identified in the NIH3T3 screen

The **Clathrin light chain B** (LCB) is part of the triskelion, a three-legged structure that reflects the monomeric form of clathrin. Each leg of the triskelion consists of

one heavy chain and two light chains (Kirchhausen 1981; Ungewickell 1981). Clathrin coated vesicles are the agents for receptor-mediated transport of macromolecules between membrane bound compartments. The promoter of LCB is not characterized yet, so we could not perform a search for LEF-1 consensus sites. How LEF-1 regulation of the LCB fits in the context with LEF-1, is a question difficult to answer, as the precise function of LCB still needs to be uncovered.

Glycoprotein A15 a member of the TM4SF family that was found to be repressed in our NIH3T3 screen, has also no characterized promoter yet. Therefore, an analysis for LEF-1 binding site was not possible. But dissecting its function, the involvement in differentiation, proliferation, and overexpression in many forms of cancer, supports the idea of a regulation by LEF-1. Although this is not a proof for interaction it is obvious that A15 is involved in similar processes as LEF-1. A15 is abundantly expressed in brain but can also be detected in heart, lung, kidney, colon, and muscle (Hosokawa 1999). LEF-1 is not detected in the same compartments but is mainly expressed in spleen, thymus, and the lymph nodes. Although the expression profiles are not overlapping, this finding still supports the regulation of A15 by LEF-1, as this was identified as a downregulated target. The absence of A15 in tissues with high levels of LEF-1 points to a strong repression of A15 through LEF-1.

As described before **MAGED2** belongs to the big MAGE family. The family members share a coiled coil domain, termed MAGE domain and are only expressed in a variety of tumors but not in normal cells. As the function of those proteins is completely unclear we cannot judge how close the connection between LEF-1 and MAGED2 is.

The oncofetal gene **pem** is a well characterized protein. It is expressed in a stage-specific manner during embryonic development. Pem can be detected at high levels in T-lymphoma cells and is not detectable in normal lymphoid tissue (Mac Leod 1990). Pem encodes a helix-turn-helix (HTH) motif, structural similar to HTH transcription factors (Scott 1989). Within our analysis we could see that the expression pattern has on the one hand big differences compared to the expression pattern of LEF-1, as for example the absence of pem in lymphoid cells, but it can be detected in lung in similar regions as LEF-1 (Sasaki 1991). Because

pem is also a gene repressed by LEF-1, this findings are as expected, especially as the complete signal is lost in our northern blot analysis after CatCLEF expression. The gene is alternatively spliced resulting in two different splice variants (Maiti 1996) with two different promoters. As the androgen dependent promoter was already well characterized (Maiti 1996; Barbulescu 2001) we tested if this promoter is active in our cells and is therefore involved in the regulation. By PCR reaction we could show that not the splice variant belonging to this promoter but the androgen independent one is expressed in the cells used for the screen (data not shown). As the androgen independent promoter was not characterized yet we could not search for LEF-1 consensus sites to get greater evidence for direct regulation of pem through LEF-1.

Spi2, another target that was found to be repressed by LEF-1, is as described before an acute phase reactant that belongs to the protein superfamily of serine proteinase inhibitors (serpins). The activity of the spi-2 gene is controlled by several regulatory elements located in the promoter region (Simar-Blanchet 1996) and by transcriptional repressor sites within the 3' untranslated region (Paul 1998). Screening both elements for LEF-1 binding sites uncovered that the promoter has one perfect LEF-1 consensus site, making a direct regulation possible (Figure 31A). Furthermore showed the study of the 3' untranslated region three for repression important elements (Paul 1998). At least two of the sites show homologies to the LEF-1 consensus sequence, suggesting that a direct regulation could also be mediated through this silencer. Another support for this theory is that the silencer function is not active in NIH3T3 cells, confirming again our findings that spi-2 is expressed in the RXR control cell line and loss of the expression occurs after LEF-1 induction.

The **IGF-IIR** encodes a multifunctional membrane-spanning glycoprotein. It is involved in targeting lysosomal enzymes from their sites of synthesis in the Golgi to an acidic prelysosomal compartment. The IGF-IIR promoter was already characterized (Liu 1995) and showed potential binding sites for c-myc within an E-box. As we could also find LEF-1 consensus motifs within the sequence (Figure 31A), two possible regulation processes were hypothesized. On the one hand could the regulation be secondary mediated through c-myc, a verified LEF-1 target

(He 1998), on the other hand we were testing for direct regulation. Although we were trying different methods, transient transfection assays, stable integration, and infections we could not detect direct effects on the promoter. This does not mean necessarily that the interaction is indirect, it just showed that the regulation is not acting through the promoter. It was demonstrated for many other genes that the regulation could be provided through enhancer regions that can be placed everywhere on the gene as for example the 3' UTR in the *spi-2* gene just described, or in the fibroblast growth factor 4 (FGF4) gene, where the regulatory element that interacts with LEF-1 was also found in the 3' UTR (Kratochwil 2002).

5.4.2. Target genes identified within the T cell screen

MCP-1 (Yoshimura 1989; Leonard 1990), a cytokine identified in the NIH3T3 screen and RANTES (Schall 1988), a cytokine identified within the T cell analysis, belong to the same family of chemokines. The superfamily of small proteins consists of about 50 so far identified proteins. These molecules share a secondary structure with a flexible N-terminal segment followed by three antiparallel β-sheets, and a C-terminal α-helix. According to the position of the cysteine residues they have been subdivided in four families: CXC, CC, C, and CX3C (Zlotnik 2000). The chemokines activate a family of seven transmembrane G protein-coupled receptors, called chemokine receptors (Murphy 1996). Both identified target genes belong to the CC chemokine family and are located on the same chromosome cluster. They share overlapping functions and belong to the inducible form of CC chemokines that are made in response to diverse signals. The first function that was uncovered was their ability to recruit leukocytes on demand, in response to inflammatory, infectious, and immunological signals. Furthermore have recent investigations shown that they also play an important role in T cell differentiation (Luther 2001). As they only start to play a role in already matured T cells where LEF-1 is not expressed any more this finding is not contradictionary to the finding that LEF-1 represses both chemokines. Moreover we were analyzing if the

induction of RANTES in stimulated PBMCs was triggered by the absence of LEF-1. Our data suggest as LEF-1 is in all stages of stimulated PBMCs only weakly expressed, that the discontinuation of a repressor does not lead necessarily to upregulation of the gene. This can be easily explained by the fact that regulatory processes are mostly not simple, but they consist of a network of negative and positive stimuli. In this case there is RFLAT needed as a positive factor to obtain the upregulation of RANTES (Song 1999).

Search of the MCP-1 and RANTES promoter for LEF-1 consensus sites revealed that MCP-1 has one strong and several weaker LEF-1 consensus sites (Figure 31A). As the promoter region of RANTES is not conserved between mouse and human we studied both and could show by EMSA, binding of LEF-1 to two sites of the mouse promoter. For the human promoter strong binding to two sites and weaker binding to two additional sites was proven. The cumulative appearance of LEF-1 binding sites in the different chemokine promoters raises the question, if there are more members of the inducible CC chemokine family regulated through LEF-1, than the so far discovered MCP-1 and RANTES.

As a model for the regulation of CC chemokines we performed a more detailed analysis of the RANTES gene. First we were checking the endogenous RANTES levels in Jurkat and Hut78 cell lines. It appeared that RANTES was highly expressed in cell lines where almost no LEF-1 is expressed and vice versa. Those findings of endogenous regulations motivated us to dissect the mouse and the human promoter in a promoter analysis. Although the mouse promoter is active in the human T cell line Hut78, we could detect only a very weak response to LEF-1 and β-catenin overexpression. In NIH3T3 fibroblast we could discover activation of the mouse RANTES promoter. But this activation was not mediated through the LEF-1 binding sites as it was still occurring when transfecting the truncated construct of the RANTES promoter, which is missing all LEF-1 binding sites. Next we were also testing the human promoter, as there was the possibility that there were differences in the regulation because of the diversity between human and mouse and we were carrying out most experiments in human cells. The human promoter could be shown to be downregulated up to two fold in Hut78 cells and PBMCs. Further analysis of the repression in PBMCs revealed that we could

observe an upregulation after m5LEF-1 expression for the mutated promoter in contrast to the repressive effects detectable with the wildtype promoter. This might be due to secondary activating effects that we also could observe in NIH3T3 cells. The network of direct repressive and indirect activating effects can be explained by the fact that *in vivo* LEF-1 is not expressed in the cell types examined. Therefore, the direct repressive effect of LEF-1 has to overcome the diametrical indirect activation observed. The fact that in NIH3T3 cells the activation is also observed for the wildtype promoter suggests that LEF-1 has to interact with a co-repressor not expressed in NIH3T3 cells (Figure 30). Organization

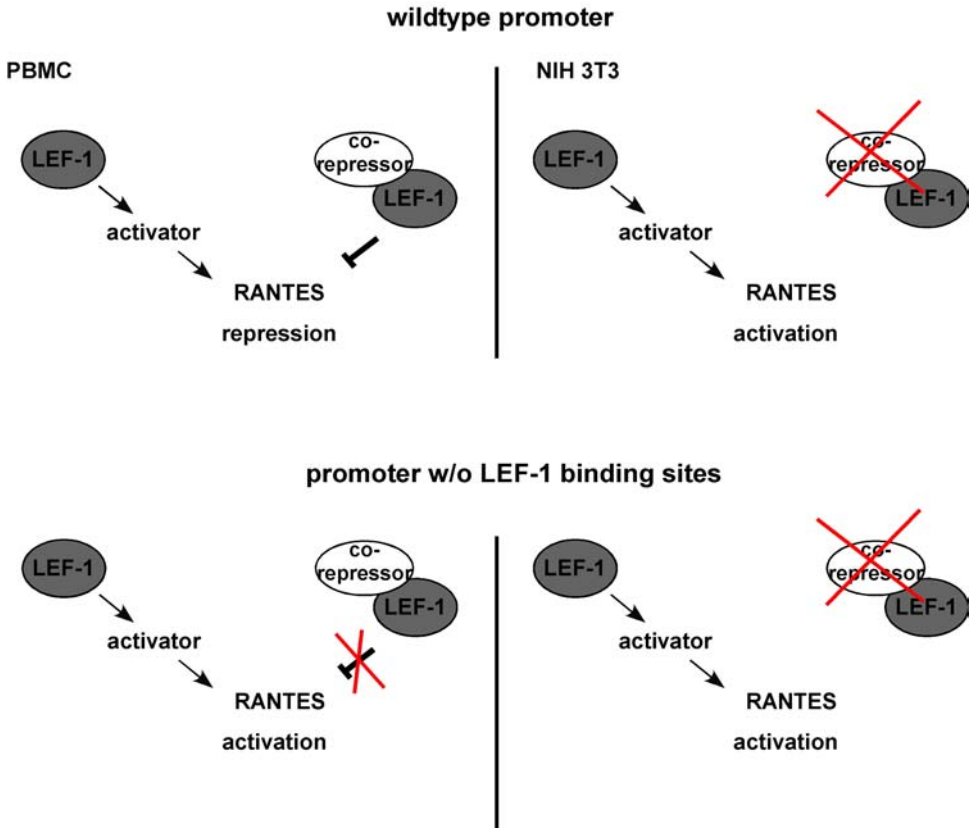


Figure 30. Model for regulatory effects affecting RANTES promoter activity.
In PBMC's LEF-1 mediates directly the downregulation of the RANTES promoter through its LEF-1 binding sites together with a co-repressor. The effect is weakened by a secondary unnatural effect of LEF-1 on an activator, which can upregulate RANTES. The mutated RANTES promoter, missing the four LEF-1 binding sites, can not get repressed by LEF-1 due to the absence of binding sites and the secondary, positive effect is observed. In NIH3T3 cells, the necessary co-repressor is missing and wildtype and mutated promoter are detected upregulated due to the secondary effector.

As the downregulation already occurred after expression of m5LEF-1 and β-catenin was not necessary, but the help of a co-repressor was most likely, taking the results of the NIH3T3 cells into account, we were examining the effects of Groucho-1 addition. Nevertheless, RANTES was not responding specific to LEF-1 and Groucho but in M12 cells a repression could be achieved already with Groucho alone. We could not delineate if this effect is specific or not.

Taken together we could provide evidence of specific repression of the RANTES promoter through LEF-1, although we could not identify a co-repressor. But as predicted in our model (Figure 29), the repression is mediated without the interaction of β-catenin.

The second repressed gene that we identified and validated in our T cell screen is **FasL** (Pinkoski 1999), a tumor necrosis facto (TNF) related type II membrane protein. It can bind to Fas and is expressed in highly activated T-lymphocytes (Nagata 1999). FasL and Fas are mediators of apoptosis and can downregulate the immune response. In an analysis of developing B cells of wildtype and *Lef1*$^{-/-}$, it was shown that Fas is deregulated (Reya 2000). With the T cell screen we now identified the interplayer of Fas, FasL to be regulated. FasL is also known to be a marker for apoptosis (Brunner 1995; Nagata 1999). By identifying Fas and FasL in two different studies and different tissues, it becomes most likely that the Fas/FasL expression levels in all tissues are strongly depending on LEF-1 expression. This theory is supported by the identification of one potential LEF-1 binding sites in the FasL promoter (Figure 31A). As for RANTES it could be shown for FasL that its endogenous levels are only high in cell lines expressing low levels of LEF-1.

One of the two validated activated genes in our T cell screen is **SATB1**, a MAR-binding protein that was shown to regulate expression of multiple genes during T-cell development. Mice, sufficient for SATB1, also develop a T cell block, but this block occurs later in development as seen for the *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ mice (Alvarez 2000), making it most likely that SATB1 is downstream of LEF-1. Another evidence for this model was provided by Matsuzaki and co-workers. They could provide evidence, that the *Drosophila* homolog of the Satb genes, Dve, is regulated by Decapentaplegic (Dpp), the *Drosophila* homolog of BMP4, or Wingless (Wg), the *Drosophila* homolog of Wnt within different target cells of the gut (Nakagoshi 1998).

Dve expression responds differentially to either of them in distinct parts of the midgut. In the middle midgut, dve expression was shown to depend mainly on Dpp. This analysis was conducted with abdominal-A (abd-A) mutants, where the Dpp but no Wg is expressed. Nevertheless *dve* could still be detected in the middle midgut. In contrast, the *dve* expression in the anterior-most midgut depends on Wg, but not on Dpp. A mutant fly that does not express Wg in this region was found to also have no *dve* expression. It could be strong evidence provided, that at least in some target cell the regulation of the Satb homolog is regulated through Wg, respectively Wnt. This is in line with our observation, that SATB1 is found to be activated by LEF-1, as most of the activating processes of LEF-1 are mediated through the Wnt signaling pathway.

For SATB1 several different EST variants can be found in the database, sharing all the second exon, but showing different non-coding first exons (Figure 31B), resulting in different splice variants but the same translated protein. This can be necessary to obtain a diverse regulation in different tissues as it was also observed for Dve. We could identify 4 different exon 1 for the mouse and the human SATB1. As there are more than 20 kb between the different first exons, we concluded that there are most likely at least four different promoters. None of them is characterized yet, but by searching upstream of the putative 5' end of the different exon 1, we could identify several LEF-1 binding sites upstream of one of the splice variants (Figure 31B). The other two putative promoter regions did not have any LEF-1 binding sites, reinforcing the theory, that the regulation of SATB1 is cell type specific. The control of endogenous levels in different cell lines revealed that SATB1 is highly expressed in cell lines that also have high LEF-1 levels. To reduce the possibility that SATB1 is only a secondary target we could show in a transfection assay that already 12 hours after transfection of *Lef1* and β-catenin the endogenous levels of SATB1 are up-regulated more than 8-fold. For a homologue of SATB1, termed SATB2, that was identified in our lab (G. Dobreva, unpublished data), an upregulation of the endogenous levels was also detected when tested after 24 hours. This finding suggests that not only SATB1 is a target of LEF-1 but that also SATB2 is regulated by LEF-1. As the induction time of 12 hours for

protein production is quite short, this finding strongly indicates that the regulations of SATB1/2 are tightly connected to LEF-1 and are most likely direct.

CD5, the second validated gene of the T cell screen that was found to be activated, is a pan-T marker present on helper T cell and on suppressor or cytotoxic T cells. CD5 function is still unclear, but a function in T cell proliferation was suggested (Hollander 1981; Loydberg 1985). CD5 was identified in a T cell analysis before (Okamura 1998). There a downregulation was examined in *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ mice in comparison to wildtype mice. But as the northern blot of this analysis was performed out of unsorted thymic cells, the effect was accounted to the absence of later stages of T cells and the change of the compartment. As outlined before we have performed the analysis with sorted cells, just to rule out any secondary effects due to the changed cell populations. But as we can still detect differences of CD5 expression between wildtype and *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ mice, we can predict that the changes are really due to the withdrawal of LEF-1/TCF-1. Although the analysis of the promoter did not show any good LEF-1 binding sites (Figure 30B), we were testing the endogenous regulation after transfection of *Lef1* and β-catenin. As seen for SATB1 we could also detect a more than 12-fold upregulation of CD5 after 12 hours. This again indicated a tight regulation of CD5 through LEF-1.

Most of the genes validated could be shown to have either LEF-1 binding sites in the promoter, or to have similar functions and expression profiles as LEF-1. Taken together the identified target genes are mostly involved in essential processes, and the knowledge of a regulation through LEF-1 will help to define their role more precisely in the future.

5.4.3. Target genes identified within the skin screen

We did not validate genes of the skin microarray analysis so far. Nevertheless we could identify in this analysis a number of very interesting targets as for example

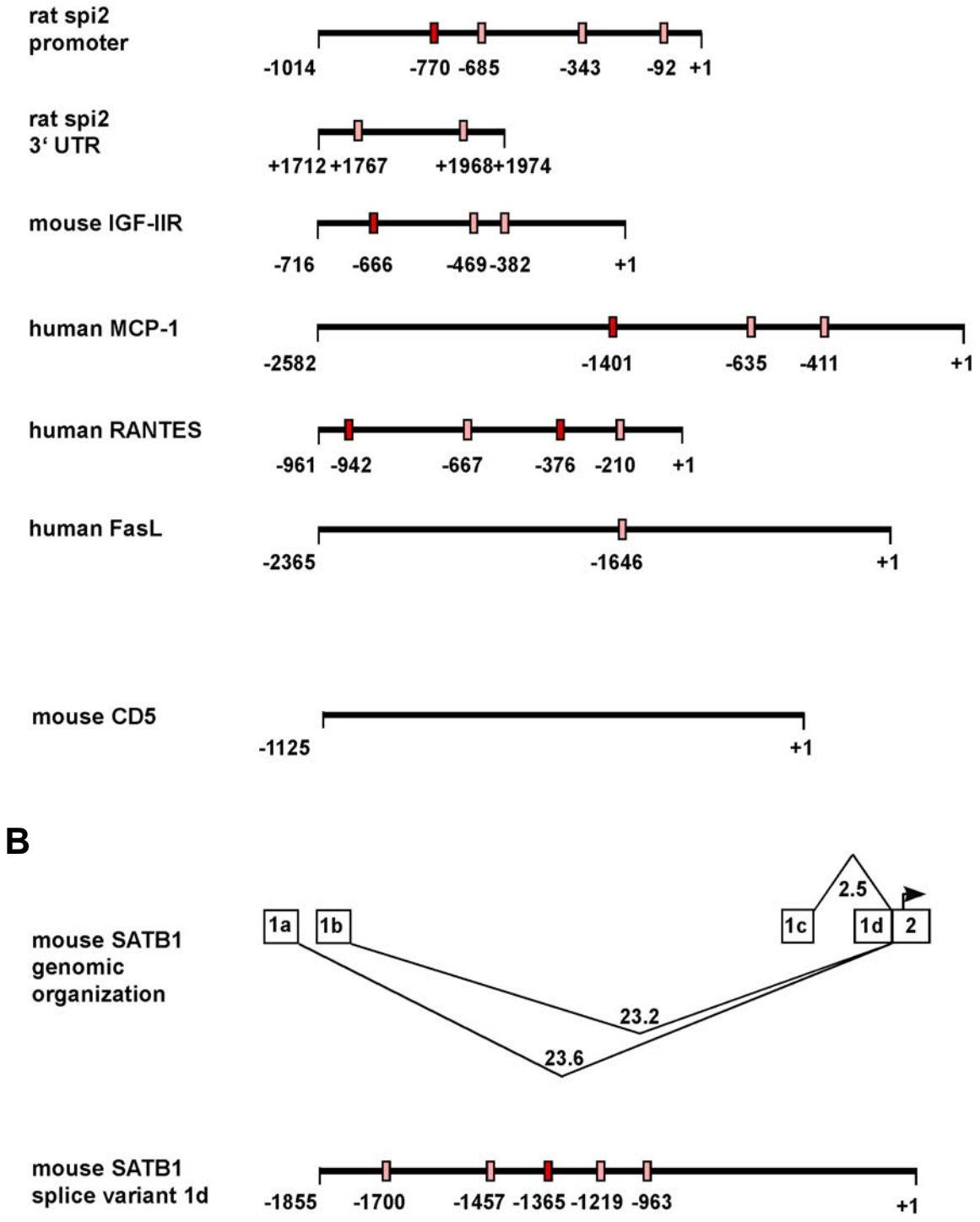


Figure 30. Promoters and genomic organization of the validated genes.
(A)The characterized promoters of the validated genes of both screens were searched for LEF-1 binding sites. The strong LEF-1 binding sites are indicated in dark red, the weaker binding sites in light red. For the spi2 LEF-1 binding sites could be detected in the promoter region and in the 3’ UTR. +1 indicates the transcription start site.
(B) Scheme of the genomic organization of the 5’ region of mouse SATB1. The different exons are indicated by numbers (1a-2). Distances between the different exons are given as kilobases. The putative exon of splice variant 1d is indicated with the LEF-1 binding sites.

BMP2 that was found to be positively regulated through LEF-1 and β-catenin. Many studies already revealed that there is a connection between the BMP and the Wnt signaling pathway, as for example the study of Birchmeier and coworkers (Soshnikova 2003), where they describe the interplay of the Wnt/β-catenin and BMP signaling during the formation of the apical ectodermal ridge (AER) and of the dorsal-ventral axis of the limbs. They come to the conclusion that in the AER formation β-catenin acts downstream of the BMP, but upstream or in parallel by the dorsal-ventral patterning. Moreover it was shown recently that the BMP receptors, termed Smad, can directly interact with Dishevelled-1, a positively regulator of the Wnt signaling (Warner 2003). Another study could show that LEF-1 can regulate BMP-target genes synergistically through the interaction with Smads (Hussein 2003). Those findings make a further analysis of BMP-2 very interesting as it could provide another level of cross-talk between the Wnt and the BMP signaling.

Taken together the studies give a great overview of the diversity of LEF-1 regulated processes in the cell. It shows that many pathways are maybe closer linked than suggested so far. We could also provide evidence for the different functions of LEF-1, acting either as an activator or as a repressor. Many of the targets are promising targets and should be subjects of further analysis.

6. Summary

In this analysis we were able to provide great insight into many genes regulated by LEF-1. We encircled the question for LEF-1 target genes from three directions. One gain of function experiment, where we overexpressed a constitutive active form of LEF-1, and two loss of function experiments, where we compared different forms of LEF-1 mutant mice with wildtype mice, were accomplished. All approaches were conducted with Affymetrix chip technology.

On the one hand we could identify many target genes that were differentially expressed in NIH3T3 cells overexpressing CatCLEF in comparison to a control cell line without CatCLEF expression. Nine of the identified genes, Clathrin light chain, Glycoprotein A15, MAGE D2, pem, Spi2, IGF-IIR, and MCP-1, were validated by northern blot analysis.

On the other hand, we were able to find LEF-1 target genes in sorted ISP $CD8^+$ T cells, comparing wildtype and *Lef1*$^{-/-}$*Tcf1(V)*$^{-/-}$ cells. Four genes, SATB1, CD5, FasL, and RANTES, were validated and we could show that the endogenous expression levels in cell lines revealed the regulation observed. After overexpression of LEF-1 and β-catenin in a LEF-1 negative T cell line, we could show that endogenous levels of SATB1 and CD5 were induced. Furthermore, we could provide evidence that LEF-1 is able to bind within the RANTES promoter region and thus can directly repress the RANTES promoter. This effect is mediated β-catenin independent by LEF-1.

As a third approach we were investigating the differences between regulations mediated through the interaction of LEF-1 and β-catenin and regulations occurring without β-catenin interaction. Therefore we were performing a screen of skin from wildtype, *Lef1*$^{-/-}$, and *Lef1*$^{m5/m5}$ E16.5 embryos. The analysis showed that most of the activating events are mediated through the canonical Wnt pathway, where LEF-1 and β-catenin interact. However, the greater number of repressive effects is happening without β-catenin cooperation.

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