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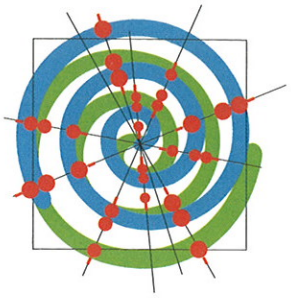

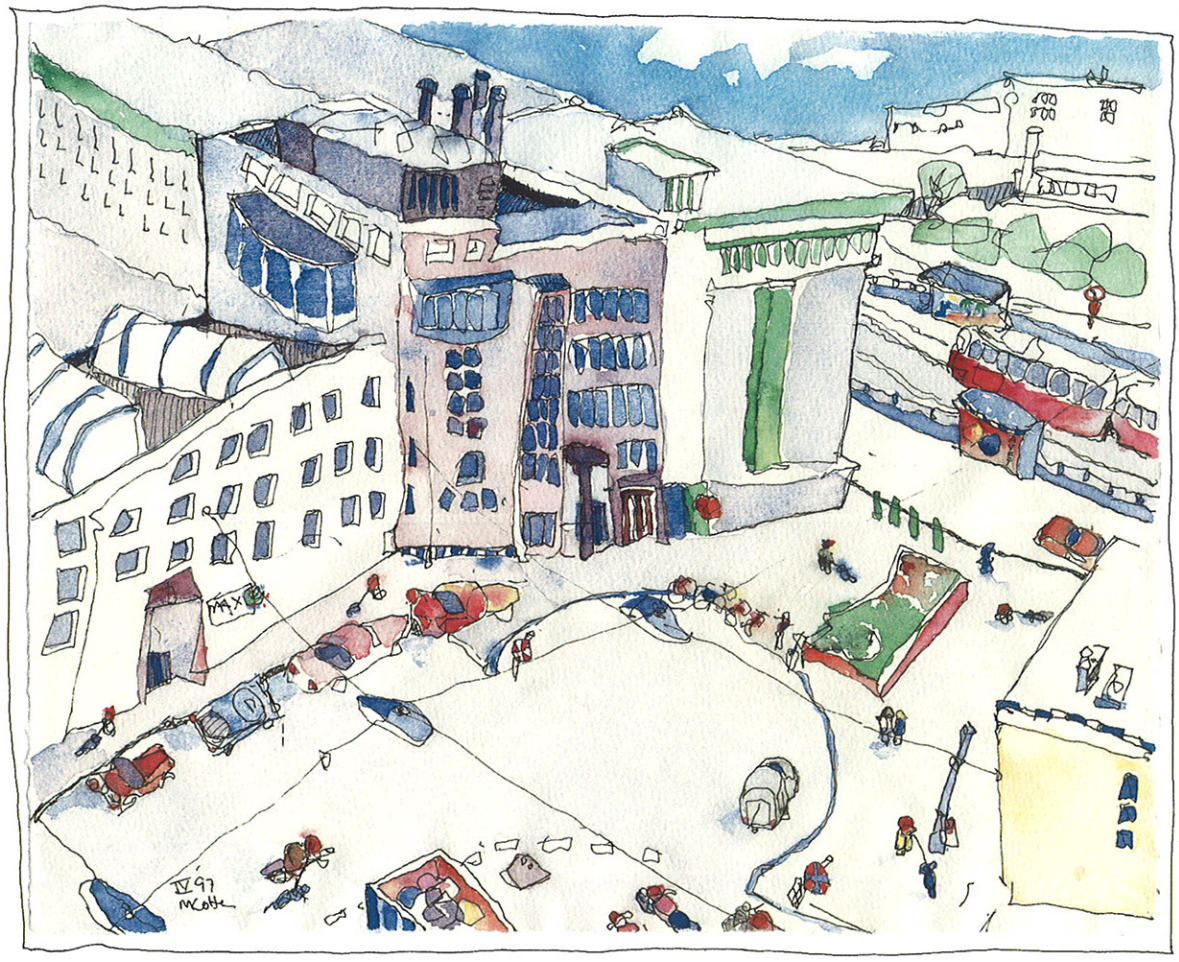

ten years IMP

Research Institute of Molecular Pathology

VIENNA BIOCENTER

Cover: Painting by Matt Cotten entitled "Amelia and Michaela, outstanding, in front of the IMP"

Scientific Report
1996

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Introduction

The late Sol Spiegelman once said that there were two kinds of honors. The "dry" kind by which he meant honors with no personal moneys attached and "wet" prizes where at least one part was for personal use.

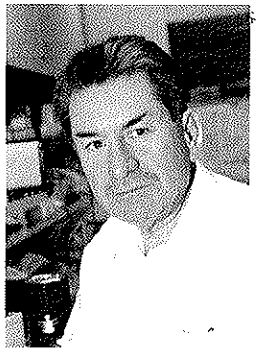
As the IMP entered its 10th year of existence from modest beginnings with an office in the City of Vienna and some provisional labs at Bender & Co (another Austrian subsidiary of Boehringer Ingelheim International) there was a rich harvest of honors for several members of the IMP. Prof. Erwin F. Wagner was awarded the "dry" Ludwig Wittgenstein Prize by the Austrian Science Foundation which came with the equivalent of Mio 1.5 dollars for research.

Erwin also got a call as Professor to the University of Innsbruck (Austria) but after long soul searching and to everybody´s delight at the IMP he decided to stay on for the time being. Equally "dry" was the appointment of Prof. Kim Nasmyth as corresponding member to the Austrian Academy of Sciences and as a member of the EMBL Scientific Advisory Council. Kim was also selected by the Royal Netherlands Academy of Arts and Sciences to receive the Unilever Science Award which, rumor has it, was very "wet". Yours truly was admitted to an Honorary Degree of Doctor of Science by the University of Edinburgh, where I had spent nine years from 1963 to 1972. In addition I received from the Austrian Government the "Grosse Silberne Ehrenzeichen für Verdienste um die Republik Österreich", which I share with Max Perutz who received the same honor one year earlier. I regard this award as a tribute to the achievements of the IMP as a whole and recognition of its role as a center of scientific excellence in Austria. Last, but not least Meinrad Busslinger and Hartmut Beug were elevated to Honorary Professors at the University of Vienna which means that by now all Senior Scientists of the IMP have been recognized by the University of Vienna.

In January 1996, one of the pioneers of the IMP from nearly its very beginnings, Denise Barlow left us to direct her own group at The Netherlands Cancer Institute in Amsterdam where she will continue her work in Developmental Biology. All of us wish her much success in this new venture. In fall, Jan Michael Peters, coming from Kirschner´s lab, joined the IMP to strengthen Cell Biology in the area of cell cycle control. 1996 was a good year in terms of productivity and a specially high number of publications appeared in best journals. Late in 1996 the Phase I trial with terminally ill melanoma patients at the University Clinics of Vienna and Würzburg (Germany) under the supervision of Profs. G. Stingl and E.B. Bröcker was concluded for which the IMP did much preparative work in animal models. A short account of this undertaking is given in Chapter "Tumor Vaccines."

In the preface of the Yearbook 1995 I explained how the IMP was trying to keep abreast with the newest developments in Molecular, Cell Biology and Molecular Pathology by ensuring turn-over at all levels excepting the Senior Scientists, the administration and the group of supporting staff such as technicians, workshop, library etc. Having built up the IMP from nothing and having served as director to the IMP for 10 years I thought it necessary to also have some turn-over and to have some fresh blood at the top. Consequently I resigned my job as at the end of 1996 and made way for Kim Nasmyth who has taken over the directorship of the IMP as from the beginning of 1997. Having followed with great admiration his steep career in the field of the genetics of the cell cycle, I now wish him well and great success in running the Institute.

Max L. Birnstiel



Emeritus Director
Max L. Birnstiel

Managing Director
Kim Nasmyth

Chemically defined, generic and cell-free tumor vaccines

Managing director	Max L. BIRNSTIEL
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Postdoc	Michael BUSCHLE
Postdoc	Tamás SCHWEIGHOFFER (until Jan. 96)
Laboratory technician	Manfred BERGER
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Laboratory technician	Barbara TRSKA (since June 96)

Introduction

Despite much progress made in the treatment of certain human malignancies the overall cancer mortality is still increasing. For the case of malignant melanoma of the skin, a cancer type which has held our attention in the past years, overall mortality has jumped by 34,1% in the USA in the last twenty years. Although the systemic administration of high doses of recombinant alpha interferon has recently been registered for the treatment of this disease, this clinical intervention requires careful selection of patient types and exhibits significant drug related mortality.

Worldwide "tumor vaccines", i.e. autologous tumor cells secreting cytokines, have been shown to be highly efficacious in rejecting of tumor challenges in mouse models. During 1996 we followed closely a Boehringer Ingelheim phase I trial of autologous tumor vaccines producing relatively high levels of interleukin-2 which was carried out in the clinical departments of both Prof. G. Stingl (Vienna, Austria) and Prof. E.B. Bröcker (Würzburg, Germany) on terminally ill melanoma patients. Although some clinical effects were seen, nothing about the effectiveness of such vaccines can as yet be concluded, except that they are well tolerated. One drawback immediately emerged from this study. Even the most skillful preparation of melanoma cell cultures from patients by Dr. Ernst Wagner and his team at Bender + Co. (Vienna), a precondition for vaccine preparation, yielded a vaccine only in ca. 55% of 43 patients from whom tumor tissue was obtained. This means that the vaccination strategy will not be available for nearly half of the melanoma patient population and the situation would be even more untractable with stage I to III patients where tumor tissue will become limiting. Also, the preparation of IL-2 secreting, autologous tumor cells is cumbersome and expensive and requires 6 to 8 weeks to prepare for a disease, where quick intervention would be desirable.

In view of these findings, Boehringer Ingelheim is planning second generation treatments with allogeneic tumor vaccines consisting of a mixture of extensively characterized tumor cell lines at low passage number prepared by the Bender & Co team, while at the I.M.P. we have developed a cell-free, chemically defined vaccine based on the IMP transloading procedure (see below).

For a long time, human tumors were considered to be largely non-immunogenic, but recent molecular and immunological investigations have identified an ever increasing number of tumor antigen peptides presented by a variety of tumor cells in a MHC class I restricted manner. The idea emerged that it should be possible to vaccinate cancer patients with residual disease using such tumor antigen peptides. The pioneering work by T. Boon at the Ludwig Institute (Brussels) has shown that simple injection of tumor antigen peptides of the "Mage" family of tumor antigens elicits clinical beneficial effects in a small minority of terminally ill melanoma patients. It is found that a T cell response is readily obtained with viral peptide antigens applied with an appropriate adjuvant but with tumor antigen peptides this is difficult, and apart from Feldmans laboratory (Revohot) successful vaccinations even in mouse model systems have not as yet been reported. We have recently discovered a procedure which enhances the immunodominance of small tumor antigen peptides by the use of our IMP "transloading procedure".

The emergence of transloading of tumor antigen peptides can be traced back to the AVET (adenovirus enhanced receptor mediated transferrinfection) technique developed at the IMP and is an offshoot of this gene transfer method described extensively in earlier yearbooks. In this IMP AVET protocol plasmid DNA is condensed with polylysine and combined with inactivated adenovirus. We noted that also short tumor antigen peptides (eight or nine amino acids long), ligands for matched MHC-class I receptors, could be introduced into tumor cells by AVET where the DNA was simply an "empty" plasmid. The

question arose whether such a transfer could be effected by less complete AVET complexes. It soon transpired that peptide transfer could be elicited without adenovirus and without DNA and yielded the unexpected answer that polylysine alone was the key ingredient for peptide transloading into cells. During the last year, as a last step, we also eliminated the tumor cells and to our great delight discovered that injections of tumor antigen peptides in conjunction with the adjuvant polylysine or other polycations (see below) afforded protection against tumor take in mice (see below).

Finally, in our last chapter, we decribe the adaptation of AVET to the transfection of primary cultures of macrophages, a cell type which is otherwise very refractory to transfection. A possible application of this approach is the genetic modification of this cell type for generation of cellular tumor vaccines.

Transloading of tumor antigenpeptides into antigen presenting cells

Michael Buschle, Walter Schmidt, Wolfgang Zauner, Karl Mechtler, Barbara Trska, Helen Kirlappos, and Max L. Birnstiel

The discovery of a steadily increasing number of tumor antigens (TA) has made generic, cell-free, peptide-based cancer vaccines an attractive alternative to cytokine-transfected autologous cellular cancer vaccines. The major drawback of peptide vaccines, however, is the poor immunogenicity of peptides, i.e. their poor ability to induce anti-cancer cytotoxic T cell responses. Most known TA derived peptides are generally but not exclusively presented in a major histocompatibility (MHC) class I context where 8-10 amino acids long peptides bind to a cleft in the MHC molecule.

It is commonly thought that for the induction of an effective anti-cancer immune response antigen-presenting cells have to display TA derived peptides to naive T lymphocytes **(Fig. 1)**. Therefore, efficient delivery of peptides to antigen presenting cells is the key event for priming of T lymphocytes with TA derived peptides. TA peptide-primed, tumor specific cytotoxic T lymphocytes (CTL) in turn destroy cancer metastases in the periphery **(Fig. 1)**.

Our group has shown that short MHC class I binding peptides can be very efficiently delivered to tumor cells in conjunction with polylysine, a procedure we termed "transloading" and several other polycationic amino acids and histones have in the past been employed to enhance transport of proteins into cells. In a systematic study, the ability of different cationic polymers to "transload" fluorescence tagged peptides to antigen presenting cells was investigated.

We were able to demonstrate that prototype antigen presenting cells generated from mouse bone marrow can indeed be transloaded with a mixture of polylysine and MHC class I binding peptide at very high efficiency **(Fig. 2)**. Furthermore we identified

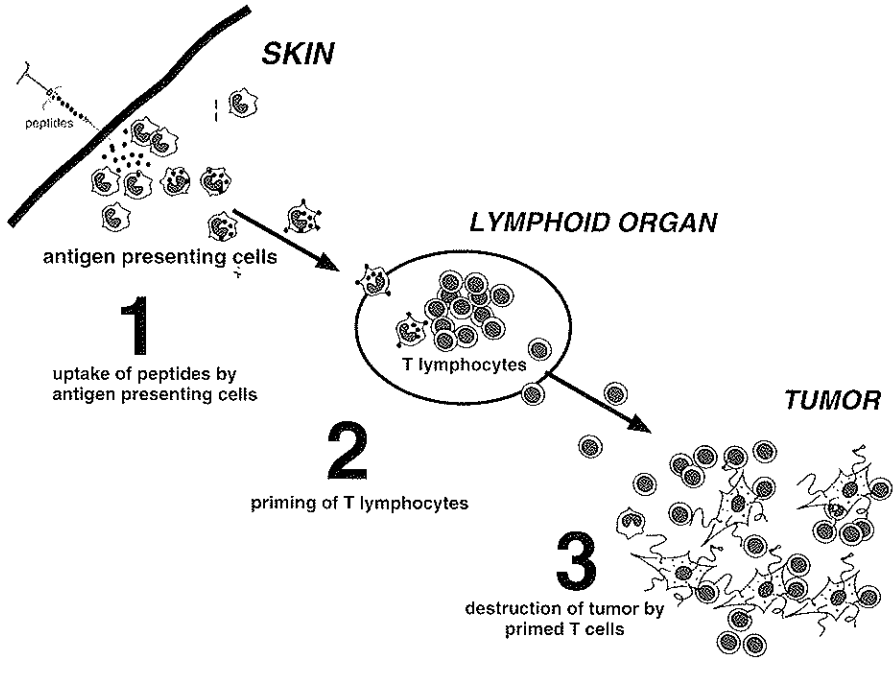

Fig. 1: Peptide vaccines

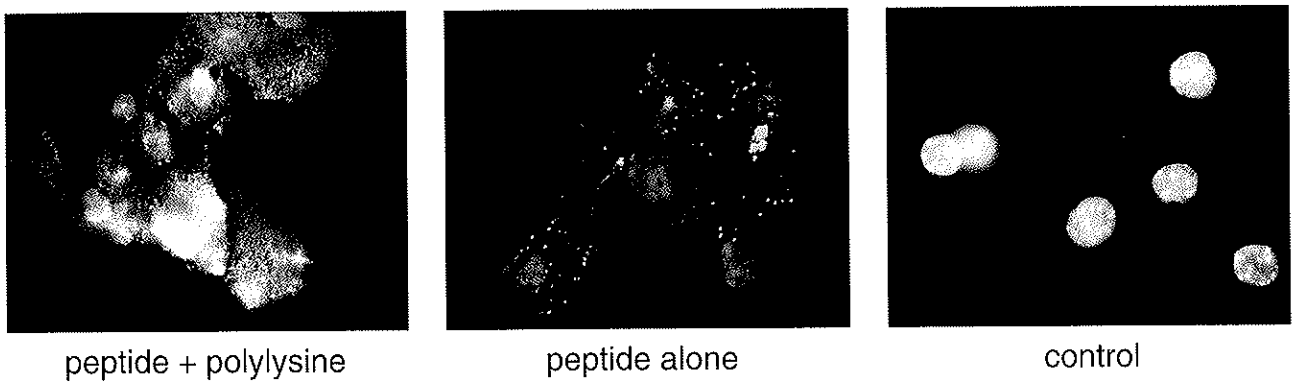

Fig. 2: *Peptide delivery to bone marrow-derived antigen presenting cells.*
Bone marrow derived APC were generated by culturing bone marrow cells in the pesence of GM-CSF for 10 days. Cells were incubated with a nine amino acids long fluorescein-labelled peptide alone or a mixture of peptide and polylysine for 30 minutes on coverslips, washed extensively and fixed. Nuclei were counterstained with DAPI (blue fluorescence).

several other compounds enhancing uptake of fluorescence-labelled peptides by antigen presenting cells to different degrees to an antigen presenting cell line **(Fig. 3)**. The most efficient compound identified, polyarginine, enhanced peptide delivery by up to three orders of magnitude as compared to cells treated with peptide alone, while polylysine treatment resulted in approximately 10-fold increased levels of fluorescence **(Fig. 3)**. Augmentation of peptide uptake was concentration dependent and the molecular weight of polyarginine or polylysine also influenced peptide delivery. As expected, highly negatively charged peptides appear to be delivered with higher efficiency although neutral peptides were also taken up at enhanced rates.

Tumor antigen derived peptides applied as cancer vaccines in conjunction with polycations afforded anti-tumor protection in animal models. The ability of

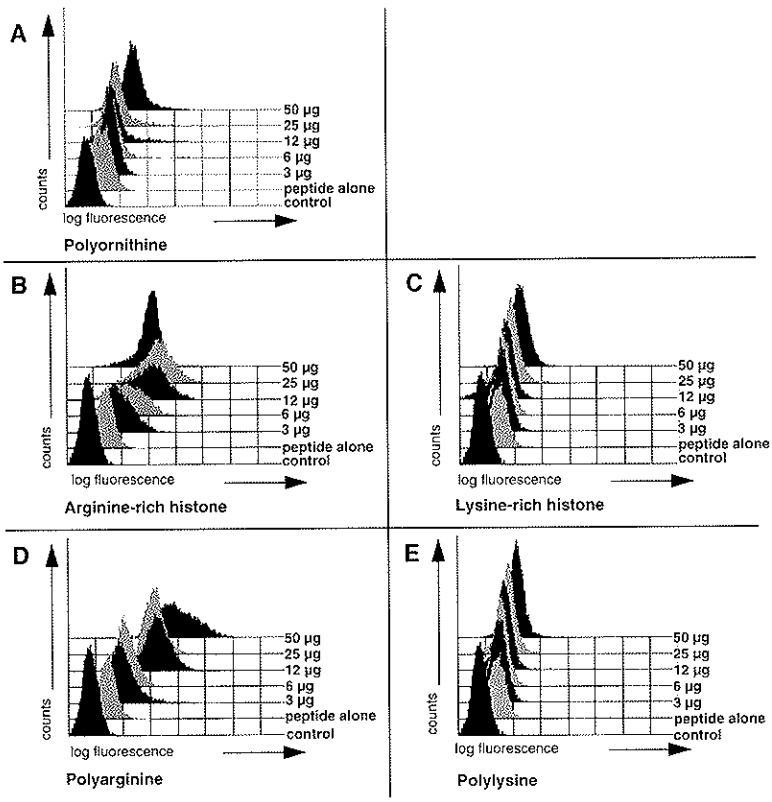

Fig. 3: *Polycationic amino acids and histone preparations greatly enhance peptide delivery to cells.*
The mouse APC cell line P388D1 was transloaded with a constant amount (5 µg/ml) of the fluorescein-tagged model peptide LFEAIEGFI alone or a combination of peptide and serial dilutions of polycationic amino acids or a mixture of peptide and histone preparations at concentrations indicated. Prior to analysis by flow cytometry, cells were washed extensively to remove free peptide. The following compounds were tested: (**A**) polyornithine (average Mr 110,000 , chain length 580), (**B**) arginine-rich histone, (**C**) lysine-rich histone, (**D**) polyarginine (average Mr 100,000 , chain length 490), and (**E**) polylysine (average Mr 94,000 , chain length 450).

polyarginine to deliver peptides much more efficiently to APC as compared to polylysine *in vitro* was mirrored in animal experiments where vaccines consisting of a mixture of polyarginine and TA peptide were superior in the induction of anti-tumor immunity as compared to treatments with polylysine together with TA peptide (see following chapter W. Schmidt).

First experiments addressing the mechanism of enhanced peptide uptake revealed that polylysine may mainly exert its function by at least transiently permeabilizing cell membranes as indicated by a substantial release of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the tissue culture medium following transloading with polylysine **(Fig. 4)**. By contrast, virtually no LDH release was observed following transloading with polyarginine, suggesting that in this case peptides are ingested by means of endocytic processes.

Future experiments will address at the cellular level how peptides are transloaded into different cellular compartments and are finally released into the cytosol and bound to MHC molecules for presentation in antigen presenting cells. We will also examine in more detail the requirements for immunogenic peptides.

Understanding these processes may give insight into basic immunological mechanisms and will be essential for further improving peptide vaccines.

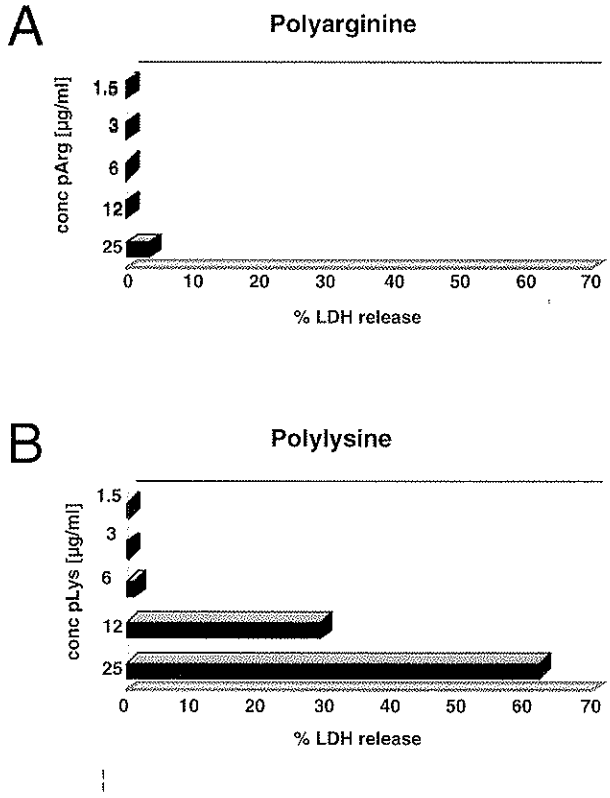

Fig. 4: *Determination of LDH release following treatment of cells with polylysine or polyarginine.*
P388D1 cells were incubated with polylysine (average Mr 94,000 , chain length 450) or polyarginine (average Mr 100,000, chain length 490) at concentrations indicated using identical conditions as for flow cytometric transloading assays and LDH levels released into the medium determined. LDH was not detectable in cells treated with peptide alone. LDH release into the medium is expressed as percentage of LDH levels of samples which underwent 3 freeze/thaw cycles.

Antitumor Efficacy of Peptide-Based Cancer Vaccines in murine tumor models

Walter Schmidt, Michael Buschle, Wolfgang Zauner, Helen Kirlappos, Karl Mechtler, Barbara Trska, and Max L. Birnstiel

Tumor antigen-derived epitopes, usually presented by major histocompatibility complex (MHC) class I molecules of tumor cells, are essential for the eradication of malignant cells mediated by specific cytotoxic T lymphocytes (CTLs). The generation of such tumor specific CTLs is mediated by professional antigen presenting cells (APCs) which propagate tumor antigens into small peptides. Upon presentation of the peptides naive T cells are stimulated to expand and differentiate into CTLs which are capable of reaching and eliminating tumor cells in peripheral tissues. The central role that tumor antigen-derived peptides play in this process makes them ideal candidates for peptide-based cancer vaccines. The pre-

ceding report by Michael Buschle demonstrates that transloading, the polycation-mediated peptide delivery protocol, very efficiently applies antigen-presenting cells *in vitro*. Postulating that the transloading procedure might effect peptide uptake by APCs also *in vivo* we tested this approach for the generation of peptide-based cancer vaccines in two murine tumor systems, mastocytoma P 815 and melanoma M-3.

In the murine mastocytoma cell line P815 an unusually large proportion of approximately 5% of the MHC class I molecules in the H-2Kd haplotype present the single peptide SYFPEITHI (Falk *et al.*, 1991; Harpur *et al.*, 1993). This peptide displayed prominently on cancer cells is derived from murine tyrosine kinase JAK-1. We chose this prominent "self"-epitope, to test the peptide vaccine approach in the P815 tumor model. Mice were vaccinated three times at weekly intervals and subjected to a contralateral challenge with 10^4 live tumor cells one week after the last vaccination. Animals vaccinated with irradiated P815 cells, stably transfected to secrete GM-CSF, were used as positive controls. Either 16 µg or 100 µg of the JAK-1 peptide was injected together with a transferrin-coupled polylysine (Wagner *et al.*, 1990) (dp,ca. 200 lysines). One group of animals was applied with 100 µg of peptide/mouse emulsified in IFA. As shown in **figure 5a**, this latter treatment did not confer antitumor protection, since all animals developed tumors rapidly with only a small delay as compared to untreated animals. However, when the JAK-1 peptide was applied with polylysine, antitumor protection was achieved. 2/8 animals were protected in the group which received 16 µg of the peptide and 3/8 animals when 100 µg were applied. Treatment with the cellular, GM-CSF-producing vaccine, resulted in a protection efficiency of 50% (**Fig. 5a**). In a second experiment in the P815 mastocytoma model, two unmodified polylysines of different chain lengths were compared, a short one consisting of only 16 lysine residues (pLys16) and a longer one with 240 residues (pLys240). Peptide control groups were injected with 100 µg peptide/mouse either dissolved

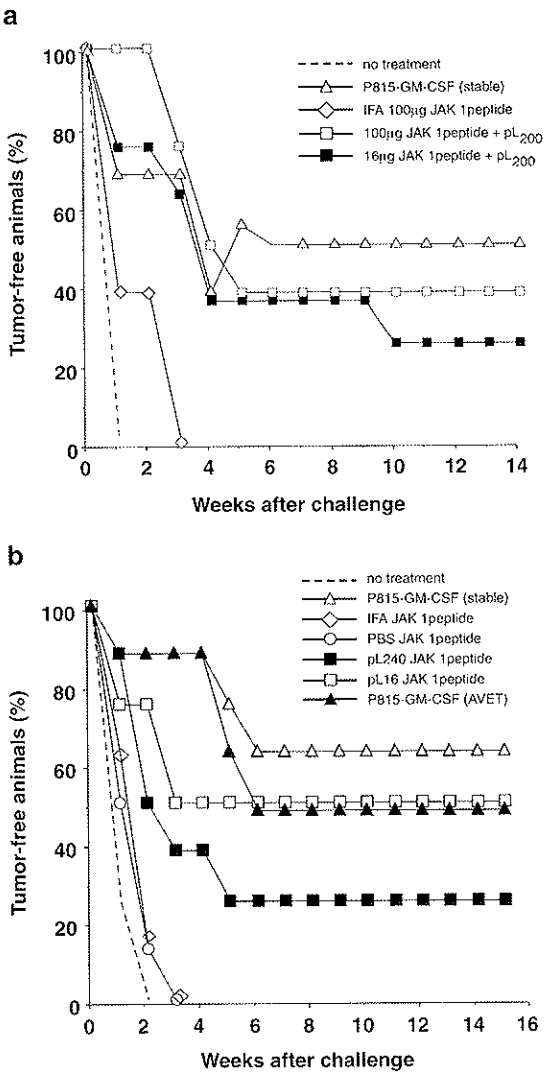

Fig. 5.: *Vaccination of DBA/2 mice against P815 tumor challenge with the JAK-1 peptide SYFPEITHI.*
(a) The indicated amounts of peptide were administered per animal. The ratio between peptide and TfpLys200 referenced on polylysine was 6.25:1 (w/w). (b) Vaccination with JAK-1 peptide and two polylysines with dp 16 (pLys16) or dp 240 (pLys240). Per animal, 100 µg of peptide were applied in conjunction with 75 µg of pLys16 or 7.5 µg of pLys240.

in PBS or emulsified in IFA. Two GM-CSF secreting cellular control vaccines were used. The stably transfected P815 cells as well as a second vaccine generated by the AVET transient transfection protocol (Wagner *et al.*, 1992) were used as a positive control. Both whole cell vaccines conferred similar protection efficiencies with 4/8 and 5/8 animals (**Fig. 5b**). The peptide-based vaccines, consisting of peptide only or peptide admixed with IFA, showed no protection, all animals developing tumors rapidly upon challenge. However, when applied in conjunction with polylysine the peptide vaccine did protect animals against tumor challenge. 2/8 animals were protected when the long polylysine 240 was used and 4/8 animals in case of the short polylysine 16. These results show that a single peptide, when applied with polylysine as adjuvant, can evoke efficient antitumor protection comparable to one of the most potent cytokine-secreting whole cell vaccines which has become the gold standard for anti-tumor vaccination (Dranoff *et al.*, 1993; Schmidt *et al.*, 1995). In the P815 mastocytoma system, we vaccinated mice by subcutaneous injection of a single, known natural peptide derived from JAK-1 kinase. Whereas vaccination with peptide alone or mixed with incomplete Freund's adjuvant (IFA) was ineffective, application of the peptide in conjunction with the polycation polylysine protected a significant number of animals against tumor challenge. Depending on the type of polylysine applied protection against tumor take was comparable to that achieved with irradiated whole cell vaccines, genetically modified to secrete GM-CSF.

In human melanoma, melanocyte differentiation antigens like tyrosinase, trp-1 or gp-100 have been found to be targets for specific CTLs and CTL epitopes have been identified (Brichard *et al.*, 1993; Bakker *et al.*, 1994; Kawakami *et al.*, 1994b; Robbins *et al.*, 1994; Wölfel *et al.*, 1994; Wang *et al.*, 1995).

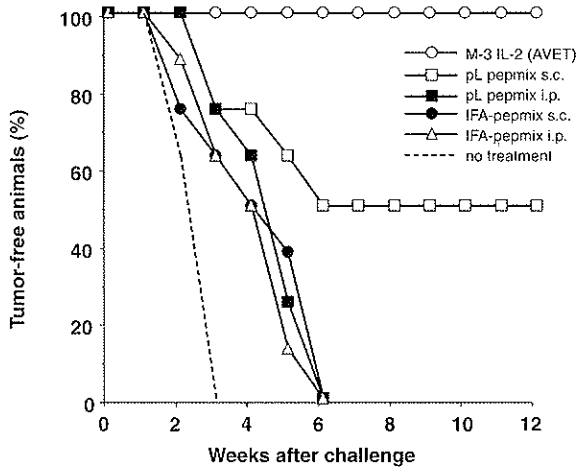

Fig.6.: Vaccination of DBA/2 mice against M-3 melanoma challenge with a combination of four peptides (see Materials and Methods) and pLys240. A total amount of 100 µg of peptide mix was applied per animal, consisting of equal amounts of each peptide. pLys240 was used as in **figure 5b.**

The murine M-3 melanoma [H-2^d(Zatloukal *et al.*, 1995)] expresses tyrosinase and trp-1, as determined by RT-PCR (Schmidt *et al.*, 1996, and M. Buschle, unpublished). Since neither CTL epitopes nor immunodominant tumor antigen peptides have been identified in this tumor model so far, we decided to use a mixture of four putative H-2K^d-restricted peptide ligands. The selection of the peptide motifs was based on requirements for H-2K^d-restricted anchor amino residues as described (Rotzschke & Falk, 1991; Rammensee *et al.*, 1993a; Rammensee *et al.*, 1993b; Rammensee, 1995; Rammensee *et al.*, 1995). Two of the peptides were derived from tyrosinase, two from trp-1 (see materials and methods). In a first experiment the peptide mix was applied to vaccinate naive DBA/2 mice against tumor challenge (**Fig. 6**). A cellular vaccine, consisting of irradiated M-3 cells, AVET-modified to produce optimal amounts of IL-2 (Schmidt *et al.*, 1995) has been shown to induce very potent antitumor immunity and was therefore chosen as positive control. As for the P815 experiment (**Fig. 5**), three vaccinations were applied in weekly intervals followed by a s.c. challenge one week after the last vaccination. Four groups of animals were applied with the peptide mix-based vaccine. Two groups received the peptides emulsified in IFA either s.c. or i.p. The other two groups were administered with the peptides in conjunction with polylysine (dp 240 lysines) via the s.c. or the i.p. route. As shown in **figure 6**, the peptide mix in conjunction with the polylysine protected a significant number of animals (50%) against tumor challenge of 10^5 live tumor cells as compared to untreated animals in which solid tumors were formed quite rapidly. Interestingly, the peptides conferred antitumor protection in conjunction with polylysine after s.c. vaccination. When ap-

plied i.p. the peptide polylysine mix was as ineffective as the IFA-based vaccines. Here, tumor challenge was not rejected and tumors grew with only a small delay compared to untreated control animals. These results demonstrate that the tyrosinase/trp-1 derived peptide vaccines can induce antitumor protection when administered s.c. in conjunction with polylysine. However, this vaccine is only half as efficient as the IL-2-based cellular vaccine which, consistent with recent reports, protects up to 100% of the animals against the tumor challenge with 10^5 live M-3 cells (Zatloukal *et al.*, 1993; Zatloukal *et al.*, 1995).

We next tested our peptide-polylysine approach under more stringent experimental conditions for the treatment of pre-existing M-3 "micrometastases" established by s.c. injection of 10^4 live tumor cells five days prior to the onset of the vaccination cycle. Since the i.p. vaccination was found to be ineffective in the prophylactic treatment (**Fig. 6**), we decided to vaccinate only via the s.c. route. Control groups received the tyrosinase/trp-1 derived peptide mix dissolved in PBS without adjuvant or with IFA. Unmodified polylysine 240 was applied as an adjuvant. Receptors for the binding of carbohydrate-terminated glycoproteins, involved in the phagocytosis of pathogens, are widely expressed on phagocytotic cells of the skin (Lanzavecchia, 1996). We therefore reasoned that it might be possible to improve targeting of such cells with fucose-modified polylysine. Therefore, peptides mixed with fucose-modified polylysine 200 were also administered. For comparison, a group receiving the IL-2-based cellular vaccine was included in this experiment. As shown in **figure 7a** the polylysine-peptide vaccine was effective for the treatment of pre-existing M-3 deposits. However, a significant cure rate was achieved only with the fucose-modified polylysine. With this treatment, 50 % of the animals rejected the metastases which compares favourably

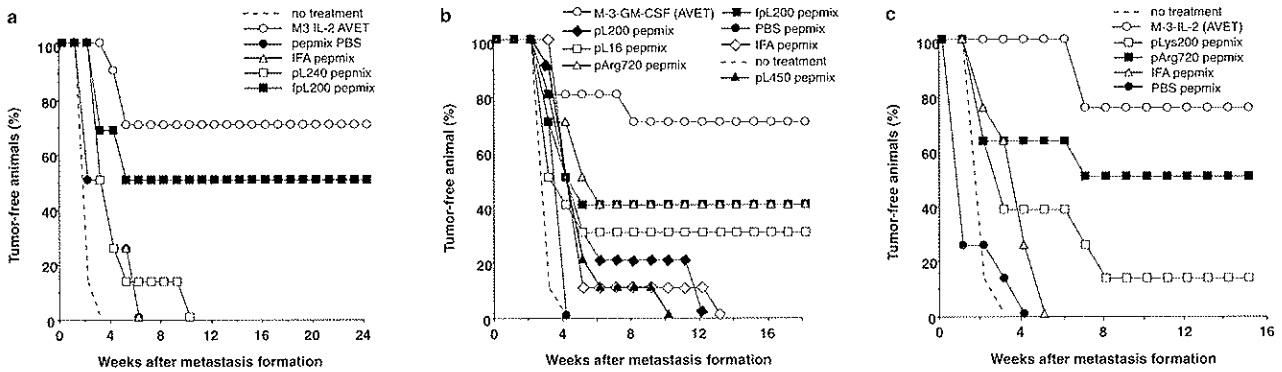

Fig. 7.: Cure of M-3 micrometastases-bearing mice with four peptides and polycations as adjuvant. Peptides were used as described in figure 6. The following amounts of polycations were used per 100 µg of peptide mix: pLys16, 37.5 µg; fpLys200 (fucose-modified polylysine dp 200), pLys240, pLys450 and pArg720 7.5 µg, respectively.

with the IL-2-based vaccine curing 70 % of the mice in this instance. Unmodified polylysine-peptide vaccine, efficient against tumor challenge in the prophylactic model (**Figs. 5 and 6**) was not very effective in the therapeutic model. Compared to the untreated control group it achieved only a delay in tumor onset but no complete rejection. When treated with peptides alone or peptides in IFA, all mice developed tumors without delay.

We further studied the effect of alterations and modifications of the polycation on the antitumor efficiency of our peptide-based vaccine in the M-3 metastasis model (**Fig. 7b**). In addition to unmodified and the fucose-modified polylysine 200 we also tested the short, unmodified polylysine pLys16, a long polylysine pLys450 and a different polycation, polyarginine pArg (dp 720). As positive control, a cellular vaccine secreting GM-CSF was applied (Schmidt *et al.*, 1996). Again, control groups receiving the peptide mix in IFA or without any adjuvant were also included. As expected from the experiment shown in **figure 7a**, the best peptide-mediated efficacy was achieved when fucose-polylysine was applied as adjuvant (**Fig. 7b**). In this group, 40% of the animals rejected the metastases compared to 30% in the group administered with the short polylysine 16. In all other peptide groups there was only a delay in tumor onset as compared to untreated animals, except for peptides which were admixed with polyarginine. Interestingly, this unmodified polycation was as effective as the fucose-modified polylysine enabling the rejection of the M-3 deposits in 4 of 10 animals. **Fig. 7c** shows the reproduction of this polyarginine effect in an independent experiment. Again vaccination with tyrosinase/trp-1 derived peptides in conjunction with polyarginine showed antitumor efficacy

in 4 out of 8 treated animals. These results identify polyarginine as a very interesting adjuvant for the application of peptide-based cancer vaccines.

While it is evident from the experiments described above that vaccination with a combination of peptides and polycations leads to protection of mice, we wished to address the question whether T cells were activated by this treatment. Cytokine secretion upon exposure of splenocytes from vaccinated animals to parental M-3 cells was taken as a surrogate marker for T cell activation (Kawakami *et al.*, 1994a). Only splenocytes from vaccinated animals secreted substantial levels of IFN-γ (**Fig. 8**) upon co-cultivation with M-3 cells *in vitro* while no IFN-γ was detectable from splenocytes of naive animals.

In the future we plan to optimize the cell-free transfer of peptides in the mouse models. Other modifications of the polycations will be tested and the mechanism(s) by which T cell immunity is generated will be studied. We hope that this ultimately will lead to application of these new principles in man.

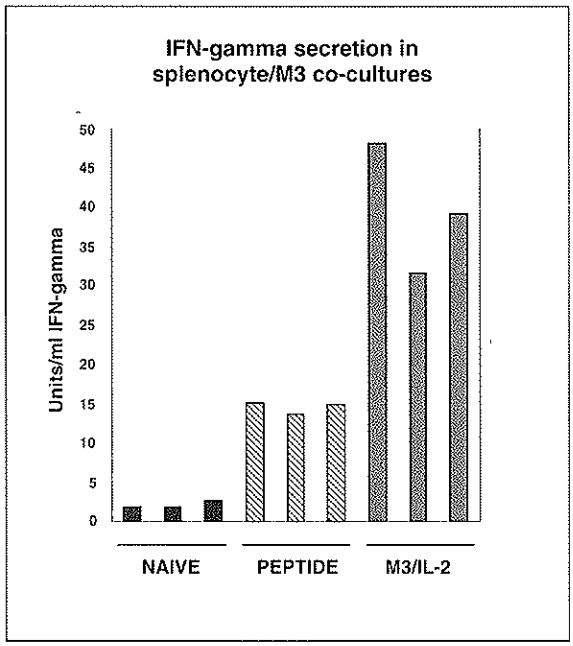

Fig. 8.: IFN-γ release in response to M-3 target cells by non adherent splenocytes from DBA/2 mice. 24 weeks after metastases formation splenocytes of disease free mice cured by the IL-2-secreting cellular vaccine (M-3-IL-2) or fpLys peptide mix treatment (**fig. 7a**) were used in the assay. An age-matched naive mouse was used as negative control. Triplicate measurements of IFN-γ production are shown.

The macrophage mannose receptor as a target for receptor mediated gene transfer

Michael Buschle, Wolfgang Zauner, Karl Mechtler, Walter Schmidt, Barbara Trska, and Max L. Birnstiel

Numerous gene transfer systems have been described for the transfection of cell lines. Gene transfer to non-transformed primary cells, however, is only achieved with a limited set of vectors and with very low efficiency. Thus, improved methods for gene transfer to primary cells *in vivo* and *in vitro* are needed for future gene therapy applications.

A cell type which is very difficult to transfect with common techniques are fully differentiated primary macrophages. Mature macrophages cycle at only low frequency, which may explain why many gene transfer techniques are ineffective. This cell type plays a central role in cell-mediated immunity. Important functions include antigen presentation and clearance of bacterial and parasitic infections. Macrophages also display tumoricidal activity. Therefore, these cells are excellent candidates for immunotherapy. Gene transfer with for example cytokine genes may ultimately enhance cell function and viability and improve overall efficacy of therapeutic applications. Alternatively, genes coding for tumor antigens may be transfected into macrophages in order to elicit specific immune resonses to multiple MHC class I and class II epitopes contained within a given protein sequence.

In an attempt to identify a method allowing transfection of macrophages, a panel of different techniques was tested initially on the mouse mono-

cyte-macrophage cell line P388D1/M. These included different cationic liposomes, the poly-cation polyethylenimine, glycerol mediated gene transfer or adenovirus-enhanced receptor mediated transferrin-fection (AVET). Only AVET resulted in significant activity of a luciferase reporter gene.

Macrophages express at high levels the mannose receptor allowing endocytosis of captured mannosylated/fucosylated antigens at very high frequency. At low pH endocytosed particles are released freeing the receptor from its cargo. Replacement of transferrin conjugated to polylysine with mannosylated/fucosylated polylysine chains in the basic AVET protocol resulted in significantly higher reporter gene expression levels following transfection of P388D1/M cells. Approximately 20-30% of cells synthesized the green fluorescent protein (GFP) reporter gene as assessed by flow cytometry (**Fig. 9**). Unexpectedly, cell viability following transfection was improved when mannosylated/fucosylated polylysine chains were included in the gene transfer complexes as compared to polylysine chains alone or transferrin-polylysine. No gene transfer was observed in the absence of adenovirus particles.

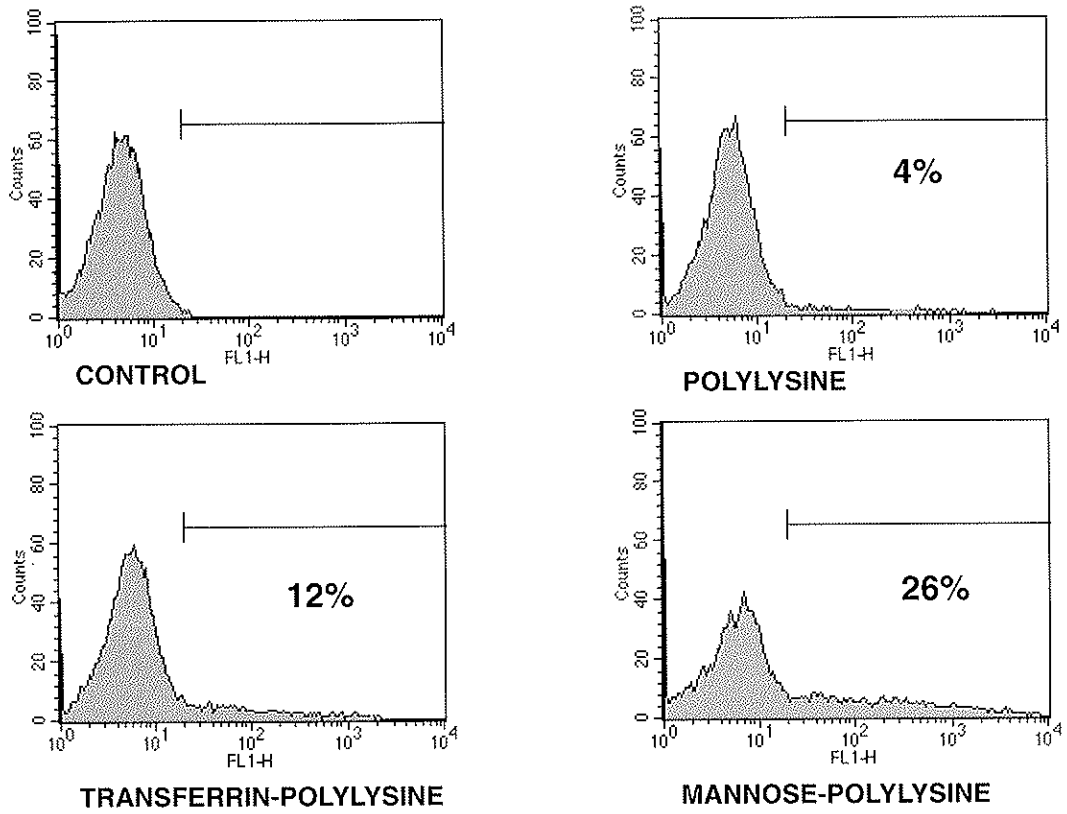

Fig. 9: *FACS analysis of green fluorescent protein (plasmid peGFP) transfected P388D1/M macrophages.*
Cells were transfected by adenovirus-enhanced receptor mediated gene transfer using polylysine, transferrin-polylysine or mannose-polylysine. No gene transfer was obtained without adenovirus. GFP expression was measured 24 hours after transfection.

Primary, fully differentiated macrophages were generated from mouse bone marrow within a period of 2 - 3 weeks. At this stage <0.1% of the primary macophages incorporated bromodeoxyuridine (BrdU).

Again, inclusion of mannosylated/fucosylated polylysine chains resulted in improved GFP expression compared to transferrin polylysine or polylysine alone: approximately 1 - 5% of cells were positive for a GFP reporter gene after transfection (**Fig. 10**). Preincubation of cells with mannan, a mannose polymer, reduced gene expression to levels obtained without specific ligands (<0.1% GFP positive cells). In the absence of adenovirus, no detectable reporter gene was synthesized.

In order to investigate whether gene expression was only transient or whether cells transfected via the mannose receptor express transferred genes for prolonged periods of time, macrophages were transfected with a cDNA coding for mouse GM-CSF and cell supernatants assayed for cytokine contents for a period of 5 weeks. GM-CSF is an important activator of antigen presenting cells, including macrophages.

Control cells transfected with a luciferase reporter gene secreted no measurable GM-CSF into the medium. Cytokine secretion by GM-CSF transfected macrophages peaked 24 hours after gene transfer ranging from 70-86 ng GM-CSF/24hours/10^6 cells. GM-CSF was detectable for 5 weeks with relatively constant production levels of 4-12 ng

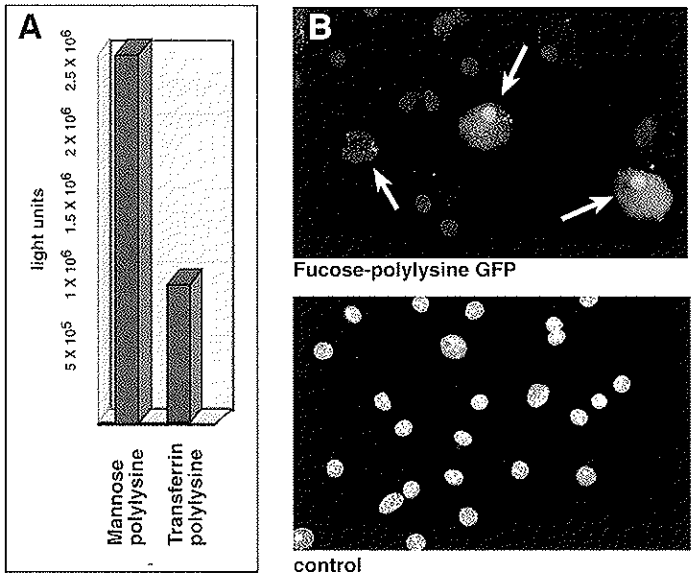

Fig. 10a: Primary bone marrow-derived macrophages were transfected with a luciferase encoding plasmid using adenovirus enhanced receptor mediated gene transfer and transferrin-polylysine or mannose-polylysine.

Fig. 10b: Photomicrograph of macrophages expressing a GFP reporter gene (plasmid pCSeGFP). Transfections were carried out using a fucose-polylysine conjugate. Typically, GFP expression ranged from 1-5% . Gene expression 24 hours after transfection is shown. Mannose and fucose bind to the macrophage mannose receptor with the same affinity. Fully differentiated macrophages were generated from mouse bone marrow by culturing cells for 2-3 weeks in GM-CSF. The cycling rate of the cultures at the time of transfection was low: <0.1% of cells incorporated BrdU. No reporter gene exression was detected without adenovirus.

GM-CSF/10^6 cells/24 hours **(Fig. 11)**.

Taken together, receptor mediated endocytosis is a suitable method for transferring genes into resting primary macrophage cultures. Studies are under way to determine whether other cell types expressing the mannose receptor including dendritic cells can also be transfected using this method. Mannose receptor mediated gene transfer may be a useful system for future therapeutic applications.

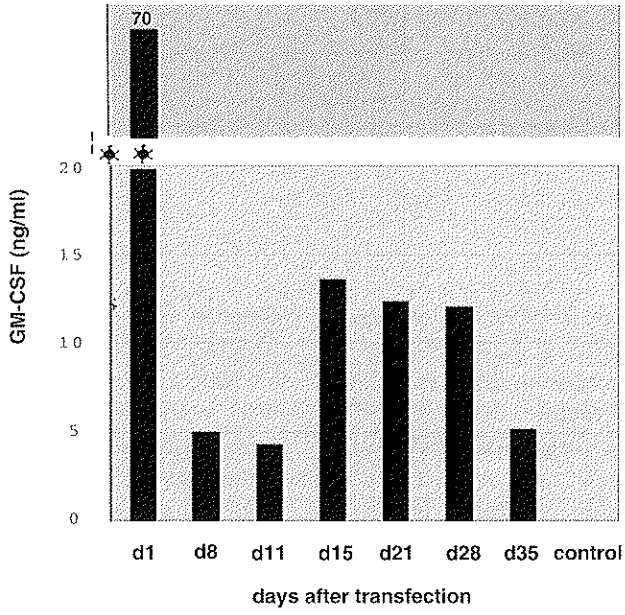

Fig. 11: *GM-CSF expression following mannose receptor mediated gene transfer.*
Primary bone marrow macrophages were transfected with the gene coding for murine GM-CSF using adenovirus enhanced receptor mediated gene transfer and mannose-polylysine. Cell supernatants were assayed for cytokine contents for a period of 5 weeks. No GM-CSF was found in cell supernatants transfected with a luciferase plasmid. Cells were cultured in medium without added growth factors.

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Leukemogenesis and carcinogenesis: Protooncogene function in avian, human and murine *in vitro* systems

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PhD student	Oliver WESSELY (until Oct. 96)
PhD student	Georg MELLITZER
PhD student	Anton BAUER
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Technician	Evi DEINER
Technician	Gabi STENGL
Guest	Iris KILLISCH (April - July 96)
Guest	Claudia PETRITSCH (from April 96)

Overview

In 1996, we further continued our work on both established and new areas within our two major fields of interest: **(i)** normal hematopoiesis and leukemogenesis in vitro; and **(ii)** mammary carcinogenesis, using a mouse model (see Report 1995). In the first system, i.e. normal and leukemic hematopoietic progenitors, proliferation and differentiation is regulated by two distinct gene families, e.g. receptor tyrosine kinases and nuclear hormone receptors. Here, we are increasingly shifting our focus to use cell culture model systems of human and mouse origin to demonstrate more general relevance to our findings in chicken cells and support them by genetic data (see Report 95). We also successfully completed a study aimed at using chicken erythroid cells as a primary, heterologous system to analyze the *in vitro* cooperation of three oncoproteins known to cooperate *in vivo*, i.e in a well-known murine erythroleukemia model.

In the second project, we concentrate on a model for oncogene cooperation in mammary carcinogenesis. Specifically, we analyze the cooperation between the Ha-Ras oncoprotein and the endogenous TGFβ receptor in contributing to tumor progression and invasion of tumorigenic mouse mammary epithelial cells. We have also started a project, where we try to replace Ha Ras in its cooperation with TGFβ with overexpressed normal and mutated receptor tyrosine kinases of the human epidermal growth factor receptor (HER) family.

A. Normal and leukemic erythropoiesis

The **first** major interest of our group are mechanisms controlling normal hematopoiesis and leukemogenesis. Presently, our main emphasis is on two questions. Firstly, we identify and functionally characterize the molecular players in normal hematopoietic progenitors, which are involved in maintaining and regulating the balance between proliferation without apparent differentiation (here referred to as "self renewal") and differentiation to more mature progenitors. Secondly, we try to characterize the mechanisms in leukemia, which abnormally "tip" this balance between self renewal and differentiation towards uncontrolled proliferation of leukemic progenitors. We find that in both processes, receptor tyrosine kinases located at the plasma membrane have to cooperate in an intricate, specific fashion with one or more members of the nuclear hormone receptor family. It also becomes increasingly clear that the latter ligand-dependent transcription factors represent ligand-operated molecular switches. A subgroup of these nuclear receptors, i.e TRα/ RARα may function as more effective "switches", acting as gene regulators with different, often opposing activity both in the absence and presence of ligand (For more detailed information, see Beug *et al.*, 1996; Beug *et al.*, 1994 and Report 1995).

1. Molecular players in balancing self renewal and differentiation

1.1 Avian erythroid progenitors: Glucocorticoid receptor acting as a crucial player in induction and maintenance of progenitor self-renewal

Oliver Wessely, Marieke von Lindern, Anton Bauer, Evi Deiner and Hartmut Beug.

One group of molecular components of the machinery regulating self renewal versus differentiation in normal erythroid progenitors had been identified as several receptor tyrosine kinases [RTKs; e.g. the stem cell factor (SCF) receptor c-Kit or the avian epidermal growth factor receptor c-ErbB, which utilizes mammalian transforming growth factor (TGF) α as a high affinity ligand]. These RTKs have to cooperate with a second group of proteins, i.e. nuclear hormone receptors. In 1995, we showed that at least two members of this nuclear receptor family were required: the estrogen resceptor (ER) and the glucocorticoid receptor (GR). The GR clearly played a major role, since addition of a specific GR antagonist (ZK 112993) to normal erythroid progenitors expressing ligand-activated c-Kit, c-Erb and ER receptors still completely inhibited their long-term self renewal. Thus, the GR ligand Dex was absolutely required for both the development of long-term self-renewing erythroid progenitors from c-ErbB-negative precursors and their proliferation in the c-ErbB ligand TGFα in media depleted for serum steroid hormones (Steinlein *et al.*, 1995; Wessely *et al.*, 1996).

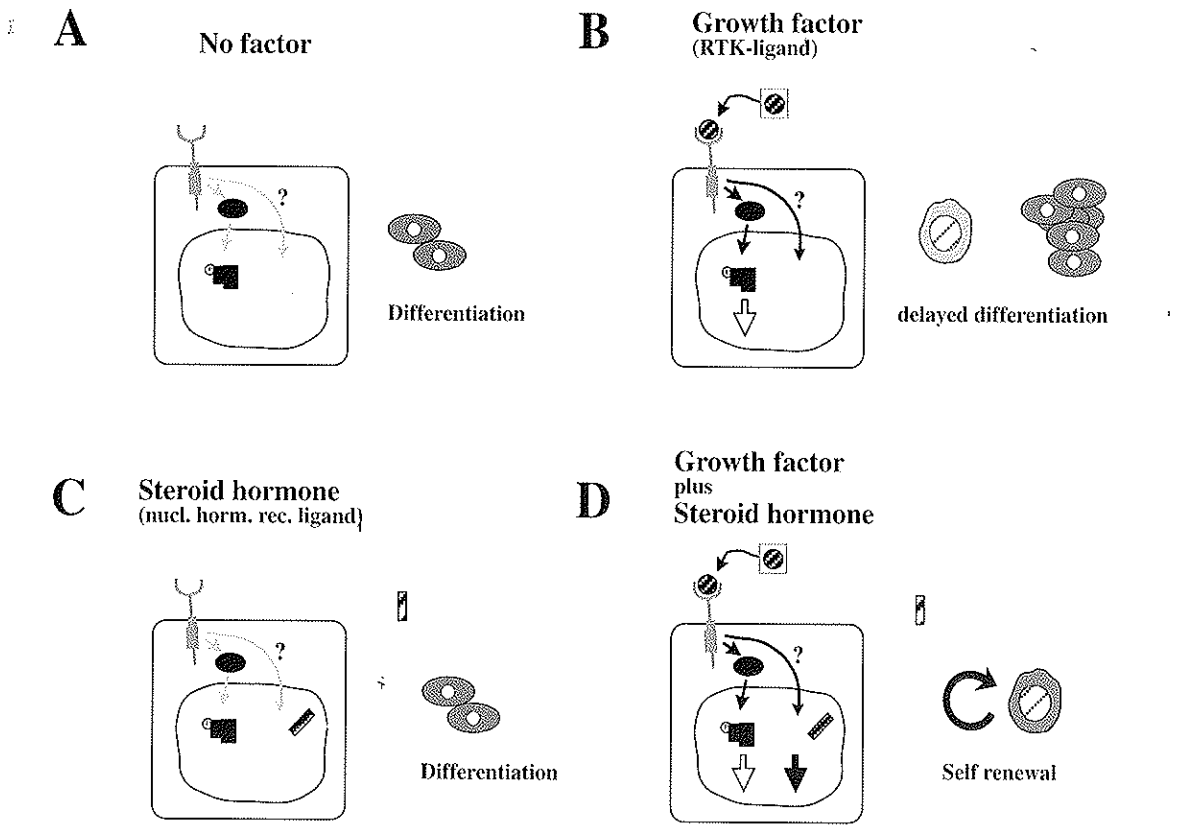

Fig. 1: *Regulating the balance between proliferation and differentiation: cooperation of receptor tyrosine kinases and nuclear hormone receptors.*
These schemes depict our current ideas about the interdependence of receptor tyrosine kinases (dark grey plasma membrane receptor symbol) and nuclear hormone receptors (light grey nuclear symbol) in regulating erythropoiesis. (**B**) Ligand-activated (hatched circle/ arrow) receptor tyrosine kinases activate transcription factors (open arrow) via signal transduction intermediates (black cytoplasmic and nuclear symbols), causing stimulation of proliferation but no differentiation arrest. (**D**) In contrast, the nuclear hormone receptors require both ligand activation (hatched rectangle, light grey arrow) and a co-operative signal from the receptor tyrosine kinase (RTK; black arrow with question mark) to be active (black arrow) causing both proliferation and differentiation arrest (self renewal, erythroblast symbol with black circular arrow). (**C**) Without a co-operative signal from the receptor tyrosine kinase (dark grey arrows), the ligand-activated nuclear hormone receptor is inactive, similar as in cells receiving no self renewal factors (no factor, **A**).

We also demonstrated that the activated GR (in cooperation with an active ER) would arrest erythroid differentiation induced by avian erythropoietin and insulin. Again, this activity of the GR required cooperation with c-Kit. Finally, a molecular analysis of how the GR regulated self renewal versus differentiation was initiated. Results obtained in 1995 suggested that the GR did not act through transrepression and that it required an intact DNA binding domain for function. Finally, first attempts to isolate GR-regulated target genes relevant for proliferation induction and differentiation arrest suggested the c-Myb gene as a possible candidate.

In 1996, these studies could be completed and submitted for publication. The following picture emerges. On the **cellular level**, the primary actors known to tip the balance between proliferation and differentiation towards cell growth are ligand-regulated receptors involving members of two gene superfamilies: receptor tyrosine kinases (c-ErbB and c-Kit) and nuclear hormone receptors (ER and GR). In contrast, members of the cytokine receptor family (EpoR, Il-3Rα/β, see below), together with the insulin receptor, the thyroid- and/or retinoic acid receptor and further suspected or unknown proteins direct the fate of the erythroid progenitors towards differentiation.

Importantly, progenitor self renewal, i.e. proliferation in the virtual absence of differentiation, absolutely requires the cooperation between receptor tyrosine kinases and nuclear hormone receptors. On its own, neither receptor type can cause this phenotype in erythroid cells. The interplay between the members of the two gene families is intricate (**Fig. 1**). In the absence of active steroid hormone receptors, the tyrosine kinases stimulate cell proliferation. This retards but does not inhibit terminal differentiation. As a result, more erythrocytes are produced per erythroid progenitor (**Fig. 1**). Like the tyrosine kinases, the steroid hormone receptors alone show little effect on the erythroid proliferation / differentiation program. Only if activated by ligand and cooperating with a simultaneously active receptor tyrosine kinase, the steroid hormone receptors are able to carry out their differentiation-arresting function (**Fig. 1**, Beug *et al.*, 1996).

Analysis of GR function has also progressed at the **molecular level**. The GR has to bind to a glucocorticoid-responsive element (GRE) and requires a functional AF-2 transactivation domain for proliferation stimulation and differentiation arrest. This was clearly shown by expressing an ER mutant (HE82), in which the P-box sequence of the ER was changed to the corresponding sequence of the GR (E203G, G204S, A207V). This mutant ER transactivates GRE-containing reporter genes upon activation by E2 (see Report 1995). When expressed in primary erythroblasts, the HE82 receptor functioned like a bona-fide, but estrogen-activated GR, i.e. it stimulated proliferation in the presence of the GR-antagonist ZK (plus SCF,TGFα and estradiol). In contrast, a further mutant ER construct bearing additional inactivating point mutations in its AF-2 transactivation domain (HE82LALA) was completely inactive. This indicated that directing a conserved, steroid-hormone receptor transactivation domain (AF-2) to GRE-containing target genes was sufficient to stimulate proliferation and arrest differentiation in erythroid progenitors (Wessely *et al.*, 1996).

In a first trial to identify GR target genes, the protooncogene c-Myb was identified as a candidate. c-myb mRNA levels were rapidly induced upon stimulation of normal erythroid progenitors with Dex. A possible function of Myb in proliferation stimulation and differentiation arrest could be demonstrated by the fact that v/c-Myb, an activated form of the c-Myb protein, functionally replaced the GR when overexpressed in erythroblasts. Like the GR, v/c-Myb was only active if a ligand-activated c-Kit or c-ErbB receptor tyrosine kinase was also present (Wessely *et al.*, 1996).

Taken together, the identification and functional analysis of molecules acting together in regulating the erythroblast´s decision between proliferation and terminal differentiation has identified a number of key players as well as first glimpses on how they interact (**Fig. 2**). As also seen below, even the current picture is most likely still oversimplified as well as incomplete. In particular, the possible cooperation of c-Kit with c-Myb or upstream players regulating its activity remains to be investigated (**Fig. 2**).

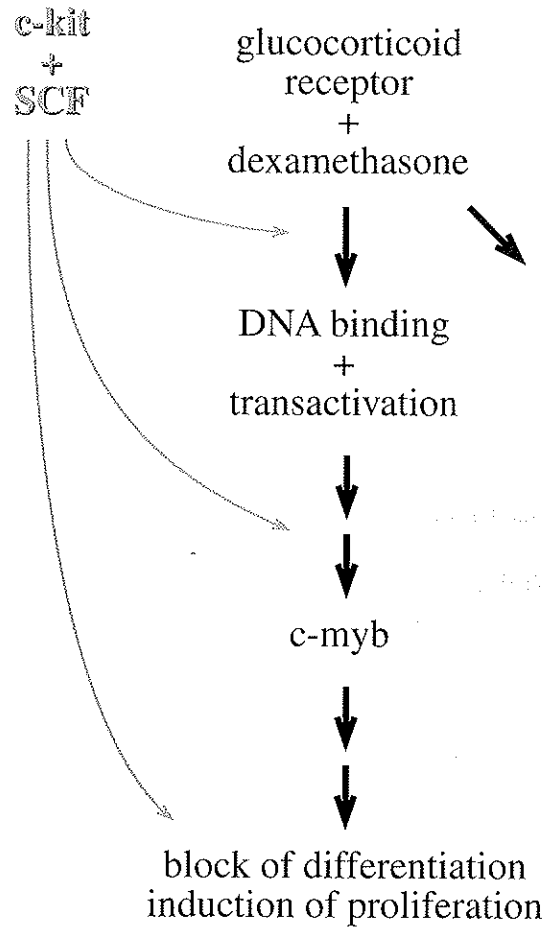

Fig. 2: *Regulation of self renewal by the GR: molecular players involved.*
This simple flow diagram illustrates our current view of possible molecular mechanisms involved in regulation of erythroblast proliferation and differentiation by the GR. The vertical steps shown in black illustrate molecular events supported by experimental data. It remains to be investigated, at which level and by what mechanisms c-Kit feeds into this pathway (dark grey arrows). Additional, currently open questions are shown in light grey.

1.2. The v-ErbA oncoprotein fully substitutes for steroid hormone receptor function in erythroid progenitor self renewal

Anton Bauer, Marieke von Lindern and Hartmut Beug, with H. Stunnenberg, Heidelberg and B. Vennström, Stockholm

Last year we demonstrated that the oncoprotein v-ErbA, a mutated version of the avian thyroid hormone receptor α (c-ErbA/TR-α), fully substitutes for the biological functions of the steroid hormone receptors ER and GR in erythroid progenitors. This is true both for induction of sustained proliferation and differentiation arrest in these cells, and for the complete dependence of these functions on the cooperation with endogenous, activated receptor tyrosine kinases such as c-Kit or c-ErbB. We also showed by means of respective v-ErbA mutants, that specific DNA binding was indispensable for v-ErbA function. If heterodimerization to the co-receptor RXR was restored in v-ErbA by mutation, the ability of c-ErbA (and RAR) to overcome the v-ErbA-induced differentiation arrest after ligand activation was abolished. Finally, phosphorylation of v-ErbA (shown to be required for ErbA function in cooperation with oncogenic receptor tyrosine kinases like v-ErbB or v-Sea; (Glineur *et al.*, 1990) was not important for v-ErbA cooperation with c-Kit in normal erythroid progenitors (see Report 1995).

In 1996 we tried to narrow down the mutations required vor v-ErbA function as opposed to c-ErbA. For this, a whole series of different v-ErbA proteins, in which the six most N terminal mutations (two N terminal of the P-Box mutation S61G, this site as well as the D box mutation in the first and second zinc finger as well as the two adjacent c-terminal mutations were changed , singly or in pairs or larger groups, to the c-ErbA configuration. Except for one mutation (v/c-ErbA 1-6), containing all 6 residues in the c-ErbA configuration) and another, presumably toxic mutant (this protein could never be expressed at

sufficient levels), all these mutants were able to stimulate proliferation and arrest differentiation more or less as the wild-type protein. Under appropriate culture conditions, v/c-ErbA 1-6 was still able to induce sustained erythroblast proliferation. It differed, however, in the tightness of the differentiation arrest induced: when cells expressing this protein were compared to wt v-ErbA erythroblasts (carrying mutations to the c-ErbA configurations at positions 5 and 6 (c-terminal mutations adjacent to the zinc fingers), v/c-ErbA 1-6 only caused a partial differentiation arrest.

Despite this differential action of v/c-ErbA 1-6, suggesting that the mutations in the DNA binding region of v-ErbA influence its phenotype, the fact that this protein was at least partially active cast doubt on the idea that c-ErbA was unable to induce self renewal and arrest differentiation like v-ErbA (Zenke *et al.*, 1990). In these older studies, an overexpressed gag-c-ErbA protein was unable to stimulate proliferation in absence of ligand, rather, it seemed to be toxic. In addition, it only caused an aberrant, partial differentiation arrest. However, while the TRα/c-ErbA ligandT3 had been removed from the sera in these experiments, no attempt had been made to remove retinoids, which could atimulate the TRa/RXR heterodimer by binding of RXR ligands. Pilot experiments indeed suggest that removal of both T3 (by ion exchangers) and retinoids (by a combined freon/ charcoal treatment) from all sera including anemic serum is feasible and that under such conditions (i.e. complete absence of T3 / RXR ligands) an overexpressed gag-c-ErbA may indeed function like v-ErbA, i.e. stimulate proliferation and arrest terminal differentiation. This new possibility is under intense study right now, since it would offer an easy explanation how v-ErbA can substitute for GR/ER function. Rather than assuming that due to its DNA binding mutations v-ErbA binds to regulatory DNA elements normally employed by the ER/GR (an unlikely event because of the differences between TREs and GRE´s) the GR may function upstream of c-ErbA, regulating its expression and stabilizing it in a ligand-deprived, proliferation inducing conformation.

1.3. Self renewal induction in primary human and murine erythroid progenitors

Oliver Wessely, Marieke von Lindern, Peter Steinlein, Georg Mellitzer and Hartmut Beug, (with W. Fritsch, St. Anna Kinderspital, Vienna, K. Huber, AKH, Vienna, M. Sibilia, IMP and G. Schütz, DKFZ, Heidelberg, Germany).

Human erythroblasts: In 1995, we established that the self renewal of committed progenitors found in the chicken (violating a dogma in hematopoiesis that only the pluripotent stem cell can self renew; Keller, 1992) could also be demonstrated for human erythroid progenitors. In the presence of human recombinant (hu-r-) Epo, murine SCF, estradiol, dexamethasone (Dex) and insulin-like growth factor (IGF)-1, erythroid progenitors from human cord blood underwent self renewal for 20-25 generations, resulting in a 10^5-10^6 fold net increase in the number of erythroid cells. These cells could be clearly characterized as immature, proerythroblast-like cells by colony assays in semisolid medium and FACS analysis for a large number of lineage markers (see Report 1995). In contrast, cells resembling the c-ErbB expressing, rare erythroid progenitor found in the chicken could not be found so far in the human system. The human erythroid progenitors could be induced to terminal differentiation into enucleating erythrocytes. Despite extensive characterization of the factor requirements of erythroid differentiation in vitro (see Report 1995), a number of serum factors influencing these differentiation events still remain to be characterized.

In 1996, these studies have been completed. Most importantly, we could show by limiting dilution analysis that a large proportion of erythroblasts from the initial culture is able to undergo 15-20 cell divisions without apparent maturation. While each clone generates some differentiating offspring throughout its lifespan, immature, self renewing cells are maintained until the end of the lifespan of the clone. Outgrowth is possible from bone marrow or peripheral blood buffy coat cells of essentially every healthy human donor tested. Outgrowth can occur from $CD34^+$ and $CD34^-$ progenitors with similar efficiency. Even committed erythroid progenitors (BFU-E) purified by repeated rounds of cell sorting for different markers will grow for 20-22 generations.

We have also determined which of the factors combined to activate long-term proliferation of human erythroid progenitors was essential, determining the consequences of omitting one or the other factor. Epo, SCF and Dex were absolutely essential for long-term outgrowth. While omission of SCF or Dex caused premature differentiation of the cultures into erythrocytes, cultivation in absence of Epo led to death of the culture by apoptosis. Omission of estradiol had no detectable effects, while IGF-1 was clearly beneficial in that it caused more rapid proliferation of the cells for a somewhat enhanced number of generations.

Finally we have tested, whether human erythroid progenitors are responsive to ligands specific for receptors of the HER (EGF-receptor) family. We chose human heparin-binding EGF (hbEGF), since this ligand was claimed to be active on hematopoietic cells. While we failed to generate erythroid progenitors capable of growing for >50 generations (as

predicted for cells resembling avian SCF/TGFα progenitors), we could clearly show that hbEGF both increased the lifespan of limiting dilution clones from human cord blood for 3-5 generations and strongly increased the frequency of long-living clones. Thus, human erythroid progenitors are in principle responsive to HER ligands and simple absence of HER-type receptor tyrosine kinases from human erythroid cells cannot be the reason why we have so far failed to demonstrate this cell type in humans (see below, 1.4.).

Mouse erythroblasts. Recently, the group of G. Schütz in Heidelberg, Germany, has produced mice, in which the gene for the GR had been inactivated by homologous recombination (Cole *et al.*, 1995). This would allow to obtain genetic evidence that an activated GR is required for the proliferation of erythroblasts, the respective cells could also be grown from murine fetal liver (GR-/- mice die around birth). We found that murine erythroblasts positive for the erythroid markers ter-119, CD71 (transferrin receptor) and c-Kit, as well as negative for lymphoid (B 220) and myeloid markers (Mac-1/GR) could be grown from suspensions of fetal livers from 14.5 day-old embryos. For this, murine SCF, human recombinant Epo and the GR ligand Dex were absolutely required, similar as for human erythroid cells (**Fig. 3A**). The lifespan of murine erythroblasts grown out from fetal liver was much shorter as seen for human cells, in accord with a much shorter "Hayflick limit" of murine fibroblasts (7-15 generations) than respective human fibroblasts (40-60 generations. Nevertheless, enough cells could be grown out for characterization (cells express c-Kit, EpoR, GR and IGF-1R) and limited trials of differentiation induction (enucleating, very small erythrocyte-like cells are formed from the erythroblasts in 1-2 days with few divisions in between). Indeed, omission of dexamethasone was sufficient to induce differentiation of the murine erythroblasts, demonstrating the requirement of these cells for an active GR (**Fig. 3B**).

To unequivocally show that the GR is required for erythroid cell self renewal, we tried to grow fetal liver cells from GR -/- mice under the same conditions as from the GR +/+ control mice. All cultures from the GR +/+ controls showed the expected outgrowth, but no outgrowth of erythroid blasts from fetal liver cells of GR -/- was observed. Furthermore, the nongrowing erythroid cells present in these cultures were totally unresponsive to GR ligands (**Fig. 3B**). Current work is now concentrating on the analysis of these mice with respect to the question of the potential *in vivo* role of the GR in the regulation of erythropoiesis. Pilot experiments using FACS analysis suggest that c-Kit expression may be impaired in the erythroid blasts from the GR -/- mice and that these cells may proceed through fewer cell divisions while maturing into erythroctes.

1.4. Cooperation of c-Kit with the EpoR can replace c-ErbB in GR-dependent erythroblast self renewal.

Oliver Wessely, Georg Mellitzer, Peter Steinlein and H. Beug, with C. Tran-Quang, Orsay, France

Our studies of self-renewing erythroblasts in mice and man showed a few important differences to avian erythroblasts, with respect to the factors required by these cells for self renewal (**Fig. 4**). In the chicken, an initial, transient outgrowth of erythroblasts could be obbtained with SCF plus steroids. However, an upregulation of c-ErbB triggered by the simultaneous presence of SCF, TGFα, estradiol and dexamethasone was required for long-term self renewal of avian erythroblasts (Steinlein *et al.*, 1994).

In contrast, neither human nor murine erythroblasts grew out at all from the available sources in SCF plus steroids alone. Also, no outgrowth or upregulation of c-ErbB was observed in SCF, estradiol, dexamethasone and various ligands for human members of the HER family. Rather, the mammalian cells were completely dependent on exogenous Epo for efficient outgrowth. Furthermore, the activated ER played no important role in the mammalian erythroblasts, while clearly required in the chicken system for efficient differentiation arrest.

This divergence in factor requirement between avian and mammalian erythroid progenitors could either represent species-specific differences or reflect the fact, that different combinations of plasma membrane- and nuclear hormone receptors could cause the same phenotype. To analyze if the latter idea was correct, we determined, whether a combination of the EpoR and c-Kit would be able to cause self renewal also in chicken cells, when cooperating with an activated GR. Since neither chicken Epo nor its receptor is available in purified form, we analyzed if the murine EpoR (previously shown to be active in avian erythroblasts; Steinlein *et al.*, 1994) could cooperate with endogenous c-Kit and the activated GR in avian erythroblasts. After expression of a murine EpoR c-DNA in normal erythroid progenitors via a suitable retrovirus, these cells underwent sustained self renewal when grown in human recombinant Epo (hu-r-Epo), SCF, Dex and IGF-1. These cells were no longer dependent on an activated ER, as shown by their insensitivity to the ER antagonist ICI 164384. Also, these cells grew completely independent of an activated c-ErbB, since they were not affected in their long-term proliferation by a fully inhibiting dose of a c-ErbB-specific tyrosine kinase inhibitor (PD 153035, Fry *et al.*, 1994). More interestingly, these cells completely failed to upregulate c-

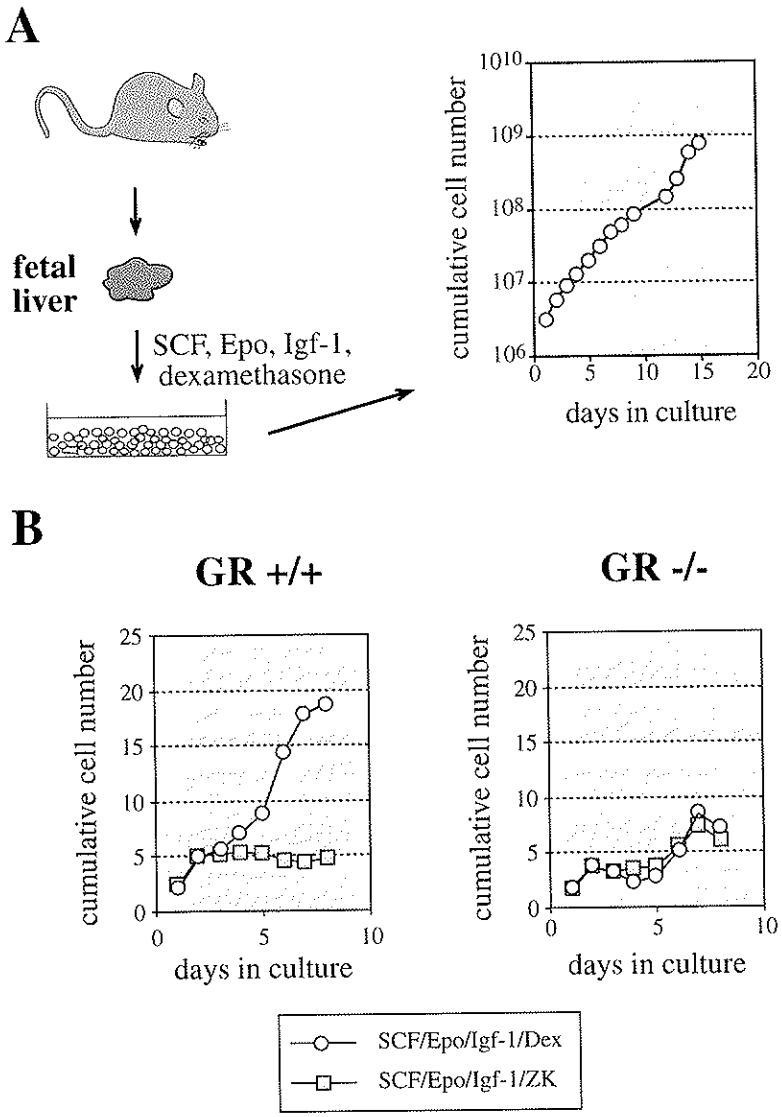

Fig. 3: *The GR is required for outgrowth of murine erythroblasts in culture: Genetic evidence.*
(A) Outgrowth of murine erythroblasts from fetal livers of 14.5 day-old mouse embryos. A fetal liver cell suspension is seeded in medium containing hu-r-Epo, murine SCF, Dex and IGF-1. Cells are counted at the time points indicated and cumulative cell numbers plotted. **(B)** Outgrowth from 14.5 day old embryo fetal livers obtained from mice in which the GR has been deleted by homologous recombination (GR-/-) and from control mice (GR +/+). Cells are cultivated in the above medium (see A) containing (circles) or lacking (squares) dexamethasone. Note Dex-dependent outgrowth in the GR+/+ cultures, while no significant, Dex-stimulated outgrowth is seen in the GR knockout mice (GR-/-).

ErbB, even if cultivated in the presence of its ligand (e.g. in hurEpo, SCF, TGFa, Estradiol, Dex and IGF-1). Strikingly, no c-ErbB detectable by Western blot, P-tyr-Blot or bioassay was found in these cells even after 30 days in culture.

Perhaps due to the constitutive expression of the mu-EpoR , these cells were not fully dependent on exogenous hu-r-Epo, since they grew with reduced speed even in the absence of hu-r-Epo. Nevertheless, traces of Epo are required for outgrowth, since sustained proliferation was completely inhibited after addition of an Epo-neutralizing antibody active both against chicken and human Epo.

Finally, we determined if the endogenous avian EpoR could also cooperate with c-Kit to induce self renewal in the presence of an activated GR. For this, normal erythroid progenitors were grown in the presence of anemic serum, SCF, estradiol, Dex, IGF-1 and PD 153035 (to inhibit endogenous c-ErbB otherwise activated by unknown ligands in anemic serum). Sustained self renewal for up to 20-22 days was clearly obtained under these conditions. Limiting dilution experiments showed that the *in vitro* lifespan of these cells was somewhat shorter (15-18 generations) than in the respective, c-ErbB expressing SCF/TGFα progenitor clones (20-30 generations, Steinlein *et al.*, 1995). In summary, self renewal of erythroid cells in the chicken can be induced by exactly the same growth factor/steroid hormone combinations as in the respective human and murine erythroid cells (**Fig. 4**).

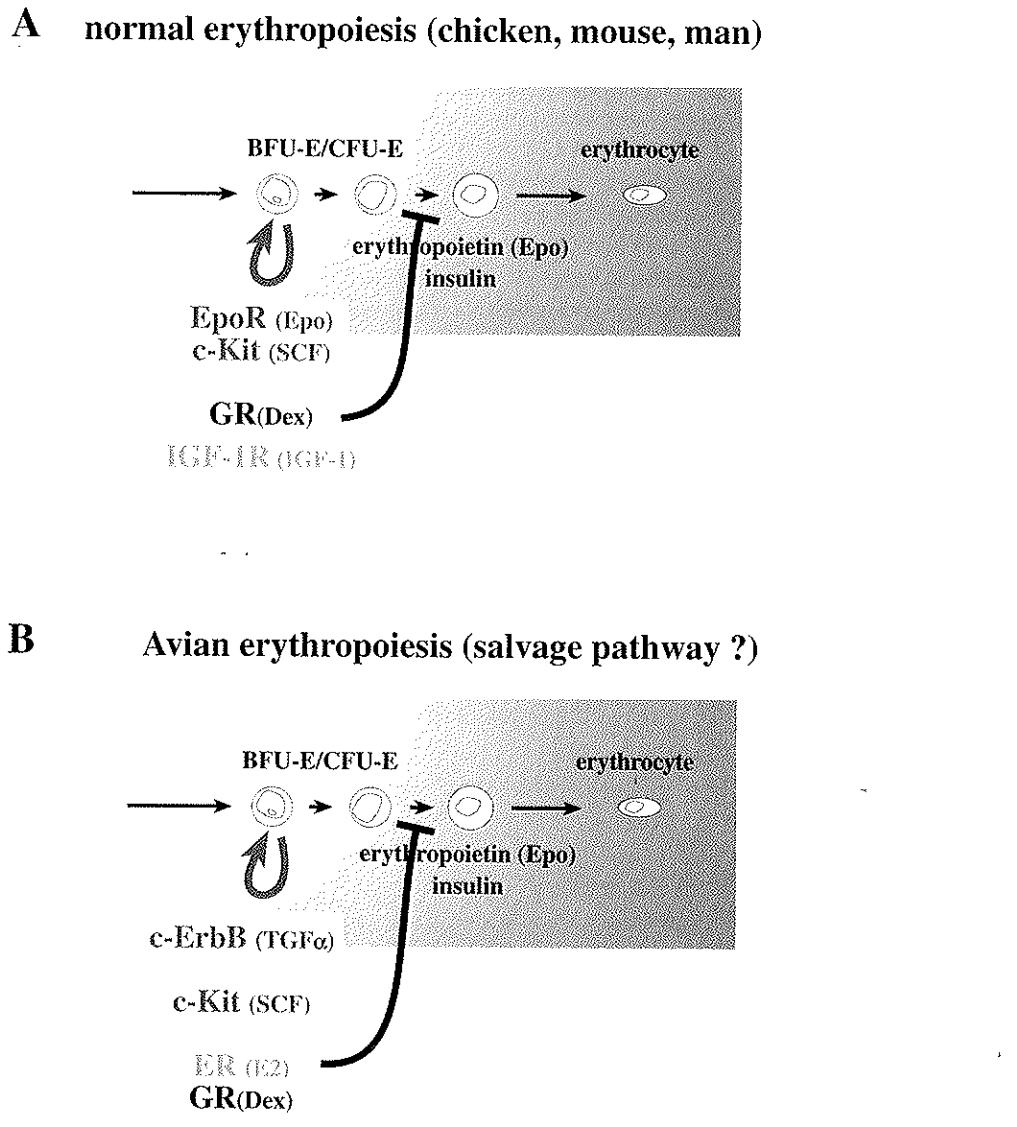

Fig. 4: *Growth factors and hormones required by different avian, human and murine erythroid progenitors for sustained self renewal*
(A) This simple scheme depicts the "standard" set of growth/differentiation factors and steroid hormone receptors which cooperate in regulating self renewal and differentiation in primary avian, human and murine erythroid progenitors. Epo plus SCF stimulate proliferation (dark grey circular arrow, Dexamethasone arrests differentiation (black inhibitory symbol) and IGF-1 speeds up proliferation and protects from apoptosis. **(B)** Similar scheme for the rare, c-ErbB-expressing erythroid progenoGors undergoing long-term self renewal in SCF, the c-ErbB-ligand TGFα, estradiol and dexamethasone. Again, the receptor tyrosine kinase ligands stimulate proliferation, while the nuclear hormone receptor ligands arrest differentiation (see panel **A** and **Fig. 1**)

1.5. Self renewal and differentiation: opposite effects of Stat 5b in avian erythroid and myeloid cells ?

Georg Mellitzer, Oliver Wessely and Hartmut Beug, with M. Kieslinger, I. Woldmann and T. Decker (Vienna Biocenter) and M.J. Hayman (Stonybrook, USA).

Erythroid cells. In pursuit of the question, which molecular mechanisms are responsible for the fact that c-ErbB can induce sustained self-renewal in erythroid progenitors, while c-Kit alone is unable to do so, we demonstrated in 1995 that c-ErbB, but not c-Kit was able to induce tyrosine phosphorylation and DNA binding of a member of the **s**ignal **t**ransducer and **a**ctivator of **t**ranscription (Stat) family, Stat 5b, in avian erythroblasts. In addition, the activated Stat 5b caused transactivation of suitable reporter constructs [(Mellitzer *et al.*, 1996), see Report 1995].

In 1996, we sought to complete these studies, (i) by analyzing the effect of the EpoR on Stat 5 activation, (ii) by analyzing activation of other signal transduction pathways by c-Kit and c-ErbB (iii) by testing the effects of Ras-inhibitors on Stat 5 transactivation and (iv) by construction and testing of dominant-negative versions of Stat 5b. We found that the EpoR could activate Stat 5b DNA binding upon stimulation with Epo. However, no transactivation of

reporter genes was induced by the activated EpoR, either in human erythroblasts expressing endogenous EpoR or in avian erythrovblasts expressing an exogenous, murine EpoR.

To analyze if other signal transduction pathways were activated selectively by c-ErbB, we tested activation of Ras by measuring GTP/GDP exchance ("Ras loading") and tyrosine phosphorylation of the RTK adaptor protein Shc in cells activated by either c-ErbB- or c-Kit ligands. While c-ErbB- and c-Kit activation stimulated Ras loading to a similar extent and with similar kinetics, Shc became tyrosine phosphorylated by c-ErbB only. However, this was most likely not important for self renewal induction, since the c-ErbB specific inhibitor AG30 (which abolishes self renewal by acting downstream of the c-ErbB receptor kinase rather than by inhibiting the kinase itself such as PD 153035) did not affect Shc phosphorylation. Interestingly, AG30 did inhibit induction of DNA binding by c-ErbB, while AG 555, which inhibits both c-ErbB and c-Kit, did not affect Stat 5 DNA binding.

Recent results on Stat 1 and Stat3 suggest that tyrosine phosphorylation of Stats is sufficient to induce their binding to DNA but that they have to be phosphorylated on serine/threonine residues as well to transactivate reporter genes. The latter phosphorylation is believed to be mediated by kinases activated by the Ras-MapK pathway. To ask, whether Ras-dependent modification was important for transactivation by Stat 5b, cells were stimulated with TGFα in the presence or absence of B581, a specific inhibitor of Ras farnesylation. Indeed, Ras-inhibition led to an almost complete inhibition of TGFα-induced transactivation, while TGFα-induced DNA binding was not affected. These results raise the interesting possibility that c-Kit might restore transactivation to Stat 5b after activation of DNA binding by the EpoR. We have already generated suitable cells overexpressing both EpoR and exogenous avian c-Kit to analyze, if the EpoR together with c-Kit may be able to cause Stat 5b DNA binding and transactivation, thus mimicking the effects of c-ErbB also on the biochemical level.

Finally, we generated various mutants of Stat 5b to generate a dominant-negative (dn) version, suitable to repress the activity of endogenous Stat 5 in cells. One dnStat 5 construct, also active in other systems, was clearly able to completely suppress transactivation of reporter genes by Stat 5 after stimulation with TGFα, when transfected into avian erythroblasts. Presently, we try to introduce such constructs stably into suitable erythroblasts, using inducible retovirus vector systems. This should allow to answer the question, if such dnStat5 proteins are able to abolish c-ErbB-stimulated self renewal of avian erythroblasts. By such an approach, we eventually would like to prove or disprove the concept that signal transducers of the Stat family are causally responsible or involved in the induction of erythroblast self renewal.

Myeloid cells. Erythroid and myeloid progenitors differ crucially in their response to receptor tyrosine kinases such as v- or c-ErbB, v- sea, v-fms and v-src, as well as to downstream signal transducers such as Ha-Ras. In all cases, these genes seem to arrest differentiation and promote proliferation in erytrhroid progenitors, while they have an opposite effect, i.e. induction of differentiation in myeloid progenitors. Hints for a possible molecular explanation of this phenomenon have come from work in T. Deckers laboratory. It was shown that various human myeloid cell lines show activation of Stat 5 during their drug-induced differentiation. These immortalized cell lines, however, are limited in their usefulness as an experimental system. They fail to differentiate in a normal fashion, and do not show a normal dependence on cytokines, which are the main known inducers of various signal transduction pathways involving Stats and which regulate cell proliferation as well as differentiation.

We therefore analyzed Stat 5 activation in primary, nonimmortalized myelomonocytic progenitor cells of the chicken, transformed by the *ts*21-E26 avian retrovirus. At 37°C, the temperature-sensitive oncoprotein of the *ts*21-E26 virus ($p135^{gag\text{-}myb\text{-}ets}$) causes a differentiation arrest at the myeloblast stage. This block is released by a shift to the nonpermissive temperature (42°C), leading to terminal differentiation into macrophages. Both proliferation and differentiation of these cells require the continuous presence of chicken myelomonocytic growth factor (cMGF).

At both the permissive and nonpermissive temperature, cMGF rapidly caused activation of Stat5 tyrosine phosphorylation, DNA-binding and reporter gene transactivation in these cells. Stat5-dependent transcription, but not its activation for DNA-binding activity, were inhibited by the B581 Ras farnesylation inhibitor. In addition to cMGF treatment, the temperature shift-induced onset of myelomonocytic differentiation also led to the appearance of activated Stat5. Differentiation as well as Stat 5 acivation after temperature shift required the presence of cMGF, since cells shifted to 42°C in the presence of IGF-1 neither differentiated nor showed Stat 5 activation. These data indicate that cMGF-responsive tyrosine kinases and Ras-dependent signals converge on Stat5. Moreover, they suggest a contribution of Stat5 to the process of myelomonocytic differentiation or to the functional changes that accompany the maturation of myeloid progenitor cells to a terminally differentiated stage.

2. Reconstructing murine Friend erythroleukemia in avian erythroblasts

Oliver Wessely and Hartmut Beug, with C. Tran-Quang and J. Ghysdael, Paris.

Friend leukemia, an acute murine erythroleukemia caused by a murine retrovirus (the Friend leukemia virus complex) is caused by at least three cooperating oncoproteins. Firstly, the SFFV component of the Friend virus complex expresses a modified envelope glycoprotein (p55 env) which constitutively activates (and probably modifies the bioactivity of) the Epo-receptor. Secondly, the retrovirus activates the (probably ectopic) expression in erythroid progenitors of an ets-family transcription factor (PU1/Spi-1) by promoter insertion mechanisms. And finally, the tumor suppressor oncoprotein p53 becomes deleted or functionally inactivated by mutation in most, if not all leukemic clones. Since 1993, we try to use the chicken cell system as a heterologous system to study how the various oncoproteins cooperating in Friend leukemia affect the erythroid phenotype on their own, and by which mechanisms they cooperate. The advantage of such a heterologous system is, that the proteins in question are analyzed in primary cells capable of normal terminal differentiation. The factors required for proliferation and differentiation of these cells can be rigorously controlled, since they are mostly chicken-specific and thus not present in the foetal calf serum required for the growth of these cells. We could show before that Spi-1 exhibited a marked ability to arrest erythroid differentiation, particularly when able to cooperate with endogenous or exogenous receptor tyrosine kinases. In contrast, mutant p53 profoundly altered the growth requirements of normal erythroid progenitors, i.e. their dependence on receptor tyrosine kinase activation (Tran Quang *et al.*, 1995).

In 1995 and 1996, we have performed and completed an analysis, how all three Friend virus oncoproteins act and cooperate in avian erythroid cells, reversibly transformed by the ts-v-sea oncoprotein. We used retroviruses expressing PU1/Spi-1, a temperature-sensitive mutant of p53, exhibiting mutant conformation at 39°C and two versions of the murine EpoR. Besides the wild-type receptor (unable to contribute to leukemia *in vivo*), a receptor, which had been rendered constitutively active by a substitution of Arg 129 for Cys in the extracellular domain of the EpoR was used. Apparently, this mutation results in constitutive dimerisation of the receptor, activating its mitogenic potential in several immortalized cell lines. Furthermore, the activated receptor [EpoR(R129C)] caused polycythemia and splenomegaly in mice infected with a respective retrovirus and thus mimics the gp55-dependent activation of the wt-EpoR characteristic of Friend erythroleukemia.

Using retroviral vectors expressing these three genes, we sought to analyze whether overexpressed Spi-1 and mutated p53 cooperate with EpoR(R129C) to deregulate the differentiation and proliferation of primary erythroid progenitors, in a fashion mimicking the Friend disease. For this, novel replication defective and replication competent retroviral vectors were developed, allowing the separate or simultaneous transfer of EpoR (R129C), mutant p53 and PU1/Spi-1 into ts-v-Sea transformed cells (see Report 1995). We actually obtained the result hoped for, i.e that the coexpression of these three proteins in ts-v-Sea erythroblasts induces an increased survival of immature progenitors tightly blocked in their differentiation. While cooperation of EpoR(R129C) with PU1/Spi-1 alone results in an efficient, but incomplete differentiation arrest accompanied by significant apoptosis, addition of mutant p53 to these two oncoproteins resulted in complete differentiation arrest of cells protected from apoptosis. In each case, differentiation arrest was dependent on the mutant EpoR(R129C) cooperating with PU1/Spi-1 or PU1/Spi-1/ts p53, the respective combinations with the wild-type EpoR showed no effect of PU1/Spi-1 or PU1/Spi-1/ts p53 and differentiated normally. Finally, we show that ligand-induced activation of endogenous c-Kit in the EpoR(R129C)/Spi-1 or EpoR(R129C)/Spi-1/ts p53 expressing, differentiation-arrested erythroblasts induces them to massive, sustained proliferation.

In conclusion, we could show that a mutated, constitutively activated EpoR (probably forced into such a mutant conformation by the Friend glycoprotein gp55) is central to the induction of the leukemic phenotype by the Friend leukemia "oncoproteins". Obviously, signal transduction by this mutant receptor is required for the overexpressed PU1/Spi-1 transcription factor to arrest differentiation (rather than apoptosis, as would be expected for a myeloid cell-specific transcription factor extopically expressed in erythroid cells.). The role of p53 most likely is to further reduce apoptosis and [as suggested by our earlier work, (Tran Quang *et al.*, 1995)] to recruit more immature progenitors as target for the Friend leukemia specific oncogenic alterations.

B. Cooperation of signal transducers in mammary carcino genesis and normal mammary gland development

The **second**, major interest of the laboratory is how defined oncoproteins contribute to the changes occurring in epithelial cells upon carcinogenesis. In several well-studied examples, formation of dedifferentiated, invasive tumors from normal epithelia was shown to be the result of multiple, cooperative genetic changes. Our group therefore concentrates on the question how single oncogenes involved in signal transduction (e.g. growth factor receptors, intracellular signaling proteins and transcription factors activated by intracellular signaling) affect defined aspects of the epithelial cell phenotype.

We use mammary epithelial cells of the mouse that retain normal epithelial polarity in culture, i.e. the ability to direct different proteins to the apical or the basolateral face of the epithelial cell. In this system we analyze oncoprotein-induced alterations in *epithelial polarity* and *epithelial-fibroblastoid conversion*, i.e two aspects of the regulated modulation of the polarized epithelial phenotype occurring during embryogenesis or tissue remodeling (Hay, 1990). Our long-term aim is to gain insight into related mechanisms operative in human mammary carcinomas, which frequently contain overexpressed c-Ras and/or overexpressed and/or mutated receptor tyrosine kinases of the EGF receptor family (HER-1, HER-2, HER 4).

3.1 Ha-Ras cooperates with TGFβ1 to induce epithelial/fibroblastoid conversion and invasiveness in polarized mammary epithelial cells

Martin Oft and Hartmut Beug, in collaboration with E. Reichmann and J. Peli, Lausanne

The current model. In this project, we concentrate on Ha-Ras transformed mouse mammary epithelial cells that form rapidly growing tumors upon injection into syngeneic mice. During 1994/1995, we showed that this tumor forming ability of the Ha-Ras epithelial cells is due to a highly plastic epithelial cell phenotype. Polarized epithelial Ep-ras cells undergo epithelial-fibroblastoid conversion (EFC) during tumor formation in mice, a process which can be reproduced *in vitro* by cultivating the cells in serum-free collagen gels, after addition of serum or transforming growth factor (TGF)-β1. This effect is specific for TGF-β1 and for Ras transformed cells. Ras cells grown in collagen gels in the absence of TGF-β1 retained a polarized phenotype. Furthermore, TGF-β1 induces morphogenesis or apoptosis in normal epithelial cells, but never causes EFC. After EFC the cells produce and deposit TGF-β1, while the parental, epithelial Ha Ras cells failed to produce TGF-β1, similar to normal control cells.

We also demonstrated that the fibroblastoid cells obtained after EFC maintain their fibroblastoid phenotype via an autocrine loop involving TGF-β1. The same antibody also neutralized the activity in serum that induced EFC. This led to the concept that TGF-β1 has two important, specific effects on Ha-Ras epithelial cells (i) induction of EFC and (ii) induction of an autocrine TGF-β1 loop in the converted cells. Finally, limited proof for this concept to be valid *in vivo* could be obtained [see Report 1995, (Oft *et al.*, 1996)].

Extension to human tumor cells. In 1996, we attempted to extend this concept to other tumor models and gain evidence for its applicability to human carcinoma. Firstly, in cooperation with K.H. Heider at Bender, Vienna, we analyzed if processes like EMT and concomitant TGF-β1 production also occur in in human cancer. One indication for EMT having occurred in a tumor would be the coexpression of the mesenchymal marker vimentin with the general epithelial marker cytokeratin by the tumor cells. While human renal cell carcinomas showed a very high incidence of vimentin/cytokeratin double expression (> 70% in more than 30 tumors), various stages of human breast carcinomas showed vimentin/cytokeratin double expression in 25-66% of the cases (n=65). A tendency for better expression of both markers in dedifferentiated tumors was also visible.

We also tried to detect TGF-β1 mRNA and protein in the same tumors, using immunohistochemistry, in-situ hybridization and PCR. To our surprise, the tumor cells were positive in 100% of the cases. Also, as expected from our mouse results, the tumor stroma, consisting of mesenchymal cells, was negative or low in TGF-β1 expression

In another approach, we tried to determine if signal transduction emanating from TGF-β receptors contributes to invasiveness in human carcinoma cell lines. A number of such lines of different tissue origin was grown in serum-free collagen gels, in the presence or absence of TGF-β1. While the cells readily invaded the collagen gel in the presence of TGF-β1, they formed tight clumps of non-invasive cells in the presence of TGF-β-neutralizing antibody (**Fig. 5A**).

Advanced mouse models. The use of TGF-β-neutralizing antibody for *in vivo* experiments is difficult or impossible due to the large amounts of antibody required. As an alternative approach to abolish signal transduction from the TGFβ receptor in tumor

cells, we overexpressed a dominant negative TGF-β receptor type II (TβRII-dn) in Ha-v-Ras expressing mammary gland epithelial cells, using suitable retrovirus vectors. While control cells readily formed exponentially growing tumors in nude mice, the TβRII-dn expressing cells formed only very small tumors, which exhibited an epitheloid phenotype after isolation from the animal.

Even more interestingly, overexpression of TβRII-dn in a highly tumorigenic mouse colon carcinoma cell line (CT26, Figure 5B) caused the reversion from a focus-forming, spindle-shaped phenotype to a cuboidal, dome-forming epithelial one. This morphological conversion from a mesenchymal to an epitheloid phenotype (mesenchymal-epithelial transition, MET) was accompanied by the reexpression of epithelial markers lost in the original tumor cells, e.g. E-cadherin and ZO-1. In addition, TβRII-dn overexpression completely abolished or retarded tumor outgrowth *in vivo* from the great majority of infected clones .

Inhibition of epithelial mesenchymal transition or reversion of mesenchymal tumor cells to an epithelial phenotype should be particularly important for invasiveness of the tumor cells during metastasis. Accordingly, CT26 induced tumors were surgically removed from control and experimental animals at a certain size. All control animals receiving unmodified CT26 cells died from lung metastases a few weeks later. However, all animals treated with the TβRII-dn expressing CT26 cells have remained tumor-free for 3 months already (Figure 5B). These results suggest that a functional TGF-β receptor is required for formation of lung metastases in this tumor model. Taken together, our results clearly indicate that TGF-β-induced signal transduction plays a key role in promoting uncontrolled invasiveness of tumor cells already expressing other oncogenes and/or tumor suppressor genes.

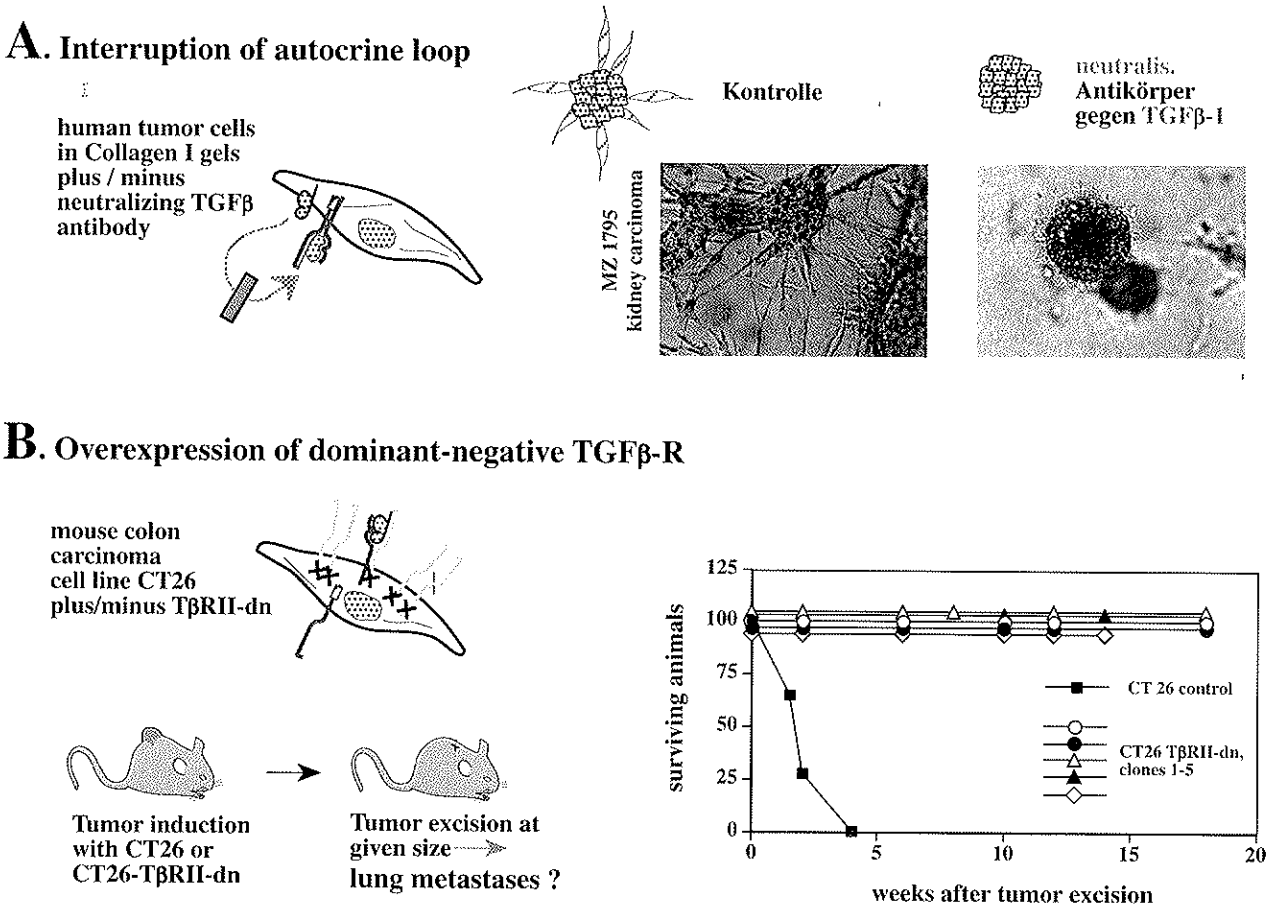

Fig. 5: *Tumor cell invasiveness in vitro and in vivo requires signal transduction from the TGFβ receptor: additional human and murine models*
(A) TGF-β-receptor signaling is required for invasive growth of human carcinoma cell lines (MZ 1795 kidney carcinoma, micrographs, KB nasopharyngeal carcinoma, not shown). Tumor cells were seeded into serum-free collagen gels receiving either TGF-β1 (5 ng/ml) or TGF-β-neutralizing antibody. Note that the invasive outgrowth of spindle-like cells from the clumps is totally inhibited by the TGF-β neutralizing antibody. **(B)** Expression of a dominant negative TGFβ-receptor II (TβRII-dn) in the murine colon carcinoma cell line CT26 (see scheme, receptor chain II symbol with cross indicates the kinase-dead dominant negative version used). This cell line displays a mesenchymal phenotype, coexpressing basal cytokeratins and mesenchymal markers (vimentin). It rapidly forms tumors which metastazise to the lung after excision of the primary tumor, causing rapid death of the animals (CT 26 control, solid squares). Five different clones expressing TβRII-dn gave rise to delayed tumor growth. When these primary tumors are excised at similar sizes as in the control animals, no metastases were seen in the animals even after 18 months. In some animals, the primary tumor recurred 14 weeks after excision (open diamonds).

3.2. Role of TGF-β1 in normal mammary gland development.

Martin Oft

Since the TGFβ family of growth factors is highly involved in morphogenetic processes during embryonic development we also re-investigated the role of TGFβ1 on normal mammary gland development. Last year, we showed that *in vitro* organogenesis in collagen gels was totally abrogated in the complete absence of TGFβ1, but strongly stimulated by low concentrations of the factor. At higher concentrations of TGFβ1 (required to induce EFC in the Ep-Ras cells), normal epithelial cells underwent growth arrest and apoptosis. In analogy to these *in vitro* findings, we could also show that TGF-β1 production by mesenchymal cells played a possible *in vivo* role during endbud outgrowth of the normal mammary gland, as well as in the massive apoptosis of mammary epithelial cells during involution of the mammary gland after weaning.

In 1996, we have started to investigate the factors that regulate differentiation of primary mammary gland mesenchymal cells *in vitro*. These mesenchymal cells are mainly preadipocytes and/or adipocytes, developing from fibroblastoid progenitors. The epithelial cells in the end buds of the developing tubular structures interact with these mesencymal cells while the mammary gland colonizes the fat pad. This development is controlled by various growth factors as well as by steroid hormones.

Primary cultures of mammary gland mesenchymal cells resemble fibroblasts or preadipocytes when cultured in ordinary, serum-containing media. When seeded in collagen gels containing dexamethasone and insulin (factors required for adipocyte differentiation), these cells differentiate into mature adipocytes and cease to produce TGF-β1. We presently try to expose these mammary preadipocytes to various epithelial growth factors and steroid hormones, which are supposed to be involved in the induction of mammary gland development. Pilot experiments suggest that factors like FGF´s and TGFα, together with steroid hormones, prevent or even revert the development of mammary gland mesenchymal cells into adipocytes. Rather, these cells retain or assume a fibroblastoid morphology and also continue to produce high levels of TGF-β1. These initial findings are the subject of ongoing, intense research.

Taken together, our results suggest that activated Ras (or overexpressed receptor tyrosine kinases activating the Ras pathway) cooperate with theTGF-β1 receptor in both normal development and carcinogenesis. This seems to involve processes like induction/activation of stromal TGF-β1 by interaction of epithelial and mesenchymal cells and epithelial-fibroblastoid conversion induced and maintained by TGF-β1. The main difference between normal and tumor cells thus seems to be that normal regulation of these processes is disrupted in the tumor cell.

3.3. Cooperation of HER receptor tyrosine kinases with the TGFβ receptor in mammary carcinogenesis.

Alexandra Pacher, Martin Oft and Hartmut Beug, together with T. Metz and T.v. Rüden, Bender, Vienna

In human breast cancer, ras mutations are relatively rare, but overexpression of nonmutated cellular Ras and/or activation of upstream receptors hyperactivating signal transduction pathways, (e.g. receptor tyrosine kinases of the EGF receptor family) are frequent events (for review see Clark and Der, 1995). It was therefore of interest to determine, whether or not activated receptor tyrosine kinases of the HER family (HER 1-4) were also able to cooperate with the endogenous TGF-β-receptor to induce EMT and invasiveness. Among the members of the HER family, HER2 seems to play a central role. Ligands for HER2 homodimers are unknown despite intense research. Rather, HER2 seems to cooperate with either HER1 (EGF-receptor) or with HER3/HER4 by forming heterodimers, which bind HER1 ligands (EGF, TGFα, amphiregulin, heparin-binding EGF etc.) or HER3/4 ligands (members of the Heregulin/glial growth factor family) much more avidly than the respective HER1 or HER3/4 homodimers, respectively.

For these reasons, we introduced a retrovirus expressing a constitutively activated HER2 (HER2*) into mammary epithelial cells. After demonstrating HER2* expression by Northern- and Western blot analysis, the cells were injected into nude mice. All mice receiving HER2* cells developed tumors. These tumors grew significantly slower than control tumors generated by injecting Ha-Ras-transformed mammary epithelial cells. However, the isolated tumor cells after establishment in tissue culture showed a similar fibroblastoid morphology to Ha-Ras ex tumor cells, while the cells displayed an epitheloid morphology before injection. This suggested that the HER2* cells had also undergone EMT during tumor formation.

Currently, we are characterizing these HER2* epithelial cells in more detail. First, we try to establish if the cells can undergo EMT in collagen gels in response to TGFβ1. Pilot experiments suggest that this is indeed the case. If seeded into serum free collagen gels in the absence of TGF-β1, HER2* epithelial cells formed branched structures, that were however essentially devoid of lumina, in contrast to

Ha-Ras epithelial cells. After addition of TGF-β1, however, the cells formed unordered cords and loose networks of fibroblastoid cells. This suggests that the cells underwent EMT in response to TGF-β1, however, this notion requires to be confirmed by more detailed characterization of these cells.

We also did a preliminary characterization of the HER2* cells before and after isolation from tumors. The epitheloid HER2* cells before injection expressed cytokeratins, E cadherin, ZO-1 and β-catenin more or less in the expected distribution. In contrast, the fibroblastoid HER2* cells isolated from tumors essentially lacked E-cadherin detectable by immmunofluorescence, whereas the distribution of cytokeratins, ZO-1 and β-catenin was clearly altered towards a less polarized expression pattern. However, the HER2* ex tumor cells did not express highly elevted levels of mesenchymal markers such as vimentin or fibronectin, while these are clearly upregulated in Ha-Ras control cells.

A preliminary conclusion from these results would be, that HER2* cooperates with the TGF-β-receptor in a fashion similar, but not identical to Ha-Ras. Future work will have to show if the HER2* cells are really comparable to the Ha-Ras epithelial cells, e.g. if they also exhibit an autocrine loop involving TGF-β1 and if this is responsible for an invasive phenotype. More importantly, we also try to establish whether or not overexpressed but unmutated versions of HER2 or other HER family members can also cooperate with the TGF-β-receptor, resulting in EMT and alterations in cell invasiveness.

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Transcription factors involved in early development, differentiation and oncogenesis

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Introduction

Early development and differentiation depend on differential gene expression which is brought about by the interplay of tissue-specific and ubiquitous transcription factors. We are interested in the role of the **pa**ired bo**x**-containing (Pax) transcription factors in these biological processes. The vertebrate *Pax* gene family consists of nine members which are expressed in spatially and temporally restricted patterns during embryogenesis and which are known to play important roles in early development and morphogenesis. These genes can be divided into subfamilies according to their sequence similarity and expression pattern (**Fig. 1**). The most unusual and characteristic feature of mammalian *Pax* genes is their haploinsufficiency which results in the frequent association of heterozygous *Pax* gene mutations with human disease syndromes and mouse developmental mutants (**Fig. 1**). The function of these transcription factors is therefore thought to be particularly sensitive to gene dosage, as mutation of one allele already results in developmental abnormalities. In addition to these loss-of-function mutations, two genes, *PAX-3* and *PAX-7*, participate frequently in chromosomal translocations which are causally involved as gain-of-function mutations in the genesis of alveolar rhabdomyosarcoma.

We are particularly interested in elucidating the role of the subfamily consisting of *Pax-2*, *Pax-5* and *Pax-8* (**Fig. 1**). In the past, we have studied in detail the *Pax-5* gene which codes for the transcription factor BSAP (reviewed by Busslinger and Urbánek, 1995). *Pax-5* is expressed in the developing midbrain of the embryo as well as in all B-lymphoid tissues and testis of the adult mouse (Adams *et al.*, 1992). Consistent with this expression pattern, gene inactivation in the mouse germline demonstrated that *Pax-5* plays an essential role in B-lymphopoiesis and midbrain development (Urbánek *et al.*, 1994). The human *PAX-5* gene has furthermore been implicated in oncogenesis, as deregulated expression of this gene was consistently observed in medulloblastoma, the most common brain tumour of childhood (Kozmik *et al.*, 1995).

In the last year we have gained further insight into the B cell and midbrain phenotype of the *Pax-5* mutant mouse. Detailed analysis revealed an essential role for Pax-5 at early stages of pro-B cell development. Moreover, development of the midbrain and cerebellum was shown to depend on the cooperation of both Pax-2 and Pax-5. The further characterization of the B-cell phenotype also led to the surprising discovery that *Pax-5* is expressed only from one of its two alleles. This monoallelic expression pattern of *Pax-5* is responsible for the haploinsufficient phenotype of heterozygous *Pax-5* mutant mice and, by extrapolation, can explain the frequent disease association of mammalian *Pax* genes. Moreover, *PAX-5* was shown to be recruited as an oncogene by a specific chromosomal translocation in two patients with non-Hodgkin lymphoma. Finally, in a collaboration with Walter Gehring's laboratory (Basel), we have identified and characterized *twin-of-eyeless*, a second *Pax-6* gene of *Drosophila*, which acts upstream of *eyeless* as a master control gene in eye morphogenesis.

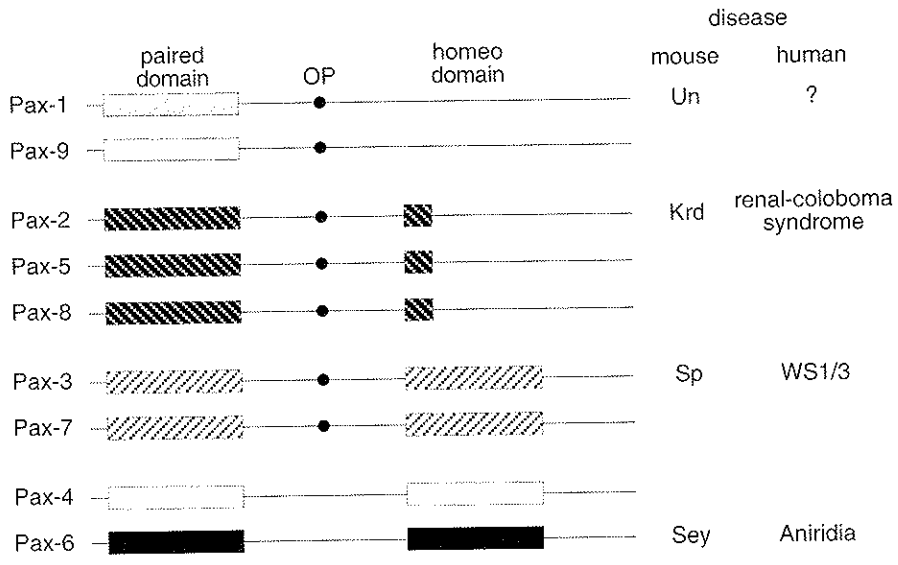

Fig. 1: Mammalian *Pax* genes and their association with disease.
The sequence similarities of the different Pax proteins are highlighted. Abbreviations: OP, conserved octapeptide; un, *undulated*; Krd, *Kidney and retinal defects*; Sp, *Splotch*; Sey, *Small eye*; WS, Waardenburg's syndrome.

Essential functions of Pax-5 (BSAP) at early progenitor stages of B cell development

Stephen Nutt and Claire Thévenin, in collaboration with Antonius Rolink (Basel Institute for Immunology)

B-lymphocytes develop from hematopoietic stem cells through an orderly process of differentiation which results in antigen-responsive B cells with individual immunoglobulin surface receptors. This developmental process can be dissected into different stages according to the expression of specific cell surface markers and the sequential rearrangement of immunoglobulin heavy (*IgH*) and light (*IgL*) chain genes (**Fig. 2**). We previously demonstrated that Pax-5 is essential for early B-lymphopoiesis (Urbánek *et al.*, 1994). Subsequently, we have determined the precise developmental stage of this differentiation block by extensive analysis of the expression profile of cell surface proteins and the rearrangement status of immunoglobulin genes in Pax-5-deficient B-lymphocytes (Nutt *et al.*, 1997). These experiments revealed a differential requirement for Pax-5 in fetal and adult B-lymphopoiesis. In the bone marrow, B cell development is arrested at the early pro-B cell stage (**Fig. 2**). These Pax-5-deficient pro-B cells fail to express the BSAP target gene *CD19* (**Fig. 3**) and are capable of long-term proliferation *in vitro* in the presence of stromal cells and IL-7. Interestingly, B-lymphoid progenitors could not be detected in the fetal liver of *Pax-5* mutant embryos. However, Pax-5-deficient fetal liver cells gave rise to the development of pro-B cells in bone marrow upon transplantation into lethally irradiated mice. These data indicate different functions of Pax-5 in the distinctive microenvironments of fetal liver and adult bone marrow (**Fig. 2**). As shown by PCR analyses, the pro-B cells in Pax-5-deficient bone marrow have undergone D_H-to-J_H rearrangement of the *IgH* locus at normal frequency. In contrast, V_H-to-D_HJ_H rearrangements were ~50-fold reduced in Pax-5-deficient pro-B cells, suggesting a role for Pax-5 in the developmental pathway leading to V-DJ recombination (Nutt *et al.*, 1997).

An important checkpoint in B cell development is the transition from the pro-B to the pre-B cell stage (**Fig. 2**). Productive rearrangement of the *IgH* gene initiates this transition by signaling through the pre-B cell receptor complex which is composed of the rearranged μ chain, the surrogate light chain proteins λ5 and VpreB, and the signal-transducing proteins Igα and Igβ. The pro-B to pre-B cell transition is abrogated by targeted inactivation of genes which either code for components of the pre-B cell receptor (μMT, λ5, Igβ) or prevent immunogobulin gene rearrangement (RAG-1 and RAG-2). Moreover, expression of a functionally rearranged μ transgene can complement the recombination defect of RAG-deficient mice, thus resulting in expression of the pre-B cell receptor and progression to the pre-B cell stage. The transition from the pro-B to the pre-B cell stage can be monitored by flow cytometric analysis of specific cell surface markers in vivo and by the loss of IL-7 responsiveness in vitro.

The *Pax-5* gene mutation also prevents transition from the pro-B to the pre-B cell stage and interferes with V_H-to-D_HJ_H rearrangement at the *IgH* locus. In addition to the absence of μ chain synthesis, Pax-5-deficient pro-B cells express also lower levels of Igα, but normal levels of all other components of the pre-B cell receptor complex. The question there-

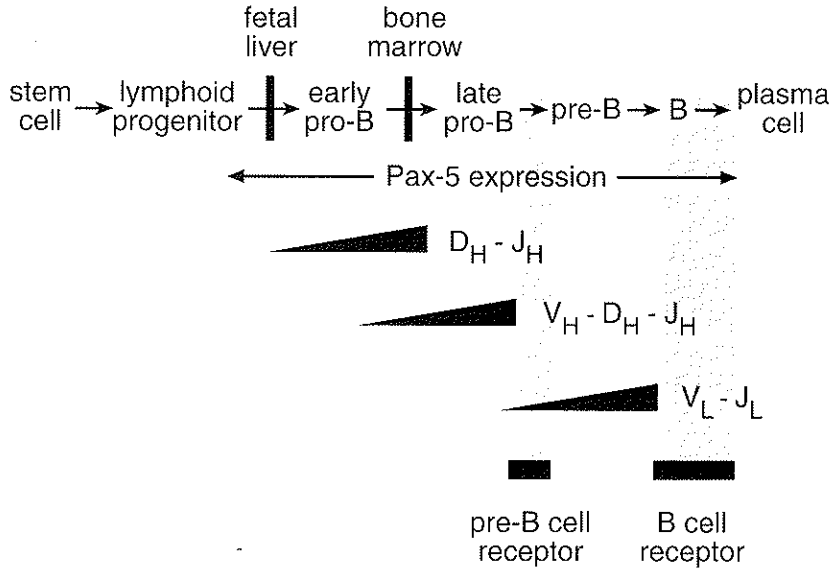

Fig. 2: Schematic diagram of B cell development.
The developmental block observed in fetal and adult B-lymphopoiesis of Pax-5 (-/-) mice is indicated together with time window for D_H-J_H and V_H-D_HJ_H rearrangement of the immunoglobulin heavy-chain locus and for V_L-J_L rearrangement of the light-chain loci.

fore arises whether the defect in μ chain and/or Igα synthesis could explain the block at the pro-B cell stage in Pax-5-deficient mice.

To address these questions, we have introduced functional immunoglobulin transgenes into Pax-5-deficient mice and analyzed their effect on B cell development by flow cytometry and analysis of in vitro cell clonability. By both criteria, neither expression of a functionally rearranged μ transgene nor expression of a μ-Igβ fusion gene were able to advance B cell development to the pre-B cell stage in *Pax-5* mutant mice. Interestingly, the μ-Igβ fusion protein is able to signal the transition from the pro-B to pre-B cell stage even in the absence of μ, Igα and Igβ proteins. The inability of the μ-Igβ transgene to complement the Pax-5 defect therefore demonstrates that neither the absence of V_H-to-D_HJ_H rearrangement nor the reduced Igα expression are responsible for the developmental block in *Pax-5* mutant mice. We conclude therefore that Pax-5 exerts its effect on early B cell development through (so far unknown) target genes which are not involved in the synthesis of the pre-B cell receptor complex.

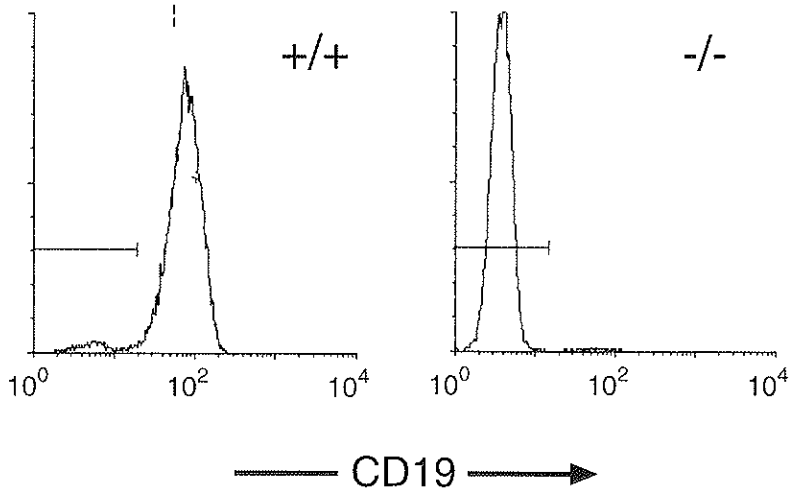

Fig. 3: *CD19* is a direct target gene of BSAP (Pax-5).
CD19 expression was analyzed by flow cytometry on wild-type (+/+) and Pax-5 (-/-) pro-B cells which were grown *in vitro* in the presence of stromal cells and IL-7.

The haploinsufficiency of *Pax-5* is caused by monoallelic expression.

Stephen L. Nutt and Susanne Vambrie, in collaboration with Peter Steinlein and Andreas Weith

As discussed in the introduction (see **Fig. 1**), *Pax* genes are frequently associated with mouse mutants and human disease syndromes due to their hapoinsufficient nature, since mutation of one allele often results in phenotypic abnormalities. Our detailed analysis of B-lymphocytes from heterozygous *Pax-5* (+/-) mutant mice has recently revealed that the *Pax-5* gene is in vivo transcribed from only one of its two alleles in most expressing cells. Consequently, mutation of one *Pax-5* allele already results in the loss of a subpopulation of *Pax-5* expressing cells due to the complete abrogation of Pax-5 function at the single-cell level. Therefore, monoallelic expression appears to be responsible for the haploinsufficiency of *Pax-5*.

A direct test of the monoallelic expression hypothesis depends on the possibility to follow the expression of each *Pax-5* allele in vivo. This possibility was provided by analysis of B-lymphocytes from heterozygous *Pax-5* (+/-) mice. First, expression of the B-cell surface protein CD19 is strictly dependent on the presence of the transcription factor BSAP (Pax-5) (see **Fig. 3**). Hence, only cells expressing the wild-type *Pax-5* allele can be stained with an anti-CD19 antibody and are subsequently detected as CD19$^+$ cells by flow cytometric analysis (**Fig. 4A**). Second, the inactivated Pax-5 allele has been tagged by an in-frame *lacZ* insertion in exon 2, and its expression can thus be followed by flow cytometric analysis of β-galactosidase activity (**Fig. 4A**). Analysis of the B-cell compartment of heterozygous *Pax-5* (+/-) fetal liver and bone marrow indicated that the majority of B-lymphocytes express the CD19 protein, yet display no β-galactosidase activity (**Fig. 4B**). Hence, most heterozygous B-lymphocytes express exclusively the wild-type *Pax-5* allele in vivo, supporting the hypothesis of allele-specific regulation of the *Pax-5* gene. Moreover, this monoallelic expression pattern of *Pax-5* was independent of the parental origin of the *Pax-5* alleles.

To further investigate the phenomenon of monoallelic *Pax-5* expression, we established pro-B cell lines from heterozygous mice in vitro. Single cell cloning expriments and time course analyses demonstrated that individual cell colonies were able to switch expression between alleles within 2 weeks.

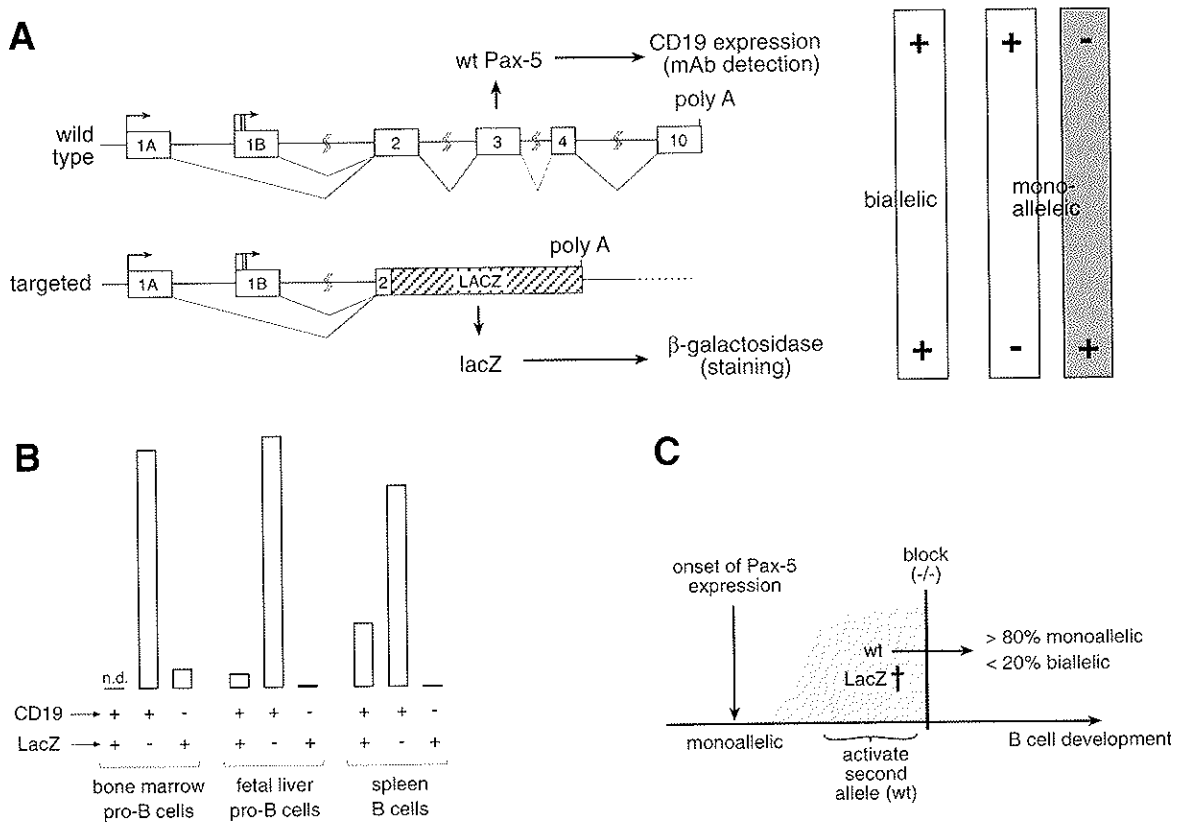

Fig. 4: Monoalllelic expression of *Pax-5* in B-lymphocytes of heterozygous *Pax-5* (+/-) mice.
A) Schematic diagram of the wild-type and disrupted *Pax-5* allele. **B)** Percentages of bi- and mono-allelically expressing B-lymphocytes in fetal liver, bone marrow and spleen. The bar graph indicates the different percentages of CD19 and/or lacZ expressing cells which were determined by flow cytometric analysis of the respective B-cell compartment. **C)** Model to account for mono- and bi-allelically expressing B-lymphocytes.

Moreover, replication timing analysis by fluorescence in situ hybridization (FISH) demonstrated that the *Pax-5* alleles are synchronously replicated during S-phase in B-cells of heterozygous mice. In summary, the allele-specific regulation of *Pax-5* is stochastic, reversible, independent of parental origin and does not correlate with asynchronous replication in contrast to the monoallelic expression of genomically imprinted genes. Monoallelic expression of *Pax-5* generates a haploinsufficient phenotype at the cellular level in heterozygous *Pax-5* mutant mice (see **Fig. 4C**). Cells which express only the mutant (*lacZ*) allele are absent in the B-lymphoid lineage past the developmental block of the *Pax-5* mutation. Hence, heterozygous pro-B cells which initially switch on the mutant *Pax-5* allele can only participate in later development, if they also activate the wild-type allele early on. These B-lymphocytes give rise to the small percentage of biallelically expressing cells (**Fig. 4C**).

Based on the results obtained with *Pax-5*, we propose the following model (**Fig. 5**) to account for the haploinsufficiency and frequent disease association of *Pax* genes. *Pax* genes are usually transcribed from only one allele, which leads to division of the *Pax*-expresssing cells into two subpopulations (A and B). Both cell populations can normally participate in development and differentiation, as long as the two alleles contain wild-type sequences. However, if one allele is mutated, one subpopulation of cells lacks the function of the respective *Pax* gene. These cells may be immediately lost, if the function of a *Pax* gene is essential right from the onset of expression. Alternatively, these cells may switch on the wild-type allele, if a time window exists between the onset of expression and functional requirement of the *Pax* gene. As a consequence, about half of the *Pax*-expressing cells may be unable to participate in tissue formation. Such 'functional hemizygosity' could explain the frequent association of *Pax* gene mutations with mouse developmental mutants and human disease syndromes.

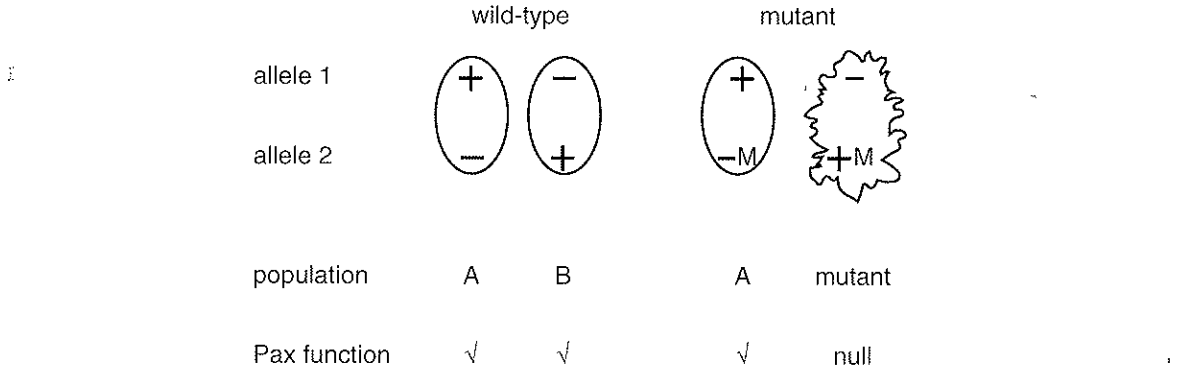

Fig. 5: The monoallelic expression hypothesis can account for the haploinsufficiency and disease association of *Pax* genes. The presence or absence of expression is indicated by + and -, respectively. M; mutated allele. For further explanations, see text.

Oncogenic activation of *PAX-5* by translocation in a subset of non-Hodgkin lymphomas

Aline Morrison, in cooperation with Uli Jäger (Vienna University Hospital)

In the last year we have demonstrated that *PAX-5* can be activated as an oncogene by specific chromosomal translocations in a subset of non-Hodgkin's lymphomas. The discovery of *PAX-5* at the breakpoint of a chromosomal translocation resulted from a detailed study of the human *PAX-5* locus (Busslinger *et al.*, 1996). This analysis revealed that the *PAX-5* gene is transcribed from two distinct promoters, resulting in splicing of two alternative 5' exons (1A and 1B) to the common coding sequences of exons 2-10. Previously, we have located the human *PAX-5* gene on chromosome 9 in region p13 which is involved in t(9;14)(p13;q32) translocations in a small subset of non-Hodgkin lymphoma. A molecular analysis of such a t(9;14) translocation breakpoint from a diffuse large-cell lymphoma (KIS-1) demonstrated that the *IgH* locus on 14q32 was juxtaposed to chromosome 9p13 sequences of unknown function (Ohno *et al.*, 1990). We have now localized the KIS-1 translocation breakpoint 1807 base pairs upstream of exon 1A of *PAX-5*. As a consequence, the potent Eμ enhancer of the *IgH* gene was brought into close proximity of the *PAX-5* promoters, resulting in deregulation of *PAX-5* transcription (see **Fig. 6**).

A second translocation was identified in the *PAX-5* locus by screening patients with CD5^{low} non-Hodgkin lymphoma. One patient (MB) diagnosed with marginal zone lymphoma was shown by Southern blot analysis to contain a translocation in the *PAX-5* locus. The malignant B-cells of this patient were present in the peripheral blood where they comprised up to 80% of all mononuclear cells. Clon-

ing of the breakpoint revealed that this novel translocation occurred within exon 1B of *PAX-5* and the Sμ switch region of the *IgH* locus (**Fig. 6**). This translocation brought the *PAX-5* gene under the control of a new promoter which is located in the Sμ switch region and which is responsible for increased synthesis of *Pax-5B* transcripts in the malignant B-cells. Together these data indicate therefore that *PAX-5* can be activated as an oncogene by enhancer or promoter insertion. To directly demonstrate a causal role of *PAX-5* in tumor formation, we are currently generating transgenic mice containing the *PAX-5* gene under the control of the Eμ enhancer (KIS-1 configuration).

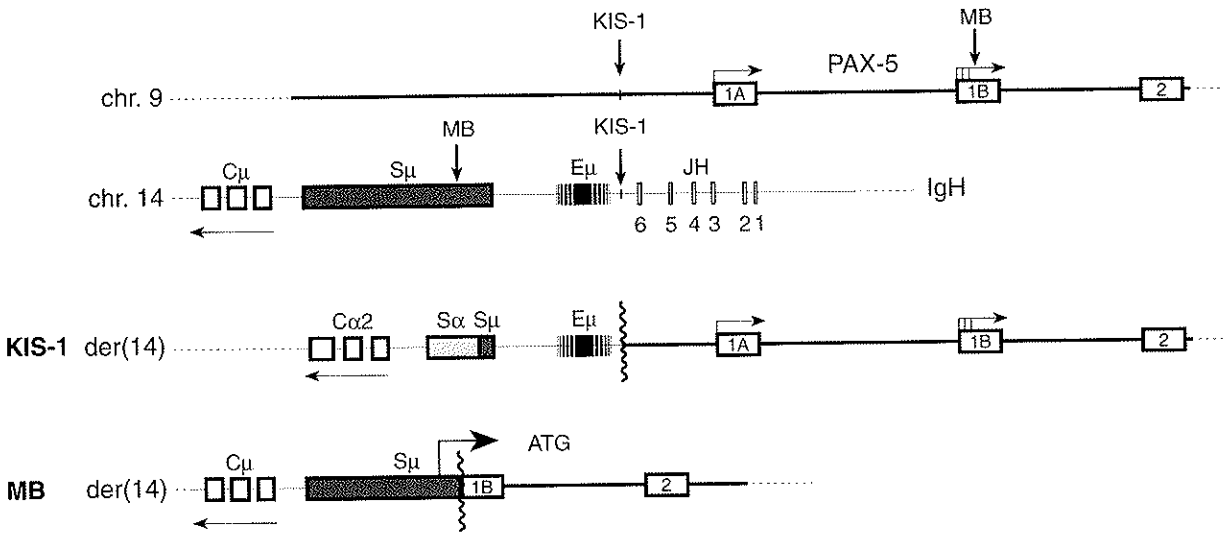

Fig. 6: Chromosomal translocations between the *PAX-5* and *IgH* loci in two patients with non-Hodgkin lymphoma.
The 5' region of the *PAX-5* gene, the J_H-to -Cμ region of the *IgH* locus and the corresponding translocation breakpoints present on the derivative chromosome 14 in the KIS-1 and MB lymphomas are schematically diagrammed.

Cooperation of *Pax-2* and *Pax-5* in midbrain and cerebellum development

Pavel Urbánek

The development of the midbrain and cerebellum is known to depend on an organizing center which is located at the midbrain-hindbrain junction of the vertebrate embryo. Expression of the two closely related transcription factors Pax-2 and Pax-5 overlaps spatially and temporally in this region of the developing CNS. During the last two years we have studied the interaction between Pax-2 and Pax-5 in midbrain and cerebellum patterning by generating *Pax-5 Krd (Pax-2)* double mutant mice. Deletion of one *Pax-2* allele by the transgene-induced *Krd* mutation had no effect on midbrain and cerebellum development in heterozygous *Krd* (+/-) mice. Moreover, only minor developmental defects were previously observed at the midline of the inferior colliculus and anterior cerebellum in mice which were homozygous for a targeted *Pax-5* mutation (Urbánek *et al.*, 1994). Similar morphological alterations were observed in 80% of all compound heterozygous *Pax-5* (+/-) *Krd* (+/-) mice. However, in the remaining 20% of compound heterozygotes, the inferior colliculi were missing, and the vermis of the cerebellum was severely disrupted due to the failure of the cerebellar primordia to fuse at the midline. Inactivation of the second *Pax-5* allele in *Pax-5* (-/-) *Krd* (+/-) mice resulted in complete loss of the posterior midbrain and cerebellum. As shown in **Fig. 7**, the tissue originating from the midbrain-hindbrain boundary region was deleted in the mouse embryo as early as day 9.5. These data therefore indicate that cooperation of Pax-2 and Pax-5 is required for the development of a functional organizing center at the midbrain-hindbrain junction.

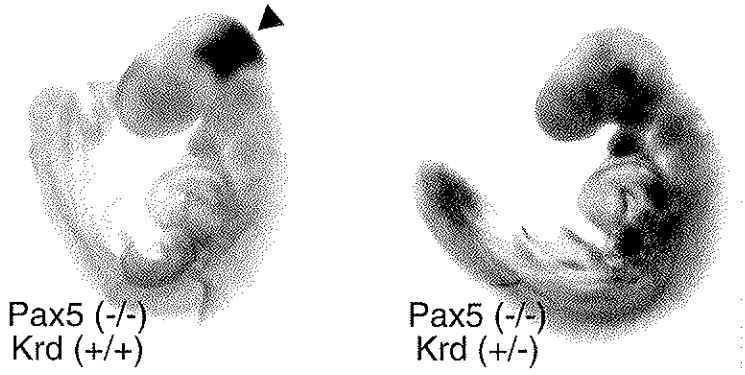

Fig. 7: Early deletion of midbrain-hindbrain boundary tissue in *Pax-5* (-/-) *Krd* (+/-) embryos.
β-Galactosidase staining of 9.5-day-old embryos of the two indicated genotypes. The *Pax-5* gene was inactivated in the mouse germline by the insertion of a *lacZ* gene which is thus brought under the transcriptional control of the endogenous *Pax-5* locus (see **Fig. 4A**). *LacZ* gene expression in *Pax-5* mutant embryos can therefore be used to trace cells originating from the midbrain-hindbrain boundary region. Note that all *Pax-5* expressing tissue is lost in *Pax-5* (-/-) *Krd* (+/-) embryos.

Delineation of a neuronal enhancer region of the *Pax-5* locus

Peter Pfeffer

The analysis of *Pax-5 Krd* double mutant mice revealed that *Pax-5*, in cooperation with *Pax-2*, plays an essential role in midbrain and cerebellum development. *Pax-5* is expressed in the CNS from embryonic day 8.25 (3-5 somites) onwards at the midbrain-hindbrain junction and in the spinal cord, but can no longer be detected in adults. To address the regulation of *Pax-5* in the developing CNS, we have first mapped two distinct promoters which differentially regulate *Pax-5* expression (see **Fig. 6**). The distal promoter drives expression predominantly in the B-cell lineage, whereas transcription from the proximal promoter is observed in all tissues expressing *Pax-5*. To determine the location of CNS-specific enhancer elements, a trangenic approach was employed. Initially a genomic fragment containing 25 kb of sequence upstream of exon 2 was fused in frame to *lacZ*. Subsequently, enhancer-trap vectors containing a minimal β-globin promoter driving *lacZ* expression were used. Successive deletions have narrowed down an element to 600 bp, which is responsible for correct mid-hindbrain expression.

The use of cross-species comparisons should provide a powerful tool for high resolution mapping of critical regulatory sequences. Comparison between the relevant mouse and human *Pax-5* sequences pointed to a 400 bp region which is more than 90% identical. Comparison with an evolutionary more distant species such as fish should result in a better definition of conserved sequence elements for further functional tests in transgenic mice. To this end, pufferfish (*Fugu*) *Pax-5* cosmids were isolated, and upstream regions are presently being analyzed.

Twin of eyeless, a second *Pax-6* gene of *Drosophila*, acts upstream of *eyeless* in the control of eye development.

Thomas Czerny, in collaboration with Georg Halder, Patrick Callaerts and Walter Gehring (University of Basel)

The *Pax-6* gene, which codes for a transcription factor containing both a paired domain and a homeodomain (**Fig. 1**), has been implicated as a master control gene of eye development by several lines of evidence. Loss-of-function mutations in the *Pax-6* gene cause the *Small eye* phenotype in mouse and aniridia in humans. Moreover, the *eyeless* (*ey*) gene of *Drosophila* has recently been identified as a homolog of the vertebrate *Pax-6* gene. Most interestingly, gain-of-function experiments in *Drosophila* demonstrated that ectopic eyes can be induced by targeted misexpression of the *ey* gene (reviewed by Halder *et al.*, 1995).

In a screen for new *Drosophila Pax* genes we have now isolated a second *Pax-6* homolog from the *Drosophila* genome which we refer to as *twin of eyeless* (*toy*) (Czerny *et al.*, 1997). This novel gene codes for a protein that is more closely related to vertebrate Pax-6 proteins than to the *Drosophila* Ey protein. During embryogenesis, *toy* is expressed

earlier than *ey*. *toy* transcripts are first detected at the blastoderm stage in the posterior procephalic region which includes the anlagen of the optic lobe and the brain. At later developmental stages, *toy* and *ey* are similarly, but not identically expressed in the developing visual system, the brain, and the ventral nerve cord. Interestingly, the expression of the two genes is, however, indistinguishable in the eye imaginal discs.

The similar expression pattern in the developing visual system suggests similar functions for the two *Pax-6* genes in eye morphogenesis. Nevertheless, *toy* cannot compensate for the loss of *ey* activity in ey^2 and ey^R mutants. Careful analysis demonstrated that the *toy* gene is not mutated and is furthermore normally expressed in the two *ey* mutants. Hence, *toy* and *ey* must fulfill non-redundant functions in eye morphogenesis. Interestingly, targeted expression of the *toy* gene in different imaginal discs also induces the formation of ectopic eyes on legs and wings, demonstrating that *toy*, like *ey*, can initiate the eye developmental pathway (**Fig. 8**). Furthermore, targeted expression of *toy* in these imaginal discs induces the expression of *ey*, whereas ectopic expression of *ey* does not activate *toy* transcription. Together these data demonstrate that *toy* acts upstream of *ey* in the genetic pathway of *Drosophila* eye development. In a PCR screen for a second *Pax-6* gene in different animal species, we found two *Pax-6* genes only in holometabolous insects, but not in primitive insects, echinoderms and vertebrates. These data imply therefore that the gene duplication leading to *ey* and *toy* presumably occurred late during insect evolution.

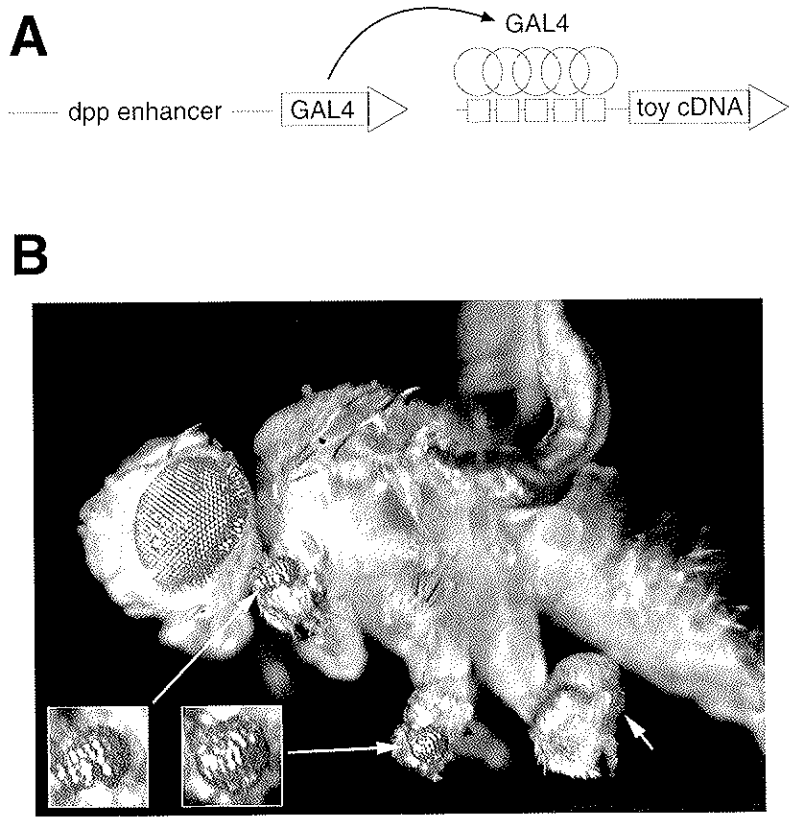

Fig. 8: Targeted misexpression of *toy* induces ectopic eyes in *Drosophila*.

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Cell cycle control and differentiation

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Introduction and Overview

Duplicating and segregating chromosomes

Cell proliferation is the basis of all life. To perform this remarkable act, cells must duplicate all their constituents and then segregate them equally to two daughter cells. Most of these constituents can be synthesized *de novo* under direction from the genome (the chromosomes) and the cell does not need to take particular care in their duplication or segregation. It must, however, take enormous care to duplicate fully all its chromosomes and to segregate each duplicate (known as a sister chromatid) to opposite poles of the cell prior at cell division. It must also ensure that no DNA sequence in the genome is replicated more than once during each round of division.

It is thought that the earliest forms of life on this planet would have consisted of cells whose enzymes were RNA molecules which are capable of replicating themselves or helping the replication of other RNA molecules. In those days, enzymes were both workhorses and templates for their own replication. There was no separation between genome and enzyme. Such anarchic systems would have faced many problems; not least how to ensure that all enzymes were replicated equally and with high fidelity and at least one copy of each enzyme inherited at cell division. The evolution of genomes like those of modern bacteria solved these problems. Each enzyme is encoded in double stranded DNA (the gene), which is chemically more stable than RNA, and all genes are joined together in a single DNA molecule (the bacterial chromosome), which is replicated bidirectionally from a single origin of replication. Bacteria also have means of segregating the two products of this duplication (sister chromatids) to opposite poles of the cell prior to cell division. We know rather little about how bacteria segregate sister chromatids, but the mechanism is probably no Rolls Royce. With all its genes on a single chromosome it does not need to be. The worst that can happen if sister chromatids are missegregated is that cells lose a bit of their cytoplasm. With only a single chromosome, the "society of genes" always stay together.

The bacterial genome has served bacteria well. They are still the most numerous life form on this planet. But they are not the most visible and this is because their mechanism for reproducing the genome has its limitations. Without further "development", animals, plants, and fungi would have never evolved. The genome of the bacterium *E. coli* consists of 4.7 Mbp of DNA, which contains about 4,000 genes. The human genome, in contrast, consists of 3,300 Mbp and contains around 70,000 genes. Fish have a similar number of genes and it is reasonable to presume that you could not produce anything resembling either a fish or a human with fewer. Much of our genome is almost certainly junk, but even if we

eliminate this part, we are left with at least 500 Mbp, which is about 100 times as large as an average bacterial genome.

Using their single bi-directional replication origin, it takes a bacterium 40 minutes to complete replication of its genome. By similar means, replication of a human genome, even when stripped of its junk DNA, would take 70 hours! A second problem with the bacterial solution is that a single chromosome containing 3,300 Mbp would be so large that it would be next to impossible to segregate sister chromatids away from the plane of division unless cells also became enormous. The solution adopted by eukaryotic cells was simple. Split the genome into multiple chromosomes and have each one replicated from multiple origins. Using these innovations, some embryonic cells take only 7 minutes to replicate their genomes. Indeed, embryogenesis in most animals, mammals excluded, would be quite impossible without the rapid cleavage divisions that such rapid chromosome replication enables.

Multiple replication origins and multiple chromosomes bring their own problems. How do cells ensure that each origin behaves itself and fires only once per cell division cycle? The cell had to invent a "Democracy" in which each origin fires once and only once. The second problem is that splitting the genome into multiple chromosomes means that the cell now faces a much more serious problem ensuring that every daughter cell inherits a complete set of chromosomes. If the segregation of each chromosome were correct 99% of the time, a human cell, which contains 46 chromosomes, would inherit the correct complement of chromosomes in only 60% of its cell divisions. A new way of segregating chromosomes, which presumably has far greater fidelity than the system used by bacteria, was invented by the ancestor of eukaryotic cells and this is called mitosis.

Having duplicated its chromosomes, the eukaryotic cell generates a bipolar mitotic spindle, attaches sister kinetochores to microtubules that associate with opposite poles of this spindle, and by means of tension exerted on sister kinetochores aligns each pair of sister chromatids on the metaphase plate. Chromosome alignment during metaphase depends not only on "splitting" forces exerted by microtubules on kinetochores but also on an opposing "cohesive" force exerted by tethers that hold sister chromatids together. This Newtonian action and reaction is the essence of mitosis for it is the means by which cells determine which DNA molecules are sisters. The subsequent separation of sister chromatids to opposite poles, known as anaphase, marks the point of no return in the mitotic cycle. Two sorts of processes are involved: loss of cohesion between sister chromatids, which leads to movement of sisters to opposite poles, and changes in the dynamics of spindle growth and disassembly that enable separation of spindle poles (see **Fig. 1**).

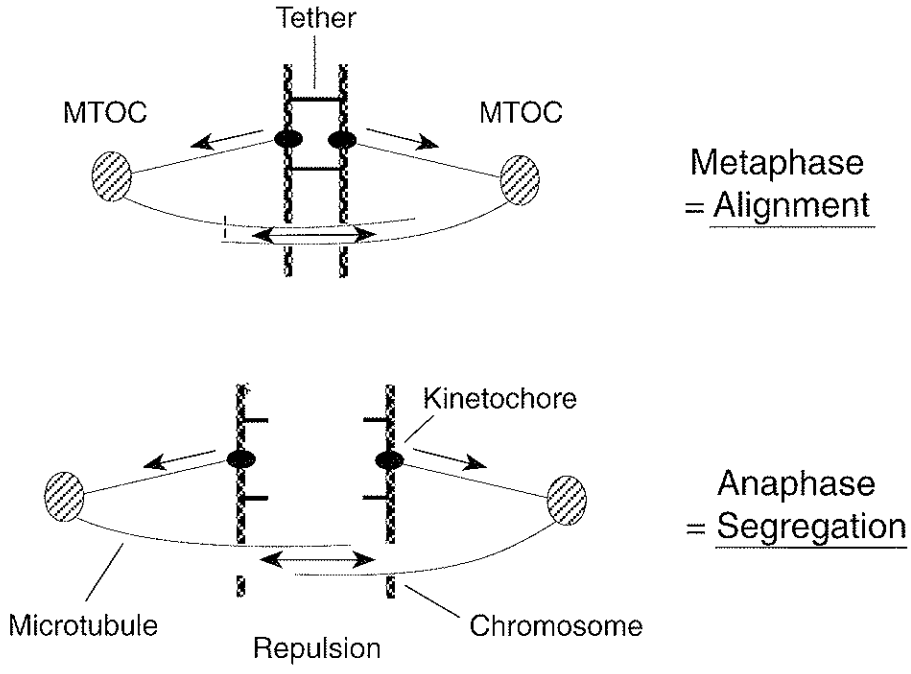

Fig. 1: Mitosis is a two step process: first alignment and second segregation of chromosomes

One of the "holy grails" of cell cycle research has been to understand how chromosome duplication and segregation are coordinated so that origins fire once and only once for each round of chromosome segregation at mitosis (this is known as the alternation problem; see **Fig. 2**). Mistakes in this process lead to polyploid genomes. This sounds bad. It is! It means no sex and worse still, probably cancer. To understand alternation, we and many others have sought to identify the signals that trigger chromosome duplication and segregation. Having identified them, we hoped then to study how cells only send these signals in a defined order once per cycle.

The periods of chromosome duplication and segregation are known as S (synthesis) and M (mitosis) phases and the intervals between these two phases are known as G1 (the gap between M and S) and G2 (the gap between S and M). Work by many labs around the world has shown that chromosome duplication and alignment of sister chromatids on the metaphase spindle are triggered by the activation of two related and rather special types of protein kinase, which alter the activity of other proteins by attaching phosphate groups to them. These kinases are called cyclin dependent kinases (CDKs) because their activity depends on the kinase's association with a "cyclin" regulatory subunit, whose abundance oscillates during the cell cycle. Chromosome duplication (S phase) is triggered by the accumulation of S phase cyclins and the destruction of proteins (CKIs) that inhibit S phase-CDKs. Alignment of chromosomes on the mitotic spindle is triggered by accumulation of M phase cyclins and activation of a mitotic or M phase-CDK (see **Fig. 2**). Successive waves of S- and

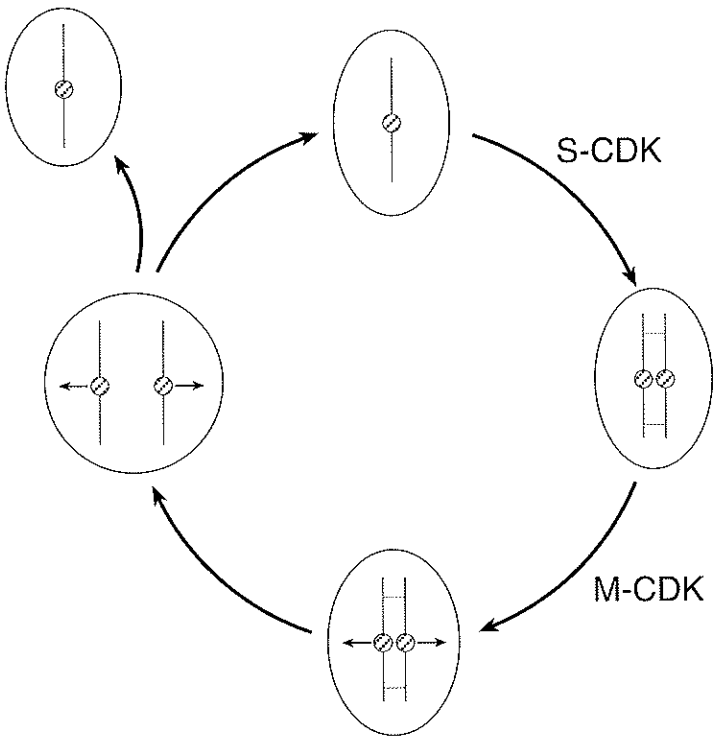

Fig. 2: Duplication+alignment triggered by S- + M-CDKs

M-CDKs trigger chromosome duplication and alignment on mitotic spindles, but as we shall see, they are far from the whole story. Some other signal, for instance, must be responsible for triggering the separation of sister chromatids at anaphase.

One of the goals of my lab during the past five years has been to characterize exactly which CDKs trigger S and M phases in the budding yeast *Saccharomyces cerevisiae*. Once we understood how the budding yeast CDK waves were regulated, we were able to reverse their order. We expected that induction of an M phase CDK in a G1 cell would cause it to enter mitosis instead of S phase, but were very surprised to find that it in fact triggered chromosome duplication and only later mitosis. We concluded that CDKs capable of triggering chromosome duplication were in fact present from the beginning of S phase till the end of M phase but, despite this, origins fired once and only once during this interval of the cell cycle. The implication was that the state of chromosomes must vary during the cell cycle. CDKs trigger the chromosomes of G1 cells but not those of G2 cells to enter S phase.

Work from many labs, my own included, now

suggest that chromosome duplication is in fact a two step process (see **Fig. 3**). During G1, prior to the activation of S phase CDKs, the cell assembles a large complex of proteins at future origins of DNA replication. Having formed this "pre-replication complex" (pre-RC), cells activate S phase CDKs, which then trigger the replication process. We discovered that S phase CDKs not only trigger initiation from origins that have formed pre-RCs but also prevent the *de novo* assembly of pre-RCs. M phase CDKs also inhibit this process, which means that pre-RCs cannot be formed from the point of S phase CDK

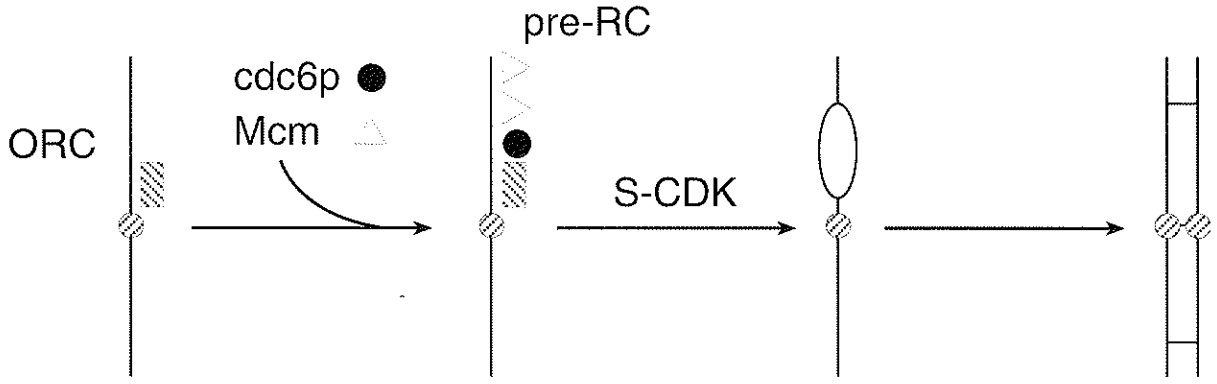

Fig. 3: Origin firing is a two step process

activation in late G1 until the degradation of M phase cyclins at anaphase (see **Fig. 4**). Initiation of DNA replication therefore depends on a period of low CDK activity during which pre-RCs can be assembled followed by a period of high CDK activity, which permits origin firing (see **Fig. 5**). The two opposing effects of CDKs prevent conditions ever arising that permit both the assembly of pre-RCs and firing of origins that have formed them. The consequence is that origins cannot fire more than once during an S/M CDK cycle.

This model suggests that the key to re-duplicating chromosomes is the drop in M phase CDK activity (due to cyclin destruction) that occurs during anaphase. If we were to understand how cells coordinate chromosome duplication and segregation, we would need to understand the relationship between M phase cyclin destruction and the separation of sister chro-

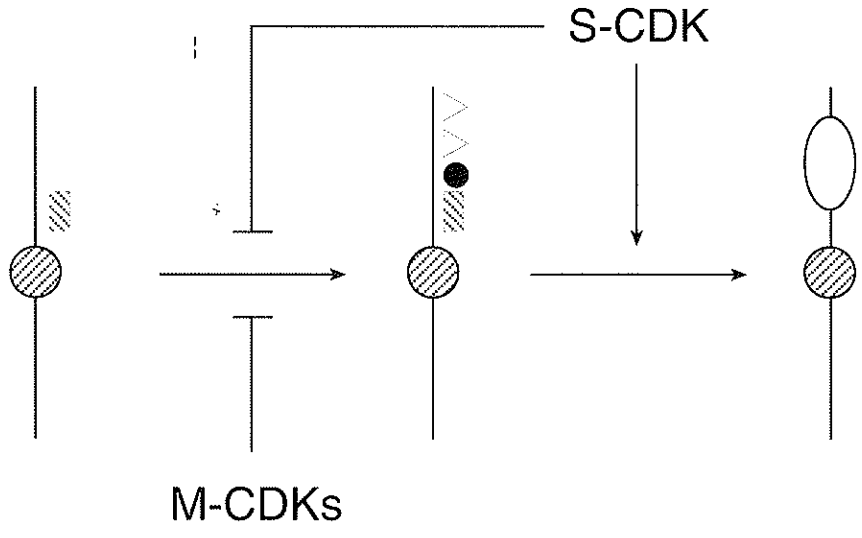

Fig. 4: CDKs trigger firing + block preRC formation

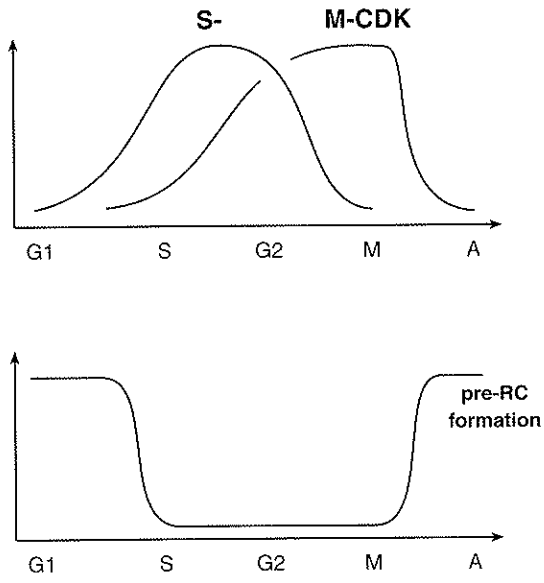

Fig. 5: Re-firing depends on M-CDK destruction at Anaphase

matids at anaphase. For a time, it was thought that M phase CDKs blocked the onset of anaphase; in other words, that mitotic cyclin destruction was the trigger for the separation of sister chromatids. This nice idea had two problems. First, it never explained the directionality of mitosis. More serious still, it was wrong! Nevertheless, cyclin destruction and sister chromatid separation do occur more or less simultaneously. Might some unknown factor be involved in triggering both events (see **Fig. 6**)? We therefore set out to identify factors required for the destruction of M phase cyclins during anaphase.

Genetic and biochemical studies from my lab and those of Marc Kirschner and Avram Hershko have recently identified a large complex of proteins conserved between yeast and man that is essential for cyclin destruction. Remarkably, the complex is also required for the separation of sister chromatids and we have therefore called it the Anaphase promoting complex (APC). The APC is composed of

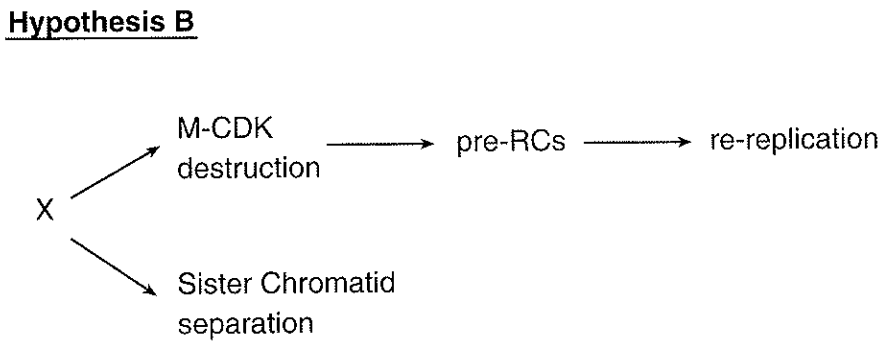

Fig. 6: How to link segregation and re-replication?

eight or more subunits and its role is to attach ubiquitin molecules to proteins which directs their degradation by another huge complex called the proteosome. It is thought that the APC mediates the destruction not only of mitotic cyclins but also of proteins whose destruction is essential for sister chromatid separation (see **Fig. 7**).

As we have seen, proteolysis of M phase cyclins relieves the block to pre-RC assembly exerted by S and M phase CDKs and this may be a key aspect of the mechanism by which eukaryotic cells ensure that re-duplication of chromosomes usually never precedes separation of sister chromatids produced at the previous S phase. By using the APC to regulate degradation of both anaphase inhibitors and M phase cyclins, the cell prevents preparations for S phase before anaphase is initiated.

The identification of pre-RCs and the APC have

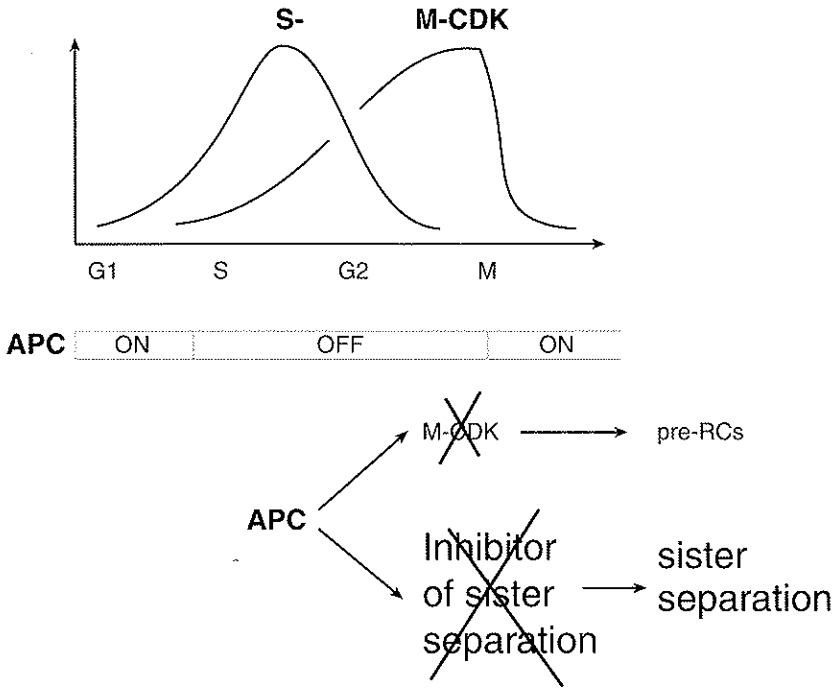

Fig. 7: The Anaphase Promoting Complex triggers sister separation and M-CDK destruction

led to a working hypothesis for how origins of DNA replication fire once and only once between successive mitoses. The key ingredient is a two step process, the second of which, activation of CDKs, also inhibits the first, formation of pre-RCs. This "reciprocating" principle might be a general feature of several cell cycle regulatory mechanisms. Mitosis also involves two fundamental steps, alignment of sister chromatids on bipolar spindles followed by splitting of sister chromatids, and the enzyme responsible for the second step, the APC, also destroys the M phase cyclins needed for the first step (see **Fig. 8**).

In conclusion, I should say that we are really only just embarking on the voyage of discovery about how cells duplicate and segregate their chromosomes. We now have the crude outlines of the logic of this process, but "the devil is in the details" and these have yet to be worked out. Understanding how our genome is duplicated and segregated to daughter cells with high fidelity may shed insight into how tumor cells make a mess of this process and this might eventually reveal their "Achilles heel".

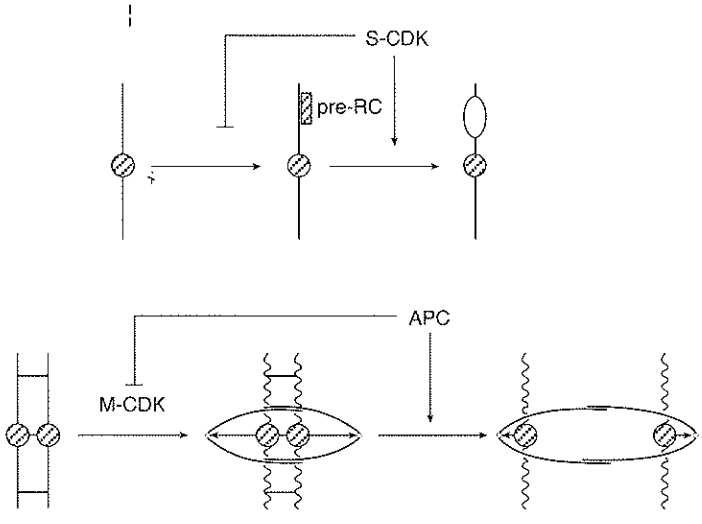

Fig. 8: Both replication and mitosis are two step processes in which the second step blocks the first

Generating differential gene expression

Localized protein synthesis is an essential feature of many living organisms. In those composed of multiple cells, it is largely achieved by differences between cells in their patterns of gene expression. These differences arise to a large extent due to cellular communication that exposes sister cells to different external signals. However, there are several cases where sister cells produced from a single cell division adopt different patterns of gene expression in the absence of any differences in their environment. Such differences in the behavior of sister cells are thought to be due to their unequal inheritance of specific "determinants" of gene expression from their mother cells. Inequality could arise either due to their mother cell's plane of division being asymmetric or due to the asymmetric localization of determinants within the mother cell immediately prior to its division. There are only a few instances where such determinants have actually been identified and there are none where the mechanism by which they become localized in mother cells is fully understood.

A good example of differential gene expression amongst sister cells occurs during the diploidization of homothallic strains of the budding yeast *Saccharomyces cerevisiae*. Upon germination, haploid spores of this yeast grow to a critical size and then produce buds. Anaphase takes place at the bud neck and thereby delivers a complete set of chromosomes to both mother and bud. Formation of a septum at the bud neck produces a mother cell that proceeds to switch its mating type and a daughter cell (the bud) that does not. This difference is due to transcription in mother cells but not in their daughters of the *HO* gene, which encodes an endonuclease that initiates site specific gene conversion of yeast mating type loci.

Transcription of *HO* in mothers but not their daughters arises due to the unequal accumulation within mother and daughter nuclei of a repressor of *HO* transcription called Ash1p. This is an unstable protein that accumulates at the end of anaphase due to the transcriptional activation of its gene. Ash1 protein appears within both mother and daughter nuclei soon after synthesis of its mRNA but it accumulates to higher levels in the nuclei of daughter cells. The differential accumulation of Ash1p within daughter cell nuclei depends on at least five *SHE* genes, one of which *SHE1/MYO4* encodes a type V myosin. It has therefore been postulated that some factor needed for Ash1p's preferential accumulation in daughter cells (or indeed Ash1p itself) might be transported by She1/Myo4 along cytoplasmic actin cables that run from mother cells into their buds. It seems unlikely that Ash1p itself is transported by She1/Myo4, because Ash1 protein never acccumulates even transiently in the cytoplasm. The mRNA for Ash1p, on the other hand, presumably does accumulate in the cytoplasm. In collaboration with Roy Long and Rob Singer (at Albert Einstein), we have now investigated, using fluorescent in situ hybridization, the distribution of ASH1 mRNAs within cells as they undergo anaphase. We have found that ASH1 mRNAs accumulate at one pole of late anaphase cells, in an arc on the distal cortex of their buds (see **Fig. 9**). This localization depends on actin cables and on She1/Myo4 a type V myosin.

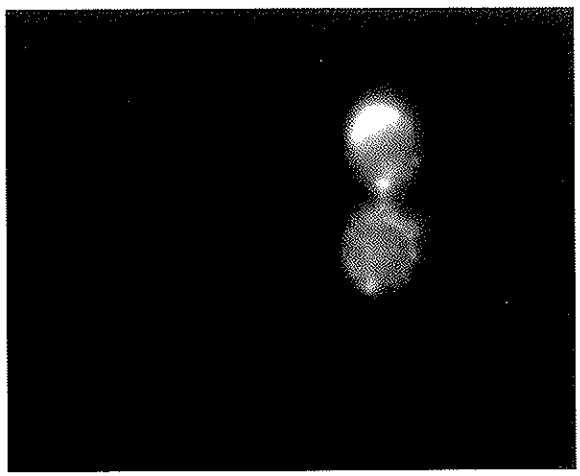

Fig. 9: Asymmetric localization of Ash1 mRNA to the distal tip of buds

These data show that asymmetric intracellular RNA localization is not confined to cells from the animal kingdom or to those from multicellular organisms. The common ancestor of fungi and animal cells presumably possessed this ability. Thus far, the only mRNAs the function of whose localization is well understood are ones that encode determinants of differential gene expression. Yeast is commonly thought of as unicellular organism but many other ascomycetes like other branches of the fungal kingdom are multicellular. The use of *ASH1* mRNA localization by yeast to set up differential *HO* gene expression in mother and daughter cells is analogous to the role of Oskar or Bicoid mRNA localization in establishing differential gene expression along an anterior-posterior axis in *Drosophila* embryos.

Our data raise the possibility that mRNA localization might be an extremely ancient property of the eukaryotic lineage that long predates the common ancestor of fungi and animals. It might for example be possessed by the earliest unicellular protozoan offshoots. If so, rather than being simply a tool for establishing differential gene expression in multicellular organisms, mRNA localization might actually have been utilized to localize protein synthesis within organsims long before the evolution of multicellularity. Multicellularity and differential gene expression is just one way of localizing protein synthesis within an organism. Much the same can be achieved on a small scale by localizing mRNAs or translation factors within a single cell. Many unicellular protozoa after all are as large and almost as spatially complex as small multicellular animals. In this light, mRNA localization might be seen as a precursor, and for some organisms a substitute, for differential gene expression. Though largely superseded in multicellular organisms, it continues to be used for generating spatial complexity within individual cells and thereby for establishing differential gene expression in sister cells.

Control of DNA replication

Simonetta Piatti and Kim Nasmyth

In eukaryotic cells, DNA replication is confined to a discrete period of the cell cycle and usually does not recur until after anaphase. In the budding yeast *Saccharomyces cerevisiae*, assembly of pre-replication complexes (pre-RCs) at future origins as cells exit mitosis (or later during G1) is necessary for subsequent initiation of DNA replication triggered by activation in late G1 of Cdc28/Cdk1 kinases associated with B-type cyclins Clb1-6. The absence of pre-RCs during G2 and M phases could explain why origins of DNA replication fire only once during the cell cycle, even though S phase promoting Cdks remain active from the beginning of S phase till the end of M phase. Formation of pre-RCs and their maintenance during G1 depends on the synthesis and activity of an unstable protein encoded by *CDC6*. We find that Cdc6 synthesis can only promote DNA replication in a restricted window of the cell cycle: between destruction of Clbs after anaphase and activation of Clb5/ and Clb6/Cdc28 in late G1. The latter corresponds to a "point of no return" after which Cdc6 synthesis can no longer promote DNA replication. Cdc6 protein can be made throughout the cell cycle and, in certain circumstances, can accumulate within the nuclei of G2 and M phase cells without inducing re-replication. Thus, control over Cdc6 degradation and/or nuclear localization is not crucial for preventing origin re-firing. Our data is consistent with the notion that cells can no longer incorporate *de novo* synthesized Cdc6 into pre-RCs once Clb/Cdk1 kinases have been activated. We show that Cdc6p associates with Clb/Cdk1 kinases from late G1 until late anaphase, which might be important for inhibiting pre-RC assembly during S, G2 and M phases. Inhibition of pre-RC assembly by the same kinases that trigger initiation explains how origins are prevented from re-firing until Clb kinases are destroyed after anaphase.

New subunits of the yeast Anaphase Promoting Complex

Wolfgang Zachariae, Tae Ho Shin, Marta Galova and Kim Nasmyth

Most events that occur when cells exit from mitosis such as chromosome decondensation, spindle-disassembly and cytokinesis require degradation of mitotic cyclins. Mitotic cyclin degradation is also a prerequisite for S-phase in the subsequent cell cycle because cyclin B-CDK activity inhibits the establishment of replication competent complexes at origins. Cyclin degradation depends on a particle, called the Anaphase Promoting Complex (APC) or Cyclosome which functions as a cell cycle regulated ubiquitin-protein ligase. The APC mediates destruction box-dependent ubiquitination and thereby targets cyclins for proteolysis by the proteasome. The isolation of mutants defective in cyclin degradation led to the identification of the tetratricopeptide repeat (TPR) proteins Cdc16, Cdc23, and Cdc27 as subunits of the APC. These proteins are required for the onset of anaphase in various organisms. Because cyclin proteolysis per se is not required for anaphase it has been suggested that the APC also targets for

destruction proteins whose proteolysis is necessary for sister chromatid separation. We have started to analyze the subunit composition of the yeast APC, which is a particle of 36S. The complex contains at least eight proteins in addition to Cdc16p, Cdc23p, and Cdc27p. The largest subunit which is encoded by the *APC1* gene is conserved between fungi and vertebrates and shows similarity to BIMEp from *Aspergillus nidulans. BimE* has been implicated in the regulation of the entry into mitosis. The *CDC26* gene encodes a small heat-inducible subunit of the APC.

Sister chromatid cohesion in yeast

Christine Michaelis and Kim Nasmyth

Chromosomal DNA must be replicated and segregated accurately during each cell division. Replicated chromosomes consist of two sister chromatids which are held together until metaphase. During anaphase A the cohesion of the sisters is relieved and in anaphase B spindles elongate. Although this is a fundamental step during the eukaryotic cell cycle it is unknown how the cohesion of sister chromatids is maintained and how their separation is initiated.

The APC (Anaphase Promoting Complex), a large protein complex which promotes the degradation of B-type cyclins during the cell cycle might control this transition. APC mutants such as *cdc16* arrest in metaphase. However, the degradation of B-type cyclins, the only presently known substrate of the APC, is essential at the end of mitosis. Therefore it was postulated that another substrate(s) must be degraded at the metaphase to anaphase transition. Because sister chromatids are separated with the onset of anaphase, the postulated protein could be involved in holding sister chromatids together.

Based on this prediction and the assumption that a defect in this postulated protein would cause chromosome loss, we performed a genetic screen. We used *cdc16-123*, a temperature sensitive APC mutant, and monitored in a primary screen the stability of a non-essential chromosome. In a secondary screen we selected 8 mutants, which could partially overcome the metaphase arrest of the *cdc16-123* mutant at the non permissive temperature. The 8 mutants fall into 4 complementation groups.

An M phase-specific protein kinase, Cdc5, is a substrate of APC

Masaki Shirayama and Kim Nasmyth

Mitosis in the yeast *Saccharomyces cerevisiae* is regulated by oscillations in the activity of the Cdc28 cyclin-dependent kinase (CDK) associated with the B type cyclins Clb1-4. The level of Cdc28 protein is constant throughout the cell cycle, but Clb2 is tightly regulated both transcriptionally and post-transcriptionally. The latter is primarily achieved by ubiquitin-mediated proteolysis, which requires a large E3 ubiquitin ligase complex referred to as the anaphase promoting complex (APC).

The regulation of mitotic transitions is attributed not only to the activity of CDK, but also to the *polo* family of protein kinases, which have been shown to have a function in controlling spindle function and chromosome segregation in various organisms. The *CDC5* gene of *Saccharomyces cerevisiae* encodes a protein kinase with a high degree of sequence similarity to the *polo* family of protein kinases. We have demonstrated that the protein level of Cdc5 oscillates in a manner similar to that of the B type cyclin, Clb2. Both protein levels are low in G1 phase, but increase during S phase, reach maximal levels during G2 and M phase and appear to decrease at late M phase. This result prompted us to examine whether Cdc5 is degraded in an APC-dependent manner. Based on the results presented here we suggest that the degradation of Cdc5 depends on APC activity. By immunofluorescent staining, Cdc5 was found to be diffusely distributed in the nucleus throughout M phase, but concentrated in the spindle pole bodies in early M phase, suggesting that Cdc5 may play a role in the operation of the mitotic spindle.

Differential gene expression and mRNA localization in yeast

Isabel Gonzales, Ralf-Peter Jansen, Uwe von Ahsen and Kim Nasmyth

The generation of cellular heterogeneity through asymmetric cell divisions is an important aspect during development. A well understood example of asymmetric gene activation occurs in the budding yeast *S. cerevisiae* where mating-type switching is confined to one of the progeny of each cell division. Switching is initiated by an unstable endonuclease encoded by the *HO* gene which is expressed only in "mother cell" and only at a certain window during the cell cycle, at START.

We have identified the determinant of mother-specific mating type switching. The *ASH1* gene encodes a putative transcriptional repressor that appears at the end of mitosis only in one of the two nuclei. In the daughter cell nucleus it represses the activation of the *HO* gene. The asymmetric accumulation of Ash1p depends on the function of at least 5 genes, *SHE1* to *SHE5,* one of which codes for an unconventional myosin. In addition, it requires a functional actin cytoskeleton. The asymmetric accumulation of Ash1p is independent of the promoter that drives its expression, ruling out the possibility that *ASH1* is only activated in daughter cell nuclei.

In a collaboration with the laboratory of Robert Singer we could show that - much like in *Drosophila* and *Xenopus* oocytes - the basis of asymmetric Ash1p accumulation is the localization of *ASH1* mRNA to the daughter cell. This localization depends on the activity of the unconventional myosin and the other She proteins.

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Gene function in mammalian development and oncogenesis

Senior scientist	Erwin F. WAGNER
Staff scientist	Maria SIBILIA (since Nov. 96)
Postdoc	Kanaga SABAPATHY
Postdoc	Koichi MATSUO
Postdoc	Emmanuelle PASSEGUÉ (since Feb. 96)
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Technician	Candice ELLIOT (since April 96)
Technician	Ingrid FETKA (until July 96)
Technician	Laura STINGL
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PhD student	Martin SCHREIBER (until June 96)
PhD student	Axel BEHRENS
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PhD student	Anton Wutz (since Feb. 96)
PhD student (BI Fonds)	Ulrich MÜHLNER
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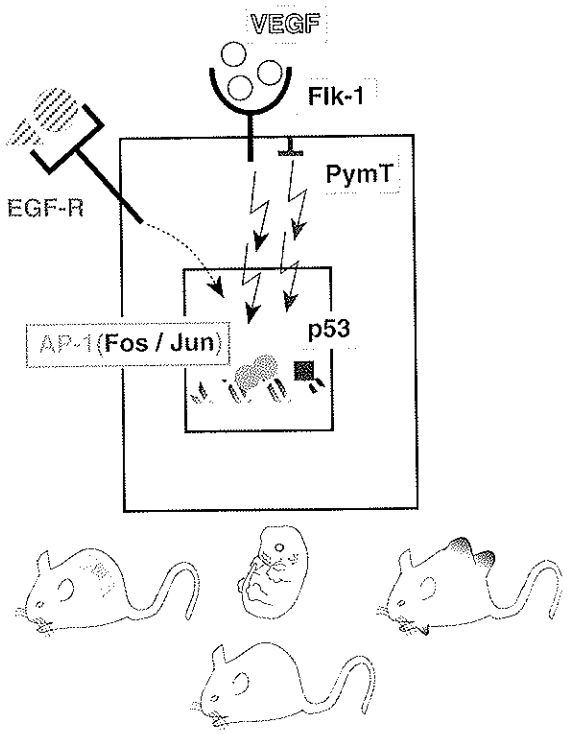


Fig. 1: Overview of research projects.

Introduction

Growth control genes coding for growth factors and their receptors together with molecules involved in signal transduction and in gene expression are essential components for the regulation of mammalian development. We are interested in the function of such genes in normal mouse development, stem cell differentiation and tumor formation (**Fig. 1**). "Gain-of-function" (overexpression) and "loss-of-function" (gene inactivation) experiments are being used to generate genetically defined strains of mice, either by micro-injection of DNA into fertilized eggs or via mouse embryonic stem (ES) cells which are introduced into mouse embryos. The aim of all these projects using the mouse as a model system is the better understanding of gene function in the control of normal and pathological development.

I. Functional analysis of the AP-1 transcription factor

Transgenic mice, embryonic stem (ES) cell chimaeras and "ES mice" generated with tetraploid blastocysts are being used to investigate the function of the AP-1 transcription factor complex. This multicomponent transcription factor appears to play a central role in controlling gene expression in cell proliferation, differentiation, development and oncogenesis. The active form of AP-1 is comprised of Jun/Fos heterodimers or Jun/Jun homodimers with its individual components (c-Fos, FosB, Fra-1, Fra-2; c-Jun, JunB and JunD). A detailed analysis of the *in vivo* function of AP-1 is being performed by generating loss-of-function and gain-of-function mutations of individual AP-1 components in mice with the aim to define the cell types and the genes which are regulated by these transcription factors.

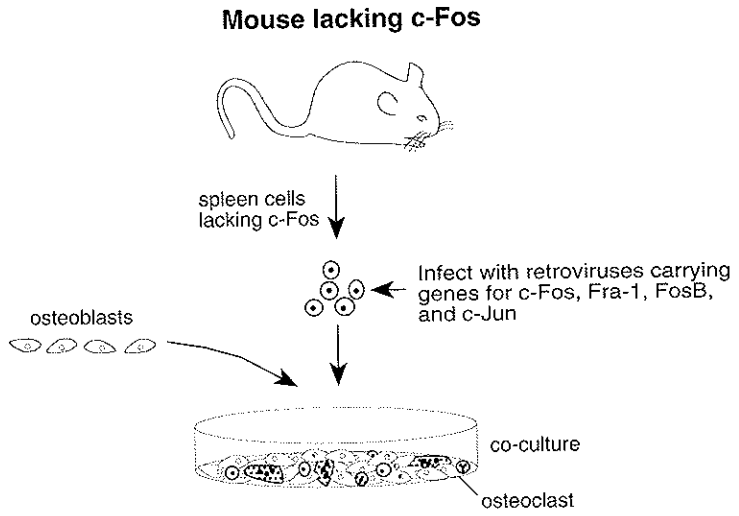
1. Function of Fos/AP-1 in bone cells

Koichi Matsuo and Martin Tonko

Mice lacking the *c-fos* proto-oncogene develop osteopetrosis due to a block in the differentiation of osteoclasts (Grigoriadis *et al.*, 1994). Bone resorbing osteoclasts are totally absent in the bones of mutant mice. A key question is how c-Fos protein is causally involved in osteoclast-macrophage lineage determination. We have so far analyzed c-Fos protein function by focusing on the regulation of potential target gene(s) and on the comparative analysis of Fos family proteins.

Several experiments have shown that the tartrate-resistant acid phosphatase (TRAP) gene, which is not expressed in c-Fos-deficient bone, is a potential target gene of c-Fos in osteoclasts. The most highly conserved motif in mammalian TRAP promoters was found to be a binding site for NFAT (nuclear factor of activated T cells) and a nearby atypical AP-1 site, suggesting a potentially important site of c-Fos binding. In bandshift and transient transfection experiments with non-osteoclastic cell lines, this NFAT/AP-1 site, termed TR-120, was shown to mediate transcriptional activation by NFAT3 in combination with c-Fos-containing AP-1 complex. We are currently attempting to show that NFAT is present in osteoclasts and that the NFAT/AP-1 site in the TRAP gene is functional in the osteoclast lineage.

Previous experiments have shown that exogenous c-Fos rescues the differentiation block in the osteoclast lineage when expressed in c-Fos-deficient spleen cells in a co-culture system. These *in vitro* rescue experiments have been extended to other AP-1 members. All Fos family members (c-Fos, Fra-1, Fra-2 and FosB), but none of the Jun family members (c-Jun, JunB and JunD), rescued the differentiation block as judged by the formation of TRAP-positive, calcitonin receptor-positive, bone resorbing osteoclast-like cells (in collaboration with T. J. Chambers lab., London, **Fig. 2**). This suggests that the major transcriptional activation domain of c-Fos, which is located in the C-terminal half, is not required for osteoclastogenesis, since this activation domain is missing from other Fos family members. Currently, we are testing various Fos mutants in order to identify critical domains essential for osteoclast differentiation.



	Relative number of osteoclasts (%)
control	0
c-Fos	100
Fra-1	170
FosB	30
c-Jun	0

Fig. 2: AP-1 dependent osteoclast development in tissue culture.

2. Defining the functions of c-Jun *in vivo*

Axel Behrens and Maria Sibilia in collaboration with Robert Eferl and Kurt Zatloukal (Graz) and Hermann Bujard (Heidelberg)

The proto-oncogene *c-jun* has been implicated in both cell proliferation and cell differentiation by many *in vitro* and *in vivo* studies. Mice harbouring a homozygous mutation at the *c-jun* locus die between day 12.5 to 13.5 of embryonic development and it has been proposed that hepatogenesis is impaired (Hilberg *et al.*, 1993). The inability of c-*jun* -/- ES cells to contribute to hepatocytes in adult chimaeras suggests that c-Jun functions cell-autonomously in liver development. The expression levels of more than 20 liver-specific mRNAs were analyzed in c-*jun* -/- livers at different embryonic stages and no significant differences were detected, suggesting the presence of functional hepatocytes in the mutant fetuses. Histological analysis of fetal livers revealed apoptosis of cells of the erythroid lineage, implicating a role of c-Jun also in fetal liver hematopoiesis. However, when transplanted into lethally irradiated adult recipient mice, c-*jun* -/- fetal liver cells were able to reconstitute all hematopoietic lineages. Therefore, in mutant fetal livers the observed apoptosis in the erythroid lineage is most likely caused by an altered micro-environment which is unable to support hematopoiesis. Our current hypothesis is that proliferation and/or survival of fetal liver cells is regulated by different signal transduction pathways in hepatoblasts and differentiated hepatocytes.

To investigate the role of c-Jun in embryonic liver development we have decided to conditionally inactivate the *c-jun* gene using the cre/LoxP recombination system in combination with tetracyclin-induced gene expression. This strategy allows the deletion of the gene of interest in a tissue- and time-dependent manner. By homologous recombination we have generated ES cell clones and mice carrying the *c-jun* gene flanked by two LoxP sites ("floxed" *c-jun*), the recognition sites of cre recombinase. To express the cre recombinase specifically in liver we use a transgenic line carrying the cre gene under the control of a tTA-responsive promoter. When this line is bred with a second transgenic line expressing the tetracyclin controlled transactivator specifically in liver (LAP-tTA), c-*jun* will be deleted in an inducible manner only in liver. In offspring derived from intercrosses between the three above mentioned strains cre-LoxP site-dependent recombination will occur in cells where the cre transgene is expressed, thereby deleting the *c-jun* gene only in liver.

Phosphorylation at serine 63 and 73 through the c-Jun amino-terminal kinases (JNKs) strongly augments the transactivation potential of c-Jun and is required at least *in vitro* for a variety of biological processes such as cell transformation, apoptosis and T-cell activation. Using ES cell technology we are in the process of generating mice carrying a mutant *c-jun* allele which has the two serines converted to alanines to test the importance of c-JUN phosphorylation *in vivo*.

3. The Role of Jun N-terminal kinases *in vivo*

Kanaga T. Sabapathy and Martin Schreiber in collaboration with
Tuula Kallunki and Michael Karin (UCSD, San Diego)

Phosphorylation of c-Jun at Ser-63 and Ser-73 is an important event in the activation of c-Jun, and the kinases involved have been identified as the Jun N-terminal kinases 1 and 2 (JNK1 and JNK2). JNK2 has 25 times higher affinity for c-Jun than JNK1. To determine the role of JNKs in development, we have generated JNK2 -/- mice by gene targeting in ES cells. These mice are viable and do not express JNK2 mRNA. Primary embryonic fibroblasts lacking JNK2 do not show any proliferation defect, in contrast to fibroblasts lacking c-Jun. Presently we are analyzing the effects of JNK2 inactivation in mice and cells and are also targeting the JNK1 gene in ES cells to investigate the effects of loss of JNK1 in mice.

4. Essential overlapping functions of JunB and Fra-1 in early extra-embryonic mouse development

Martin Schreiber and Zhao-Qi Wang in collaboration with Marina Schorpp and Peter Angel

To analyze the individual function of JunB and Fra-1 in embryonic development we applied gene targeting in ES cells to generate mice lacking either of these proteins. Either one of the mutations results in severe growth retardation and embryonic lethality by day 10. To identify the site of the primary defect we injected junB -/- or fra-1 -/- ES cells into tetraploid wild-type blastocysts, which can only contribute to extra-embryonic tissues of the resulting chimaeras. The junB -/- or fra-1 -/- embryos complemented with wild-type extra-embryonic tissues were no longer retarded in growth and survived up to day 14.5 (JunB) or to birth (Fra-1), demonstrating that the growth retardation and the early lethality is caused by dysfunction of extra-embryonic organs. Histological analysis of the mutant embryos showed that the close contact between parietal and visceral yolk sac is disrupted. Furthermore, the placental labyrinth layer is largely avascular and reduced in size (Fra-1) or even absent (JunB). Since both these extra-embryonic organs are essential for nourishment of the embryo, the observed growth retardation and early lethality are likely due to starvation of the embryos *in utero*.

Several lines of evidence suggest that both proteins, JunB and Fra-1, exhibit overlapping if not identical functions in embryonic development: (1) Disruption of both genes results in remarkably similar phenotypes. (2) AP-1 complexes containing predominantly JunB/Fra-1 heterodimers have been shown to regulate several promoters of target genes, such as involucrin, interleukin-2 (in combination with NF-AT) and HPV 18. (3) Both JunB and Fra-1 can act as transcriptional activator or repressor depending on the promoter context and the levels of other AP-1 proteins. (4) Overexpression of a JunB transgene driven by the ubiquitin promoter enables JunB -/- and Fra-1 -/- (at low frequency) mice to survive to adulthood. (5) Preliminary results obtained from transient transfections suggest that Fra-1 is one of the very few examples of target genes that can be activated by JunB.

Our data are in agreement with the general assumption that individual AP-1 dimers exhibit unique non-overlapping functions in mouse development and we propose that JunB/Fra-1 heterodimers are major contributors to AP-1 activity in early extra-embryonic development.

5. c-Jun and JunB - is there functional compensation?

Emmanuelle Passegué, Wolfram Jochum and Zhao-Qi Wang

c-*jun* and *jun*B genes play essential roles in mouse development since mice carrying a mutation in either of them die during early embryogenesis. The lethality of the null mutation at the c-*jun* locus can be rescued by a transgene expressing c-*jun* constitutively (under the H2-promoter), giving rise to viable mice. These mice exhibit a severe eye pathology and drastically reduced fertility. Similarly, the lethality of the null mutation at the *jun*B locus can be rescued by a transgene constitutively expressing *jun*B (under the ubiquitin promoter), giving rise to viable mice with a developmental defect in coat/hair formation and poor fertility. In addition, old *jun*B-/- Ubi-*jun*B mice develop a hematopoietic disorder similar to chronic myeloid leukaemia. Hence, these experiments reveal some specific functions of c-*jun* and *jun*B during organogenesis that were masked by the embryonic lethality.

Based on the different phenotypes of the knockout mice and differences in spatial and stage-specific expression of c-*jun* and *jun*B during early embryogenesis, it seems that these two proteins have specific gene regulatory functions which are non-redundant. However, some rescued c-*jun*-/-Ubi-*jun*B em-

bryos and mice were obtained from an intercross between c-*jun*+/- and Ubi-*jun*B transgenic mice. A preliminary histological survey of organs has revealed no obvious abnormality in c-*jun*-/-Ubi-*jun*B embryo at E17.5 suggesting that *jun*B can functionally substitute for c-*jun* in the early stages of embryonic development. Two different strategies are currently used to demonstrate overlapping *in vivo* functions between these two genes: (i) a "knock-in" approach where the coding region of the c-*jun* gene will be replaced by the *jun*B cDNA using embryonic stem cell technology; (ii) a classical genetic rescue experiment in which *jun*B heterozygous knock-out mice will be crossed with Ubi-c-*jun* transgenic mice.

These *in vivo* studies are complemented by the analysis of primary or immortalized fibroblasts isolated from c-*jun*-/- and c-*jun*-/-/Ubi-*jun*B embryos. Fibroblasts lacking c-*jun* are characterized by a remarkably reduced proliferation rate. In contrast, primary c-*jun*-/-/Ubi-*jun*B fibroblasts show a proliferation rate similar to wild-type, suggesting that overexpression of *jun*B can rescue the proliferation defect of c-*jun*-/- cells. These cell lines will be appropriate tools to determine whether *jun*B can rescue other defects observed in fibroblasts lacking c-*jun* such as transformation and UV-C hypersensitivity.

6. Molecular analysis of the p53-dependent proliferation defects of fibroblasts lacking c-Jun

Martin Schreiber and Uta Möhle-Steinlein in collaboration with Andrea Kolbus and Peter Angel (Heidelberg)

We have previously shown that spontaneous immortalization of mouse fibroblasts lacking a functional c-*jun* gene is possible, but only after a remarkably prolonged crisis period. Furthermore, primary as well as immortalized mutant fibroblasts exhibit reduced proliferation rates and a decreased number of S-phase cells. This function of c-Jun is dependent on the presence of p53, since fibroblasts lacking c-Jun and p53 grow even faster than wild-type cells and become immortalized without any detectable crisis. Thus, a null mutation of p53 is fully epistatic to a null mutation of c-jun. However, this epistatic relation is not observed *in vivo*, since c-jun-/-p53-/- embryos die at the same stage as c-jun-/- embryos do. To gain insight into the interaction between c-Jun and p53 at the molecular level, we have analyzed the status of p53 in c-jun-/- cells and found that p53 RNA and protein levels are elevated, suggesting that in wild-type cells, functional c-Jun represses p53 expression by a yet unknown mechanism. To understand the molecular basis of the observed proliferation defect of c-jun-/- cells, we have analyzed mammalian cell cycle regulators and found in mutant cells higher levels of cyclin E and cyclin D1, a proposed AP-1 target gene. However, the associated kinase activities (CDK-2 and CDK-4/6, respectively) are significantly lower, particularly late in G1. Consistent with this observation, binding activity of transcription factor E2F, which is primarily regulated by the CDK-target pRb, is poorly inducible in mutant cells. As a consequence, G1-to-S-phase transition in mutant cells may be inefficient. This may explain why CDK and E2F activities are fully reverted in cells that lack c-Jun and p53. We are currently investigating whether reduced expression of the CDKs, or increased expression of CDK inhibitors such as p21 is causal to this defect.

7. Cooperativity between Fos, p53 and Rb in osteosarcoma development

Wolfram Jochum and Jody Haigh

Transgenic mice overexpressing the *c-fos* proto-oncogene develop osteosarcomas. Genetic analysis of primary human osteosarcomas demonstrated alterations of the tumor suppressor genes *p53* and *Rb* as a frequent event. Patients with Li-Fraumeni-syndrome, which is caused by germ line mutations in the *p53* gene, like mice deficient for the *p53* gene have an increased tumor susceptibility including the development of osteosarcomas. We have started to address the question whether *c-fos* can cooperate with *p53* and *Rb* in the formation of osteosarcoma *in vivo*. For this purpose we are currently generating *c-fos* transgenic mice heterozygous or deficient for *p53* and *Rb* in order to investigate the developing osteosarcomas with respect to time of onset, morphology and the expression of markers of bone and cartilage.

II. Role of p53 in stem cell proliferation and differentiation

Kanaga T. Sabapathy

The tumor suppressor gene product, p53, encodes a phosphoprotein which has been shown to play a critical role in controlling cell proliferation, growth arrest and apoptosis. Mutations in p53 as well as its functional modulation which inactivate p53 activity have been shown to result in the formation of a variety of tumors. Several studies using the temperature-sensitive p53 mutant, p53val135 (ts mutant), have indicated that p53 functional activity is associated with its conformational flexibility. Similarly, the wild-type p53 protein (unmutated gene product) has also been proposed to exist in either the wild-type or mutant conformation, depending on the physiological status of the cell, thus allowing proliferation to proceed when in a mutant conformation. Besides playing an important role in controlling proliferation and apoptosis, p53 has also been implicated in regulating cellular differentiation and several reports have shown that endogenous p53 levels and activities are modulated upon differentiation. Although p53 null mice are viable, mice lacking mdm2, a negative regulator of p53, die at an early embryonic stage, and this lethality could be rescued in a p53 null background (de Oca Luna *et al.*, 1995; Jones *et al.*, 1995). This implies that p53 is functional at an early embryonic stage, and its functional activity has to be controlled for survival of embryos and subsequent development.

In an attempt to determine the role of p53 in the early differentiation processes, we have employed embryonic stem (ES) cell lines and found that they express high levels of p53 in the wild-type conformation. Although p53 was functional as determined by its ability to bind DNA specifically and activate transcription, undifferentiated ES cells do not undergo cell cycle arrest or apoptosis. However, the presence of functional p53 in undifferentiated ES cells renders them hypersensitive to UV irradiation, indicating a protective role for p53 which presumably prevents propagation of mutations through the germline. Differentiation with retinoic acid resulted in a decrease in the levels of p53 and in a shift in its conformational status to the mutant form, with a concomitant loss of functional activity, and these differentiated cells are resistant to UV treatment. Furthermore, ES cells undergoing apoptosis during differentiation expressed functional p53. Interference with the p53 conformational change during differentiation by expression of the temperature-sensitive p53val135 mutant in ES cells resulted in a reduction of apoptosis accompanying differentiation when it adopted a mutant-conformation at 39°C, implying a regulatory role for p53 during the early differentiation process. Thus, the conformational shift appears to be a mechanism regulating p53 functional activity during differentiation. The functional inactivation of p53 allows differentiating cells to escape from apoptosis, and highlights the importance of controlling p53 activity during development for cell survival during differentiation.

As undifferentiated ES cells are able to tolerate high levels of functional p53, we investigated if the presence of p53 affects their proliferative potential. Undifferentiated ES cells lacking p53 exhibited enhanced proliferation as compared to p53 +/- ES cells and apoptosis accompanying differentiation was found to be reduced. Furthermore, differentiated p53 -/- cells also proliferate faster than p53 +/- cells. These results suggest that p53 may regulate proliferation differentially in undifferentiated and differentiated cells *in vitro,* by hitherto not well understood mechanisms. We are currently analyzing the possible mechanisms by which undifferentiated ES cells are able to overcome the effects of high p53 expression, and p53's role in regulating proliferation in undifferentiated and differentiated cells.

III. Control of endothelial cell proliferation and vascular tumor development

1. Polyoma middle T in endothelial tumor development

Kanaga T. Sabapathy, Ulrich Mühlner, Jody Haigh and Uta Möhle-Steinlein

The middle T antigen of murine polyomavirus (PymT) binds to and activates several molecules of various signaling cascades, among them the tyrosine kinases of the Src family. *In vivo,* PymT rapidly transforms endothelial cells leading to vascular malformations reminiscent of endothelial tumors or hemangiomas (Kiefer *et al.*, 1994a). PymT-transformed endothelial cells (END. cells) derived from such tumors exhibit increased proteolytic activity (i.e. urokinase plasminogen activator, uPA), implying a causal role for the PA/plasmin system in vascular tumorigenesis. However, the lack of these proteases i.e. uPA and tPA (tissue-type PA) does not render PymT incapable of endothelial cell transformation, suggesting that the proteolytic status of the host is not a critical determinant for PymT-induced vascular tu-

mor formation. In addition, the lack of either uPA or tPA activity is not limiting for the establishment and proliferation of END. cells *in vitro*, although the combined loss of both PA activities leads to a marked reduction in proliferation rates. Furthermore, the *in vitro* morphogenetic properties of mutant END. cells in fibrin gels could only be correlated with an altered proteolytic status in cells lacking both uPA and tPA (in collaboration with M. Pepper, Geneva). However, in contrast to tumors induced by PymT itself, the tumorigenic potential of mutant and wild-type END. cells was found to be highly dependent on the proteolytic status of both the tumor cells and the host (**Fig. 3**). Thus, genetic alterations in the PA/plasmin system affect vascular tumor development indicating that this system is a causal component in PymT-mediated oncogenesis.

Despite the role of uPA and Yes-Kinase in PymT-induced vascular malformations, little is known about the signaling events leading to the specific transformation event. Using RT-PCR, Northern- and Western-Blot analysis we show that the endothelial-specific receptor tyrosine kinase Flk-1 is efficiently expressed in END. cells and can be immunoprecipitated from END. cell lysates in an active complex. The expression of Flk-1 was detected in sections of PymT-virus induced hemangiomas by lacZ-staining from Flk+/- mice carrying a lacZ gene in the Flk locus. In addition, we also observed the expression and secretion of its ligand VEGF by END. cells at the RNA and protein levels.

These results obtained from *in vitro* and *in vivo* studies suggest that a VEGF/Flk-1 autocrine loop might be involved in PymT induced formation of vascular lesions. We next want to functionally interfere with this putative growth factor/receptor loop through conditional inactivation of Flk-1 by *cre/LoxP* mediated recombination and, alternatively, the inducible overexpression of a dominant-negative Flk-1 mutant (for details see next paragraph).

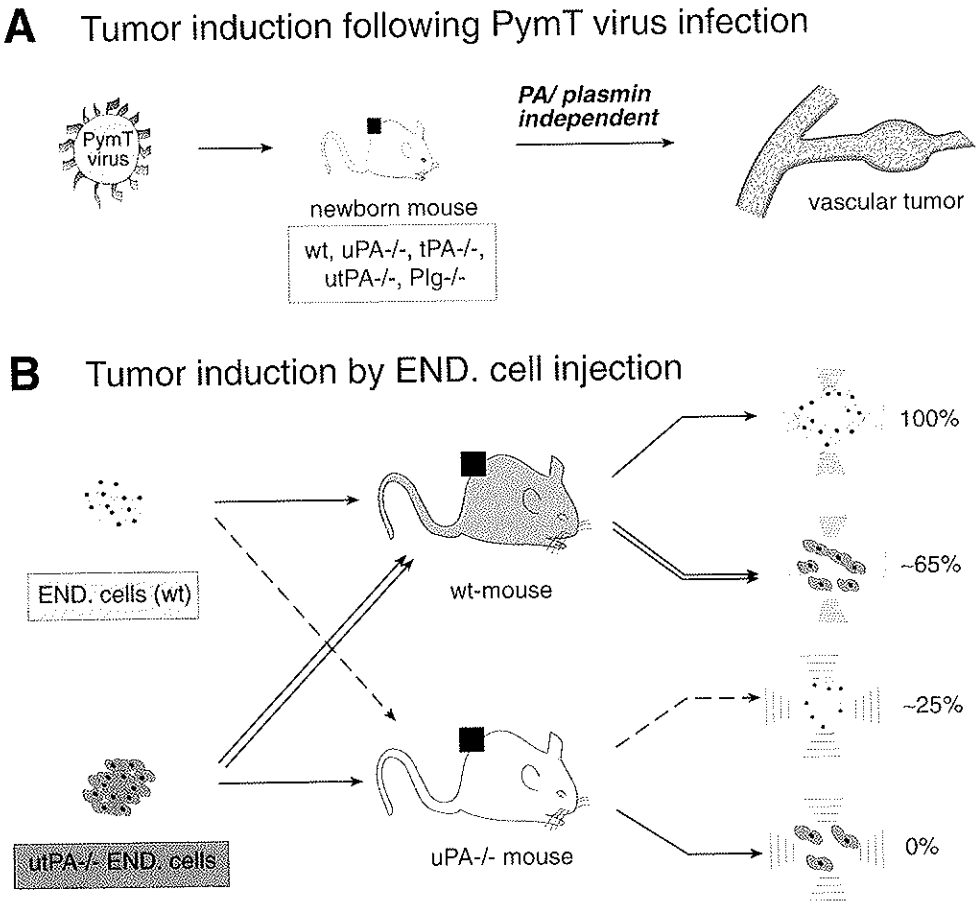


Fig. 3: Role of plasminogen activator/plasmin system in PymT-induced vascular tumors.

2. Function of Flk-1 in development and in tumor-neoangiogenesis

Ulrich Mühlner, Anton Wutz and Uta Möhle-Steinlein

The receptor tyrosine kinase Flk-1 is exclusively expressed on the surface of endothelial cells and represents a high affinity receptor for its ligand VEGF (Vascular Endothelial Growth Factor). Upon ligand binding Flk-1 becomes autophosphorylated and subsequent signaling pathways are activated. This activation of the endothelial signaling machinery leads to several cellular responses including proliferation, migration and increase of blood vessel permeability. Studies using genetically altered mice lacking either Flk-1 or VEGF demonstrated the essential need of these two molecules for proper development of the vascular system. Flk-1 -/- mice die between embryonic stage E8.5 and E9.5 without developing any blood vessels suggesting a critical role of this receptor in the early stage of blood vessel development. Since these mice die before the onset of organ development the impact of Flk-1 on the development of the vascular system cannot be studied. Therefore, we decided to apply two different strategies for the analysis of Flk-1 function in the mouse and in tumor neoangiogenesis: The first strategy is based on the cre/*LoxP* mediated recombination system (Gu *et al.*, 1994). In our case a mouse strain carrying two *LoxP*-sites in the Flk-1 locus flanking Exon 1 will be crossed with a strain expressing *cre* recombinase exclusively and inducibly in endothelial cells. We have targeted the Flk-1 locus by homologous recombination in ES cells with the targeting vector carrying the *loxP*-sites and are presently characterizing ES cell clones for the generation of chimaeric mice (in collaboration with Werner Risau, MPI Bad Nauheim).

Using the second strategy we plan to inducibly overexpress a dominant-negative mutant of Flk-1 lacking the cytoplasmic kinase domain to inhibit receptor activation. Utilizing the tetracycline-inducible expression system (Gossen *et al.*, 1992) we generated transgenic lines expressing a truncated Flk-1 under the control of the *tet*-operator. In order to investigate the expression pattern of these lines we used a bidirectional promoter to express the *lacZ* gene as well as the flk mutant. Currently we are generating transactivator lines that express the tetracycline-responsive transactivator (rtTA) exclusively in endothelial cells using a Tie2-promoter/enhancer construct (kindly provided by Tom Sato, Harvard Medical School). Breeding of these two strains will enable us to study the effect of expressing a dominant-negative Flk-1 probably at any given time-point during embryogenesis or adulthood.

3. Role of VEGF in bone and cartilage development

Jody J. Haigh

It has been well established that bone development is dependent on angiogenic processes. Early in endochondral bone development, mesenchymal stem cells differentiate into chondroblasts that secrete a cartilagenous matrix that is kept avascular through the production of anti-angiogenic substances. During the maturation of the cartilage matrix, chondrocytes become hypertrophic, cease secreting anti-angiogenic factors, and start secreting angiogenic factors which are responsible for the vascular invasion into the previously avascular cartilage core. Our interest in this biological process concerns understanding the factors which may play a role in the angiogenic process. One factor that is of particular interest is Vascular Endothelial Growth Factor (VEGF). VEGF has recently been shown to be expressed in osteoblastic cells and is upregulated in response to exogenously administered prostaglandins (Harada, 1994). In addition, high levels of VEGF immunopositive macrophages have been found in patients with rheumatoid and osteoarthritis (Koch, 1994). These results imply that upregulated VEGF expression may contribute to human inflammatory states and aberrant bone homeostasis.

We have recently detected VEGF mRNA expression in cartilaginous cell types both *in vitro* and *in vivo*. Northern blot analysis has detected VEGF mRNA transcripts in several different cartilage cell lines. More significantly, preliminary mRNA *in situ* hybridization studies have detected VEGF mRNA transcripts in chondrocytes from day 15.5 - 17.5. These data indicate that VEGF may play an important role in the early events of vascular invasion into cartilage. In order to determine the physiological effects of disturbing the balance between angiogenic and anti-angiogenic molecules on bone development a transgenic approach has been initiated to overexpress VEGF165 specifically in the cartilage primordia at an early time in development. The human Col2A1 promoter and intronic enhancer sequences have been chosen to specifically express the VEGF165 cDNA in chondrocytes. The combined *in situ* and transgenic approach will enable us to examine the possible biological role of VEGF in cartilage development.

IV. Understanding the function of the EGF-receptor

1. Characterizing the defects in EGFR mutant mice

Maria Sibilia in collaboration with Joachim Steinbach and Adriano Aguzzi (Univ. Zurich)

The epidermal growth factor receptor (EGFR) is activated by at least five ligands and belongs to a family of tyrosine kinase receptors, including erbB2, erbB3 and erbB4 which show heterodimerization *in vitro*. EGFR activation leads to the activation of multiple downstream signaling pathways which can lead to cell proliferation, differentiation and survival, but very little is known about how these different cell responses are regulated *in vivo*. In addition, amplification and overexpression of the receptor have been observed in human carcinomas and brain tumors. In order to begin to understand the function of the EGFR, mice and cells lacking functional EGFR were generated by inactivating the gene in ES cells. Mutant mice are retarded in growth and die at different stages of embryonic and postnatal development depending on their genetic background (Sibilia and Wagner, 1995). Death *in utero* results from a placental defect, whereas at birth probably from lung immaturity. All surviving mutant mice show abnormalities in various epithelia such as skin, hair follicles, eyes and lungs and, in addition, they progressively develop severe brain defects. These results suggest that EGFR primarily affects epithelial and neural cells.

We have characterized in more detail the defects in the mutant mice with regard to embryonic, perinatal and postnatal lethality. The embryonic lethality can be rescued by generating aggregation chimaeras between EGFR mutant and tetraploid wild-type embryos, the latter contributing exclusively to the extraembryonic tissues, indicating that the genetic background most likely acts on the development of the extraembryonic tissues. The expression of several "candidate" genes was investigated in mutant placentas of different genetic backgrounds, but no obvious differences could be observed. The perinatal lethality can partially be rescued by transplacental administration of dexamethasone, a hormone known to promote lung maturation, supporting the hypothesis that lung immaturity is indeed the reason why EGFR mutant mice can not sustain respiration. Using an *in vitro* organ culture system (in collaboration with Heber Nielsen, Boston) we could show that branching morphogenesis is reduced in mutant lungs and we are currently analyzing if the absence of EGFR affects the development of the epithelial or mesenchymal compartment of the lung.

Brains of all surviving mutant mice show a progressive neurodegeneration in the frontal cortex, olfactory bulb and thalamus starting around day 5 after birth (**Fig. 4**). The cortical degeneration is always accompanied by leptomeningeal hemorrhages and massive neuronal apoptosis which ultimately leads to the loss of extensive parts of the frontal cortex. Interestingly, high levels of EGFR expression can be detected in the brain vessels. Grafting experiments combined with *in vitro* cocultures of neuronal and glial cells should enable us to investigate if the vascular or neuronal compartment is affected in mutant brains.

In addition, we will try to understand the molecular mechanisms responsible for the observed phenotypes by inactivating the EGFR gene in a tissue-specific manner. This, combined with the "knock-in" of mutant human EGFRs and the tissue-specific overexpression of downstream signaling molecules should permit to dissect proliferative versus differentiation pathways *in vivo*.

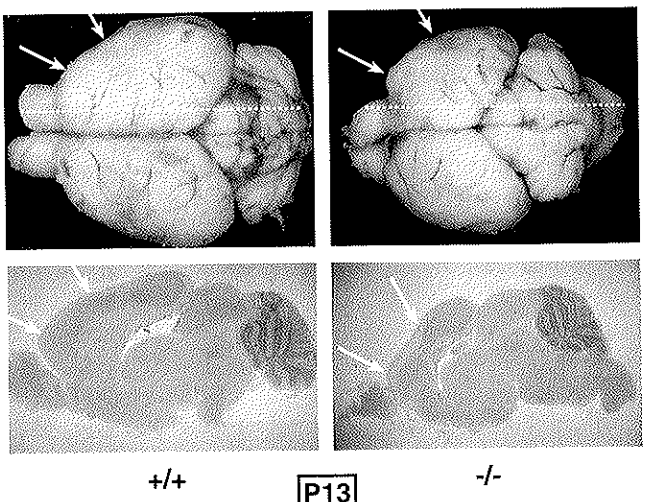


Fig. 4: Cortical degeneration in EGFR mutant brains. The upper figures represent an overview of the brains isolated from wild-type (left) and EGFR-/- (right) mice at postnatal day 13. The forebrain of the -/- is dramatically reduced in size compared to the control. The lower pictures show histological cross-sections at the level of the dotted lines of the respective brains. Note that the region of the cortex between the arrows is completely degenerated in the mutant brain.

2. Characterizing ES cells and fibroblasts lacking the EGFR

Maria Sibilia and Alexander Fleischmann

From the analysis of EGFR mutant mice as well as by several *in vitro* studies using dominant negative receptors, it appears that EGFR plays an important role during neuronal differentiation. *In vitro* differentiation of ES cells provides a powerful model system for studying the function of genes in cell differentiation. Therefore, we are using ES cells that lack both EGFR alleles to analyze whether the loss of EGFR affects the capacity of ES cells to differentiate into neurons. Preliminary data suggest that EGFR-/- ES cells are impaired in their ability to differentiate into neuron-like cells. By using specific marker antibodies we show that different neuronal- and glial-like cell types were obtained but at lower efficiency. Further studies will aim to optimize the differentiation conditions in order to prove whether the lack of EGFR affects the differentiation or survival of these neuron-like cells.

Primary embryonic fibroblasts lacking EGFR were isolated and spontaneously immortalized cell lines were established. These cells were used to study the function of this receptor in cell proliferation, UV response and transformation, processes in which EGFR had previously been reported to be involved. Although some of our results have to be considered preliminary, it seems that cells lacking functional EGFR do not display any significant alterations in the biological processes investigated. These results suggest that functional complementation between members of the erbB family may be substituting for the lack of EGFR *in vitro*.

3. Searching for downstream targets of EGFR in mice

Maria Sibilia, Alexander Fleischmann and Axel Behrens in collaboration with Joseph Carroll and Fiona Watt (ICRF, London) and Joseph Schlessinger (NYU, New York)

From the analysis of various mutant mice that are defective in EGFR signaling it has been shown that this receptor plays a key role in skin and hair follicle development. EGFR expression has been detected in the basal cells of the epidermis and in the outer root sheath of the hair follicles. In order to identify downstream components of the EGFR in these particular cell types, we want to overexpress putative signaling molecules, e.g. hSOS (human Son of Sevenless) in the skin of transgenic mice, cross them to EGFR mutant mice and ask whether the defects can be rescued.

We have generated transgenic mice that express a dominant form of the human SOS gene under the control of the keratin 5 promoter that is active in the same cells that express the EGFR. Four transgenic founders were obtained, and all of them developed a skin disease, but with different severity depending on the number of integrated transgenes. Histological examinations of transgenic skins showed that the epidermis is hyperplastic consisting of an increased number of cell layers. Immunohistochemistry with different markers such as Ki67, keratin-1, -6, and -14 suggests that there is a less differentiated epidermis with massive hyperproliferation of the basal cells, reminiscent of basal cell carcinoma.

Genetic experiments to determine whether overexpression of SOS can complement a phenotype caused by a weak EGFR allele (the waved-2 mutation) are ongoing. We are also analyzing which pathways are activated downstream of SOS with the ultimate goal to dissect proliferative versus differentiation pathways *in vivo*.

V. Understanding the mechanism of gametic imprinting of the mouse Igf2r gene

Anton Wutz in collaboration with Denise Barlow, Amsterdam

Using the Igf2r gene as a model to study the mechanism of gametic imprinting we aim to gain insight into parental specific epigenetic regulation of gene expression during embryonic development. The imprinted mouse Igf2r gene is transcribed exclusively from the maternally inherited chromosome after implantation, and embryos that inherit a mutation of the gene from their mother die perinatally. Notably, a paternally inherited mutation does not lead to pathology.

Parental specific expression of Igf2r has been proposed to be mediated by an imprinting box, a 3kb CpG island located in intron 2 of the gene (termed region2). Methylation of region2 in the maternal germline is thought to be the signal constituting the chromosome's parent-of-origin memory. However, since

the mouse Igf2r gene is located in a large chromosomal domain which has been demonstrated to replicate asynchronously, other factors in addition to region2 may play a role in imprinting.

We have generated transgenic mice by pronuclear microinjection of a 300 kb YAC (yeast artificial chromosome) containing the entire Igf2r locus (**Fig. 5**). The YAC construct was genetically engineered using homologous recombination in *S. cerevisiae*, intact high molecular weight YAC DNA was purified, and roughly one percent of the injected B6CBF1xB6CBF1 zygotes yielded transgenic offspring. Subsequent analysis of the transgenic lines showed that the YAC-Igf2r allele is subject to gametic imprinting. Expression of the transgene as well as the methylation pattern of region2 exactly match that of the endogenous locus. We conclude that the sequences constituting the gene's parent-of-origin memory are retained within the 300 kb genomic fragment containing the Igf2r gene. To address the function of region2 in imprinting Igf2r, we have constructed a YAC deleted for this element. Mice expressing this construct were obtained, and the YAC-Igf2r allele was no longer repressed when the transgene was inherited from the father (**Fig. 5**). Thus, the imprinting phenomenon of the Igf2r gene depends on region2. Moreover, these data demonstrate that region2 acts as a repressor, and suggests that its repressive potential is alleviated by DNA methylation. The YAC transgenes have also been used to confirm the existence of an RNA originating in region2, which has the opposite orientation to the Igf2r mRNA (**Fig. 5**). This region2 associated RNA is also expressed specifically from the endogenous paternal Igf2r locus, and may play a role in repression of the paternal promoter.

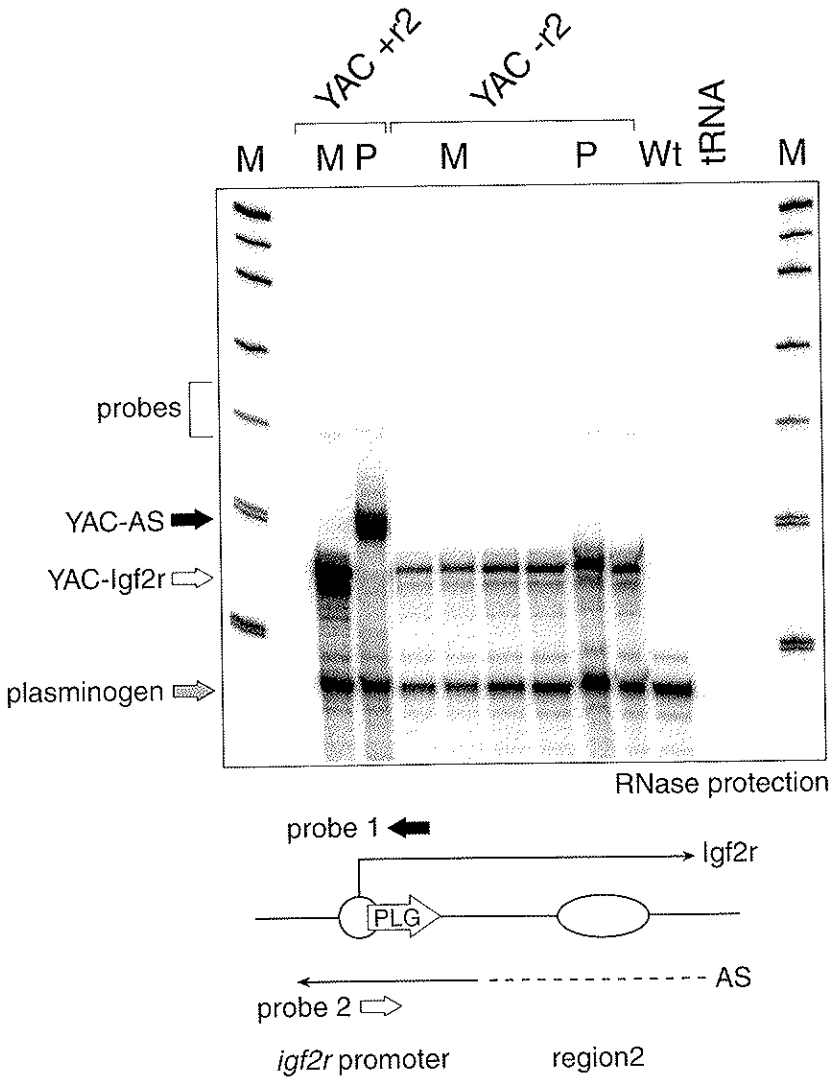


Fig. 5: Imprinted expression of YAC transgenes in 13.5 day embryos was analyzed by RNase protection. Transcripts from the YAC transgene were identified by a sequence tag from the mouse plasminogen cDNA in the first exon of the YAC-Igf2r. The YAC construct containing region2 (YAC +r2) shows maternal specific expression of the YAC-Igf2r allele (lanes M) and paternal specific expression of an AS RNA (lane P). The construct deleted for region2 (YAC-r2) shows expression of the YAC-Igf2r when inherited from either parent, and no AS RNA was observed following paternal inheritance of the transgene.

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Molecular dissection of multistage tumorigenesis in transgenic mice

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Lab technician	Petra WILGENBUS (since Nov. 1996)

Introduction

The molecular analysis of tumorigenesis is often hampered by the unavailability of tissue specimens from the multiple stages of tumor development. Thus, the genetic reproducibility and the accessibility of tissue specimens have made transgenic mice a valuable tool to study the molecular events involved in the stepwise progression towards tumor malignancy.

In one transgenic model of tumorigenesis (RIP1Tag2), the rat insulin gene regulatory region has been used to target expression of Simian Virus 40 large T antigen (Tag) to the ß cells of the pancreatic islets (Hanahan, 1985). These mice develop tumors in the ß cells of the islets of Langerhans (insulinomas) in a predictable manner and different stages of tumor progression are clearly distinguishable (**Fig. 1**). Although all the ß cells express T antigen, only about 50% of the 400 islets in a pancreas develop hyperplasia, as determined by increased proliferation index and increased size. Approximately 10% of the total islets induce the formation of new blood vessels (angiogenesis), and about 2% of the islets develop into benign tumors (adenomas). Finally, about 0.5% of the total islets progress into invasive tumors (carcinomas). It appears that additional genetic or epigenetic changes occur during the stepwise progression of tumorigenesis in RIP1Tag2 transgenic mice.

The major objective of our research is the identification and characterization of the molecular events involved in the transition between the multiple stages of tumorigenesis. In particular, we investigate the molecular mechanisms that underlie tumor cell hyperproliferation, the onset of neovascularization during tumor development (tumor angiogenesis), and tumor cell invasiveness. In addition to tumor cell lines *in vitro*, we employ transgenic mouse models to prove causal connections between the expression of a particular gene and tumor progression *in vivo*. For example, genetic complementation of RIP1Tag2 transgenic mice with mice that are genetically modified to overexpress a gene of interest or to carry a deletion of this gene might give insight into the role these genes play in ß cell tumorigenesis in particular and in cancer development in general.

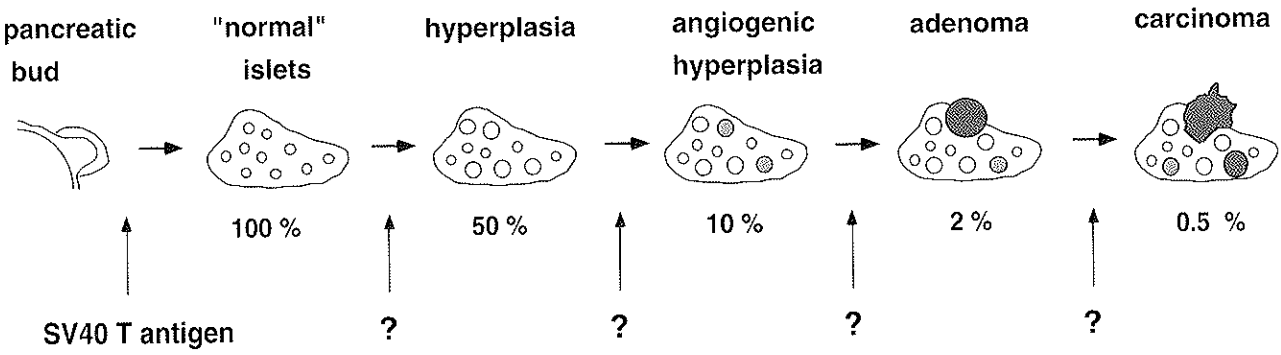

Fig. 1: Schematic representation of multistage tumor progression in RIP1Tag2 transgenic mice.

I. A paradox: diminished tumor growth in the absence of p53

Michaela Herzig, Susanne Luef, and Patrick Smith

Although in RIP1Tag2 transgenic mice SV 40 T antigen is expressed early during embryonic development, it takes several weeks after birth before ß cell proliferation is first detected. Apparently, expression of T antigen is not sufficient to directly elicit hyperproliferation; rather additional genetic or epigenetic events have to occur. It is thought that SV 40 T antigen transforms cells by sequestering the tumor suppressor gene products p53 and Retinoblastoma. It is conceivable that this sequestration may require time to come to completion. To test for this possibility we have intercrossed the RIP1Tag2 transgenic mice with p53-deficient mice. Contrary to the expected enhancement of tumorigenesis, we observed significantly diminished tumor growth in the p53-deficient RIP1Tag2 as compared to wildtype RIP1Tag2 mice (**Fig. 2A**).

Molecular analysis revealed that the incidence of tumor cell apoptosis was unaffected by the absence of p53 indicating that p53 is not required for ß tumor cell apoptosis. In contrast, proliferation of ß tumor cells was significantly reduced in p53-deficient mice (**Fig. 2B**). This behavior is recapitulated in tumor cell lines that have been derived from the ß cell tumors: the incidence of apoptosis was unchanged between the different cell lines, whereas p53-deficient ß tumor cell lines exhibited significantly reduced proliferation rates. Thus, slower proliferation may explain the decrease in tumor growth in p53-deficient mice.

What is the molecular basis for diminished ß tumor cell proliferation in the absence of p53? We found that the steady-state levels of T antigen were dramatically lower in p53-deficient cells as compared to wildtype ß tumor cells due to reduced halflife of T antigen. In order to assess a possible connection between tumor cell proliferation and T antigen levels we performed transactivation assays using promoter constructs that drive the luciferase gene under the control of p53- or E2F-responsive elements, respectively. These experiments revealed that p53 transactivation activity is completely neutralized by T antigen, comparable to the genetic absence of p53 in the p53-deficient cell lines. This indicates that p53 transactivation activity does not account for the difference in tumor cell proliferation. In contrast, E2F

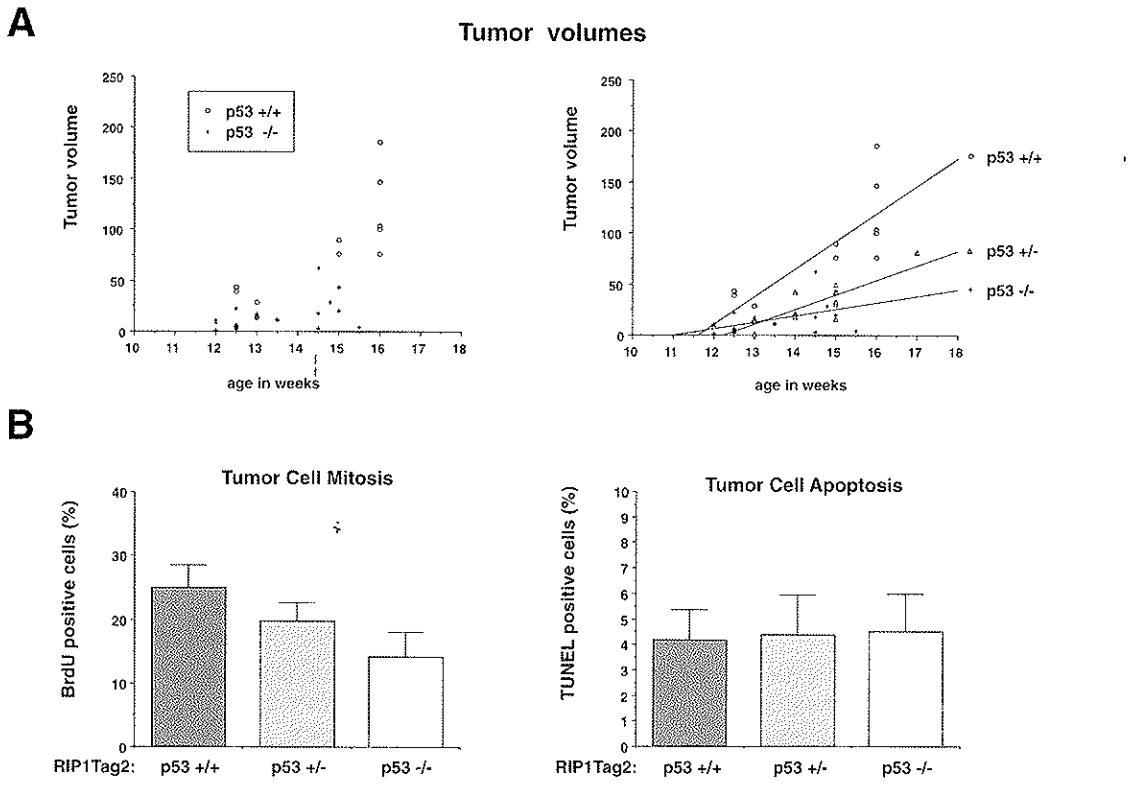

Fig. 2: *Decreased growth of ß cell tumors in p53-deficient RIP1Tag2 transgenic mice.*
(A) Histograms show tumor volumes at autopsy in a series of individual RIP1Tag2 transgenic mice either wildtype (p53 +/+), heterozygous (p53 +/-), or deficient (p53 -/-) for p53. The increase in tumor volume over time is given as calculated slopes in the histogram on the right. **(B)** The incidence of mitosis (left-hand panel) was determined by immunohistochemical visualization of cells that incorporated BrdU during a short labeling pulse prior to autopsy. The incidence of apoptosis (right-hand panel) was determined by staining histological sections from ß cell tumors using the TUNEL technique. p53 genotypes are as in (A).

transactivation activity was found to be significantly lower in the p53-deficient ß tumor cell lines as compared to the corresponding wildtype cell lines.

From our data we propose a model in which T antigen not only stabilizes p53 but conversely p53 also stabilizes T antigen. In the absence of p53 the steady state levels of T antigen drop below the threshold required for the quantitative sequestration of Retinoblastoma protein. Unsequestered Retinoblastoma protein is able to bind E2F thereby blocking its transactivation capability. Thus, in T antigen-transformed cells in the absence of p53 reduced E2F activity may be the cause for diminished tumor cell proliferation.

II. Tumor cell proliferation versus tumor cell apoptosis

We are interested in understanding the molecular mechanisms which determine the balance between tumor cell proliferation and programmed cell death (PCD) and thus, ultimately, malignant tumor outgrowth. The requirement for the close coupling of cell proliferation and PCD during tumor development is exemplified by studies of RIP1Tag2 mice carrying a homozygous disruption of the IGF-II gene. In the absence of IGF-II, ß tumor cells continue to proliferate but show a dramatically increased incidence of PCD resulting in a net reduction of tumor mass (Christofori *et al.*, 1994; Naik *et al.*, 1994). These results indicate that IGF-II is a survival factor for ß tumor cells. Our work focuses on understanding the molecular mechanisms, downstream of IGF-II binding to the IGF-1 receptor, required for tumor cell survival and tumor cell death.

IGF-II-deficient ß tumor cells are prone to apoptosis

Gábor Lamm

The balance between tumor cell proliferation and tumor cell apoptosis described above is also apparent in cultured ß tumor cell lines derived from RIP1Tag2 insulinomas. First, tumor cell lines that are deficient in IGF-II expression show a higher incidence of apoptosis as compared to wildtype ß tumor cells, even when grown under high serum conditions (**Fig. 3**). Second, apoptosis of IGF-II-deficient ß tumor cells is dramatically increased by serum deprivation or by treatment with relatively low concentrations of staurosporine, whereas wild-type ß tumor cell lines exhibit a remarkably low incidence of apoptosis when grown under these conditions (**Fig. 3**). A similar difference in the apoptotic response is found when IGF-II-deficient and wildtype tumor cells are exposed to other apoptotic stimuli or chemotherapeutic agents, such as staurosporine, tumor necrosis factor α (TNFα), ceramide, or daunomycine. These results raise the possibility of novel approaches towards cancer therapy.

In order to address the molecular link(s) between IGF-II signaling and the (anti)-apoptotic pathway, we are currently utilizing both the wildtype and the IGF-II-deficient ß tumor cell lines to test apoptotic and anti-apoptotic gene products by transient transfection assays and FACS analyses. We envisage that these experiments will give an insight into the communication pathway(s) between the IGF-II signal and the apoptotic machinery and thus, ultimately, may provide us with valuable targets for cancer therapy.

Role of cysteine proteases in ß tumor cell apoptosis

Gábor Lamm and Rüdiger Schweigreiter

Apoptotic factors which are likely candidates for IGF-II signal mediated inhibition include the family of cysteine proteases which have recently been reported to play a pivotal role in the execution of PCD (for a review see Chinnaiyan and Dixit, 1996). Our analyses have focused on several members of this protease family including ICE (Interleukin-1ß converting enzyme), CPP32ß, and Ich-1 (ICE and ced-3-like homolog).

We have exposed wildtype and IGF-II-deficient ß tumor cells to various apoptotic stimuli such as serum deprivation, staurosporine, ceramide, or TNFα in combination with both a general cysteine protease peptide inhibitor (Z-VAD) or specific peptide inhibitors for either ICE (YVAD) or CPP32ß (DEVD). Our data indicate that apoptosis in IGF-II deficient tumor cell lines can be inhibited by the general cysteine protease peptide inhibitor but not by either the ICE- or the CPP32ß-specific inhibitors. This suggests that a cysteine protease other than ICE or CPP32ß is involved in mediating ß tumor cell apoptosis.

During our studies we found that CPP32ß as well as Ich-1, including novel splice variants of Ich-1, but not ICE are expressed during ß cell tumor devel-

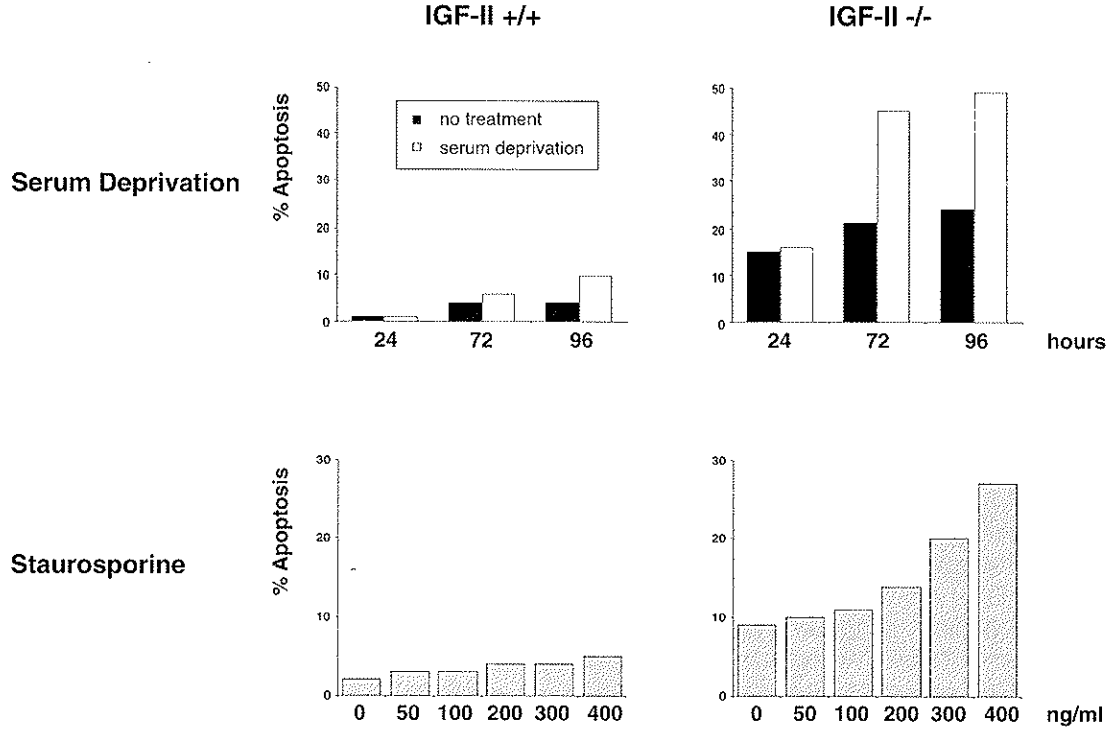

Fig. 3: *Effect of apoptotic insults on wildtype and IGF-II-deficient cell lines.*
Top Panel: Wildtype ß tumor cells (IGF-II +/+) and IGF-II-deficient tumor cells (IGF-II -/-) were grown for 24, 72 or 96 hours under normal serum conditions (no treatment/black bars) or in the absence of serum (serum deprivation/white bars). **Bottom Panel:** Wild-type and IGF-II-deficient ß tumor cells were grown for 24 hours under normal serum conditions supplemented with the indicated amounts of staurosporine (ng/ml). The percentage of apoptotic cells was determined using propidium iodide staining and FACS analyses.

opment in RIP1Tag2 transgenic mice. We are currently analyzing the effect of the different Ich-1 isoforms on wild type and IGF-II-deficient ß tumor cell lines and are interested to find out whether the novel Ich-1 isoforms are mediators of apoptosis in ß cell tumorigenesis. In parallel, we are also monitoring the cleavage pattern of cysteine protease substrates such as PARP (poly(ADP-ribose) polymerase), U1 70k and lamins. By a combination of these approaches we hope to determine which cysteine protease is involved in ß tumor cell apoptosis and to gain insight as to how IGF-II communicates with the apoptotic pathway.

III. Molecular mechanisms of tumor angiogenesis

The malignant outgrowth of all solid tumors requires the formation of new blood vessels (tumor angiogenesis). Soluble growth factors that are mitogenic for endothelial cells are thought to mediate tumor angiogenesis. Biochemical fractionation of medium conditioned by a variety of different tumor cell lines and the analysis of gene expression indicated that several angiogenic factors are expressed during ß cell tumorigenesis in RIP1Tag2 transgenic mice: vascular endothelial growth factor (VEGF) and acidic fibroblast growth factor (FGF-1).

We have reported recently that all three members of the VEGF family are constitutively expressed by normal islets of Langerhans and that their expression does not significantly change during RIP1Tag2 tumorigenesis. Our data suggested that the VEGFs may play a major role in the maintenance of permeable endothelium in islets of Langerhans (Christofori *et al.*, 1995b). In the future, we wish to determine the physiological role of VEGF in the maintenance of permeable endothelium in endocrine organs and its involvement in tumor angiogenesis. Towards this goal, in a collaboration with Ulrich Mühlner and Erwin Wagner at the IMP, we are currently constructing transgenic mice that express a dominant-negative VEGF receptor under the control of an inducible promoter in endothelial cells (see also below).

A novel pathway for the export of FGF-1 and FGF-2

Amelia Compagni and Susanne Luef

Acidic and basic fibroblast growth factor (FGF-1 and FGF-2) are potent inducers of angiogenesis *in vitro* and *in vivo*. However, their involvement as paracrine factors in tumor angiogenesis has not been unequivocally demonstrated, in part because FGF-1 and FGF-2 lack classical signal sequences for secretion. The mechanism by which they are released from cells remains to be elucidated.

We have previously demonstrated that FGF-1 is constitutively secreted by tumor cell lines derived from highly angiogenic ß cell tumors of RIP1Tag2 transgenic mice. However, secreted FGF-1 is sequestered and masked in conditioned medium in high molecular weight forms, and is only retrieved by treatment with denaturing and reducing agents. These complexes of FGF-1 are found in cell lysates as well as conditioned medium, suggesting that they represent export intermediates. The export of FGF-1 does neither involve the conventional secretory pathway nor cell lysis or apoptosis. Similarly, FGF-2 is exported in high molecular weight forms by several other tumor cell lines, including human breast carcinoma and murine fibrosarcoma, but not by normal embryo or dermal fibroblasts or by other FGF-2 expressing transformed cell lines. Our data suggest that novel forms of FGF-1 and FGF-2 are selectively exported by certain tumor cell types via a non-traditional secretory pathway.

In order to facilitate the analysis of FGF export we have begun to tag FGF-1 either by addition of epitope-tags or by fusion of FGF-1 to ß galactosidase (constructs kindly provided by Dr. Tom Maciag, Rockville, USA) or to green fluorescent protein. Stably transfected ß tumor cell lines are currently being analyzed for their capability to specifically export these FGF fusion proteins. To further substantiate the possibility that FGF-1 and FGF-2 are exported by a common yet novel export pathway we will also transfect these constructs into cell lines that have been shown to export FGF-2 (see above). Once characterized in more detail, these cell lines will be employed both to elucidate the pathway and regulation of FGF export and also to study the functional involvement of FGFs in tumor angiogenesis and tumor progression.

The functional role of fibroblast growth factors in tumorigenesis

Amelia Compagni and Anne-Karina Perl

FGFs are highly pleiotropic growth factors; they target a family of receptors on the surface of a variety of cell types resulting in different cellular responses, including proliferation, survival, and differentiation. For these reasons, their detailed role in embryonic development, tumor development and, in particular, in angiogenesis *in vivo* is not fully understood. During tumor development FGFs may induce tumor angiogenesis by targeting endothelial cells in a paracrine manner. FGFs may also stimulate tumor cell proliferation or survival by targeting the tumor cells themselves in an autocrine fashion. Finally, FGFs may influence both tumor cells and endothelial cells, or even a third cell type, for example stromal fibroblasts.

We wish to functionally dissect the different roles FGFs may play during multistage tumorigenesis by utilizing dominant-negative receptor constructs under the control of an inducible promoter, the tetracycline-inducible system (rtTA; Gossen *et al.*, 1995), in transgenic mice. We employ the insulin promoter to direct expression of the tetracycline activator to pancreatic ß cells and the tie-1 promoter to express the tetracycline activator in endothelial cells. We have generated several of these transgenic mouse lines, and they are currently being tested for their tetracycline inducibility by intercrosses to transgenic mice that carry the luciferase gene under the control of the tetracycline operator (a gift from Dr. H. Bluethmann, Basle, Switzerland). The experimental strategy and an example of the results are shown in **Fig. 4**. Currently, we are intercrossing the transgenic mouse lines that carry dominant-negative FGF receptor constructs under the control of the tetracycline operator to tetracycline activator mice. Resulting doubletransgenic mice will be treated with the tetracycline analog doxycycline and the effect of dominant-negative receptor expression will be determined during embryonic development, in adult mice, and during tumor development in a RIP1Tag2 transgenic background.

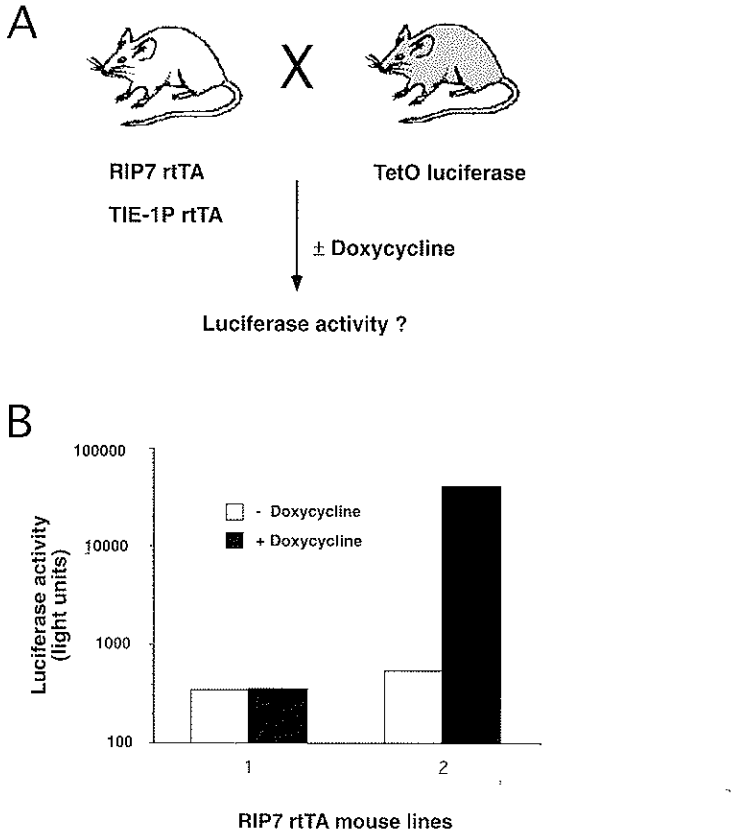

Fig. 4: *Analysis of mouse lines transgenic for the tetracycline activator.*
(A) Schematic outline of the experimental strategy to determine the inducibility of tetracycline activator transgenic mouse lines. RIP7 and TIE-1P designate the insulin promoter and the endothelial cell-specific tie-1 promoter, respectively, each driving expression of the tetracycline activator (rtTA). TetO luciferase designates a transgenic mouse line that carries the luciferase gene under the control of the tetracycline operator. In doubletransgenic mice application of doxycycline will induce the tetracycline activator to bind to the tetracycline operator sequence and to transcriptionally activate the luciferase gene.
(B) Two independent RIP7rtTA transgenic mouse lines (1 and 2) were crossed to the TetO luciferase mice and in resulting doubletransgenic mice luciferase activity was determined in isolated islets of Langerhans either from doxycycline-treated animals (black bars) or from control animals (white bars).

IV. Changes in cell adhesion and tumor invasiveness

Anne-Karina Perl and Petra Wilgenbus in collaboration with Ulf Dahl, Henrik Semb (Umeå University, Sweden), and Harold Cremer (IBD, Marseille, France)

In RIP1Tag2 transgenic mice the expression of several cell adhesion molecules changes during the outgrowth of malignant ß cell tumors. Using constitutive and inducible gene expression in established ß tumor cell lines *in vitro* and genetic complementation of transgenic mouse lines *in vivo*, we set out to address the functional involvement of these cell adhesion molecules in tumor development. In particular, we wish to address the question whether changes in a tumor cell's adhesive repertoire are cause or consequence of tumor progression.

The role of E-Cadherin in ß cell tumorigenesis

Using biochemical, histolopathological, and genetic criteria we have demonstrated that in RIP1Tag2 transgenic mice the expression of E-Cadherin is downregulated during the transition from well-differentiated ß cell adenoma to invasive, dedifferentiated carcinoma (schematically depicted in **Fig. 1**). Such a loss of E-Cadherin expression has been described in many epithelial cancers, including those of the breast, colon, and prostate. However, it remained unresolved whether the loss of E-Cadherin expression is causally involved in tumor progression or whether it is simply a consequence of tumor cell dedifferentiation.

One way to determine the role of E-Cadherin

during tumor development is to maintain its expression in ß cells throughout the different stages of tumor development in RIP1Tag2 transgenic mice. To this end we have produced transgenic mouse lines that express E-Cadherin under the control of the insulin promoter (RIP1E-Cad). Intercrosses of these mice with RIP1Tag2 transgenic mice resulted in a block of the transition from adenoma to carcinoma, also manifested in a slight reduction in tumor volume (**Fig. 5**). No effect on tumor cell proliferation or apoptosis could be detected.

E-Cadherin expression is also very low in ß tumor cell lines that have been derived from the ß cell tumors of RIP1Tag2 transgenic mice. We generated ß tumor cell lines that express E-Cadherin under the control of constitutive or inducible promoters. Forced expression of E-Cadherin did not affect tumor cell proliferation or apoptosis, but induced contact inhibition upon confluent growth. Furthermore, tumorigenicity in nude mice was only slightly reduced by the expression of E-Cadherin, thus recapitulating our findings in transgenic mice *in vivo*.

Our results indicate that the loss of E-Cadherin is a rate limiting step in the transition from benign adenoma to malignant carcinoma. In order to further validate our findings we are currently crossing the RIP1Tag2 mice with transgenic mice that express a dominant-negative form of E-Cadherin in pancre-atic ß cells (Dahl *et al.*, 1996).

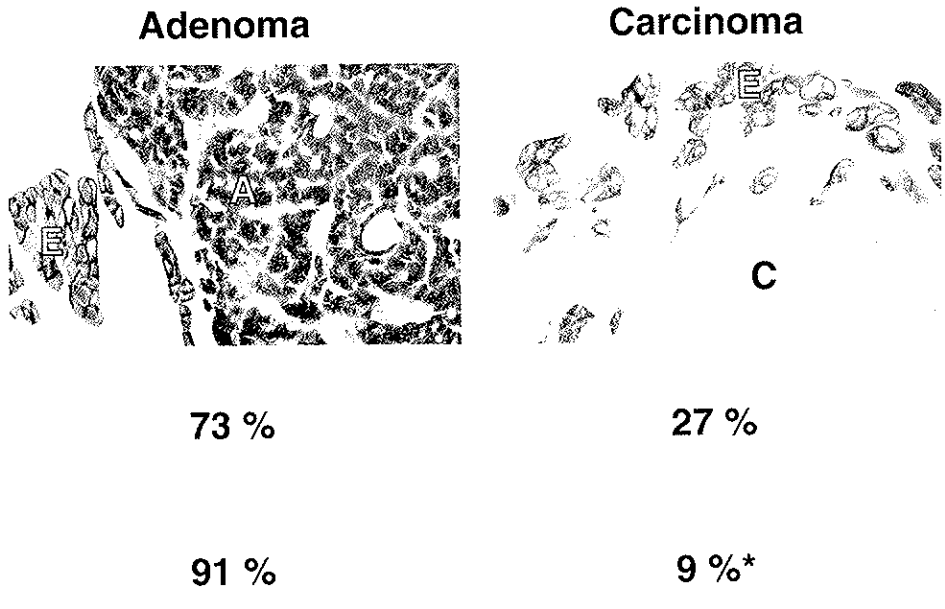

Fig. 5.: *Forced expression of E-Cadherin blocks the transition from adenoma to carcinoma in vivo.*
Tumors from RIP1Tag2 x RIP1E-Cadherin doubletransgenic mice and from their RIP1Tag2 singletransgenic littermates were grouped into adenoma or carcinoma by histopathological criteria and by immunohistochemical staining for different markers of epithelial-mesenchymal transition (staining for E-Cadherin is shown). The incidence of adenoma versus carcinoma of single transgenic RIP1Tag2 or doubletransgenic RIP1Tag2 x RIP1E-Cadherin is given in percentages of all tumors analyzed. *Note that the few carcinomas found in doubletransgenic mice also lost transgenic E-Cadherin expression. A = adenoma; C = carcinoma; E = exocrine pancreas.

Switch in N-CAM isoforms during ß cell tumorigenesis

Our studies have also revealed that the expression of neuronal cell adhesion molecule (N-CAM) changes in the later stages of ß cell tumor development. The 120 kd GPI-linked isoform on the surface of normal ß cells is almost completely replaced by the highly polysialylated 140 and 180 kd transmembrane isoforms in ß tumor cells. A similar change in N-CAM expression has been described in the development of Wilms' tumor (Roth *et al.*, 1988).

We wish to define the functional role of N-CAM in ß cell tumor development. N-CAM deficient mice are currently being employed to produce RIP1Tag2 transgenic mice that lack any functional N-CAM allele, and we are examining these mice for phenotypic changes in ß cell tumor development. We also generated transgenic mouse lines that overexpress the 120 kd isoform of N-CAM in the ß cells of the islets of Langerhans (RIP1N-CAM120). We are currently producing double transgenic RIP1Tag2 x RIP1N-CAM120 mice to determine potential changes in their tumor phenotype.

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Virus entry mechanisms and gene delivery

Group leader	Matt COTTEN
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Staff scientist	Susanna CHIOCCA (from Feb. 96)
Postdoc	Patrick HARRISON (from March 96)
PhD student	Adam BAKER
PhD student	Heike LEHRMANN
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Technical supervisor	Mediyha SALTIK

Overview

In 1996, our studies on the inflammatory and apoptoptic responses of adenovirus entry and our characterization of the chicken adenovirus CELO led to the identification of GAM-1, a novel anti-apoptotic gene. GAM-1 probably fulfils the function of the E1B-19K gene in slowing the host cell response to virus infection, blocking both apoptosis and interfering with NF-κB signalling (Chiocca *et al.*, 1997). Additional function screens have also identified a CELO gene (preliminarily, and unpoetically called Orf 8) that can activate the cellular transcription factor E2F, probably by interactions with Rb. Sequence homology led to the identification of a CELO gene encoding a functional deoxyUTPase. Further studies are in progress to identify the early gene functions of CELO. This information will be essential for constructing recombinant CELO vectors. We have also tested the upper size limit for the adenovirus-enhanced gene transfer systems using a series of bacterial artificial chromosomes (BACs). We obtain efficient delivery of 170 kb BACs, and this work has also led to the replacement of polylysine with polyethylenimine (PEI) for condensing and linking DNA to virus carriers.

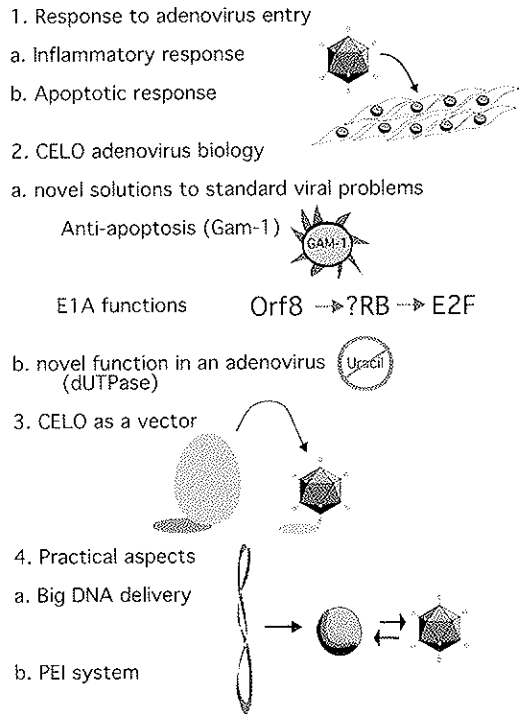

Figure 1

The identification and characterization of GAM-1, a novel anti-apoptotic protein encoded by the chicken adenovirus CELO

Susanna Chiocca

We recently reported the complete sequence of the avian adenovirus CELO (Chiocca *et al.*, 1996). A striking feature of this adenovirus genome is the lack of E1A and E1B regions identifiable by sequence homology. We presumed that like most small DNA viruses, CELO must possess genes such as those encoded by the E1A region which influence cellular proliferation and genes such as E1B 55K and E1B 19K which alter the cellular apoptotic response (reviewed in White, 1994; Shen and Shenk, 1995). Furthermore, CELO is capable of transforming cells and inducing tumors in newborn hamsters (Sarma *et al.*, 1965) demonstrating that the virus does possess transforming genes and could therefore possess functional homologs to the Mastadenovirus E1A and E1B regions. Lacking an obvious sequence homolog, we established a function assay to screen for genes in CELO that slow the apoptotic response.

Our method for identifying genes that block apoptosis is based on an established adenovirus-mediated transfection system in which adenovirus particles, linked to polylysine-condensed plasmid DNA, are used to transfer DNA into primary human fibroblasts (Wagner *et al.*, 1992). Transcriptionally silent, psoralen-inactivated virus particles (Cotten *et al.*, 1994) are used to avoid gene expression from the carrier adenovirus. We obtain high levels of transient gene expression with this system but in many cell types the expression levels decline rapidly over a 10 day period. We demonstrate here that this decline is slowed by the co-transfection of Bcl-2 or E1B-19K, two genes whose ability to block apoptosis is well-documented. We used this transfection system to identify a gene in CELO that mimics the function of E1B-19K and Bcl-2.

The gene encodes a novel 30 kd nuclear protein that we have named GAM-1 (for Gallus Anti Morte) which functions comparably to Bcl-2 and adenovirus E1B 19K in blocking apoptosis (Chiocca *et al.*, 1997). However, GAM-1 has no sequence homology to Bcl-2, E1B 19K or any other known anti-apoptotic proteins and thus defines a novel anti-apoptotic function. The nuclear localization of GAM-1 suggested that the protein might influence apoptosis by altering transcription and indeed, we find that certain promoters are potently activated by the expression of GAM-1. Mutants in the GAM-1 leucine-zipper motif impair both the survival function and the transcriptional activation of the protein, suggesting that GAM-1 blocks apoptosis by directly up-regulating the expression of protective genes. We are now analyzing how GAM-1 activates transcription and determining if the cellular target genes of GAM-1 transcriptional activation are known or novel anti-apoptotic genes.

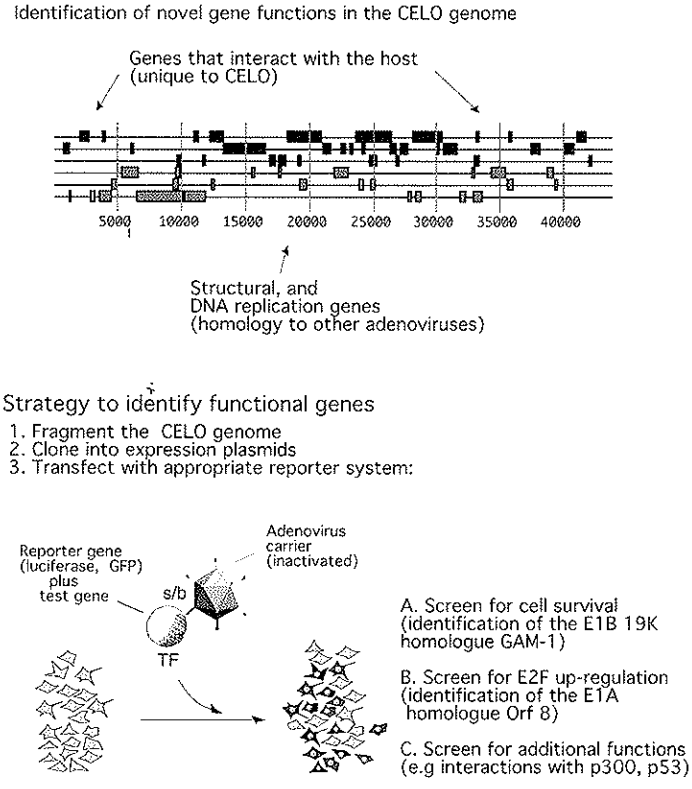

Figure 2

A novel method of activating E2F: Identification of a functional homolog of the mastadenovirus E1A gene in the CELO adenovirus

Heike Lehrmann

Most human adenoviruses are able to transform cells (Trentin *et al.*, 1962; Pope and Rowe, 1964; Sultanian and Freeman, 1966). Although the avian adenovirus CELO (Sarma *et al.*, 1965) was also found to be transforming and oncogenic in certain hosts, the sequencing of this virus (Chiocca *et al.*, 1996) revealed that CELO lacks genes comparable to E1 region, responsible for the transforming activity of other adenoviruses (Graham *et al.*, 1975; Van der Eb *et al.*, 1980; Ruley, 1983).

The molecular mechanism of transformation by human adenoviruses is also employed by several other DNA tumor viruses like SV40 and human papillomavirus. A common target for these viruses is the Rb/E2F complex (Whyte *et al.*, 1988; Chellappan *et al.*, 1992, reviewed in Moran, 1994). The release of E2F from Rb induces entry into S phase and therefore cell proliferation. Release of E2F is usually controlled by phosphorylation of Rb by cyclin dependent kinases. Upon adenovirus infection, however, E1A binds Rb and triggers the release of E2F overriding the cellular control mechanism. The cell is now trapped in a proliferating state.

We considered that CELO virus interacts with the host cell cycle in a similar manner but perhaps using gene products that, because of viral evolution, were not recognizable by normal homology searches. We set up a functional assay then to identify CELO genes that activate an E2F-dependent promoter. To do this, we constructed a reporter plasmid which carried the firefly luciferase gene driven by an E2F-inducible Ad5 E2-promoter. Transfection of this reporter construct together with CELO virus clones into chicken cells or human cells resulted in activation of the luciferase reporter by one region of the CELO genome. Further subcloning and deletion experiments identified a reading frame at the right end of the viral genome (Orf8) which functioned like Ad5 E1a in activating E2F. CELO Orf8 possesses a peptide motif, similar to the LxCxE motif found in all DNA tumor virus proteins that bind Rb. Immunoprecipitation studies demonstrate that Orf8 indeed interacts with Rb. Further studies, including mutation of the proposed Orf8 Rb-binding site and deletion of the Orf8 coding region from the viral genome, will allow us to understand the importance of Orf8 during viral infection and its interaction with the host cell cycle.

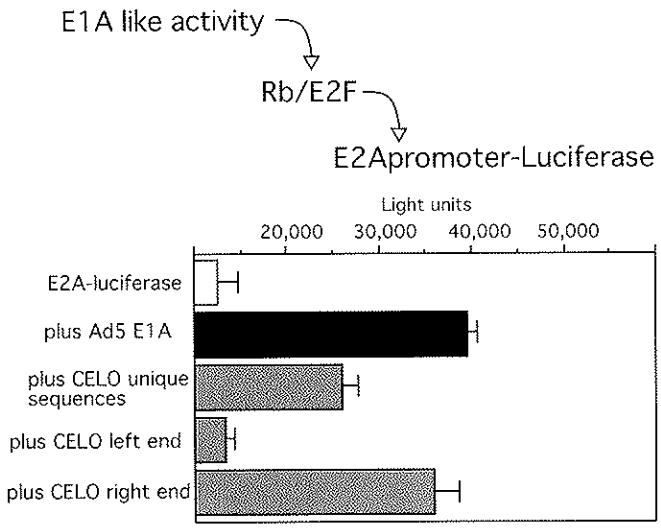

Figure 3

CELO virus encodes a functional dUTPase

Patrick Harrison

One of the CELO virus open reading frames has considerable sequence similarity to the deoxyuracil triphosphate hydrolase (dUTPase) from a number of organisms. dUTPase catalyzes the hydrolysis of dUTP to dUMP and pyrophosphate, reducing the intracellular concentrations of dUTP. The reaction provides dUMP for *de novo* dTTP synthesis, maintains the intracellular level of dTTP:dUTP at a high level, and is thought to, thereby, prevent the incorporation of uracil into DNA. We devised a genetic

complementation assay to determine if the putative CELO dUTPase ORF encodes a functional enzyme. The assay was based upon the observation that phagemids produced from dUTPase (dut⁻)/uracil N-glycosylase (ung⁻) deficient *E. coli* will have DNA with uracil (U-DNA) in place of thymine (T-DNA) (Künkel, 1985). Phagemids with U-DNA cannot grow in wild type (ung⁺) *E. coli* strains, but phagemids with T-DNA can. Using this assay, we have shown that CELO virus encoded dUTPase is functional.

CELO is the only adenovirus reported to date that encodes a dUTPase activity. However, the enzyme is encoded by herpes-, pox- and some lentiviruses. In all cases, the enzyme is dispensable for growth in dividing cells, but appears necessary for viral growth in non-dividing cells (Steagall *et al.*, 1995). To determine the role of CELO virus encoded dUTPase, we have constructed a recombinant plasmid clone of the virus that lacks the dUTPase ORF. This clone will be used to produce the mutant virus and assess its growth properties in chicken cells.

A transfection system that uses psoralen-inactivated adenovirus to deliver Bacterial Artificial Chromosomes into mammalian cells

Adam Baker

Molecular biology has many applications where the introduction of large (>100 kb) DNA molecules is required. The current methods of large DNA transfection are inefficient with regard to time and material. We reasoned that two limits to improving transfection methods with these large DNA molecules were the difficulty of preparing workable quantities of clean DNA, and the lack of rapid assays to determine transfection success. Addressing the first limit, we have used Bacterial Artificial Chromosomes (BACs) based on the *Escherichia coli* F factor plasmid system (Kim *et al.*, 1992). BACs are simple to manipulate and purify in mg quantities. In addition, because the plasmids are kept at 1-2 copies per cell, the problems of rearrangement observed with YACs are eliminated. To address the second limit, we generated two series of BAC vectors bearing the easily assayed marker genes luciferase and green fluorescent protein (GFP).

We have used these reagents to develop methods of delivering BAC DNA molecules of up to 170 kb into mammalian cells with transfection efficiency comparable to 5kb DNA. Psoralen-inactivated adenovirus is used as a carrier, eliminating the problems associated with viral gene expression with the delivered DNA linked to the carrier virus with a condensing polycation. Long range pulsed field electrophoresis demonstrated that the BACs are delivered intact into the cells where they remain in an episomal state. Expression studies also show that molecules larger than 100kb produce exceedingly stable expression in non-dividing cells. At present BAC expression is lost over time in dividing cultures probably due to its episomal state. We have developed a new series of BAC vectors that contain a mammalian origin of replication and selection marker which should maintain the BAC's in dividing cells.

Initial experiments demonstrated the utility of the adenovirus/polylysine system. However, interactions between the polylysine and the large BAC DNA molecule were plagued with solubility problems. We replaced the polylysine with the synthetic polycation polyethylenimine (PEI). The BAC/PEI/adenovirus method gives approximately 10-fold greater levels of transient gene delivery than the polylysine/adenovirus system, and 2-3 log higher levels than cationic lipid systems such as lipofectamine or transfectam.

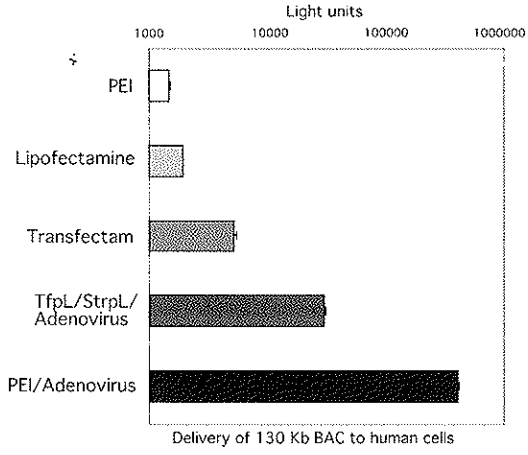

Figure 4

Polyethylenimine (PEI) is a simple, inexpensive and effective reagent for condensing and linking plasmid DNA to adenovirus for gene delivery

Matt Cotten, Mediyha Saltik, and Adam Baker

Nearly all successful DNA delivery systems include some form of polycation. The charged, extended DNA molecule is highly susceptible to mechanical shearing and to enzymatic cleavage. Cationic agents are used to neutralize the negative charge of the DNA and condense its structure. An effective method of gene delivery has been to link plasmid DNA to inactivated adenovirus particles (Wagner *et al.*, 1992; Cotten *et al.*, 1992, 1994). Our work developing large DNA delivery (see above) led to the use of polyethylenimine (Boussif *et al.*, 1995) as a reagent for condensing and binding DNA, ionically, to the carrier adenovirus capsid. We have further developed this idea (Cotten *et al.*, 1997a, 1997b) and we find that within certain limits it can be as useful for gene delivery as the previously described biotin-adenovirus/streptavidin-polylysine conjugate system (Wagner *et al.*, 1992). The advantages of the PEI system include its simplicity and the ready availability of the reagents, requiring only adenovirus (or psoralen-inactivated adenovirus) and commercially available PEI.

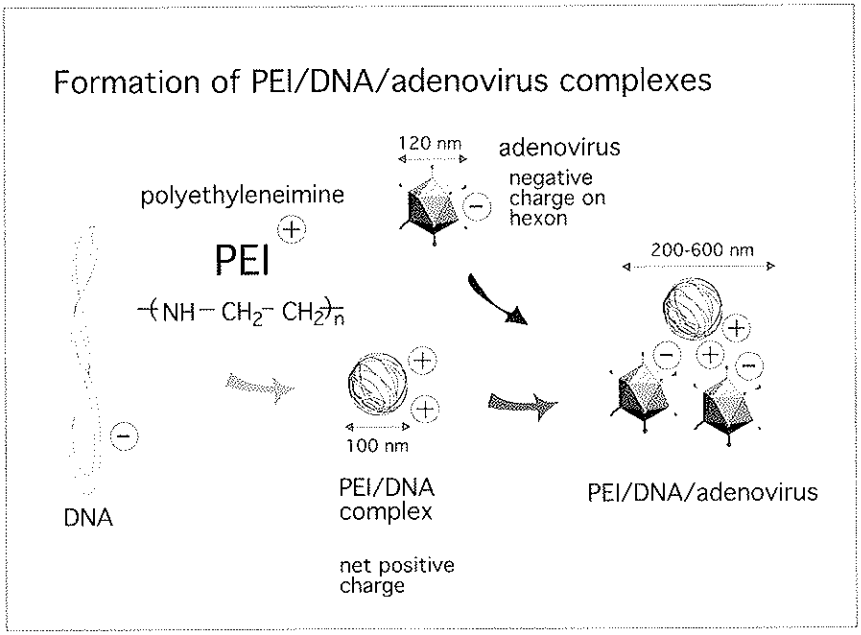

Figure 5

CELO as a vector

Matt Cotten

The avian adenovirus CELO is being developed as a vector, both for use in humans and for veterinary vaccine applications. The virus is naturally defective in human cells, and shows no significant binding to human cells, allowing cell targeting modifications. The CELO virion is remarkably stable and the virus has 8 kb greater DNA packaging capacity than the human adenoviruses. The entire 44 kb CELO genome has been cloned as a bacterial plasmid that when transfected can generate infectious virus. This cloned genome is now being used for deletion analysis in efforts to understand the biology of the virus as well as to identify useful sites for the insertion of therapeutic genes.

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Epithelial polarity and cancer

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Introduction

Cell polarity is the ultimate reflection of complex mechanisms that establish and maintain functionally specialized domains in the plasma membrane and the cytoplasm. To understand how the complex three-dimensional organization of polarized epithelial cells is established and maintained is the central question in our laboratory.

The cells in a simple epithelium are linked through junctional complexes to form a permeability barrier. These cellular sheets perform a variety of vectorial functions in absorption and secretion of ions and solutes between different biological compartments. These cellular transport functions are regulated by structurally and functionally distinct plasma membrane domains, commonly referred to as apical and basolateral, which face opposite biological compartments. These distributions appear to be regulated in two ways: first, by targeted delivery of transport vesicles from the trans Golgi network (TGN) to specific plasma membrane domains and, second, by protein sorting at the cell surface membranes and in the endocytic apparatus.

Protein sorting and transport from the TGN to the basolateral plasma membrane seems in many aspects very similar to the pathway from the Golgi to the plasma membrane bud in yeast. In previous work we could identify the small GTPase rab8, as a regulator of basolateral transport (Huber *et al.*,1993b). Rab8 is closely related to the *Saccharomyces cerevisiae* Sec4 protein and to the *Schizosaccharomyces pombe* Ypt2 protein, both regulating protein transport from the Golgi to the cell surface. Interestingly, polarized hippocampal neurons use this conserved protein sorting machinery for transport from the Golgi to the somato-dendritic surface (Huber *et al.*,1993a; Huber *et al.*,1995). Recently it was also shown that the basolateral/dendritic route requires NSF/SNAP/SNAREs (Ikonen *et al.*,1995), whereas the apical transport and sorting pathway uses different principles, lipid rafts and most likely also annexins (Fiedler *et al.*,1995).

Much less is known about the molecular machinery conferring polarity along the endocytic pathway. Since epithelial cells possess distinct apical and basolateral plasma membrane domains, separate endocytic pathways exist from each cell surface (Parton, 1991). Internalized solutes or membrane proteins are either recycled, transcytosed to the opposite domain, or delivered to lysosomes. The small GTPase rab5, regulating early steps in endocytosis, is a common component of both, the apical and basolateral endocytic machinery in polarized epithelial cells (Bucci *et al.*,1994). Recent evidence supported an important role for the cytoskeleton and motor proteins in both targeting and membrane sorting of proteins in endosomes of polarized epithelial cells (Huber *et al.*,1997), but up to now no proteins specific for either apical or basolateral endosomes have been identified.

Whereas normal cells form highly polarized monolayers, oncogene transformed cells display an unpolarized phenotype, detaching from the substratum and developing multilayers. If the cells are derived from malignant transformations they break through the basement membrane, invade the underlying mesenchyme and metastasize to different sites of the body. Of the many components involved in these processes, only a few molecules have attracted attention so far. These include cell adhesion molecules which change expression during invasion and metastasis of tumor cells, such as E-cadherin which was shown to control invasiveness of transformed epithelial cells (Behrens *et al.*,1992). A cell surface glycoprotein, CD44, was found to confer metastatic potential to carcinoma cells (Günthert *et al.*,1991), probably by regulating tumor cell migration. However, up to now a molecular definition of the general epithelial phenotype was lacking and pathol-

ogists had to use plain morphological criteria as the major way of classifying carcinomas. The situation is now changing for several reasons: first, tissue specific molecules are being defined, and second, the molecular understanding of epithelial organization is advancing.

Strategy

In our first year at the I.M.P. we set up two independent, complementary screens to identify genes and gene products involved in the generation and maintenance of epithelial polarity (**Fig. 1**). We are using a well established mouse mammary epithelial cell system (pathway A and B in **Fig. 1**), expressing an estrogen-inducible c-JunER fusion protein (Fialka *et al.*,1996). The physiological relevance of this cell system is accentuated by the ability of the parental, un-induced mammary epithelial cells to form three-dimensional and branching tubular structures in collagen-I gels, which closely resemble the ducts of mammary glands in vivo (Fialka *et al.*,1996; Fialka *et al.*,1997). As early as 20 hours after induction with hormone the lumina are disrupted, leading to irregular cord-like structures without any lumen. In general, the phenotype obtained with c-JunER cells is milder (Fialka *et al.*,1996) and, therefore, also better controllable when compared to c-FosER cells (Reichmann *et al.*,1992). Upon withdrawal of the hormone, epithelial polarity and junctional integrity can be fully restored within a few days on filter cultures as well as in collagen-I gels. All other epithelial systems, transformed with oncogenes, showed only a partial restoration or even an irreversible loss of the polarized epithelial phenotype (Schoenenberger *et al.*,1991; Reichmann *et al.*,1992; Huber *et al.*,1994a). It is also important to emphasize that

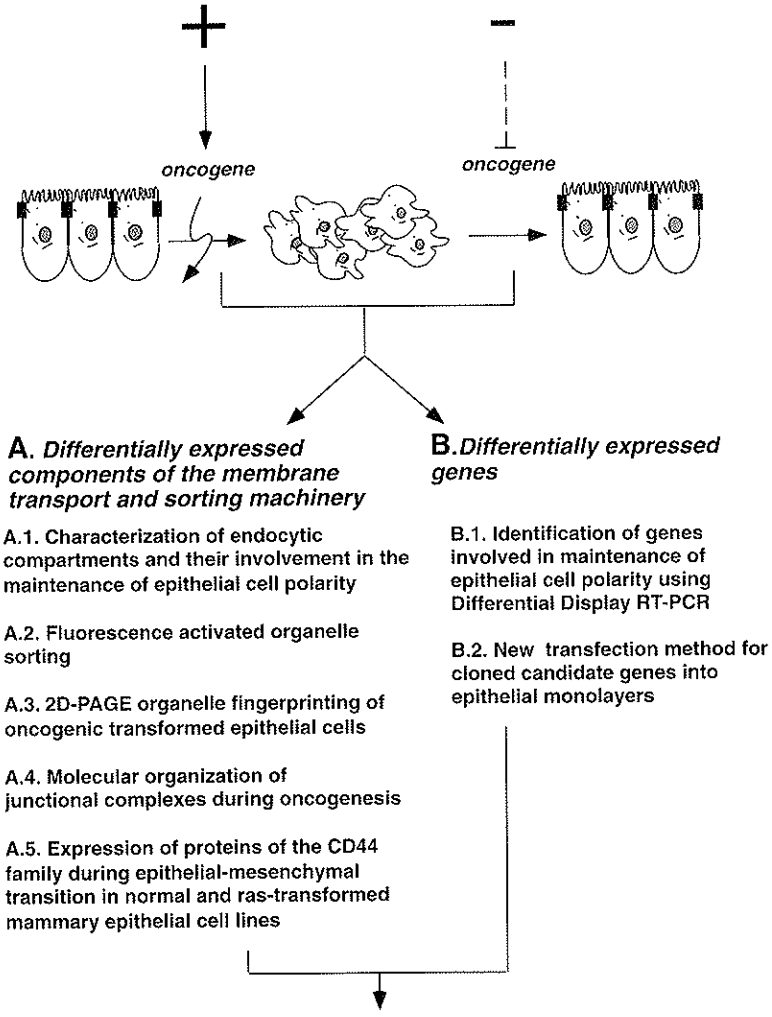

Fig. 1

hormonally activated c-JunER mimics the activation of endogenous c-Jun by cellular signaling pathways (Fialka *et al.*,1996).

One major strategy we follow is the molecular characterization of protein traffic in c-JunER cells (pathway A in **Fig. 1**). We postulate that intracellular protein transport and sorting is an early target of transformation. Therefore we aim to identify differentially expressed proteins of the intracellular protein transport and sorting machinery in purified subcellular organelles derived from normal versus oncogenic transformed epithelial cells (A.1.-3. and **Fig. 1**). We are also interested in the molecular organization of polarized microdomains in the plasma membrane. In a collaboration with Daniel Goodenough (Harvard Medical School, Massachusetts) we are studying the role and molecular interactions of ZO-1, a member of the membrane-associated guanylate kinase protein family (MAGUK), during the assembly and disassembly of tight junctions (A.4. and **Fig. 1**). In another collaboration with Hartmut Beug (I.M.P.) and Ursula Guenthert (Basel Institute for Immunology, Switzerland) we are investigating the altered expression and redistribution of CD44 and CD44 variants (A.5. and **Fig. 1**).

In the second screen (pathway B in **Fig. 1**) of the laboratory we are applying PCR-based differential display (DD) to enrich for genes that are altered in polarized versus unpolarized cells from the same cell type (B.1. and **Fig. 1**). Differential Display RT-PCR screening is complementing the protein-biochemical approach and has independently lead to the identification of several DD RT-PCR products which are known to be involved in regulation of organelle movement, gene expression, and signal transduction, respectively, during oncogenic transformation.

We are presently working on the functional characterization of candidates we identified in both screens during this first year at the I.M.P.

A. Differentially expressed components of the membrane transport and sorting machinery

A.1. Characterization of endocytic compartments and their involvement in the maintenance of epithelial cell polarity

Irene Fialka, Christian Pasquali, Michaela Haberfellner, Lukas A. Huber
in collaboration with Heinz Schwarz (MPI, Tübingen, Germany)

In our previous work we have studied the expression of small GTP-binding proteins in ras-transformed mammary epithelial cells. Members of this superfamily are known to regulate intracellular transport at all different levels. We could show that a single small GTP-binding protein, out of approximately 20 others detected in these cells, was significantly down-regulated when compared to un-transformed control cells (Huber *et al.*,1994a). By comparative mapping on high resolution two dimensional gels followed by GTP-overlay (Huber *et al.*,1994b) we could demonstrate that this small GTPase was identical to a component of the apical transport and sorting machinery from polarized MDCK cells (Huber *et al.*,1993b). In another study we investigated the effects of oncogenic transformation on the molecular organization of endocytic organelles by 2-D gel analysis. Computer-assisted mapping of polypeptide profiles revealed the differential expression of organelle-associated proteins in K-*ras* transformed cells versus control cells (Huber *et al.*,1996). These results encouraged us to initiate a systematic study of the endocytic pathway in polarized versus unpolarized, oncogenic transformed epithelial cells with the goal to identify molecules involved in polarized transport and sorting of membranes.

Using c-JunER cells in their polarized state, we have established techniques to isolate and enrich for different subcellular compartments along the endocytic pathway: early endosomes (EE), apical recycling compartment (ARC), late endosomes (LE) and lysosomes. Apical and basolateral endocytic pathways meet in a common late endosome and also in the ARC which is located above the nucleus near the apical plasma membrane in polarized epithelial cells. The ARC is accessible to fluid phase markers from both plasma membrane domains and is the compartment through which the transferrin-receptor (Tfn-R) recycles to the basolateral plasma membrane.

We were able to separate EE, LE and supposedly ARC on continuous sucrose gradients. The distribution of specific marker proteins, such as Rab4 and Rab5 (EE), Rab7 (LE), Rab11 (ARC) and Tfn-R, was corroborated by the activity of horseradish peroxidase (HRP) internalized as a fluid phase marker from both the apical and the basolateral medium (see **Fig. 2**). By analyzing marker proteins and enzymatic activities specific for other compartments, we found that most of the endoplasmic reticulum (ER) was removed. Still, due to similar physical properties, the fractions of the EE contained considerable amounts of plasma membrane and TGN (Trans Golgi Network). The plasma membrane could be partially shifted to the denser fractions as well by biotinylation

and conjugation with streptavidin-colloidal gold. Thus, we could get fractions highly enriched in all the different endosomal compartments.

The different fractions collected from sucrose gradients were used to compare the overall protein composition of different compartments on high resolution two dimensional (2D) gels (see also project A.3.). As expected, different fractions exhibit significantly different protein patterns.

Having set up these techniques we can now continue to characterize the difference between EE, ARC, LE derived from polarized versus unpolarized, oncogenic transformed cells and apical versus basolateral EE (see also project A.2.) in order to identify candidate proteins that may be involved in the regulation of polarized transport and sorting events. Receptor-mediated endocytosis of transferrin coupled to HRP (Tfn-HRP) confirmed that in polarized epithelial cells the activity can be detected in the EE and ARC only if internalized from the basolateral medium. On the other hand, estrogen-treated, unpolarized cells internalized Tfn-HRP apically as effectively as from the basolateral medium, demonstrating that there are indeed major rearrangements in unpolarized versus polarized cells. Preliminary experiments comparing the protein pattern of gradient fractions containing the different endocytic compartments on 2D-gels showed that there are not too many differences between polarized and unpolarized cells. Thus, we are confident that the few proteins which are repeatedly found to be either down- or upregulated by JunER-activation will be candidates worth investigating.

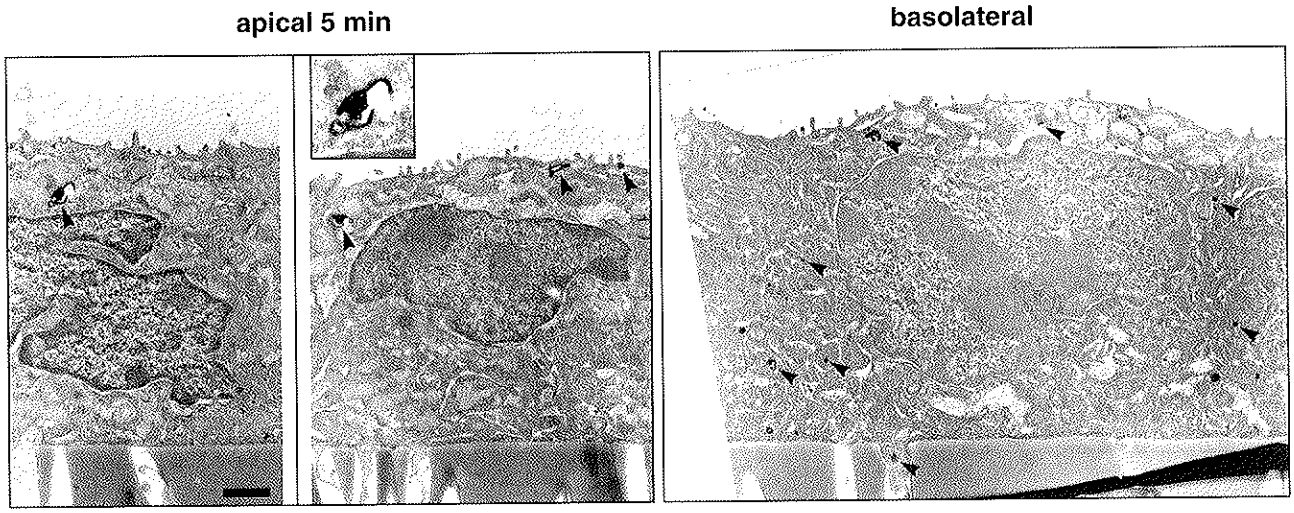

Fig. 2: *Labeling of apical and basolateral EE by fluid phase HRP.*
Polarized mammary epithelial cells were incubated 5 minutes at 37°C with either the apical (A) or the basolateral medium (B) containing 5mg/ml HRP, washed on ice and fixed. The samples were then stained with DAB and processed for EM. Note that apically internalized HRP locates solely in the apical third of the cell whereas basolaterally loaded tubulo-vesicular structures underly both the basolateral and apical plasma membrane.

A.2. Fluorescence activated organelle sorting (FAOS)

Michaela Haberfellner, Irene Fialka, Lukas A. Huber, in collaboration with Peter Steinlein (I.M.P.) and Günther Böck (Institute for General and Experimental Pathology, University of Innsbruck)

Subcellular fractionation and purification of organelles had always been a bottle neck in cell biology. Since most fractionation protocols took advantage of physical properties of intracellular membranes, e.g. their density, it was very difficult to separate organelles of similar density from each other (see project A.1.). We adapted flow cytometry to sort and analyze intracellular organelles after labeling with fluorescent dyes and combined conventional subcellular fractionation techniques with high speed organelle sorting in a flow cytometer (Böck *et al.*,1997). This new approach allowing for purification based on biological properties we call FAOS (fluorescent activated organelle sorting).

MDCK cells were grown on permeable filter supports and the fluorescent membrane dye TMA-DPH was internalized from the apical as well as from the basolateral membrane side to label the two distinct EE compartments. TMA-DPH intercalates into the outer leaflet of the plasma membrane and can be excited by UV light only if incorporated in the inner leaflet of an intact endosomal organelle after

internalization, which makes it suitable for FAOS. Cells were then homogenized and fractionated into a nuclear pellet and a post nuclear supernatant (PNS). The PNS fraction was loaded on top of a continuous sucrose gradient (10-40%) and the membrane fractions enriched in early endosomes collected after equilibrium centrifugation. Western blot analysis of the gradient fractions confirmed enrichment of the early endosomal markers (TfnR and the small GTPases rab 4 and 5) by 10-15 fold referring to the PNS. These enriched gradient fractions served as the starting fractions for FAOS that lead to highly purified apical and basolateral endosomal fractions. 2D-gel analysis proved that the apical and basolateral early endosomes differed significantly in their protein composition. In the future FAOS offers a potent tool for preparative sorting of intracellular organelles labeled with different fluorescent markers.

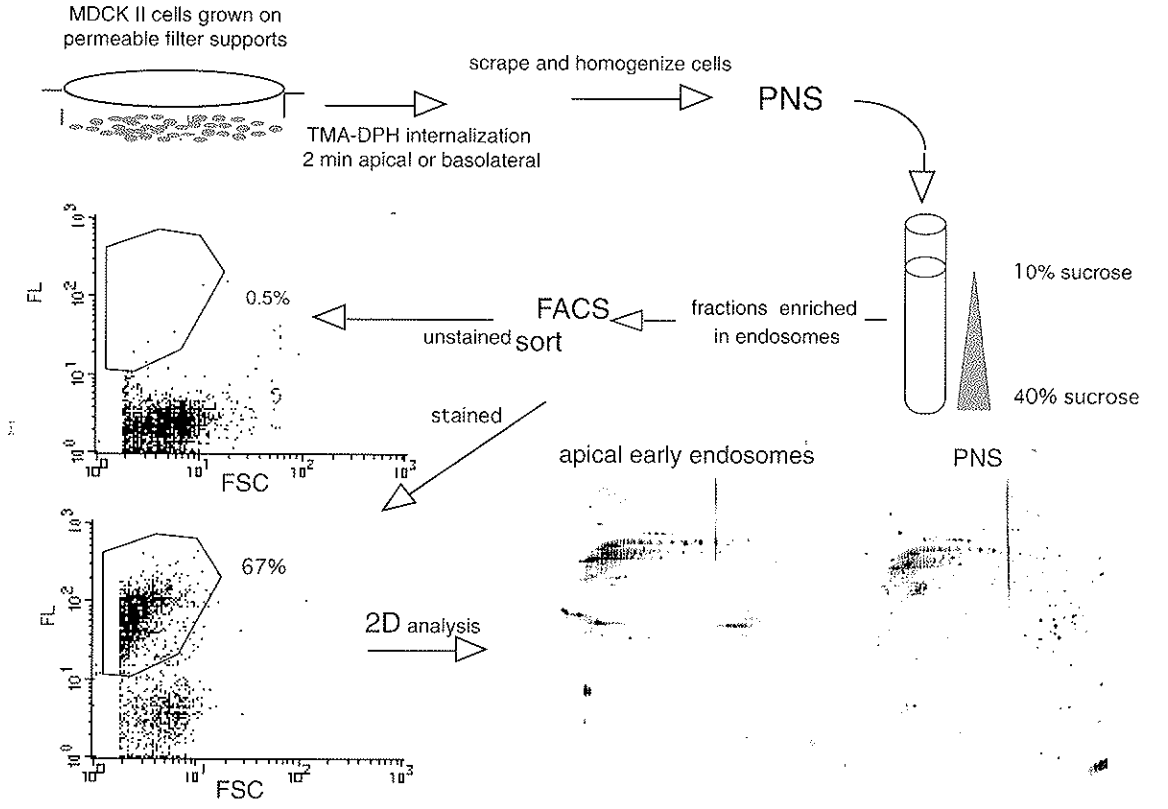

Fig. 3: *The FAOS method for organelle purification.*
Polarized epithelial cells are grown on permeable filter supports and apical or basolateral endocytic organelles are labeled with TMA-DPH fluorescent dye. Cells are scraped, homogenized and the PNS loaded on top of a continous sucrose gradient. Light membrane fractions enriched in endosomes serve as starting material for FAOS sorting. 2D-gel analysis of FAOS purified endosomal fractions shows the enrichment over the starting fraction (PNS)

A.3. 2D-PAGE organelle fingerprinting of oncogenic transformed epithelial cells

Christian Pasquali, Irene Fialka, Michaela Haberfellner, Lukas A. Huber

Electrophoretic techniques have provided an indispensable set of tools for cancer research, in particular, two dimensional (2-D) gel electrophoresis in combination with satellite technology (microsequencing, mass spectrometry, blotting and overlay techniques etc.). These have lead to the identification, cDNA cloning and analysis of proteins expressed in normal and cancer cells. In parallel, computer program systems have been designed that enable rigorous analysis of protein expression on 2-D gels. Today, many 2-D gel protein databases are linked to each other as well as to specialized databanks on genome- and proteome-mapping. Our goal is to identify differentially expressed proteins of the intracellular protein transport and sorting machinery in purified subcellular organelles derived from normal versus oncogenic transformed epithelial cells (see project A1). For this we are using 2-D gel technology with immobilized pH gradients (IPG) during the first dimension. This allows higher loading capacity, higher resolution and reproducibility than isoelectric focusing (IEF) with carrier ampholytes. The material we

analyze are highly enriched organelles isolated by sucrose gradients and FAOS (see also projects A1 and A2).

Extractions with detergents and/or carbonate result in enriched fractions of integral membrane proteins. However, due to chemical and physical limitations, the separation of integral and peripheral membrane proteins by 2D-PAGE, in sufficient amounts for microsequencing, was a difficult task. Despite using state of the art techniques, the large amount of lipids lead to low solubility, formation of aggregates and severe loss of membrane proteins during isoelectric focusing. We have established a protocol using a combination of CHAPS, chaotropic- (thiourea, urea) and reducing-agents (DTT) in order to maintain solubility and therefore accomplish protein separation at the preparative (up to mg amounts of loaded protein) range. We applied the sample during the rehydration of strips (in-gel sample loading), instead of the conventional sample-cup application procedure. Hence the separation of low copy number proteins in amounts sufficient for post-separation analysis is a difficult task, the combination of these procedures enabled us to investigate potential candidates differentially expressed in subcellular organelles from transformed or parental cells (see project A1). Maps of silverstained gels are matched against 2-D gel databases (e.g., SWISS-2D PAGE, http://expasy.hcuge.ch/) and then, for further characterization spots from preparative gels (Coomassie staining) are excised and directly microsequenced. We present here a comparison of the conventional sample loading and preparation technique of total cellular membrane proteins with our in-gel loading procedure (**Fig. 3**).

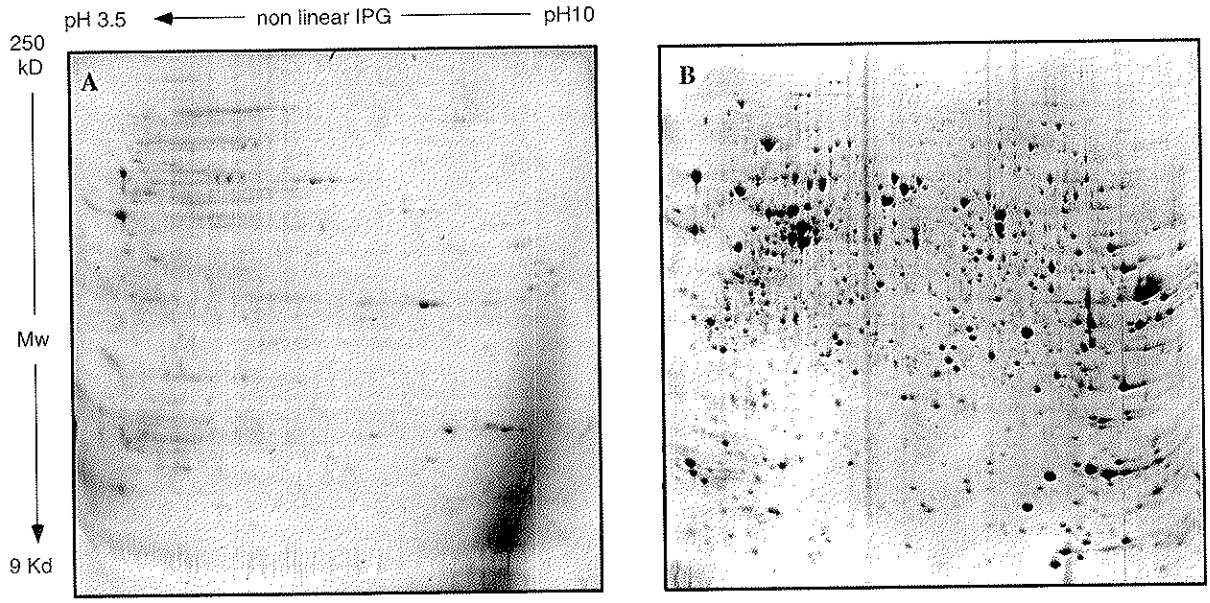

Fig. 4:
A. Coomassie stained 2-D gel of total membrane proteins from EpH4 cells. Sample was dissolved in normal lysis buffer and loaded. Membrane proteins aggregate and do not enter the gel, almost no spots are detectable.
B. Coomassie stained 2-D gel of the identical sample as in A, but the membrane pellet was solubilized in a CHAPS / thiourea / DTT buffer and loaded during reswelling. Completely solubilized membrane proteins can enter the gel and more than thousand spots could be detected.

A.4. Molecular organization of junctional complexes during oncogenesis

Thomas Bader, Nicolas Budin in collaboration with Daniel Goodenough
(Harvard Medical School, Massachusetts)

Tight junctions are present around the lateral circumference of individual epithelial or endothelial cells, forming seals between their apical and basolateral compartments.

Zonula Occludens 1 (ZO-1) is a peripheral membrane protein associated with the plasma membrane at tight junctions. ZO-1 is a member of the membrane associated guanylate kinase protein family (MAGUK). MAGUK proteins have been purified as components of important cellular structures such as tight junctions in epithelia and synaptic densities in neurons. MAGUK genes have also been identified as genes involved in cell signaling: ZO-1 is homologous to the Drosophila disc-large tumor suppressor protein (DLG) of septate junctions. Furthermore, ZO-1 binds to β-catenin/ Armadillo, a signal transduction protein implicated in

the regulation of cell-fate choices in *Drosophila* and vertebrates.

Upon JunER activation cells lose their polarity, and tight contacts between cells are loosened, leading to irregular multilayers. However, the main advantage of our cell system is that these changes are completely reversible upon hormone withdrawal, allowing the study of both assembly and disassembly of tight junctions.

Through three dimensional reconstructions of ZO-1 staining by confocal laser scanning microscopy, we could show that ZO-1 redistributes from its normal localization in tight junctions to the cytoplasm of the cells upon activation of the fusion protein. The total expression of ZO-1 protein is not modified upon activation of c-JunER.

The strategy we want to use is to compare molecular interactions of ZO-1 with other proteins, in induced vs. un-induced cells. The experimental procedure is to isolate proteins associated with ZO-1 by co-immunoprecipitation in induced and un-induced cells. With the help of several different antibodies, multiprotein complexes could already be precipitated. We were able to identify at least one protein other than ZO-1 by Western analysis, but several more are of unknown identity so far. The composition of these complexes can be compared by high resolution 2D-gel electrophoresis. The proteins of interest will then be identified by microsequencing or comparative mapping with 2D-gel databases (see also project A.3.). The next step would be cloning these proteins and reexpressing them (overexpression of wild type or mutated proteins) to study their role on the targeting of ZO-1 to the tight junctions in polarized cells or its intracellular localization in estrogen-treated cells.

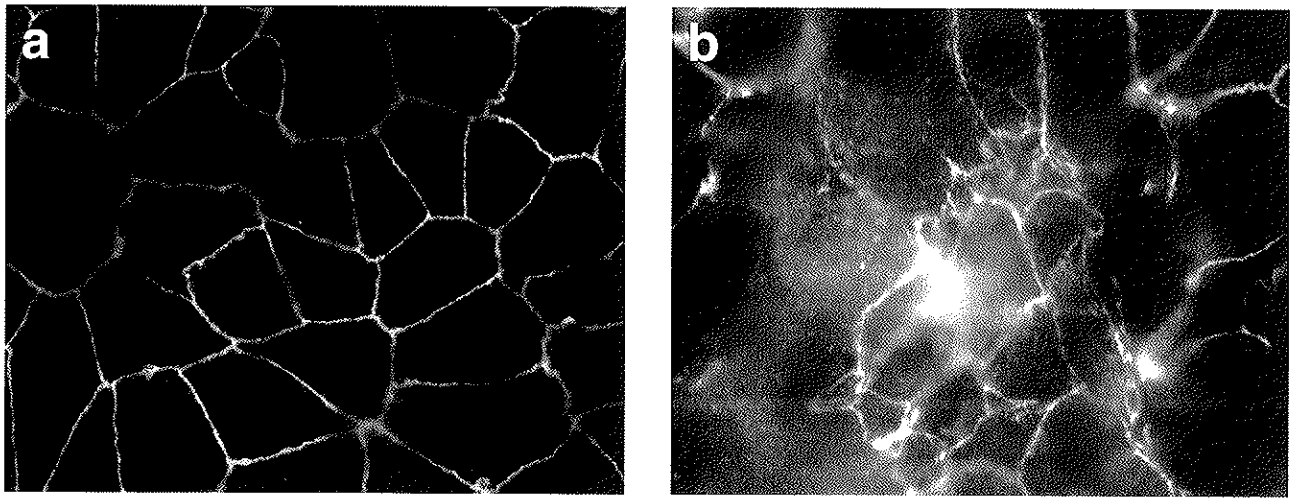

Fig. 5: ZO-1 staining of polarized c-JunER cells (A) and induced, unpolarized cells (B). ZO-1 redistributes upon induction.

A.5. Expression of proteins of the CD44 family during epithelial-mesenchymal transition in normal and ras-transformed mammary epithelial cell lines

Snezhana Oliferenko, Lukas A. Huber in collaboration with Ursula Guenthert (Basel Institute for Immunology), Heinz Schwarz (MPI, Tuebingen), Martin Oft and Hartmut Beug (I.M.P.)

Members of the CD44 family are known to be implicated in different biological processes like lymphocyte homing, leukocyte activation, development and tumor dissemination. One of the early steps of tumor progression derived from simple epithelia is the loss of polarity and/or transdifferentiation to mesenchymal-like cells, able to migrate and colonize remote tissues, thus giving rise to secondary tumors. These processes can be studied in vitro with cell systems, allowing epithelial-mesenchymal transition (Oft *et al.*,1996). During this work it was shown that expression of CD44 isoforms is highly elevated in a plastic-grown, ras-transformed mammary epithelial cell line, both at the mRNA and protein level. RT-PCR revealed strong up-regulation of the expression of all isoforms, including high-molecular weight mRNAs. Indirect immunofluorescence analysis (IF) with antibodies raised against 10th variant exon and the standard region of the protein showed a basolateral membrane localization of all isoforms of CD44 in parental cells. Significant re-distribution of the protein, involving patch-like apical expression, was observed upon ras-transformation. Immuno-EM on sections showed that in parental cells, CD44 10v-containing isoforms distribute along the basolateral membrane whereas in ras-transformed epithelial cells a

clear localization to microvilli was seen. In order to further investigate the nature of CD44 re-localization to the apical plasma membrane upon ras-transformation, we performed detergent extraction followed by Western-blotting or IF. While in parental cells CD44 was TX-100-insoluble (up to 1%), complete solubility could be achieved already in 0.1% Triton X-100 in ras-transformed cells. As a next step we analyzed mechanisms, responsible for polarized positioning of CD44 within the plasma membrane. Upon treatment of polarized cultures with the cholesterol-depleting drug β-methylcyclodextrin, CD44 redistributes to the apical side of the cells, suggesting that normally CD44 molecules locate in cholesterol-rich microdomains within the basolateral plasma membrane. The IF analysis of F-actin, using phalloidin-FITC, revealed a significant re-organization of the beta-actin cytoskeleton. Especially stress fibers were significantly diminished upon ras-transformation. To further analyze this re-distribution of CD44, upon ras-transformation or cholesterol depletion, we co-precipitated several proteins using CD44 antibodies. One of the potential candidates linking the cytoskeleton with these microdomains was annexin II, which was shown to be a part of a complex precipitated with anti-10v CD44 antibodies from normal cells. We are further analyzing these molecular interactions of CD44.

B. Differentially expressed genes

B.1. Identification of genes involved in maintenance of epithelial cell polarity using differential display RT-PCR

Ilja Vietor, Irene Fialka in collaboration with Gotthold Schaffner (I.M.P.), Brian Varnum (Amgen Center, Thousand Oaks Ca, USA) and Kurt Zatloukal (Pathology, University of Graz)

In order to identify genes that are responsible for establishment and maintenance of epithelial cell polarity we decided to analyze differences in gene expression between polarized and non-polarized epithelial cells. As a model system we use the c-JunER expressing mammary epithelial cells (Fialka *et al.*,1996).

As a screening approach we have chosen Differential Display based on PCR amplification of reverse transcribed cDNAs. This technique gives the advantage to analyze several samples at the same time, and therefore allows us to study changes in gene expression between a) polarized cells, b) cells losing polarity as a result of c-JunER activation (at different time points) and c) reverted cells (re-polarized following the removal of hormone from the culture medium).

In our experimental protocol we are using 216 combinations of anchored downstream primers ($dT_{11}VV$; V = A,C or G) with 5' arbitrary decamers. Since our attempt is to cover a spectrum of expressed mRNAs as wide as possible we are using low stringency PCR conditions which allow priming to a broad range of templates. [α-^{33}P]-dATP labeled RT-PCR products are resolved on nondenaturating PAGE, candidate cDNA bands re-amplified using the same combination of primers, cloned, sequenced and used as radioactively labeled probes for Northern blot confirmation of differential gene expression (see **Fig. 5**).

After analyzing 90 differentially expressed cDNA products, from one third of primer combinations studied up to now, we have obtained 69 sequences totally. We found 15 unique sequences, which were homologous to known genes in GenBank (using the search tool BLAST on www). Furthermore, Northern blot analysis showed that our ratio of truly positive, regulated genes to the total number of tested probes is approximately 1:4. This was in good agreement with the previously published data. Experimental approaches for further analysis of candidate genes were selected to supplement the primary DD data and are therefore neither based on the same technical principles nor on the same starting material (RNA). On RNA levels in particular, we are using multiple tissue Northern blots for i) characterization of the organ-specific expression of a given gene ii) Northern blots with samples of different breast tumors from a selected tumor bank (in collaboration with Kurt Zatloukal, Pathology, Graz), to obtain first indications for a possible tumor suppressor/oncogene role of a gene identified by DD. We routinely use immunoprecipitation and Western blots to follow the regulation on the protein level if antibodies are available. Subcellular localization of gene products upon induction is studied by immunofluorescence and confocal microscopy. We plan also to overexpress these genes by electroporation of plasmid vector constructs (see project B2) to test whether the overexpression of a particular gene will affect epithelial polarity and mimic effects of the c-Jun induction.

Among other candidate genes that we have identified so far are (see **Table 1**): primary response genes TIS7, *gly 96*, and PRG1; nuclear structure protein Lamin A, and helicase 218 kD Mi-2; anchoring filament component of the basement membrane Laminin 5 α3 chain (Nicein) and the calcium binding protein calcyclin. As one can see from this list, genes

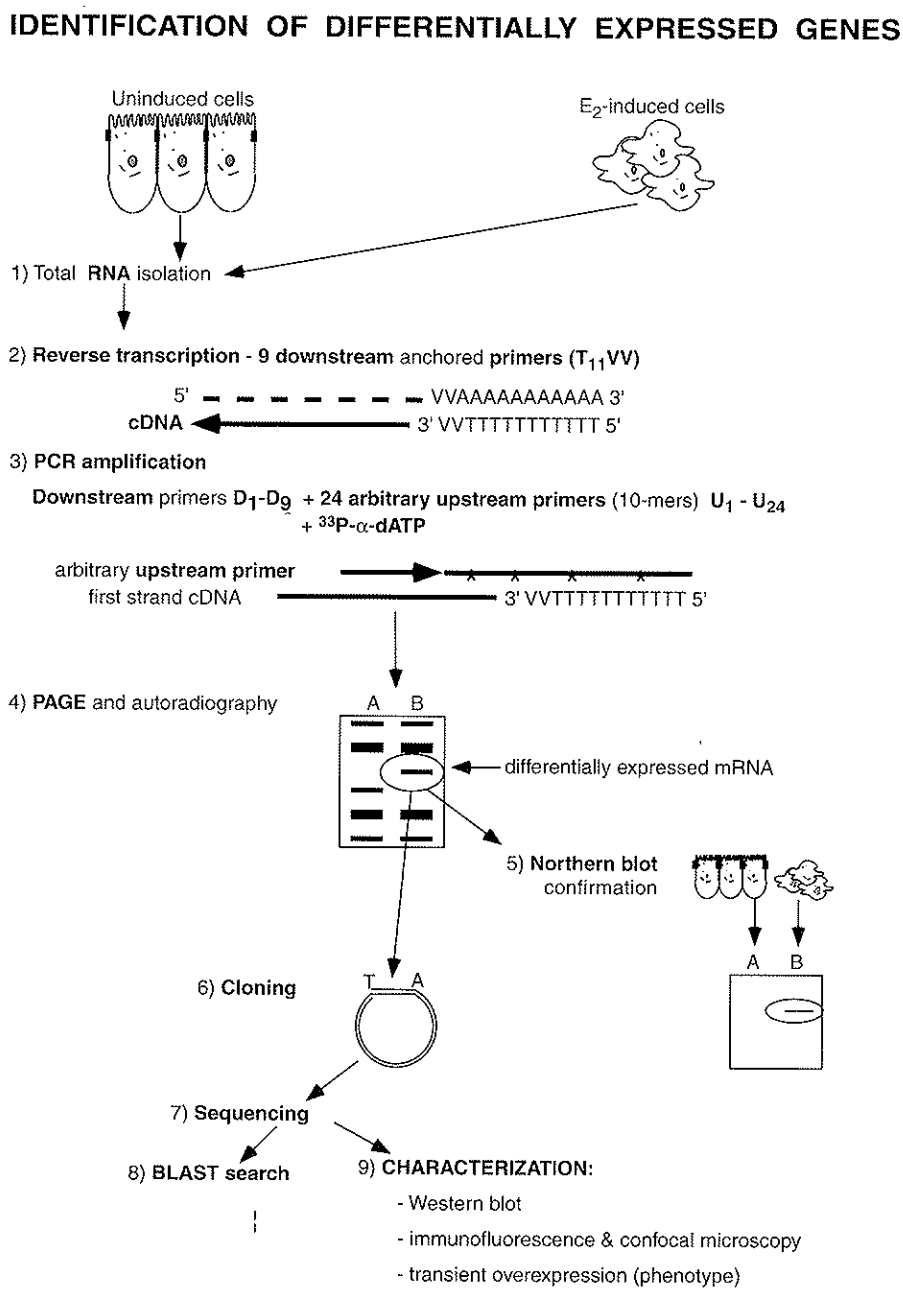

Fig. 6: Differential Display RT-PCR

identified by DD are either related to the formation and organization of the cytoskeleton or belong to the group of early response genes which might be involved in mechanisms of the transcriptional regulation. In parallel to the initial screening part of this project we are proceeding with further characterization of individual candidate genes. On several examples mentioned above we have documented that by a well controlled experimental model system as starting material for Differential Display, it becomes a powerful searchtool for identification of differentially expressed genes in the process of gain, maintenance or loss of epithelial cell polarity.

The ultimate goal of this part of the project is to link the knowledge on genes which are differentially expressed during the course of oncogenic transformation of epithelial cells with the biochemical data obtained in our other projects (see **Fig. 1**, pathway A).

LIST OF RE-AMPLIFIED, SEQUENCED, AND IDENTIFIED BANDS FROM DD RT-PCR GELS.

Band	Gene	Function	Blastdentities Score	bp	%
L 5-18/2	Mouse mRNA for **Lamin A**	Nuclear structure protein	901	181	99
L 5-19/1	H. sapiens mRNA, **218 kD Mi-2** nuclear structure protein	Helicase; transcriptional activation Role in autoimmune diseases	681	145	92
G 2-7/4	Mouse **Laminin 5 α3 chain (Nicein)**	Anchoring filament component Basement membrane protein	743	155	95
G 2- 7/5	**TIS7 / PC4;** Mouse IFN-β mRNA	Primary response gene	675	135	100
G 2-18/1	**collagen** α1 type VI; activin β -C precursor, exon 7; inbc gene, exon 1		121	37	69
L 9-16/1	Rat mRNA for ribosomal protein **L23**	60S ribosomal subunit protein	670	142	93
L 9-16/3	mouse **lipocalin**-encoding gene 24p3	Secreted transporter of hydrophobic ligands	351	71	89
G 9-13/1	mouse mRNA for **calcyclin**	Calcium binding; murine hair cycle	220	44	100
G 6-13	***gly 96*** mus musculus mRNA	Immediate early gene	1086	223	94
	PRG 1 Ratus norvegicus pankreatic carcinoma cell line	Primary-response gene	250	52	92

LEGEND: **G=gain; L=loss "2-7/5" = Upstream - Downstream primer / Number** of the **band**

Table 1

B.2. New transfection method for cloned candidate genes into epithelial monolayers

Casper Moeller-Frederiksen and Lukas A. Huber in collaboration with Martin Colombini (I.M.P., Workshop)

Once epithelial cells have established a confluent monolayer they are almost impossible to transfect transiently by conventional methods. Since we wanted to transfect candidate genes of our primary DD-RT-PCR screen transiently into monolayers of c-JunER cells at different time points before and after induction, we have developed an electroporation technique for epithelial cultures grown on permeable filter supports. The advantage of this new technique is mainly that transfections can be carried out without disturbing the established polarity of the cells by rather harsh treatments (e.g. trypsinization and transfection in suspension). The electroporation chamber was planned and constructed in collaboration with our workshop. In an initial attempt, we established a protocol to electroporate plasmid DNA and fluorescent dextrans into 85-95% of all cells from a polarized monolayer. The chamber could also be used to introduce with high efficiency synthetic peptides or antibodies into cells.

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Gene silencing by mammalian chromatin regulators

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Introduction

The stable maintenance of different patterns of gene expression is the underlying mechanism to inherit cell type identities in eukaryotes and has been correlated with the organization of chromatin domains that modulate gene activity. Paradigms for chromatin-controlled regulation of key developmental loci include *Polycomb*-group (Pc-G) dependent restriction of expression boundaries within the homeotic gene cluster in *Drosophila* and *SIR*-dependent silencing of the mating type loci in *S.cerevisiae* (for review, see Elgin, 1995). The biological significance of chromatin-mediated gene regulation, particularly for controlling mammalian development, has gained further importance by the functional analysis of murine homologues of the *Drosophila* chromatin regulators *Psc* (Pc-G) and *trx*. Both of these mammalian homologues are involved in specifying expression boundaries within the murine HOX-clusters (Alkema *et al.*, 1995; Yu *et al.*, 1995) and their deregulation has been implicated in inducing leukemia.

Recently, a 130 amino acid carboxy-terminal region of sequence conservation has been identified in *Su(var)3-9*, a dominant suppressor of heterochromatin-associated gene repression and position-effect-variegation (PEV) in *Drosophila* (Tschiersch *et al.*, 1994). This carboxy terminus has been shown previously to be shared between the *Drosophila* Pc-G gene *Enhancer of zeste* (*E(z)*) and *trx*, a positive regulator of transcriptionally active chromatin (Jones and Gelbart, 1993). Finally, this novel protein domain, designated SET (Tschiersch *et al.*, 1994), is present in gene products ranging from yeast to man (**Fig. 1**) and appears to define a new gene family of important chromatin regulators.

Using sequence information from the SET domain, we have recently isolated human (*EZH2*) and mouse (*Ezh1*) homologues of *Drosophila E(z)* and of *Su(var)3-9* (designated *SUV39H* and *Suv39h*). Our research is focused (i) on the functional analysis of these mammalian SET domain genes in the regulation of repressive chromatin regions, (ii) the biochemical characterization and chromatin association of mammalian silencing complexes containing *EZH* and *SUV39H*, and (iii) on the in vivo analysis of *Ezh* and *Suv39h* during mouse development by generating gain- and loss-of-function mutations.

I. The mammalian set domain genes *EZH* and *SUV39H* display functional conservation in regulating repressive chromatin domains

Götz Laible and Angelika Lebersorger, in collaboration with Rainer Dorn and Gunter Reuter (University of Halle, Germany)

Mammalian *E(z)* homologues are represented by two distinct loci in mouse (*Ezh1* and *Ezh2*) and man (*EZH1* and *EZH2*) which most likely reflect a gene duplication of a single ancestral locus. Over the entire length of the 760 amino acids *E(z)* protein, *Ezh1* and *EZH2* display the highest sequence identity (55% and 61%) among any of the currently known *Drosophila* and mammalian chromatin regulators. In addition to the carboxy-terminal SET domain, a cysteine-rich cluster immediately preceding the SET domain is also highly conserved (see **Fig. 1**). By contrast, mammalian *Su(var)3-9* homologues appear to be encoded by a single locus, and the respective proteins share 43% identity with the 635 amino acids fly protein. Both the human (*SUV39H*) and mouse (*Suv39h*) homologue lack 206 amino-terminal amino acids comprising a putative GTP binding domain described for *Su(var)3-9* but maintain the chromo-box - a characteristic protein motif present in several chromatin regulators.

To demonstrate involvement of *EZH2* and *SUV39H* in controlling the organization of repressive chromatin domains, we analyzed their potential to modify PEV in *Drosophila*. In collaboration with Rainer Dorn and Gunter Reuter (University of Halle, Germany), transgenic flies were established by P-element mediated transformation that overexpress *EZH2* and *SUV39H* from an inducible heat-shock promoter. As a control, we also generated transgenic flies that carry extra gene copies of *Drosophila E(z)* and *Su(var)3-9*. The transgenic flies were subsequently crossed into w^{m4} indicator strains. These indicator strains carry an inversion (*In(1)*w^{m4}) which places the *white* gene adjacent to pericentric heterochromatin, resulting in stochastically repressed, variegated patterns of gene expression that can be easily detected as red and white patches in the *Drosophila* eye. Surprisingly, w^{m4} flies, transgenic for either *E(z)* or *EZH2* (**Fig. 2**) exhibit a significant increase in the proportion of unpigmented areas in the eyes, indicating repression of w^{m4} and classifying both *E(z)* and *EZH2* as enhancers of PEV. These data provide a direct functional link between the stable maintenance of gene repression mediated by a Pc-G gene (*E(z)* and *EZH2*) and the organization of inactive chromatin domains (Laible *et al.*, 1997a). Similarly, despite the truncation of the amino-terminal third in the human homologue, *SUV39H* repressed w^{m4} activity almost as effectively as *Su(var)3-9*, demonstrating functional conservation of the encoded proteins.

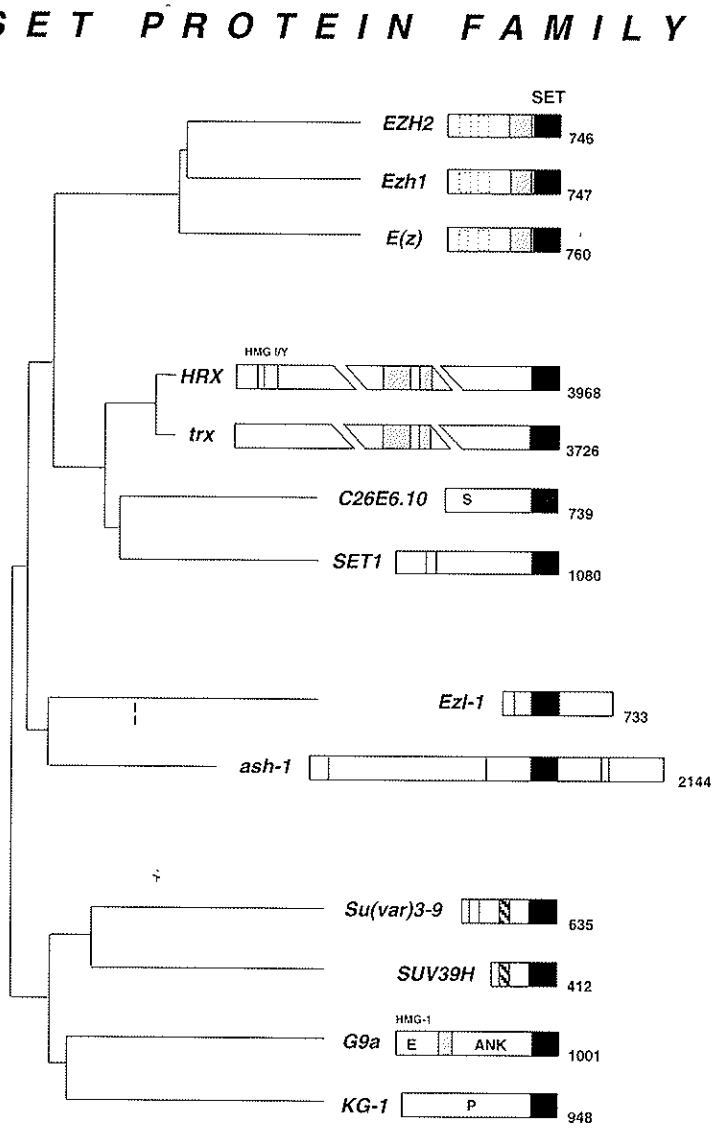

Fig. 1: Relationship of selected SET domain genes based on sequence identity within the 130 amino acid SET domain (black box). Additional sequence motifs, like the cysteine-rich regions (*E(z)* family, *S.cerevisiae Ezl-1* and human G9a) and PHD fingers (*Drosophila trx*, human *HRX*, *Drosophila ash1*) are indicated by hatched bars. The chromo-box in *Drosophila Su(var)3-9* and its human homologue *SUV39H* is shown as a stippled bar, and a putative GTP binding motif in *Su(var)3-9* and in *S.cerevisiae SET1* is indicated by a light-shaded box. ORFs encoding SET domain proteins of unknown function are *C.elegans C26E6.10* and human KG-1. Proteins are shown roughly to scale, and numbers refer to amino acid positions. References and accession numbers of the respective genes (with the exception of *Ezh1*/*EZH2* and *SUV39H*) can be found in 16.

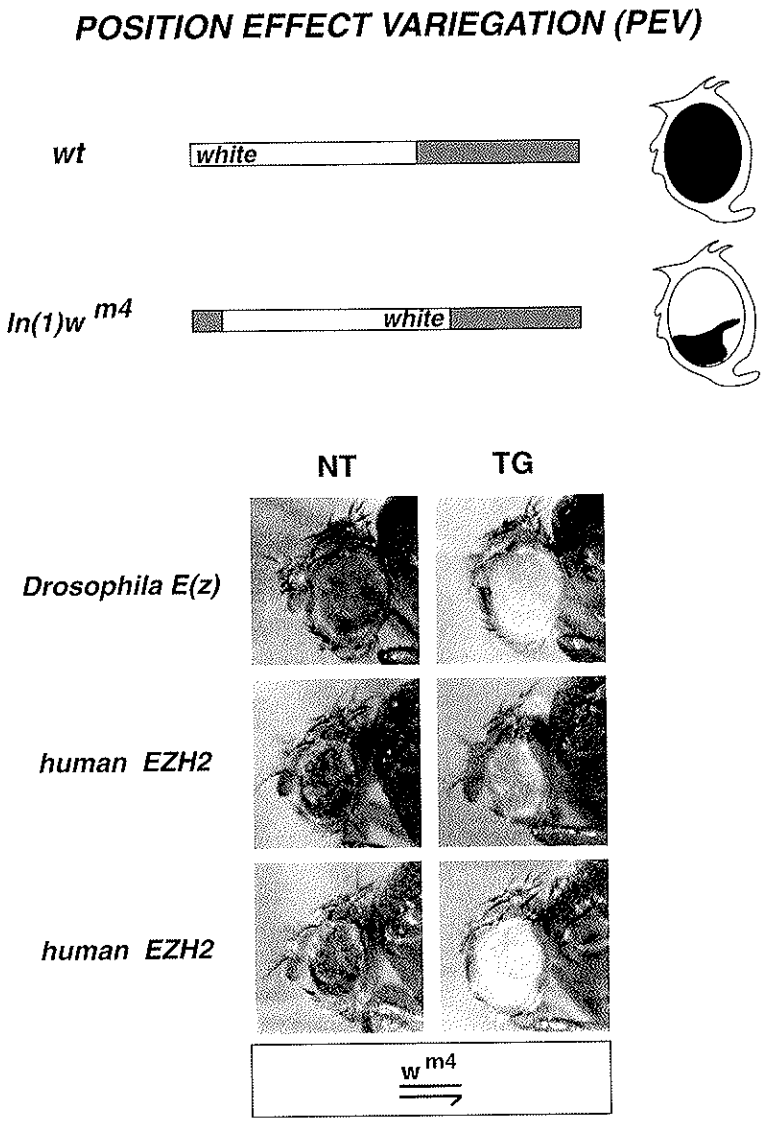

Fig. 2: Extra gene copies of *Drosophila E(z)* or human *EZH2* enhance PEV. Repression of the heterochromatin-associated *white* gene (w^{m4}) is reflected by a significant increase in the proportion of unpigmented areas in the eyes of transgenic (TG) flies. NT = non-transgenic offspring. Pericentric heterochromatin is schematically indicated as a hatched bar.

II. Chromatin association and biochemical characterization of mammalian silencing complexes containing EZH and SUV39H proteins

Louise Aagaard, Philipp Selenko, Babak Hariri and Götz Laible

In *Drosophila*, genetic data and co-localization studies of immunostained polytene chromosomes suggest that chromatin regulators are organized in multimeric protein complexes (Rastelli *et al.*, 1993; Chinwalla *et al.*, 1995; Carrington and Jones, 1996). To start analyzing chromosome association and putative complex formation of mammalian *E(z)* and *Su(var)3-9* homologues, we generated epitope-tagged versions of SUV39H and Ezh1/EZH2. Immunolocalization of $(myc)_3$-tagged SUV39H in transiently transfected mammalian cells reveals an uniform staining of metaphase chromosomes (**Fig. 3**). Together with the ubiquitous expression profile of *Suv39h* during mouse development, these data are consistent with a general role for *SUV39H* in the structural organization of mammalian chromatin and suggest that its function in gene silencing may be mediated by interactions with more specific members of the Pc-G and *Su(var)* gene families (Laible *et al.*, 1997b). By contrast, epitope-tagged Ezh1 (**Fig. 3**) and *EZH2* display a distinct, punctate staining pattern that is reminiscent of the more specific localization of *Drosophila* Pc-G and trx-G proteins which appear to decorate approximately 100 different target loci on polytene chromosomes (Rastelli *et al.*, 1993; Chinwalla *et al.*, 1995; Carrington and Jones, 1996).

Recently, the chromo-boxes of Polycomb and HP1 were shown to be required for target-specific chromatin association (Messmer *et al.*, 1992; Platero *et al.*, 1995). To investigate whether the chromo-box and/or SET-domains are necessary and sufficient in mediating target specificity and protein-protein interactions, truncated products comprising only the epitope-tagged chromo-box of SUV39H or the epitope-tagged SET-domains of SUV39H and EZH2 are currently being analyzed for chromatin association and complex formation with a variety of *in vitro* co-translated chromatin regulators containing similar protein domains. Using transient and stable transfection of mammalian cell lines, we are also extending these *in vitro* studies to investigate whether epitope-tagged SUV39H and EZH2 co-immunoprecipitate nuclear proteins *in vivo*.

In order to analyze endogenous protein complexes containing Ezh1/EZH2 and SUV39H, glutathione-S-transferase (GST) fusion proteins were expressed and purified from bacteria. The GST-fusion proteins will be attached to a solid phase and the resulting affinity matrix will be used to isolate putative interacting proteins from nuclear extracts of mammalian cells. In addition, these fusion proteins and several synthetic peptides have been injected into rabbits to raise polyclonal antibodies which will allow the biochemical purification and immunodetection of protein complexes containing endogenous Ezh1/EZH2 and SUV39H.

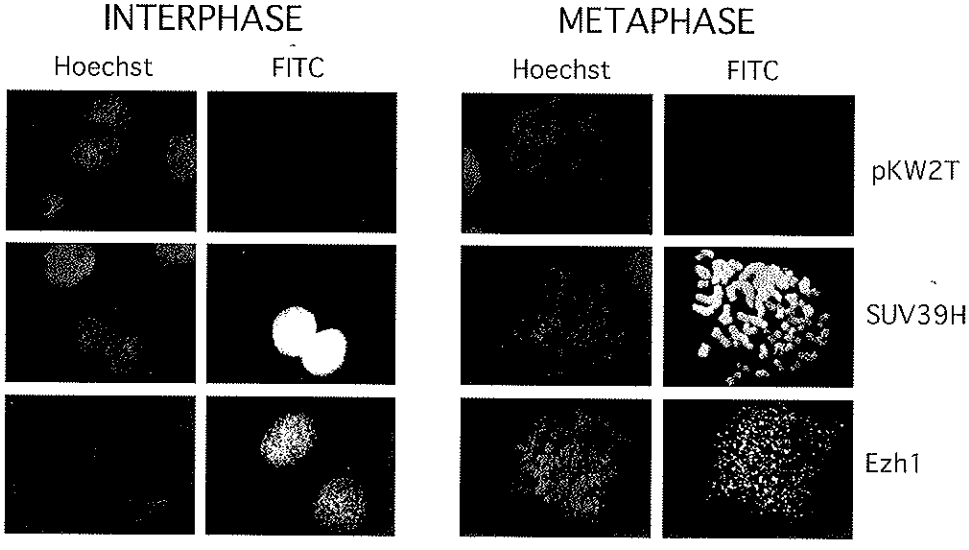

Fig. 3: Immunolocalization of $(myc)_3$-tagged SUV39H and Ezh1 at human metaphase spreads. One day after transient transfection, HeLa cells were treated with colcemid, resulting in metaphase arrest of approximately 20% of the cells. The following day, cells were fixed in 2% formaldehyde, permeabilized in PBS containing 0.5% NP-40 and processed for indirect immunofluorescence by sequential incubation with a mouse anti-myc monoclonal antibody (9E10), followed by a secondary sheep anti mouse antibody that had been conjugated to FITC. The DNA has been stained with Hoechst. pKW2T is the empty vector (CMV promoter/enhancer) control.

III. Gain- and loss-of-function of *Ezh* and *Suv39h* in the mouse

Donal O'Carroll, Götz Laible, Angelika Lebersorger and Thomas Jenuwein

Deregulation of the mammalian chromatin regulators *bmi-1* (Alkema *et al.*, 1995) and *Mll/HRX* (Yu *et al.*, 1995) in transgenic mice has been implicated in homeotic transformations and the onset of leukemia. Like *bmi-1*, *EZH2* is a Pc-G gene, and both *EZH2* and *SUV39H* share the conserved SET domain with *Mll/HRX*. All four genes are widely expressed during mouse development. Using a housekeeping promoter under the control of two strong enhancers, we generated transgenic mice for *EZH2* and *SUV39H*. Whereas several of the EZH2 lines display only low to moderate expression levels, four SUV39H mouse lines were identified that overexpress epitope-tagged SUV39H.

Among these lines, the two highest expressors exhibit transformations of vertebral identities. Taking the specific cartilage protrusions of the cervical C6 and thoracic T2 vertebrae as references, we observe posterior transformations (C7—>T1 and T1—>T2) of the axial skeleton of F_1 fetuses that persist along the antero-posterior axis (**Fig. 4**). In addition, a duplicated and partially fused R1 rib is present asymmetrically. Although the transformations are subject to variable degrees of penetrance, these data indicate that the mammalian *Su(var)3-9* homologue may participate in specifying the antero-posterior axis. Moreover, since the posterior transformations observed upon overexpression of *SUV39H* phenotypically

resemble those induced by a disruption of *bmi-1* (van der Lugt *et al.*, 1994), *SUV39H* could indirectly regulate the expression boundaries of some murine *HOX* genes.

In order to generate loss-of-function mutations, we have isolated mouse genomic clones encoding *Ezh1*, *Ezh2* and *Suv39h* sequences. The characterization of the genomic clones demonstrated a complex exon/intron structure of the murine *Ezh* loci and confirmed the 206 amino acid, amino-terminal truncation of the mammalian *Su(var)3-9* homologue, because sequence analysis predicted a GC-rich consensus promoter that precedes the 5' end of our cDNAs. Currently, we have generated the respective gene replacement vectors and are in the process of disrupting the *Ezh1/Ezh2* and *Suv39h* loci in the mouse.

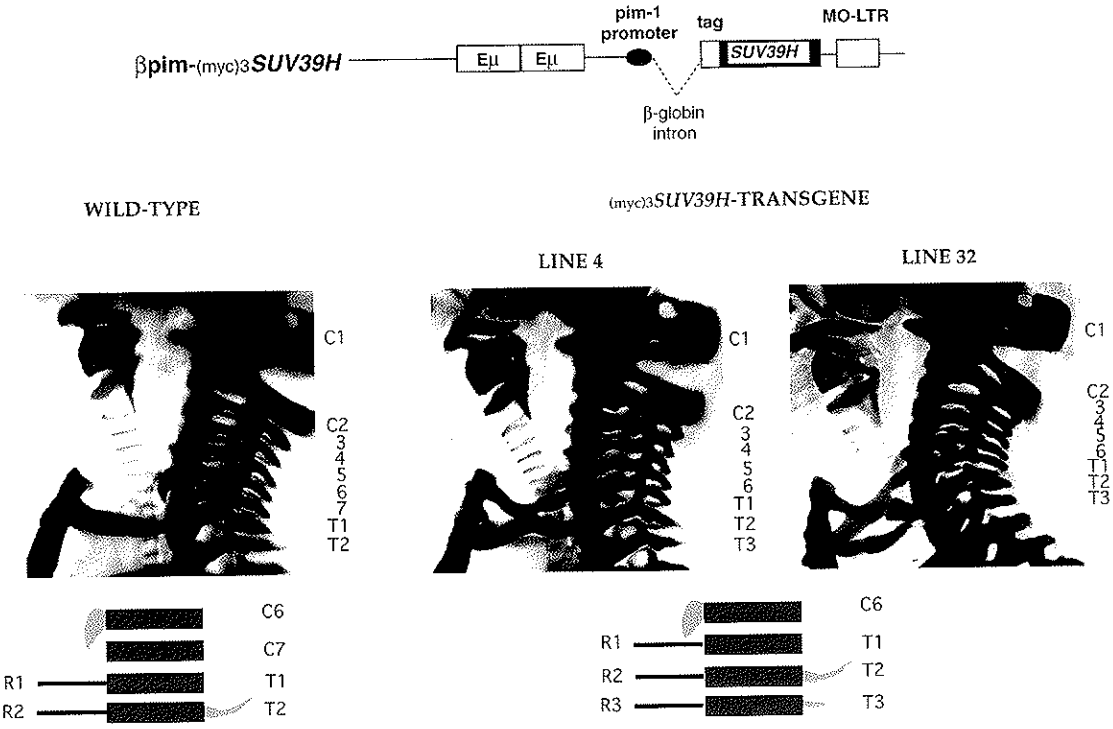

Fig. 4: Overexpression of human *SUV39H* in transgenic mice induces posterior transformations of vertebral identities. The skeletons of transgenic day 18 or newborn embryos were cleared and stained for bone (alizarin red S) and cartilage (alcian blue 8GS). Shown is a lateral view of the cervical and upper thoracic region. The characteristic cartilage extensions at C6 and T2 and the attachment of the first ribs is schematically indicated at the bottom.

IV. functional conservation of chromatin regulators from yeast to man

Andrea Wolf, in collaboration with Corey Nislow and Lorraine Pillus (University of Boulder, Colorado)

Genes that contain a SET-domain have recently been identified in yeast (see **Fig. 1**). Moreover, telomeric silencing or telomeric position effect (TPE) in yeast (Gottschling *et al.*, 1990) closely resembles PEV in *Drosophila*, suggesting that the underlying mechanism(s) for the organization of repressive chromatin domains may have been conserved during evolution. Like inversions that place marker genes adjacent to heterochromatin in *Drosophila*, insertions of the *URA3* gene at subtelomeric regions in *S.cerevisae* result in stochastic gene repression. If *URA3* is repressed, cells survive selection with the uracil analogue 5-fluoro-orotic-acid (5-FOA). Although the *SIR* genes (for silent information regulators) appear to be the major silencing genes in *S.cerevisiae*, a SET domain gene (*SET1*) has recently been described whose disruption in a telomeric *URA3* marker strain abolishes the ability of the mutant cells to grow on 5-FOA, demonstrating loss of telomeric silencing (Nislow *et al.*, 1997).

In addition to the highly homologous carboxy-terminal SET domain, *SET1* shares several other stretches of sequence similarity that are conserved in the *E(z)/EZH* gene family. Moreover, a higher resolution mapping of epitope-tagged EZH2 on human metaphase spreads seems to indicate association of EZH2 with some telomeric regions (Louise Aagaard). Using low-copy (CEN4-*LEU*) vectors, we introduced

a galactose-inducible EZH2 cDNA into the *set1 Δ/URA3*-tel mutant yeast cells. Transformants were grown to saturation and plated in five-fold serial dilutions onto -LEU and -LEU/5-FOA media (**Fig. 5**). Surprisingly, on average 50% (24/40) of primary transformants restored growth on 5-FOA, indicating complementation of *set1Δ* and the capacity of human *EZH2* to rescue telomeric silencing in *S.cerevisiae*. Moreover, subclones from initially 5-FOA sensitive colonies gradually become 5-FOA resistant, indicating that *EZH2* can induce epigenetic switching from a transcriptionally active to a transcriptionally repressed chromatin domain (Laible *et al.*, 1997a). Together with the PEV enhancement in *Drosophila* (see above), these data demonstrate a surprising functional conservation of gene repression in eukaryotes and represent the first demonstration of gene silencing in *S.cerevisiae* chromatin by a mammalian protein.

In contrast to *EZH2*, *SUV39H* did not rescue the *set1Δ*-dependent telomeric silencing defect, consistent with the more distant relationship of the SET domain in *SUV39H* with respect to *EZH2* and *SET1* (see **Fig. 1**). Additionally, the chromo-box may impart a distinct target specificity upon *Su(var)3-9* homologues, in agreement with the observation that the chromo-boxes of *Drosophila HP1* and *Pc* govern the characteristic heterochromatic (*HP1*) or euchromatic (*Pc*) distribution of the respective proteins (Platero *et al.*, 1995). Interestingly, if full-length proteins or only the chromo-boxes of SUV39H or HP1 are targeted to a modified telomere by fusing them to the GAL4 DNA binding domain, TPE is significantly disrupted, indicating function of the chromo-box in participating in chromatin-mediated gene repression. Recently, the first *S.cerevisae* chromo-box containing ORF (*YCHD1*) has been identified, and we are currently extending the functional analysis on the evolutionary conservation of chromatin regulators by disrupting *YCHD1*.

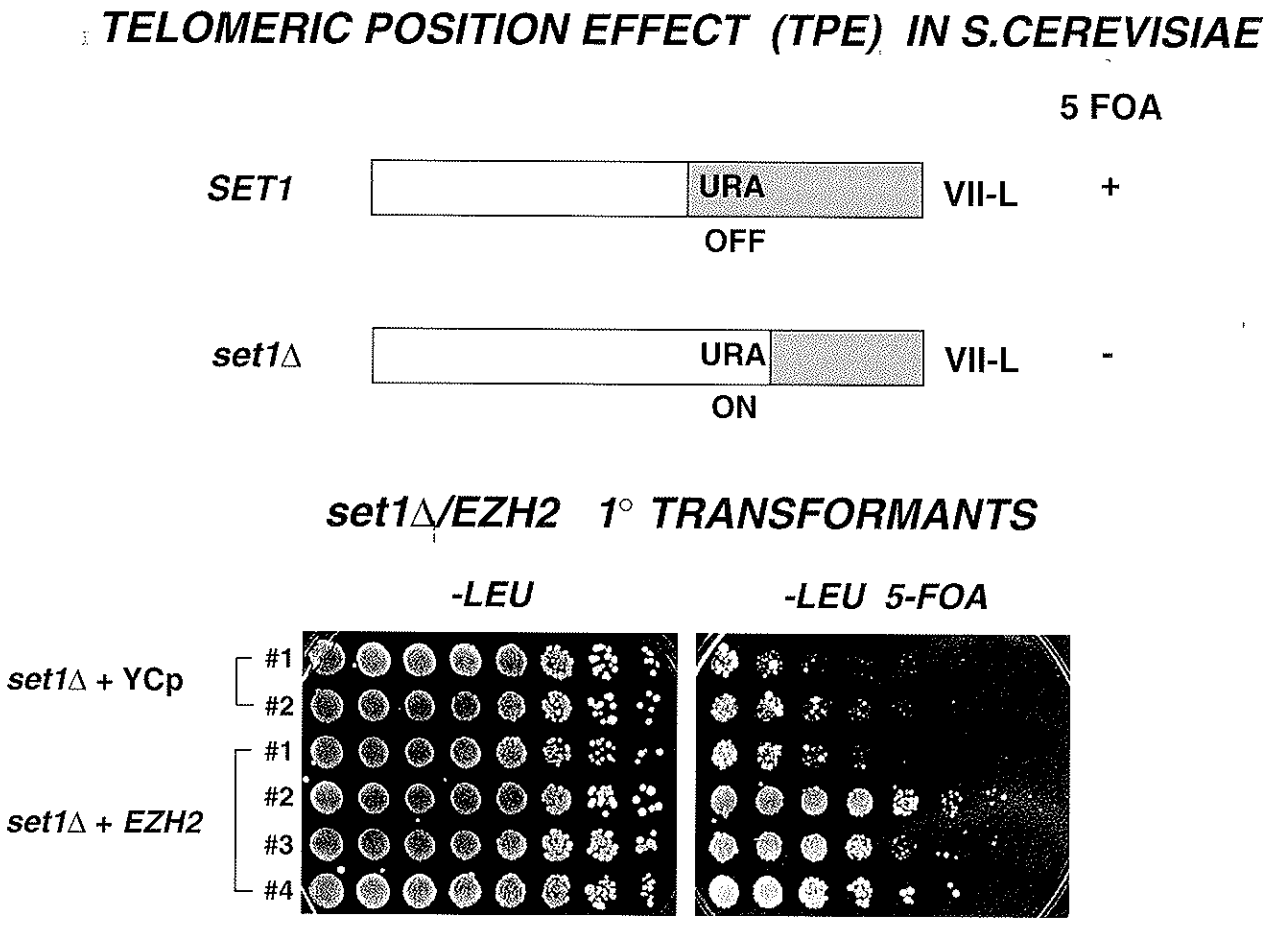

Fig. 5: Human *EZH2* restores telomeric silencing in *S.cerevisiae* mutants that are null for *SET1*. Complementation of *set1Δ*-dependent derepression of telomeric *URA3* is demonstrated by growth on 5-FOA medium. YCp is the empty vector (CEN4-*LEU*) control.

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Control of the *Xenopus* cell cycle by ubiquitin-dependent proteolysis

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Postdoc	Christian GIEFFERS (since Nov. 96)
PhD student	Edgar KRAMER (since Dec. 96)
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Introduction

The prime directive of the dividing cell is to pass on two identical copies of its genetic blueprint. This requires accurate replication of DNA and accurate segregation of replicated chromosomes. In contrast to prokaryotes, which can segregate a single circular chromosome as it is replicated, eukaryotes impose a temporal delay between chromosomal replication and segregation, establishing distinct cell cycle phases. The cell cycle control machinery regulates progression from one phase to the next, ensuring that DNA replication (in S phase) alternates with chromosome segregation (in mitosis) to maintain a constant ploidy. In most cells, growth is coordinated with division to maintain a constant average cell size; the length of the gap phases that occur before (G1) and after (G2) S phase must therefore be regulated. In contrast, early embryonic division occurs in the abscence of cell growth, so that the cell cycle consists solely of rapidly alternating S and M phases. Despite these distinct physiologies, a common biochemical machinery regulates cell division in all eukaryotic cells.

The cell cycle of amphibian embryos is driven by fluctuations in the level of the protein cyclin B, the activating subunit of the protein kinase $p34^{cdc2}$ which regulates entry into mitosis in eukaryotic organisms from yeast to man (**Fig. 1**). While cyclin B is continuously synthesized, its level is controlled by the periodic activation of a highly substrate-specific proteolytic system: cyclin B is stable during interphase, but is rapidly degraded late in mitosis as cells undergo anaphase. Overexpression of nondegradable mutants of cyclin B arrests cells in telophase with sustained $p34^{cdc2}$ kinase activity, indicating that cyclin proteolysis is necessary for kinase inactivation and subsequent exit from mitosis. It is therefore the

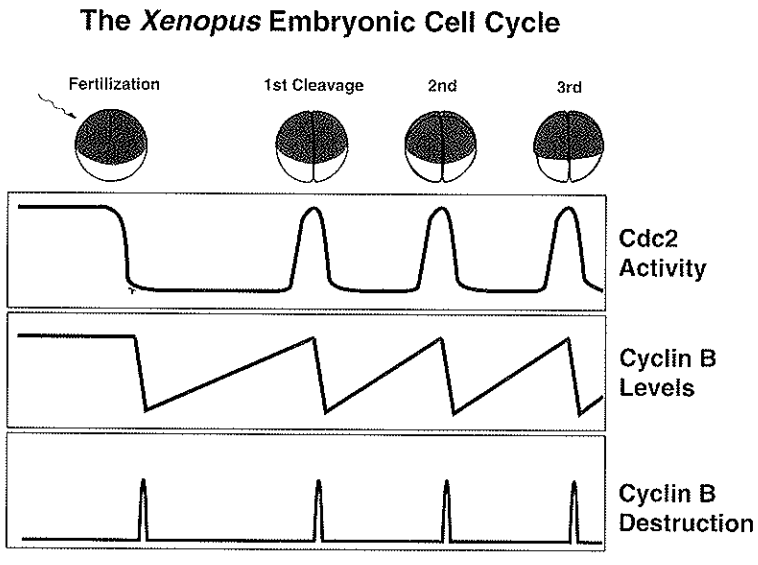

Fig. 1: *The Xenopus embryonic cell cycle.*
The cell cycle of *Xenopus* embryos is driven by fluctuations in the level of the protein cyclin B, the activating subunit of the protein kinase $p34^{cdc2}$. Cyclin B is continuously synthesized, but its level is controlled be the periodic activation of a highly substrate-specific proteolytic system in mitosis. The rapid degradation of cyclin B in anaphase leads to inactivation of the $p34^{cdc2}$ kinase and resets the "cell cycle clock" back to the ground state before a new cycle is entered.

regulated activation and inactivation of cyclin proteolysis that drives the early embryonic cell cycle.

We are interested in understanding the mechanisms of mitotic cyclin degradation and we want to find out how the activity of this proteolytic system is regulated during the cell cycle. By analyzing how the cyclin degradation machinery is naturally inhibited in special physiologic situations such as the metaphase II arrest of vertebrate eggs and the spindle assembly "checkpoint" of somatic cells we hope to obtain insight into control mechanisms that can block progression through meiosis and mitosis. We are approaching these problems biochemically using the embryonic cell cycle of the frog *Xenopus* as a model system. During early *Xenopus* embryogenesis, alternating phases of cyclin synthesis and destruction result in an oscillation of $p34^{cdc2}$ kinase activity that is often described as the "cell cycle clock". This "clock" drives a series of rapid cleavage divisions that are highly synchronous, which allows the biochemical dissection of cell cycle events during clearly defined stages.

Biochemical dissection of the cyclin degradation machinery: the anaphase promoting complex (APC)

Cyclin B is degraded during anaphase of mitosis by ubiquitin-dependent proteolysis (Glotzer *et al.*, 1991). The ubiquitin system is an enzymatic pathway that assembles chains of the small protein ubiquitin on substrate proteins and thus targets them for degradation by a large protease complex, the 26S proteasome (Peters, 1994; **Fig. 2**). The assembly of ubiquitin chains involves multiple enzymatic steps: first, ubiquitin is activated at its C-terminus by formation of a thioester bond with the ubiquitin activating enzyme, E1. Subsequently, ubiquitin is transferred to one member of a family of E2 or UBC (ubiquitin-conjugating) enzymes. Finally, ubiquitin is covalently coupled to a lysine residue of the target protein, either

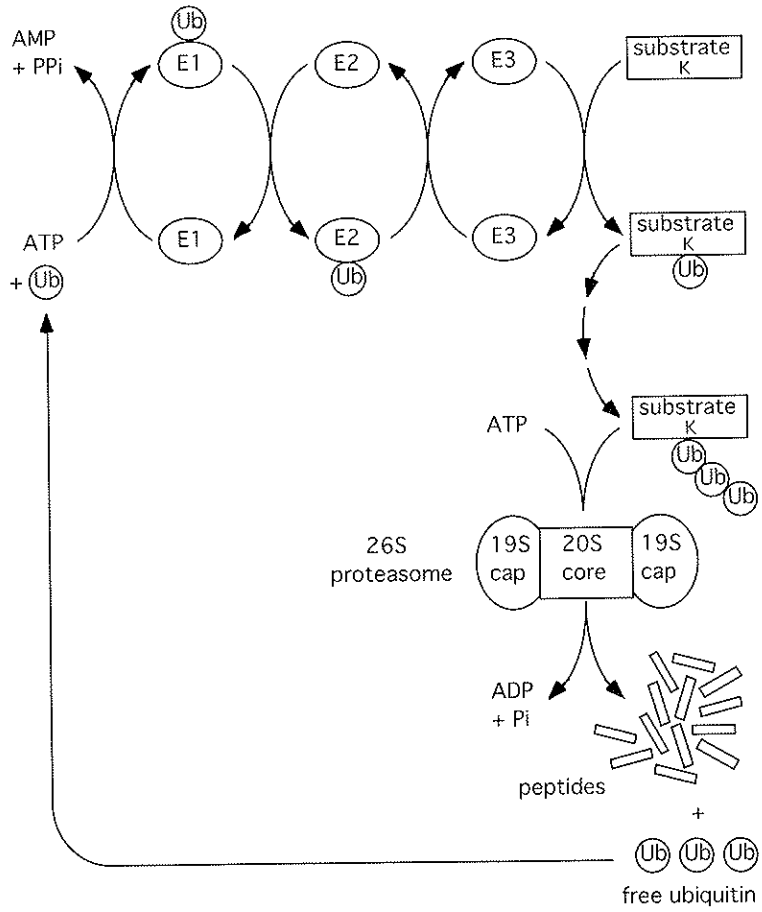

Fig. 2: *The ubiquitin-dependent proteolysis system.*
The assembly of ubiquitin chains on substrate proteins targets these proteins for degradation by the 26S proteasome. The ubiquitination reaction is mediated by three different types of enzymes, called E1, E2 and E3 (for details, see text). In some cases, E3 enzymes bind ubiquitin covalently before it is transferred to the substrate protein (Scheffner *et al.*, 1995). It is not known if this is also the case in APC-mediated ubiquitination reactions.

by direct transfer from the E2 or with the assistance of a third component, called ubiquitin-protein ligase or E3. An E3 is generally required for the formation of multiubiquitin chains on the substrate, a step that facilitates efficient recognition of the substrate by the 26S proteasome.

The cell cycle-regulated step of the mitotic cyclin degradation process is the assembly of ubiquitin chains on cyclin B, while the 26S proteasome appears to be constitutively active througout the cell cycle. The mitosis-specific ubiquitination of cyclin B can be reconstituted *in vitro* using purified factors from mitotic *Xenopus* egg extracts (King *et al.*, 1995). The cell cycle-regulated component of this system is a large protein complex which has properties of a ubiquitin-protein ligase (E3) and catalyzes the conjugation of ubiquitin to cyclin B together with unregulated E1 and E2 enzymes (E1, UBC4, UBCx). This complex is composed of eight different subunits at least six of which have homologs in budding yeast (Peters *et al.*, 1996; King *et al.*, 1995; **Fig. 3**). Because homologs of several subunits are required for the metaphase-anaphase transition in fungi and in mammalian cells (Irniger *et al.*, 1995; Tugendreich *et al.*, 1995), we refer to this complex as the Anaphase Promoting Complex (APC). A similar particle called the cyclosome has been identified in clam embryos (Sudakin *et al.*, 1995).

APC is important both for its central role in mitotic control as well as a unique example of a regulated ubiquitin-protein ligase. Accordingly, characterization of the structural and enzymatic properties of APC will illuminate both mitotic regulation and biochemical mechanisms of ubiquitin-dependent proteolysis. In collaboration with Hongtao Yu and Marc Kirschner (Boston, USA) we are planning to use cDNAs coding for individual APC subunits to express recombinant proteins for the generation of antibodies and possibly for the assembly of recombinant APC. Both will be important to elucidate the molecular mechanism, intracellular distribution and regulation of APC.

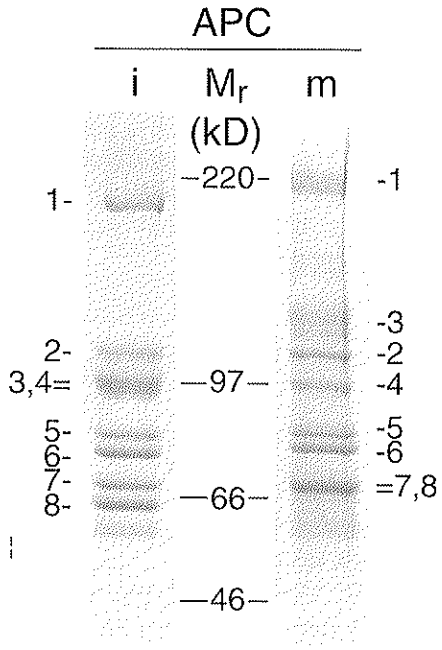

Fig. 3: *Subunit composition of the anaphase promoting complex (APC).*
APC was purified from interphase (i) and mitotic (m) *Xenopus* egg extracts and analyzed by SDS-PAGE and Coomassie blue staining. Note that subunits APC1, APC3, APC6 and APC8 have a reduced electrophoretic mobility when isolated from mitotic extracts. These mobility shifts are due to mitotic phosphorylation (for details, see Peters *et al.*, 1996).

How is APC activity regulated?

APC is also present in interphase where cyclin B is not ubiquitinated and degraded. Therefore, APC seems to oscillate in its activity during the cell cycle. At least four APC subunits are phosphorylated in mitosis immediately before cyclin ubiquitination occurs, and phosphatase treatment of purified mitotic APC results in loss of ubiquitination activity (Peters *et al.*, 1996). This indicates that mitotic phosphorylation is involved in the regulation of APC activity. We have developed biochemical assays in which the phosphorylation and activation of interphase APC can be followed, and we are using these assays to purify mitotic kinases that can activate APC. In parallel, we are directly testing the possible role of known mitotic protein kinases in APC phosphorylation and activation assays. Previous genetic studies in fungi and *Drosophila* also suggest a critical role of the protein phosphatase I in the metaphase-anaphase transition (for reviews see Peters *et al.*, 1997; King *et al.*, 1996). We are therefore also testing whether dephosphorylation events may directly or indirectly be required for mitotic activation of APC.

During the course of our APC activation experiments we noticed that purified interphase APC is not completely inactive but contains a basal cyclin ubiquitination activity (~20 % of the mitotic activity). This activity can be suppressed by fractions from an interphase extract, suggesting that inhibitors of APC contribute to the regulation of the cyclin degradation system. According to this model, stimulation of APC activity in mitosis would allow APC to overcome suppression by these inhibitors. We are presently trying to purify and characterize these inhibitory factors.

During a normal mitosis, $p34^{cdc2}$ activates the cyclin degradation system and thus initiates its own inactivation. However, during the second meiotic division of most vertebrate eggs cyclin degradation is inhibited even in the presence of high $p34^{cdc2}$ activity, leading to a cell cycle arrest in metaphase. A similar situation is found in somatic cells that have been unable to assemble their mitotic spindle properly. In these cells the so-called spindle assembly checkpoint is activated, a mechanism that leads to a metaphase arrest and thus prevents unequal chromosome segregation. Vertebrate eggs arrested in metaphase II and somatic cells arrested by the spindle assembly checkpoint mechanism cannot initiate chromosome segregation because the mitotic cyclin degradation system is inhibited. However, APC isolated from metaphase II-arrested *Xenopus* eggs is active in cyclin ubiquitination reactions *in vitro*, suggesting that the inhibition of cyclin proteolysis in these cells is not achieved by preventing the activation of APC. Therefore, other mechanisms must exist that can inhibit APC-mediated reactions in these specialized physiologic situations.

How does APC regulate anaphase?

Overexpression of nondegradable mutants of cyclin B blocks cells in telophase (Surana *et al.*, 1993; Murray *et al.*, 1989). However, mutation of APC subunit genes in yeast or inhibition of APC activity in *Xenopus* and mammalian cells arrests cells in metaphase (Holloway *et al.*, 1993; Irniger *et al.*, 1995; Tugendreich *et al.*, 1995). These two observations create an apparent paradox: blocking the APC pathway at the substrate level (by mutating cyclin B) results in a cell cycle arrest which is clearly different from the arrest induced by blocking the same pathway at the enzyme level (by mutating APC subunits). However, both observations can be explained by hypothesizing that APC function is not only required for cyclin degradation but also for the metaphase-anaphase transition, perhaps by ubiquitinating other proteins whose degradation is required for the initiation of chromosome segregation. These proteins would therefore act as inhibitors of anaphase until the activation of APC would initiate their destruction.

In collaboration with Orna Cohen-Fix and Doug Koshland (Baltimore, USA) we have recently found that the budding yeast protein Pds1p is a strong candidate for being such an anaphase inhibitor (Cohen-Fix *et al.*, 1996). Pds1p is degraded *in vivo* in yeast cells and *in vitro* in *Xenopus* egg extracts in an APC- and mitosis-dependent manner, and Pds1p is ubiquitinated *in vitro* by purified *Xenopus* APC. Importantly, yeast cells arrest at metaphase when they express nondegradable forms of Pds1p, demonstrating that the degradation of Pds1p is required for the initiation of anaphase.

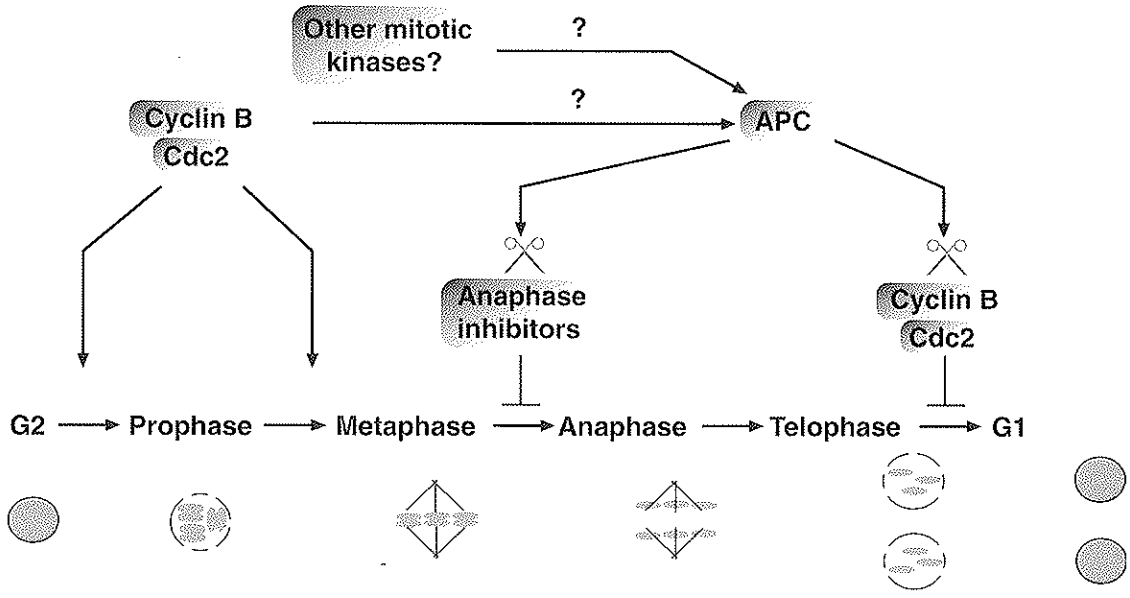

Fig. 4: The APC pathway is required for at least two distinct events in mitosis.
The initiation of chromosome segregation depends on the APC-dependent ubiquitination and subsequent degradation of anaphase inhibitors, and exit from mitosis requires inactivation of the $p34^{cdc2}$ kinase which is mediated by the ubiquitination and destruction of its cyclin B subunit.

APC is therefore required for at least two different events in mitosis: the transition from metaphase to anaphase by mediating the destruction of proteins such as Pds1p, and for exit from mitosis into the next interphase by ubiquitinating cyclin B (**Fig. 4**). However, we suspect that these two classes of substrates, anaphase inhibitors and mitotic cyclins, just represent "the tip of the iceberg" and that there are many other proteins that are degraded by the APC pathway. In support of this we have found in collaboration with David Pellman (Boston, USA) that degradation of the budding yeast microtubule binding protein Ase1p is also APC-dependent (Juang *et al.*, 1996). Overexpression of a nondegradable Ase1p mutant in telophase delays exit from mitosis, wheras overexpression in G1 arrests cells at G2/M. Degradation of Ase1p and perhaps other microtubule-associated proteins may therefore prevent premature binding of these proteins to microtubules during G1 and it may also facilitate spindle disassembly at the end of mitosis.

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Genetic principles of tumor suppression

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Introduction

Human neuroblastoma is a pediatric malignant neoplasm of neural crest origin. In search of significant genetic alterations that are associated with malignant transformation of this cell lineage, cytogenetic and molecular analyses revealed non-random aberrations in the chromosome 1p arm. Molecular mapping studies employing loss-of-heterozygosity analyses showed that these aberrations most frequently result in allelic loss of a substantial part of the sub-terminal 1p36 region. These deletions are taken as evidence for the presence of one or more tumor suppressor genes in this genomic interval. Functional studies support this idea, since the re-introduction of chromosome 1p material via microcell-mediated chromosome transfer into tumor cells has been shown to inhibit cell proliferation and to induce differentiation.

Despite the fact that a substantial amount of neuroblastoma tumors display 1p-specific allele losses, fine mapping of the critical region has been hampered by the apparent lack of sufficient DNA markers. This situation has not even favorably changed by the establishment of the most recent high-resolution genetic maps and radiation hybrid maps, since these usually reveal a significantly low marker density in the sub-telomeric region of chromosome 1p. We have previously dealt with this issue and generated a large series of 1p36-specific DNA clones by microdissection and microcloning. These clones were used both, for loss-of-heterozygosity analyses of tumors and to establish a long range restriction map of the 1p36 region. We subsequently attempted to saturate the region of interest with long insert clones as an important prerequisite for regional fine mapping of gene(s) and for gene identification strategies such as exon trapping and cDNA hybrid selection. To this aim, we screened high density gridded filters of YAC libraries, P1, PAC, and cosmid libraries with sets of 1p36-located single copy microclones and characterized and mapped the long insert clones by FISH analysis. The combined information of FISH analyses, PFGE mapping, and l.o.h analysis has led to to a detailed Integral map of the 1p36 region. We designated this map a necessary prerequisite of gene identification strategies and further fine mapping of tumor-specific rearrangements.

Since last year we also devoted considerable interest to the analysis of two balanced translocations in two cell lines derived from neuroetodermal tumors. It is suspected that - in contrast to allelic deletions - balanced translocations may dramatically increase the chances to molecularly clone the genes of interest. These rearrangements may not represent a net loss of DNA at the rearranged site but rather directly affect the important gene.

A refined neuroblastoma consensus deletion: size determination, clone content and gene map

We reported last year on three neuroblastoma-associated cell lines, SK-N-AS, CH91-074 and Kelly, which were instrumental in redetermining the minimal critical region of consistent allelic deletions in neuroblastomas. Our completed FISH analyses of long insert clones on these three cell lines have now assigned 24 ICI library YAC clones, 4 CEPH mega YACs, 9 PAC, 23 P1 and 218 Cosmid clones to the neuroblastoma consensus deletion and place this region into the 1p36.32-p36.31 band. Regarding a relative order of these clones, double hybridizations with dual color detection were unsuccessful owing to the small distances between individual clones. A sublocalization of clones was rather achieved using the cell lines NGP and SK-N-MC with balanced translocations for additional FISH mapping. This

analysis revealed that some of the clones mapped distal and others proximal of the translocation breakpoints; thus providing evidence that both breakpoints map to the consensus deletion (see last year's report). Of those clones located proximal of the NGP translocation breakpoint, several still mapped distal to the breakpoint in SK-N-MC. This provided evidence that the two breakpoints do not co-localize. Indeed, the two translocation breakpoints divided the consensus deletion into three intervalls: a telomeric "interval I", a central "interval II" and a proximal "interval III" (**Fig. 1**). Interval I was bordered distally by the telomeric deletion breakpoint of Kelly and proximally by the NGP translocation breakpoint. Interval II was delimited by the two translocation breakpoints in NGP and SK-N-MC, respectively. Interval III was delineated by the SK-N-MC breakpoint on the telomeric side and the proximal border of the deletion in SK-N-AS on the centromeric side. **Fig. 1** shows an extract of clone and marker numbers that were assigned to each of the intervals.

With the aim to further refine the relative clone order, we set out to establish clone contigs. The most effective strategy to find overlapping clones turned

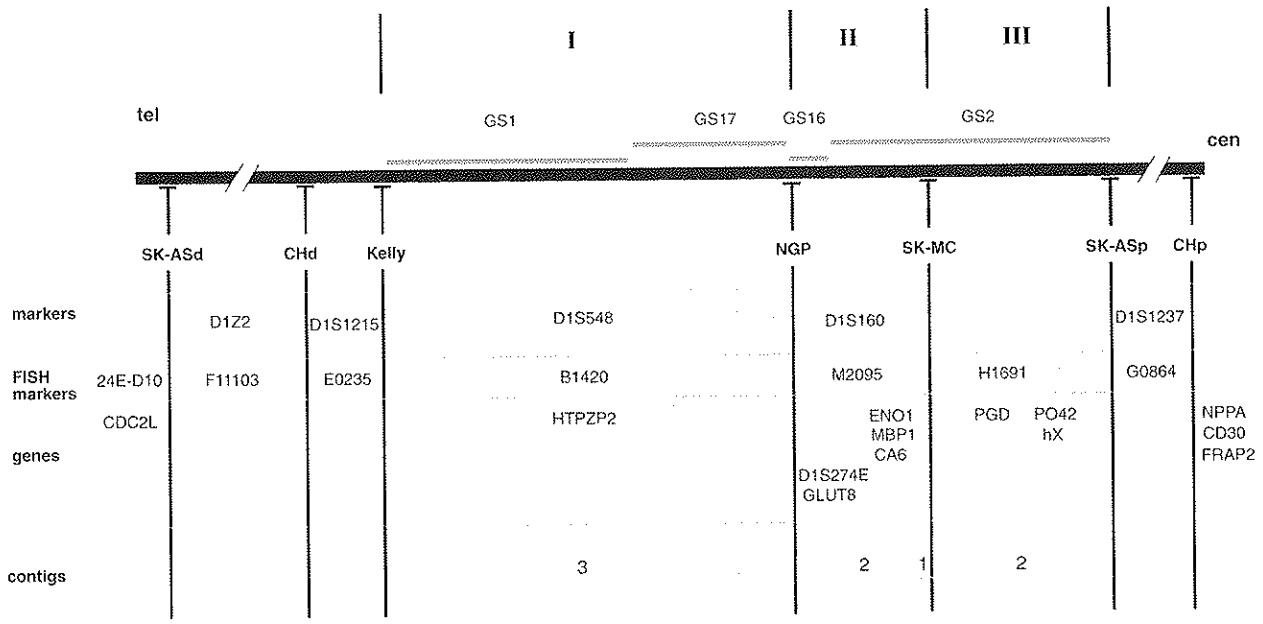

Fig. 1: *Schematic representation of the chromosome 1p36.3 consensus deletion (Grey shaded area).*
Vertical bars correspond to the positions of cytogenetic breakpoints. Roman numbers (I,II,III) indicate the intervals determined in the consensus deletion. Markers depict important genetic marker probes of the region. FISH markers correspond to representative long insert clones that may be used for FISH analysis. These clones can be obtained from the resource center of the german genome project at the Max-Planck institute in Berlin. Contigs: the numbers refer to the number of overlapping clone maps in the respective interval. Note that one of the contigs is shown to overlap the SK-N-MC breakpoint (see text).

out to be the DIRVISH (direct visualization of in situ hybridization products) technique. Applying this method in combination with restriction mapping and insert end cloning we were able to establish 7 clone contigs (**Fig. 1**).

Previous studies on long range restriction patterns have led to a partial restriction map of the chromosome 1p36 and to successful linkage of several physical markers in the region. Notably, two of the previously determined "grouped sets" of clones (see GS1 and GS2 in Barnas *et al.*, 1995) contained markers that map to the consensus deletion defined in this report. To determine the approximate size of the entire consensus deletion, we subjected all markers that map to the critical region to long range restriction analysis. Merely four "fingerprint" restriction patterns (see Barnas *et al.*, 1995) were produced: two were identical to the previously determined GS1 and 2, and another two were found with probes immediately proximal and distal of the NGP translocation breakpoint, respectively. These two were designated GS16 and GS17 (see **Fig. 1**). The four grouped sets indeed did not reveal any obvious overlap of restriction fragments, this may be due to the presence of CpG islands that characterize the ends of the GS. When calculating the maximal extent of the four GS according to previously determined parameters (Barnas *et al.*, 1995), the Bss HII fragments in GS1 amount to 1200 kbp, in GS 2 to 1400kbp, in GS16 to 160 kbp, and in GS17 to 745 kbp. In comparison, the lengths calculated from the Eag I bands were 880 kbp, 1640 kbp, 180 kbp, and

610 kbp, respectively. This indicates a cumulative length of 3505 kbp or 3270 kbp, depending on the enzyme selected. One may, therefore, assume that the entire consensus deletion encompasses approximately 3400 kbp of DNA. This will hold true provided that there is no other region within this genomic interval that is presently not covered by any of the four GS. Given the extended number of clones for this relatively small genomic interval we tend to exclude this possibility.

Our prime interest with regard to the neuroblastoma consensus deletion is the content of genetic elements. We have previously initiated several complementary approaches to identify and molecularly clone the genes contained in this region. Last year we reported an initial success using both exon trapping and direct cDNA hybrid selection for the isolation of exon fragments and partial cDNAs. This series of experiments has been extended in the course of the last year; we have now localized, identified and cloned a total of nine genes within the consensus deletion (**Tab. 1**) and mapped two of those that were newly identified last year (CD30, FRAP2) proximal to the critical region (**Fig. 1**). The genes of the consensus deletion could also be ordered relative to another according to their location relative to the intervals I, II, and III (see above) and relative to known genetic markers (**Fig. 1**). To date, none of them could be shown to reside on one of the translocation breakpoints in NGP or SK-N-MC. Furthermore, expression analyses in normal and tumor tissues led us to exclude those genes as putative tumor suppressor candidates.

Name	description	Interval (see text)
HTPZP2	human tyrosine zeta polypeptide 2	I
D1S274E	expressed sequence tag	II
GLUT8	glutaminase 8	II
CA6	carbonic anhydrase 6	II
ENO1	enolase 1 (non-neuronal)	II
MBP1	c-myc promoter binding 1	II
PGD	phosphoglycerate dehydrogenase	III
PO42	anonymous exon trap	III
hX	human xylosidase	III

Tab. 1: Genes localized to the human chromosome 1p36.3 neuroblastoma consensus deletion.

Mapping, cloning and characterization of the translocation breakpoint in the neuroectodermal tumor cell line SK-N-MC

We have previously reported on a balanced translocation breakpoint t(1;X)(p36.3;p12) in the neuroectodermal tumor cell line SK-N-MC and showed that this breakpoint could be localized by FISH to a 390 kbp YAC clone of the ICI library (see last year's report). We have also described initial attempts to identify and clone genes in the vicinity of or on the translocation breakpoint. For this purpose we had generated a locus-specific cosmid library by subcloning the YAC clone DNA. A contig had been constructed that placed a cosmid designated C31 across the breakpoint and established another cosmid, C28, as an overlapping proximal clone (**Fig. 2**). No distal clone could be generated from the YAC clone, indicating that it overlapped the translocation breakpoint to the telomeric side by maximally one cosmid length. Two genes, one of which was identified as the PGD (phosphoglycerate dehydrogenase) gene, were found to reside on the proximal side of the breakpoint on C28. Initial suggestions that the second gene, a previously unknown gene to the human genome, may bridge the breakpoint could not be confirmed, since C31 did not reveal any sequences homologous to this gene.

Since the breakpoint cosmid C31 did not disclose any apparent gene sequence when used for exon trapping or cDNA selection, we set out to perform a long-range sequencing of the 34 kbp insert

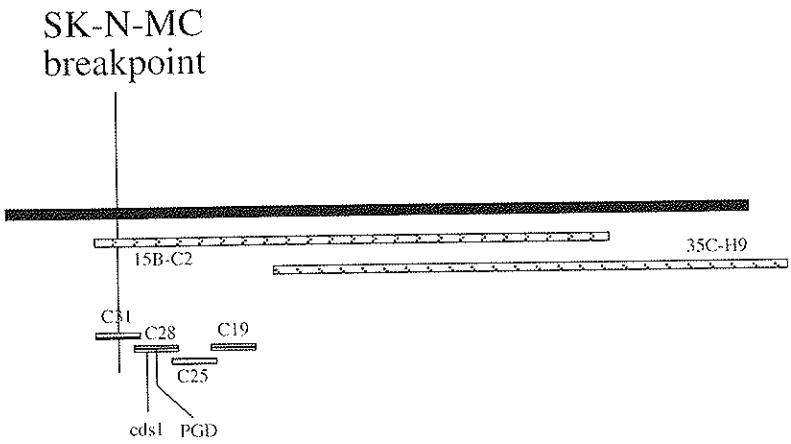

Fig. 2: Schematic representation of a YAC/Cosmid contig at the SK-N-MC breakpoint. Two mapped genes (cds1, PGD) are indicated by their putative position.

of this clone (performed by R. Kurzbauer, Service Dept.). Base perfect sequence was obtained for 33,404 bp. The sequenced fragment was shown to contain 40 Alu repeat sequences (**Fig. 3**). This corresponds to an approximately sixfold increase as compared to the average distribution of Alu's and an average spacing of approximately 600bp of non-repetitive DNA between individual Alu repeats. Using the computer program GRAIL for prediction of putative gene exons we, however, despite the extended Alu clusters discovered nine sequences in this genome region that may correspond to an exon, seven of which displayed a high probability (**Fig. 3**). These seven exon sequences were used to isolate

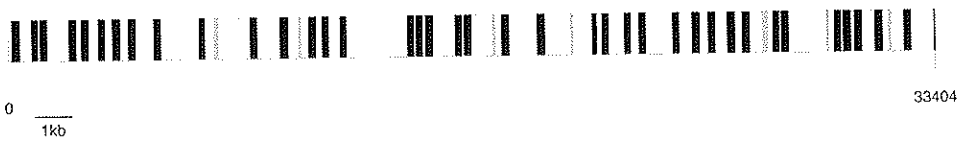

Fig. 3: Schematic representation of the C31 cosmid sequence. Black blocks correspond to Alu repeat elements, grey boxes indicate GRAIL predicted exons.

corresponding cDNAs from a human fetal brain cDNA library. Three cDNAs could be isolated from this library, which upon sequencing were shown to extensively overlap (**Fig. 4**). Distinct differences between the clones indicate the presence of alternative splice variants. When analyzing the cDNA sequence, we localized a poly(A) addition site at the very 3' end of two of the clones and also found a poly(A) tract at their end, indicating the 3' end of the gene. Further sequence analyses revealed an open reading frame of 3522 nucleotides, which may encode a protein of approximately 100 kD. We selected the laboatory designation SEQ1/1 for this gene.

Northern blot analysis displayed a fairly wide expression pattern of SEQ1/1 with a predominant transcript length of 6.5 kb and a putative alternative splice product at 6.2 kb in most tissues and a 2.5 kb transcript in sceletal muscle (**Fig. 5**). The significance

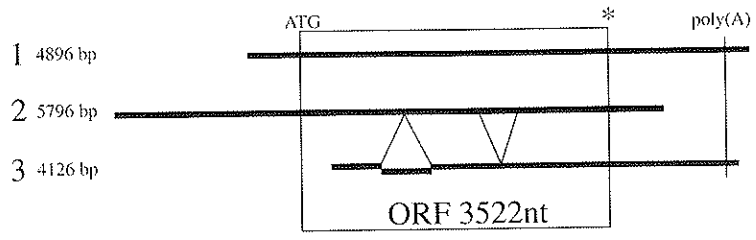

Fig. 4: cDNAs specific for the SEQ1/1 gene, isolated from a fetal brain library.

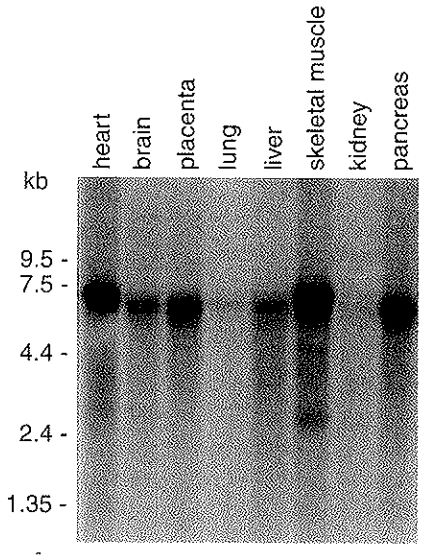

Fig. 5: Northern analysis on multiple tissues. SEQ1/1 cDNA was hybridized under stringent conditions to a "Multiple tissue blot" (Clontech laboratories).

of the alternative splice variants and their tissue distribution has not been addressed yet. When Northern blots of tumor poly(A)$^+$ RNA were probed with the cDNA probes, no obvious changes could be observed; neither transcript size nor abundance were grossly changed.

In search of the identity of this gene we performed extensive database sequence comparisons. However, no corresponding DNA sequence could be found in any of the databases. Also, the search for conserved DNA sequence motifs failed; the gene appears to encode for a protein without homology to any known functional motif. However, when using the predicted protein sequence as a search target, we discovered clear homology to two proteins, each one encoded by *C. elegans* and by *S. cerevisiae* (**Fig. 6**).

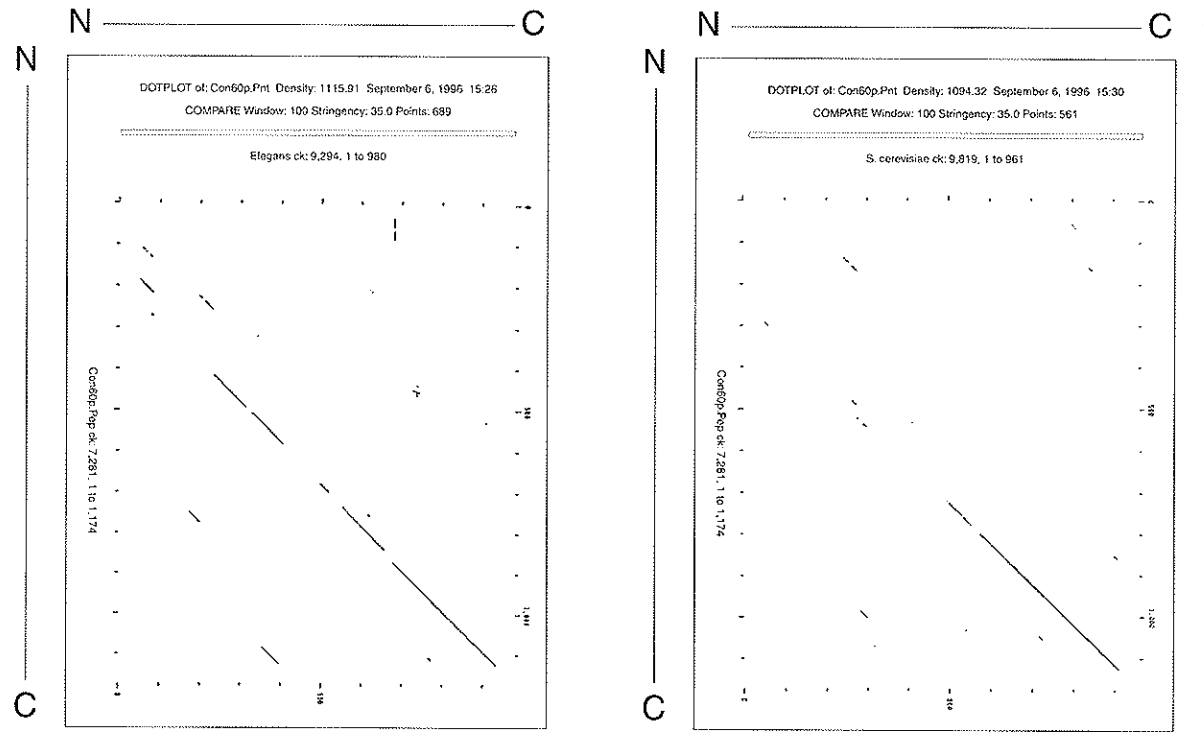

Fig. 6: Dot matrix plot comparisons between the amino acid sequences of a *C. elegans* gene (left) and *S. cerevisiae* SEQ1/1 (right). Stringency selected in both plots: 35

Owing to the missing information of important functional sites within the gene, mutation analyses of SEQ1/1 using SSCP or other high resolution analyses could not be performed to date. As a first approach to screen for tumor-specific rearrangements we, therefore, performed Southern blot analyses of tumor DNAs and corresponding normal DNAs in order to define any gross changes visible at this level of resolution. No such obvious changes could be observed in 18 neuroblastoma tumors to date. However, since we have previously described significant aberrations in the same genomic area also for primary hepatomas (e.g. Simon *et al.*, 1991), we subjected also a series of these tumors to Southern analysis. Strikingly, we could detect a clear aberration in one out of four tumors tested (**Fig. 7**). This rearrangement is currently being analyzed in detail.

As an important approach towards a detailed structural analysis of SEQ1/1 we established a cosmid contig of the entire genomic region of the gene. The SEQ1/1 locus appeared to be very large, to date we have covered more than 200 kbp of genomic DNA for the gene. From the data obtained we further suspected that the gene indeed bridges the SK-N-MC translocation breakpoint. This was deduced from the result that the breakpoint cosmid C31 covered a middle piece of the gene. In order to confirm this, we selected the most telomeric and the most centromeric cosmids of the gene contig and performed FISH on chromosomes of SK-N-MC. To our surprise we detected exclusively translocated signals; indicating that the bulk of the gene - if not the entire gene - is located distally of the breakpoint. This is in striking contrast to our initial observations; we are presently addressing the question which gene region is affected by the breakpoint. As a possible explanation of the discrepant data, the original translocation breakpoint YAC clone may include a yet un-identified deletion, which may have erroneously indicated that cosmid C31 is a breakpoint clone.

Independent of the structural analyses we have recently started to analyze the function of SEQ1/1 in order to gain insight into the possible role that the gene may have in malignant transformation. We designated this extension of our analyses important, since owing to the conspicuous size of the gene and the lack of critical functional domains a structural analysis may last inacceptably long. We attempt to both, transiently express the gene in neuroblastoma cells and to generate stable clones using inducible expression vectors. To date we have successfully generated several stably transfected clones of two tumor cell lines, clones of other cell lines are pending. The cell lines generated will be used for phenotype analyses, soft agar assays and immunocytochemical studies.

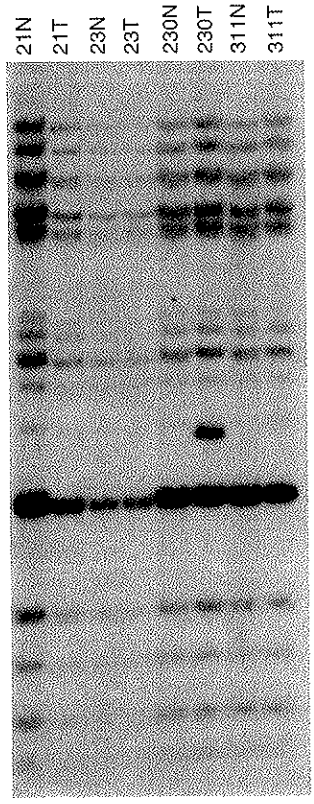

Fig. 7: Southern blot containing genomic DNA, digested with *Eco* RI, of four hepatoma patients (peripheral blood: **N**) and their tumors (**T**), and probed with a SEQ1/1 cDNA probe. Note the extra band specific to the tumor DNA in the tumor DNA of patient 230. Possible contaminations of this DNA have been excluded by hybridization with numerous other probes.

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Genomic integrity & cancer
The start-up of a transgenic mouse service

Scientist	Zhao-Qi WANG
Postdoc	Zdenko HERCEG (since March 96)
Visiting scientist	Wolfgang HULLA (since Sept. 96)
PhD student	Ciaran MORRISON
Technician	Hans-Christian THEUSSL

Introduction

Tumor development involves several genetic and epigenetic changes, including oncogene activation, repression of tumor suppressors and chromosomal translocation and deletion. Numerous studies have pointed out the importance of genomic integrity in human disease and tumor development and, thus, much effort has been made to understand the involvement of molecules which play a role in chromatin functions, such as DNA repair and recombination, in pathological conditions. The aim of our studies is to investigate the function of certain of these molecules in genomic integrity and their relation to cancer and disease susceptibility. To address these questions we are taking a genetic approach by generating gain-of-function and loss-of-function mutations. Some of the projects were performed in collaboration with Erwin F. Wagner's group.

1. The function of poly-ADP-ribosyl transferase (ADPRT/PARP)

Ciaran Morrison in collaboration with Laura Stingl and Erwin F. Wagner, Michael Jantsch (Vienna University) and Klaus Schultz-Osthoff (Freiburg University)

ADPRT is a highly abundant (10^6 molecules/cell), chromatin-associated protein and catalyzes poly-ADP-ribosylation of nuclear proteins following DNA damage, which modulates chromatin functions. This enzyme is thought to play a role in numerous cellular processes, such as cell death, proliferation, DNA repair and recombination, as well as genomic stability. After cells are exposed to ionizing radiation, alkylating agents, or free radicals, ADPRT binds to DNA strand breaks and undergoes rapid automodification leading to the formation of long, branched poly(ADP-ribose) structure. Negatively charged ADPRT subsequently dissociates from DNA ends, facilitating the DNA repair process.

To better define the function of ADPRT, mice lacking the gene were generated. Young ADPRT-/- mice display no phenotypic abnormalities but older mice originating from a mixed genetic background (129/Sv x C57BL/6) are susceptible to epidermal hyperplasia and obesity. Mutant cells and mice were used to study various chromatin functions. When compared to wild-type controls, ADPRT-/- fibroblasts grow more slowly and this difference becomes more pronounced at 39°C, implying that the lack of ADPRT either affects cell proliferation or sensitizes the cells to experimental stress. This effect on cell proliferation was also observed *in vivo* in chimeric fetuses generated by aggregating wild-type and mutant embryos. Since NAD depletion in pancreatic islet cells, caused by the activation of ADPRT, has been shown to result in cell death, we studied the role of ADPRT in diabetes. The results show that mutant mice are resistant to strepzotocin-induced diabetes, demonstrating that the activation of ADPRT mediates free radical-induced cell toxicity. The role of ADPRT in apoptosis was studied by treating ADPRT-/- cells with Fas antibody and TNF. The results showed no difference between wild-type and mutant cells, indicating that ADPRT is not an essential signal triggering the apoptotic program.

Since ADPRT is thought to play a role in DNA repair, recombination and chromosomal stability, we examined the capacity of mutant cells in double strand break repair, immunoglobulin class switching and V(D)J recombination and found no difference between them and wild-type controls. However, spontaneous sister chromatid exchange (SCE) frequency in ADPRT-/- cells is increased 2–3-fold, and they contain more micronuclei following treatment with γ-irradiation and mitomycin C *in vitro*, demonstrating a role for ADPRT in maintaining genomic integrity. Although ADPRT mutant mice have dramatically increased SCEs and micronuclei, they fail to develop malignancies after 2 years. The relationship of the observed genomic instability to tumor susceptibility of ADPRT-/- mutant mice is under investigation.

2. Role of ADPRT in apoptosis

Zdenko Herceg in collaboration with Anton Wutz

Apoptosis is the process of programmed cell death by which a cell activates an intrinsic suicide mechanism. The ICE-Ced 3 family of cysteine proteases act as the principal effector of apoptosis, presumably through their proteolytic action on specific targets. A common target of ICE-like proteases is ADPRT. Proteolytic cleavage of ADPRT by several ICE-Ced 3 family members occurs at the specific site (DEVD214-G215) and results in the separation of the two zinc fingers DNA-binding motifs from the C-terminal catalytic domain and functional inactivation of the enzyme.

Although ADPRT was specifically cleaved during apoptosis, mice lacking this molecule show a normal apoptotic response. Therefore, the significance of this cleavage is not understood. To study the function of ADPRT cleavage in apoptosis, we have used site-directed mutagenesis to generate an ADPRT resistant to proteolytic cleavage. Firstly, several point mutations were introduced into human ADPRT cDNA and wild-type and mutant ADPRT constructs were used for protein synthesis by *in vitro* transcription/translation. Following incubation with human recombinant CPP32, wild-type ADPRT was cleaved to generate two fragments of 89 kDa and 24 kDa, whereas the cleavage of mutant ADPRT was inhibited partially or completely depending on the mutation introduced (**Fig. 1**).

To test if overexpression of mutant ADPRT affects cells undergoing apoptosis, ADPRT-/- cells overexpressing wild-type and mutant ADPRT have been isolated and are currently being analyzed following exposure to various apoptotic stimuli (TNF–α, anti-Fas antibody, staurosporine, UV). Further, to study the effect of cleavage-resistant ADPRT *in vivo*, 5 transgenic mouse lines were generated using myc-tagged wild-type and mutant human ADPRT. These transgenic mice will be useful for investigations of various apoptotic pathways.

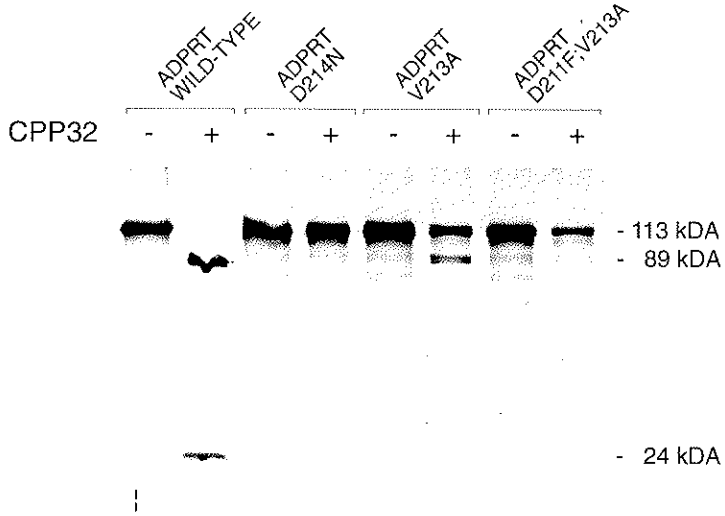

Fig. 1: SDS PAGE analysis of cleavage of ADPRT by CPP32. While wild-type and one mutant ADPRT (V213A) show complete or partial cleavage respectively, mutant ADPRTs (D214N and D211F/V213A) are resistant to CPP32 cleavage.

3. T cell development in mice lacking ADPRT and functional DNA-dependent protein kinase (DNA-PK)

Ciaran Morrison in collaboration with Laura Stingl, Erwin F. Wagner, and Graeme C.M. Smith and Stephen P. Jackson, Cambridge University

ADPRT is thought to play a role in recombination and the maintenance of chromosomal stability. Cells lacking the gene have elevated levels of SCE, indicating a higher rate of rejoining of chromosomal breaks, though mutant mice have no obvious defect in developmentally-programmed chromosomal rearrangements, such as immunoglobulin class switching and V(D)J recombination. The DNA end-binding activity of ADPRT suggests that its role in DNA rearrangements might be mediated through interplay with other end-binding proteins such as DNA-PK. A mutation in the C-terminal end of the kinase domain

of the catalytic subunit of DNA-PK (DNA-PKcs), leads to defective processing and rejoining of V(D)J recombination intermediates and a block in B and T cell development in the murine *scid* mutant. Since the absence of ADPRT results in increased chromosomal rejoining, we speculate that its absence might lead to processing and ligation of V(D)J recombination intermediates that accumulate in *scid*. We generated mice lacking both ADPRT and DNA-PK to examine the effects of the absence of both activities. These animals, though fertile, showed a marked perinatal mortality and size reduction compared to littermates (**Fig. 2**). While double mutant B cells failed to develop further than in *scid* animals, up to 50% of

Fig. 2: Mice lacking both ADPRT and DNA-PK activity (left) display marked perinatal lethality and growth retardation compared to wild-type littermate (right).

total thymocytes were positive for both CD4 and CD8 coreceptor molecules (**Fig. 3**), and thymic cellularity was significantly increased, showing that these cells had bypassed the *scid* block in T cell development. This was only a partial rescue, as they failed to express the mature CD3 marker and surface T cell receptor (TCR), while TCRγ sequences showed *scid*-like joins. In lymph nodes of 6-week old double mutant mice, we found CD4 and CD8 single positive cells expressing the mature CD3 marker and TCRβ, demonstrating further development. Possibly as a consequence of the aberrant accumulation of thymic T cells, T cell lymphoma developed in 9 out of 12 double mutant mice between 12 and 21 weeks of age. Finally, biochemical analyses reveal a biochemical link between ADPRT and Ku, a component of the DNA-PK holoenzyme. These results demonstrate genetic and biochemical interactions between ADPRT and DNA-PK in T cell and lymphoma development. Further genetic experiments are underway to probe the role of ADPRT in ensuring that the generation of antigen receptor diversity does not result in aberrant rejoining and lymphoma.

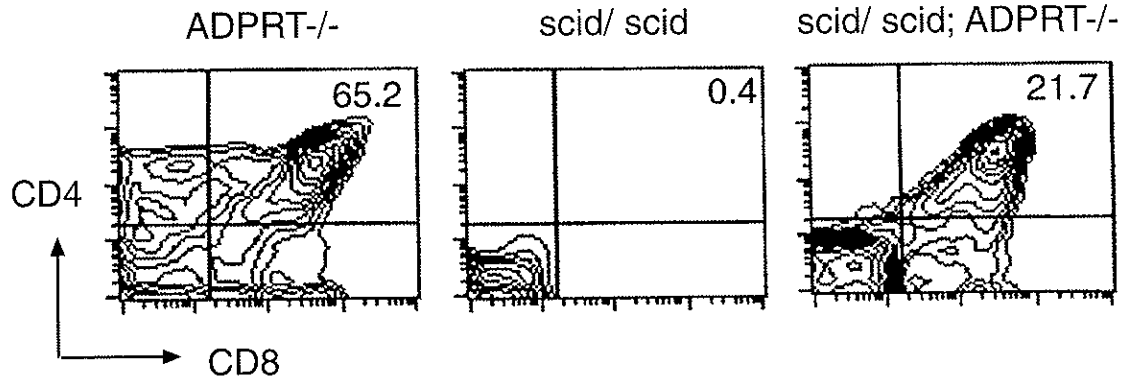

Fig. 3: Double mutant mice (*scid/scid*; ADPRT-/-) have partially rescued T cell development as demonstrated by the appearance of thymocytes expressing both CD4 and CD8 coreceptors.

4. Cooperation between c-*fos* and *jun*B in tumor development

Wolfgang Hulla and Hans-Christian Theussl in collaboration with Erwin Wagner

Overexpression of c-*fos* in transgenic mice results in osteosarcoma formation with high frequency, whereas the overexpression of other members of the transcription factor complex AP-1, such as c-*jun*, *jun*B or *fos*B does not produce a distinct phenotype. Previous studies demonstrated specific cooperation between c-*fos* with c-*jun* in tumorigenesis, resulting in enhanced osteosarcoma development compared to c-*fos* single transgenic mice and in transformation of osteoblasts and/or cells interactive with osteoblasts. We were interested in studying the cooperative effect of c-*fos* with another *jun*-related gene, namely *jun*B, in oncogenesis *in vivo* and, therefore, generated mice overexpressing both protooncogenes. These animals develop osteosarcomas at a similar frequency to c-*fos* single transgenics but additionally display nodal tumors in the tail and other soft tissues beginning at 3-4 weeks of age. Palpatory and radiological examination as well as morphological and histological analysis revealed that the tumors were fibrous with an additional osseous component, implying that the overexpression of *jun*B together with c-*fos* transforms not only osteoblasts but also, perhaps, a cell type from a different cellular compartment. Cell lines have been established from these tumors and will be used to study the molecular basis of cooperation between c-*fos* and *jun*B in tumorigenesis.

5. Establishment of transgenic mouse service

Hans-Christian Theussl

In order to facilitate research projects involving mouse experiments at the IMP, we started a service program at the end of 1995. This includes training on embryo manipulation, such as collection of embryos, embryo transfer, blastocyst injection, and embryo freezing. This expertise was used for various projects by different groups using transgenic and knockout mice. During the year, we set up an embryo freezing procedure and have frozen 1118 embryos from 9 mutant as well as wild-type mouse strains. We have also transferred and rederived 9 mutant mouse strains which were imported into the IMP animal house. Furthermore, we have provided technical help for the generation of chimeric mice by injection of embryonic stem (ES) cells into blastocysts. For example, we have helped Erwin F. Wagner's group to generate JNK2 mutant mice and contributed to the project of Meinrad Busslinger to generate mice lacking the CDP gene encoding a CCAAT displacement protein.

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Grigoriadis, A.E., Wang, Z.-Q., and Wagner, E.F. (1996). Regulation of bone cell differentiation and bone remodelling by the c-Fos/AP-1 transcription factor. In: Principles of Bone Biology 15-24, Academic Press inc.

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Wang *et al.* (1995). *Genes & Dev.* **9**, 509-520.

Wang *et al.* (1995). *Cancer Res.* **55**, 6244-6251.

Animal house

Scientific coordinator	Erwin F. WAGNER
Veterinarian	Karl SCHELLANDER (Univ. Vienna) (until Sep. 96), Matthias MÜLLER (since Oct.96)
Chief technician	Melanie KING
Technician	Mijo DEZIC
Technician	Susanne GRATZL
Technician	Ryzard KUPINSKI
Technician	Erika WEISSENGRUBER
Technician (temporary)	Johannes PIRKER (since Nov. 96)
Part-time assistant	Dominik MAYR (until Oct. 96)

The IMP animal house provides technical support for the various research programs. The largest section by far is the mouse one, and there are also chicken and *Xenopus* sections.

The requirements of the research groups altered during 1996, negating the need for in-house rabbit experiments, and instead requiring an expansion in the mouse facilities. The rabbit room was enlarged and converted to a mouse room which is now fully utilized by five research groups.

A new room has also been added to the animal house to provide a holding area for mice with a lower health status than our in-house animals which need to be imported for new research work. These animals can then be safely transferred into the main animal house by embryo transfer. Further building work is also being carried out to provide a large *Xenopus* facility which will be in operation in 1997.

Sixteen different strains of mice are bred routinely, which amounts to approximately 400 mice per week, thus providing a regular and rapid supply of animals for the research groups to use. These mice include mutant, immuno-compromised, congenic and hybrid strains as well as standard inbred and outbred strains. Mice are also bought-in from commercial suppliers to supplement the numbers required. 80 chicken eggs are produced each week from our own closed chicken flocks. These eggs are for either hatching or for embryo work. The *Xenopus* are purchased from external suppliers when required.

Breeding, stock and experimental animals, including transgenic and knock-out mice, for the various scientists are maintained by the animal house staff. The animal house staff also perform a variety of technical procedures for the research projects such as blood collection, tumor implantation, and the administration of substances by various routes e.g. intra-venous, intra-peritoneal and sub-cutaneous. All procedures are performed to a high standard under the appropriate anaesthetic regime and in conjunction with the necessary project licences.

The animals are used by the following scientists: Mouse studies: Hartmut Beug, Max Birnstiel, Meinrad Busslinger, Gerhard Christofori, Thomas Jenuwein, Erwin Wagner and Zhao-Qi Wang. Chicken studies: Hartmut Beug. *Xenopus* studies: Max Birnstiel.

Protein modeling

Scientist	Anton BEYER
PhD student	Aron MARCHLER-BAUER (until April 96)
PhD student	Bianca HABERMANN

Introduction

The main topic of our work is the relationship between primary sequence and tertiary structure of proteins.

In his thesis Aron Marchler-Bauer has developed new methods to construct a potential of mean force for proteins, using data from known crystal structures and information from sequence data alone. With this method, for a given sequence the probability of finding the most related protein in a database of known structures is significantly increased. The use of such potentials for threading has gained great acceptance in the last few years and is heavily used by several groups worldwide. Threading algorithms are used to find possible folds for a given sequence in case the structure is not yet known. We will continue to work along this line in the future. One goal will be to find reliable estimates of the quality of such structural predictions.

Bianca Habermann is working on a project concerning DNA binding proteins. Currently she is investigating correlations between sequence specificity of DNA binding and the sequence of the corresponding protein. This will be done mainly on the sequence level but also in terms of the three dimensional structures of the complexes involved. This is possible because recently several structures of this kind have been solved by various groups either by x-ray or by NMR. This project is a joint cooperation with the group of Harmut Beug at the IMP. Her work is described in more detail in the following paragraph.

Establishment of an interaction code for C_4-zinc finger proteins

Bianca Habermann, Anton Beyer, Hartmut Beug

I. Introduction

The specific interaction between two molecules depends on the physical properties of the two partners involved. Therefore, the nature of the interaction can either be electrostatic, in case of polar or charged molecules, hydrophobic, due to the Van der Waals forces, or a combination of the above. In case of specific protein/DNA interaction, the chemical properties of the side chains of the protein involved in binding the DNA and the base pair sequence of the recognized DNA element are responsible for the specific interaction between a protein and its corresponding DNA element. Changing the sequence, and therefore the chemical properties of one of the two partners will alter their binding behavior.

Because of the complex nature of protein DNA-interaction a detailed understanding of the rules involved is not yet available. One way of identifying the code of interaction between a specific DNA-binding protein and the corresponding DNA-element is the approach of random mutagenesis of the two partners involved, that is the part of the protein that is involved in the binding of the DNA and the DNA-element that is recognized by the protein. Such an approach has been carried out successfully for the classical zinc finger proteins by Yen Choo in Aaron Klug's group (Choo and Klug, 1994a; Choo and Klug, 1994b). Comparable experiments will be carried out for the C_4-zinc finger proteins and their corresponding DNA-response elements.

II. The Superfamily of the Nuclear Receptors

The class of the C_4-zinc finger proteins are ligand inducible regulators of transcription and are involved in the regulation of gene expression of tissue- and developmentally- specific genes. The superfamily of the C_4-zinc finger proteins, or nuclear receptors, include the steroid and thyroid hormone receptors, the retinoic acid and retinoic X receptor, the vitamin D receptor and a large number of receptors whose ligands have not been found yet, which are called the orphan receptors. Upon ligand binding, the nuclear receptors bind to their response elements as dimers, whereby the formation of homo-, as well

as heterodimers is possible. The nature of their cognate DNA element, which is generally referred to as *'Hormone Response Element'* (HRE), is palindromic, whereby inverted, reverted and tandem repeats are found.

The DNA-binding domain of the nuclear receptors consists of two zinc fingers. Each zinc ion is coordinated by four cysteines. The DNA-binding helix, as seen in the crystal structure, is located in close neighborhood of the first zinc finger (Luisi *et al.*, 1991). The last four residues of the first zinc finger, the P-box, also directly interact with the DNA. As was shown previously, mutations in the P-box lead to the recognition of a different response element (Zilliacus *et al.*, 1991; Zilliacus *et al.*, 1992). The residues within this stretch of the protein must therefore be involved in the specific binding of the nucleic acid. Analysis of the crystal structure indicates contacts between the side chains of these residues with the base pairs of the DNA helix. However, mutational analysis was mainly performed in a rational way, changing the amino acid sequence of one nuclear receptor to the sequence of another. A thorough analysis of the interaction code, covering a larger part of the sequence space, has not been done. The question also remains, if and how the helix following the first zinc finger, that also shows close contact to the DNA, is responsible for specific binding of the DNA. Random mutagenesis of the whole DNA binding region and of the hormone response element will be useful in identifying not only the residues that are responsible for the specific DNA contact, but also to analyze the code of interaction between these two molecules.

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Service Department

Scientist	Gotthold SCHAFFNER
Technician	Elisabeth AIGNER
Technician	Ivan BOTTO
Technician	Robert KURZBAUER
Technician	Karl MECHTLER
Technician Media Kitchen	Christa CZAPKA
Technician Media Kitchen	Gabriele BOTTO

Introduction

The Service Department offers a variety of rapid services to IMP scientists. A large portion of our effort involves DNA sequencing, oligonucleotide synthesis and peptide synthesis. In addition, a modest amount of service is provided to institutions outside the I.M.P. We sequenced DNA probes, synthesized oligonucleotides and provided oligopeptides for several university institutes in Vienna.

We also provide assistance in designing immunization protocols, we organize rabbit immunization and antisera production by a company outside the IMP and we have initiated the production of monoclonal antibodies in hybridoma cell lines. The IMP peptide facilities have been fully integrated into the Service Department, thus expanding our synthesis activities.

During 1996, a successful reorganization and expansion of space for preparing diverse reagents and cell culture media took place so that we could keep the standard of quality of various solutions prepared by our Media Kitchen staff at a high level. This is vital for the continuous success of cell culture and other exacting work done in the institute.

We also prepare many selected reagents such as DNA molecular weight markers, enzymes, a variety of transformation-competent *E.coli* strains and we maintain a stock of cloning vectors, primers etc.

Oligonucleotide synthesis

After an increase of more than 700 oligonucleotides in 1995 we expected only a moderate increase in 1996. Nevertheless, there were an additional 150 oligonucleotides synthesized, to give a total number of 2650 this year (**Fig.1**). This increase was again largely due to PCR primers and primers for large sequencing projects.

Peptide synthesis and production of monoclonal antibodies

Peptide synthesis is now part of our services. The increasing numbers of peptides produced at the IMP over the past 3 years (**Fig. 2**) demonstrates that synthetic peptides became more and more important in IMP research activities. Yeast alpha-factor in large quantities, a great variety of petides coupled to PEG or KLH for immunization purposes, and many peptides attached to Biotin or FITC have been synthesized this year.

For the "Tumor-Vaccines-Project" we synthesized several peptides to be tested in peptide-"Transloading"-experiments (see report of group Birnstiel).

In late fall we started to produce, and subsequently purify by HPLC, large amounts of monoclonal antibodies from hybridoma cell-lines. Many of these antibodies are essential for cell depletion studies in mice and we have used some to construct ELISA kits for special needs. This has resulted in considerable financial saving for the institute.

As one can easily predict, we will not be able to provide sufficient amounts of oligopeptides in the near future with the present equipment at the IMP. We have therefore tested various synthesizers and have decided to invest in a SYRO II instrument (MultiSynTech, Bochum, Germany) late in the year. The Syro II is capable of synthesizing up to 60 oligopeptides simultaneously with reasonably high quality. As the capacity of our HPLC systems is at its

limit as well, we have decided to upgrade our PerSeptive BioCad Workstation and to apply for an additonal HPLC system. The Vision Workstation of PerSeptive Biosystems seems to be the most convenient system for us.

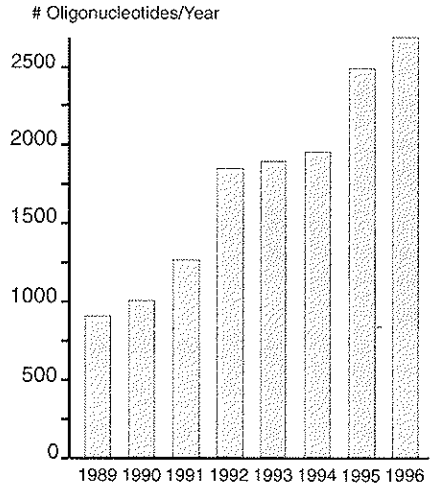

Fig. 1: Total amount of oligonucleotides synthesized per year.

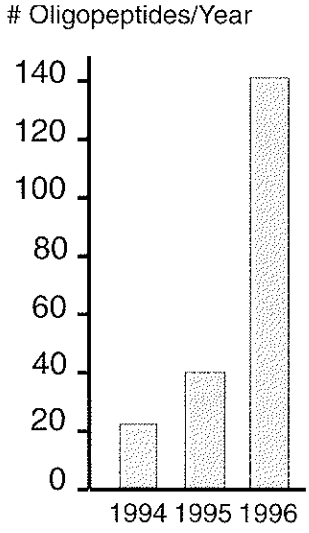

Fig. 2: Total amount of oligopeptides synthesized per year.

Sequencing and DNA isolation

The ABI PRISM 377 DNA sequencer does a great job and contributes to about 60 % of the 8200 samples (**Fig. 3**) analyzed this year. The upgraded ABI 373 A is still heavily used. In many samples one is able to analyze the sequence closer to the sequencing primer with the "old" sequencer, probably because of the slightly thicker gels used with the system.

In 1997, we expect many more samples to be analyzed. We therefore plan to use a robotic system to prepare DNA from plasmids. This will be necessary for two reasons. Firstly, we have still too many samples where the sequence analysis failed in the first round because of bad DNA quality, and secondly, we expect many more DNA samples from the "Tumor-Vaccine-Project", where an expression cloning project for isolating mouse tumor antigens is ongoing.

The robot, schematically shown in **Fig. 4**, starts with the bacterial cultures shaking for a given time in the incubator (I, step 1) on the platform. The robot arm (RA) transfers the individual bacterial clones to a 24-well microtiter-(MT)-plate on the vacuum station (V, step 2). Bacteria are then vacuum-filtrated onto filters at the bottom of the 24 vials. For lysis, the 24-well MT plate is transferred by the robot gripper (RG) to the shaker (S, step 3). After neutralization of the samples the MT plate is put back to the vacuum station and the lysis buffer containing the DNA

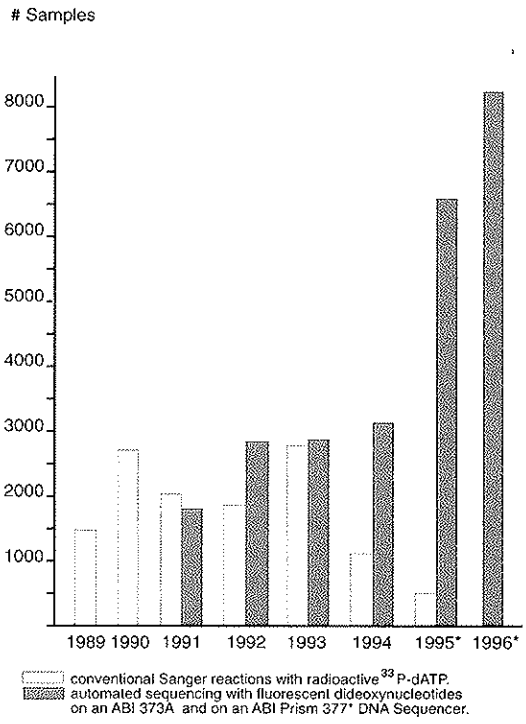

Fig. 3: Total numbers of samples sequenced in 1989 - 1996. The darker filled bar of 1995 * and 1996 * represents numbers of automated sequencing done on both ABI 373A and 377 DNA sequencers.

samples are subsequently filtered on adsorbing 96-columns-MT-plates (V, step 4), washed and eluted to second adsorbing columns (V, step 5). In the last step the clean DNA samples are eluted and filtered into a standard 96-well MT-plate (V, step 6). After measuring the concentration of the individual DNA samples a separate robot system will use the Excel-sheet values to calculate the dilutions and will prepare the sequencing reactions. Furthermore, the second robot system will be used for other laborious assays like ELISAs, cytotoxic T-cell assays and many others.

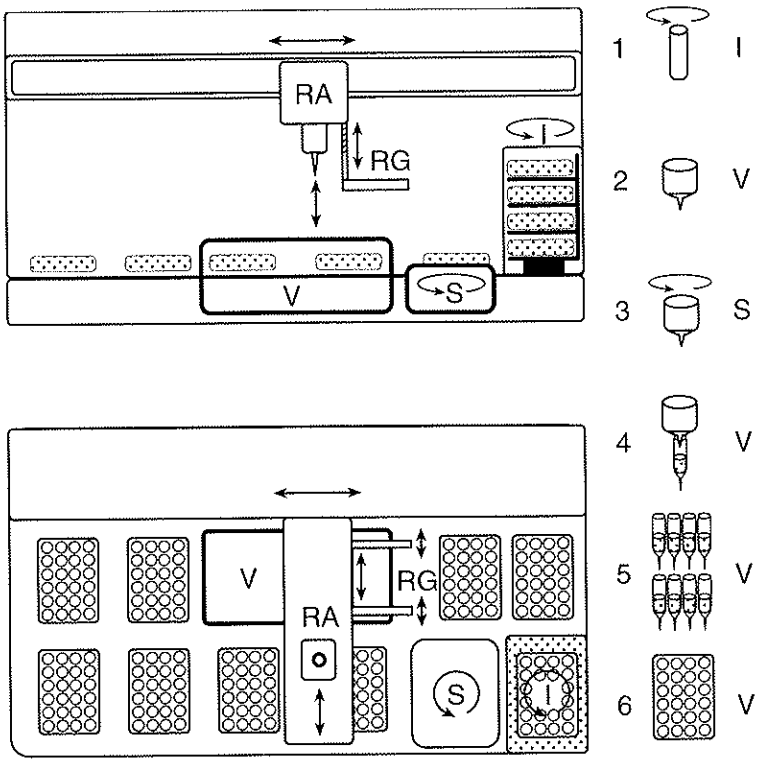

Fig. 4: *Schematic view of the automated DNA preparation.*
Step 1 I : Incubation of bacterial clones in the incubator (I) for microtiter-plates. Step 2 V: Transfer of the microtiterplates containing bacterial clones with the robotic gripper (RG) to free place on the platform, pipetting of media containing bacteria with pipet-tips on the robotic arm (RA) onto 24-well vacuum filtration unit (V). Step 3 S: Transfer of bacteria to the shaker (S) and subsequent lysis of the bacteria. Step 4 V: Transfer back to vacuum unit (V) and filtration of the lysate to the ionexchanger column. Step 5 V: Elution of DNA and absorption on DNA binding column. Step 6 V: After desalting DNA is eluted and collected in 96-well microtiterplates.

Publications during the year

Chiocca, S., Kurzbauer, R., Schaffner, G., Baker, A., Mautner, V., and Cotten, M. (1996). The complete DNA sequence and genomic organization of the avian adenovirus CELO. *J. of Virology* **70**, 2939-2949.

Ellmeier, W., Barnas, Ch., Kobrna, A., Kleiner, E., Kurzbauer, R., and Weith, A. (1996). Cloning and characterization of CpG islands of the human chromosome 1p36 region. *Genomics* **32**, 155-158.

Mechtler, K., and Wagner, E. (1997). Gene transfer mediated by influenza virus peptides: the role of peptide sequences. *New J. Chem.* **21**, in press.

Plank, Ch., Mechtler, K., Szoka, F.C., and Wagner, E. (1996). Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. *Human Gene Therapy* **7**, 1437-1446.

Schmidt, W., Steinlein, P., Buschle, M., Schweighoffer, T., Herbst, E., Mechtler, K., Kirlappos, H., and Birnstiel, M.L. (1996). Transloading of tumor cells with foreign major histocompatibility complex class I peptide ligand: A novel general strategy for the generation of potent cancer vaccines. *Proc. Natl. Acad. Sci. USA* **93**, 9759-9763.

Public Relations Department

Public Relations Officer Heidemarie HURTL

The IMP's PR-department serves as the interface between the scientists' work and the public. It has been the IMP's philosophy from the beginning to conduct no secret research but to publish all results. To assist those whose scope of knowledge is exceeded by scientific papers, the PR-department provides information at a different level, or establishes contact with the people who could give such information.

Over the past years, we have noticed a growing interest in topics relating to gene-technology. Public awareness has been stirred by spectacular experiments, by controversial ethical views and by the sheer wealth of information and techniques that are available today. Unfortunately, the complex matters of molecular biology are sometimes oversimplified in an attempt to get the news across to the general public. As a result, various different fields of research such as reproduction biology, gene technology and bio-engineering are not being distinguished from each other, and this confusing mixture creates uncertainty and distrust with regard to modern science in general. Being a basic research institution active in the field of molecular biology, the IMP sees it as its duty to keep up the dialogue with the public and the media and to have an open door to visitors.

Every year, a number of school-classes, usually in the last year of secondary education, come to the IMP to add some real-life experience to their theoretical knowledge. We try to match our tours with the school's curriculum and therefore maintain close contact to biology- and chemistry-teachers. It is good to see that our young visitors are usually very well prepared and have a high level of understanding in the biological sciences.

In 1996, apart from introducing the IMP to a number of school-classes, we organized a day for a group of journalism-students from the University of Vienna who were probing their skills as science-writers. Later in the year, we gave a tour to genetic-students from the University of Brno (Czech Republic), who were spending a day at the "Vienna Biocenter".

Another major group of visitors to the IMP are colleagues and collaborators within the Boehringer Ingelheim Group. We notice a growing interest in the IMP by B.I. employees from various backgrounds other than research. This reflects the increased awareness that Boehringer Ingelheim is essentially a research-driven company, and that future products are likely to come from such places as the IMP.

In April 1996, together with our sister-company Bender we organized a presentation for 120 external staff in the field of cardiology. Other B.I. visitors included colleagues from the PR-departments in Ingelheim and Biberach, a number of company-executives and the president of Nippon Boehringer Ingelheim.

Under the name of "Vienna Biocenter", the IMP also organizes joint events with the University of Vienna, such as the presentation of a book on gene-technology which was followed by a lively discussion with high-ranking scientists and politicians. The event was open to the public and drew a lot of attention.

At the beginning of the year, a press-conference was held at the IMP to present the achievements of the "Vienna Biocenter" to journalists and to announce the results of an evaluation-report by the European Molecular Biological Society.

Together with Bender, we organized the press-coverage of a workshop on Gene Therapy in Krumbach in April 1996. Not surprisingly, the talks were received with great interest by the media.

Accompanying press work was also done for our annual Spring Conference in May. Reports of the conference appeared in the main newspapers, as well as on TV and on Vienna's English speaking radio station.

In addition to covering these various events, the PR-department publishes press-releases, newspaper articles and the yearly Scientific Report. We also keep a video- and photo-archive, maintain contact to the media and help journalists with their research whenever possible.

Diploma- and PhD-theses of I.M.P. students in 1996

Aron Marchler-Bauer (Group Anton Beyer)
"The Calculation of Efficient Potentials of Mean Force and their Application in Fold Recognition." PhD-thesis, submitted to the University of Vienna.

Rüdiger Schweigreiter (Group Gerhard Christofori)
"Role of cysteine proteases in the regulation of apoptosis in murine ß tumor cells." Diploma-thesis, submitted to the University of Vienna.

Martin Schreiber (Group Erwin Wagner)
"Functions of the mammalian transcription factor AP-1 in mouse development, cell proliferation and the UV-response." PhD-thesis, submitted to the University of Vienna.

Oliver Wessely (Group Hartmut Beug)
"The control of proliferation and differentiation in avain erythroblasts." PhD-thesis, submitted to the University of Vienna.

Judith Antonia Lummerstorfer (Group Andreas Weith)
"Regional Fine Mapping of the Critical 1p36 Neuroblastoma Consensus Deletion with DIRVISH." Diploma-thesis, submitted to the University of Vienna.

Awards and nominations in 1996

Hartmut Beug
was appointed Professor at the University of Vienna.

Max L. Birnstiel
was admitted to the Honorary Degree of Doctor of Science by the University of Edinburgh (July 1996).

Meinrad Busslinger
was appointed Professor at the University of Vienna.

Kim Nasmyth
was appointed corresponding member of the Austrian Academy of Sciences and member of the EMBL Scientific Advisory Council and received the Unilever Science Award (November 1996).

Erwin F. Wagner
was awarded the Ludwig Wittgenstein Prize by the Austrian Science Fund (October 1996).

I.M.P./BIOCENTER seminar list 1996

JANUARY

12.01.96
MARK PTASHNE (Harvard)
"Chromatin constituents and gene activation and repression"
Host: Thomas Jenuwein

19.01.96
REINHARD LÜHRMANN (Marburg)
"Structure of UsnRNPs and their contribution to nuclear pre-mRNA splicing"
Host: Max Birnstiel

23.01.96
MAXIME BOUCHARD (Quebec)
"Early transcriptional regulation of the Drosophila segment polarity gene gooseberry-distal"
Host: Meinrad Busslinger

31.01.96
MATTHIAS WABL (UCSF)
"Hypermutability at the Immunoglobulin locus"
Host: Gerhard Christofori

FEBRUARY

05.02.96
ZDENKO HERCEG (St. Andrews, UK)
"Neoplastic transformation of human epithelial cells in vitro"
Host: Zhao-Qi Wang

08.02.96
MIGUEL BEATO (IMT Marburg)
"A promoter modulated by its structure in nucleosomes"
Host: Hartmut Beug

22.02.96
HERBERT JÄCKLE (Göttingen)
"Pattern formation in the Drosophila blastoderm"
Host: Meinrad Busslinger

29.02.96
JEAN-MARC EGLY (Strasbourg)
"TFIIH, at the crossroad of transcription, DNA repair and cell cycle regulation-studies of the ternary kinase complex: cdk 7, cyclin H and MAT1"
Host: Zhao-Qi Wang

MARCH

07.03.96
ROBERT WHITE (Univ. Glasgow)
"Retinoblastoma protein and the cell cycle regulation of RNA polymerase III transcription"
Host: Kim Nasmyth

08.03.96

GEORGES CARLE (Nice)
"Physical and genetic mapping of the centromeric region of mouse chromosome 19"
Host: Erwin F. Wagner

14.03.96

FIONA WATT (ICRF)
"Role of integrins in regulating keratinocyte growth and differentiation"
Host: Erwin F. Wagner

15.03.96

OLGA OKLADNOVA (Münster)
"Promoter analysis of mammalian Pax-8 genes"
Host: Meinrad Busslinger

21.03.96

RON T. HAY (St. Andrews)
"Control of NFκB activity by the IκB family of ankyrin repeat containing proteins"
Host: Matt Cotten

22.03.96

MARTA IZQUIERDO (Madrid)
"Gene therapy for malignant gliomas"
Host: Max Birnstiel

27.03.96

ROLF HEUMANN (Ruhr-Univ. Bochum)
"Transgenic activation of p21 ras in neurons"
Host: Erwin F. Wagner

28.03.96

QINGBO XU (Innsbruck)
"MAP kinase signal pathways in cardiovascular diseases"
Host: Zhao-Qi Wang

29.03.96

PETER GRUSS (MPI, Göttingen)
"Genes involved in the development of the visual system"
Host: Erwin F. Wagner

APRIL

10.04.96

EDGAR SCHMITT (Univ. Mainz)
"Cytokine regulated establishment of Th1 cells - a potential anti-tumor helper/effector T cell subtype"
Host: Michael Buschle

11.04.96

THEODORE FRIEDMANN (UCSD)
"Modified retroviruses for efficient gene transfer"
Host: Max Birnstiel

11.04.96

UNO LINDBERG (Stockholm)
"Can the profilin:actin structure tell us anything about cell motility and muscle contraction?"
Host: Gerhard Wiche

15.04.96
SABINE WERNER (Munich)
"Mesenchymal-epithelial interactions during tissue repair"
Host Gerhard Christofori

25.04.96
JOACHIM FRANK (Albany, NY)
"Three-dimensional visualization of tRNA in the ribosome by cryo-electron microscopy"
Host: Andrea Barta

25.04.96
RENÉ BERNARDS (Amsterdam)
"Cell cycle regulation by E2F transcription factors"
Host: Hartmut Beug

26.04.96
GIOVANNI SPINELLI (Palermo)
"Enhancer blocking function from sea urchin to man and temporal regulation by spacer sequences of the early H2A histone gene"
Host: Max Birnstiel

MAY

02.05.96
JOHN DOONAN (Norwich, UK)
"Cyclin and cdk gene expression in flowering plants"
Host: Erwin Heberle-Bors

09.05.96
IAIN MATTAJ (EMBL)
"A nuclear cap-binding protein complex; a connection between nuclear export and nuclear import"
Host: Andrea Barta

10.05.96
MICHAEL HENGARTNER (Cold Spring Harbor)
"Genetic control of programmed cell death in the nematode *C. elegans*"
Host: Kim Nasmyth

15.05.96
MARTIN BÄHLER (Tübingen)
"Unconventional myosins in signalling by small G-proteins of the rho subfamily"
Host: Lukas Huber

29.05.96
WALTER HUNZIKER (Lausanne)
"Signals and coat proteins in membrane traffic in polarized and non-polarized cells"
Host: Lukas Huber

30.05.96
DANIEL HABER (Harvard)
"Functional properties of the Wilms tumor suppressor gene WT1"
Host: Gerhard Christofori

JUNE

05.06.96
MICHEL DESJARDINS (Montreal)
"Phagolysosome biogenesis: the roles of GTPases and the vacuolar H^+ATPase"
Host: Lukas Huber

14.06.96
INGRID GRUMMT (DKFZ, Heidelberg)
"Cell cycle-dependent regulation of mammalian ribosomal gene transcription"
Host: Max Birnstiel

17.06.96
KARL MATTER (Geneva)
"Structure and function of tight junctions"
Host: Lukas Huber

20.06.96
DAVID LEVY (NYU Medical Center)
"Activation of gene expression by interferons and other cytokines"
Host: Thomas Decker

21.06.96
DOUGLAS HANAHAN (UCSF)
"Biological parameters of tumor development"
Host: Erwin F. Wagner

21.06.96
JUNYING YUAN (Harvard)
"Mechanism and functions of the ICE family in apoptosis and others"
Host: Kim Nasmyth

24.06.96
ERIC MEFFRE (Marseille)
"Identification and characterization of human pro-B cells in normal and pathologic bone marrow samples"
Host: Meinrad Busslinger

27.06.96
PETER-HANS HOFSCHNEIDER (Martinsried)
"Analysis of hepatitis B virus-related hepatocellular carcinogenesis"
Host: Max Birnstiel

28.06.96
HISAYOSHI NYM NAKAZAWA (IARC, Lyon)
"Negative regulation of telomerase activity in skin"
Host: Zhao-Qi Wang

JULY

03.07.96
DORIS APT (Singapore)
"Epithelial cell-specific regulation of human papillomavirus oncogene expression"
Host: Hartmut Beug

04.07.96
CHRISTIAN LEHNER (Tübingen)
"Cell cycle control in Drosophila"
Host: Kim Nasmyth

05.07.96
RODRIGO BRAVO (Princeton)
"The Rel/NF-κB family of transcription factors"
Host: VBC PhD-Program Students

15.07.96
KARL MATLIN (Mass. Gen. Hospital)
"Cell-substratum adhesion and epithelial cell polarization"
Host: Lukas Huber

17.07.96
DAVID SACKS (Harvard)
"Integration of Calmodulin in the insulin signalling pathway"
Host: Lukas Huber

23.07.96
JÜRGEN KNOBLICH (UCSF)
"Spindle orientation and asymmetric localization of Numb and Prospero during cell division in the developing Drosophila nervous system"
Host: Kim Nasmyth

25.07.96
LOUIS SMITH (Baylor College, Houston)
"DNA delivery using lipophilic peptides"
Host: Matt Cotten

26.07.96
MATTHEW L. FERO (Seattle)
"Abnormal growth in $P27^{kip1}$ knockout mice"
Host: Ernst Müllner

AUGUST

29.08.96
DAVID BENTLEY (Toronto)
"The role of the RNA pol II C-terminal domain in transcription and RNA processing"
Host: Kim Nasmyth

SEPTEMBER

02.09.96
TOM SATO (Harvard)
"Role of TIE1 and TIE2 in vascular development"
Host: Kim Nasmyth

09.09.96
JULIEN J. GHISLAIN (Toronto)
"Type I interferon signal transduction"
Host: Meinrad Busslinger

09.09.96
RICHARD LOSICK (Harvard)
"Asymmetric division and cell fate in a simple organism"
Host: Alexander von Gabain

23.09.96
SANDRA SCHMID (La Jolla)
"Dynamin, a "Blue Collar" GTPase"
Host: Karl Kuchler

OCTOBER

04.10.96
JAN VILCEK (New York Univ.)
"Activation of MAP kinases by TNF and IL-1"
Host: Ilja Vietor

10.10.96
BERNHARD DOBBERSTEIN (Heidelberg)
"Regulation of protein translocation across the membrane of the endoplasmic reticulum"
Host: Erhard Wintersberger

10.10.96
ARNIM PAUSE (NIH, Bethesda)
"Functional characterization of the von Hippel-Lindau tumor suppressor gene product"
Host: Kim Nasmyth

11.10.96
SUZANNE EATON (EMBL)
"Control of cytoskeletal polarity by Rac1 and Cdc42 in the wing epithelium of Drosophila"
Host: Kim Nasmyth

14.10.96
TONY HYMAN (EMBL)
"Mitotic spindle assembly and chromosome segregation"
Host: Kim Nasmyth

16.10.96
IVAN DIKIC (NYU Medical School)
"Tyrosine kinase Pyk2 couples extracellular signals with MAPK and JNK activation"
Host: Kim Nasmyth

17.10.96
RICHARD MAAS (HHMI and Harvard, Boston)
"Control of vertebrate lens induction by Pax6"
Host: Meinrad Busslinger

18.10.96
FRANK SAUER (UC Berkeley)
"TBP-associated factors (TAFs) are required for the transcriptional activation of *Drosophila* segmentation genes *in vitro* and *in vivo*"
Host: Kim Nasmyth

24.10.96
JOLA BOGUCKA GLOTZER (EMBL)
"Localization of oskar RNA in Drosophila oocytes"
Host: Kim Nasmyth

24.10.96
IRMA THESLEFF (Helsinki)
"Cell-cell signaling in the regulation of organogenesis"
Host: Erwin Wagner

25.10.96
MICHAEL GLOTZER (EMBL)
"Coordination of multiple cytoskeletal elements during cytokinesis"
Host: Kim Nasmyth

30.10.96
FRIEDRICH LOTTSPEICH (MPI Martinsried)
"Mass spectrometry methods in proteome research"
Host: Lukas Huber

31.10.96
DANIEL ST. JOHNSTON (Cambridge, GB)
"The origin of anterior-posterior polarity"
Host: Kim Nasmyth

NOVEMBER

05.11.96
TOM SATO (Harvard)
"Molecular analysis of vascular system development"
Host: Kim Nasmyth

06.11.96
JÜRGEN KNOBLICH (UCSF)
"Asymmetric cell division in the developing *Drosophila* nervous system"
Host: Kim Nasmyth

07.11.96
WILLIAM EARNSHAW (Edinburgh)
"INCENPs: where chromosomes and cytokinesis meet"
Host: Kim Nasmyth

14.11.96
STUART ORKIN (Boston)
"Regulatory circuitry for blood cell development"
Host: Erwin Wagner

21.11.96
EDMUND C. LIN (Harvard)
"Regulation of aerobic and anaerobic metabolism by the Arc system in *Escherichia coli*"
Host: Alexander von Gabain

25.11.96
MIRELLA GONZALEZ-ZULUETA (Baltimore)
"Identification of novel neuroprotective pathways in Huntington's disease"
Host: Erwin Wagner

DECEMBER

02.12.96

JEFF SCHATZ (Basel)
"ATP-dependent proteases as molecular chaperones"
Host: Kim Nasmyth

05.12.96

DAVOR SOLTER (Freiburg)
"Control of gene expression during early mammalian development"
Host: Thomas Jenuwein

06.12.96

ALAN BERNSTEIN (Toronto)
"The molecular genetics of hematopoiesis"
Host: Andreas Weith

12.12.96

JOSEPH CARROLL (ICRF London)
"Mis-expression of integrins and ifn-gamma in transgenic mouse skin: insights into disease and development"
Host: Erwin Wagner

I.M.P. Patent applications 1996

Addition CASE 14/032

"Method for generating potent anti-tumor immunity"
Inventors: M. Birnstiel, M. Buschle, W. Schmidt, T. Schweighoffer, P. Steinlein

Addition CASE 14/032

"Method for generating potent anti-tumor immunity"
Inventors: M. Birnstiel, M. Buschle, W. Schmidt, P. Steinlein

CASE 14/033

"Method for generating potent anti-tumor immunity".
Application: "Tumor vaccines and techniques for production"
Inventors: M. Birnstiel, W. Schmidt, P. Steinlein

CASE 14/034

"Compounds for the transfection of higher eukaryotic cells"
Inventor: K. Mechtler

CASE 14/035

"Drugs for the treatment of tumor diseases"
Inventors: H. Beug, M. Oft, E. Reichmann

CASE 14/036

"CELO Virus"
Inventors: A. Baker, M. Cotten, S. Chiocca, R. Kurzbauer, G. Schaffner

CASE 14/037

"Compounds that interfere with DNA replication in rapidly proliferating cells for use in therapy and methods for screening for such compounds"
Inventors: K. Nasmyth, S. Piatti

CASE 14/038

"Method for producing mammals with defined genetic characteristics"
Inventors: E.F. Wagner, Z.Q. Wang

I.M.P. Publications in 1996

1. Bartunek, P., Pichlikova, L., Stengl, G., Boehmelt, G., Martin, F.H., Beug, H., Dvorak, M., and Zenke, M. (1996). Avian stem cell factor (SCF): Production and characterization of the recombinant, His-tagged SCF of chicken and its neutralizing antibody. *Cytokine* **8**, 14-20.

2. Beug, H., Bauer, A., Dolznig, H., von Lindern, M., Lobmayer, L., Mellitzer, G., Steinlein, P., Wessely, O., and Müllner, E. (1996). Avian erythropoiesis and erythroleukemia: towards understanding the role of the biomolecules involved. Published on-line Oct. 16th, 1996, *BBA Rev. Cancer* **1288(3)**, M35-M47.

3. Birnstiel, M.L. (1996). Gene Therapy, Article in *European Review* **4**, 335-356.

4. Birnstiel, M.L. (1996). The Genetic Revolution 1950-1996: An Introduction. Article in *European Review* **4**, 333-334.

5. Bobola, N., Jansen, R., Shin, T., and Nasmyth, K. (1996). Asymmetric accumulation of Ash1 in post anaphase nuclei depends on a myosin and restricts yeast mating type switching to mother cells. *Cell* **84**, 699-709.

6. Busslinger, M., Klix, N., Pfeffer, P., Graninger, P. G., and Kozmik, Z. (1996). Deregulation of *PAX-5* by translocation of the Eμ enhancer of the *IgH* locus adjacent to two alternative *PAX-5* promoters in a diffuse large-cell lymphoma. *Proc. Natl. Acad. Sci. USA* **93**, 6129-6134.

7. Chiocca, S. and Cotten, M. (1996). Cellular responses to adenovirus entry. In: Gene Therapy for Diseases of the Lung. Ed. K.L Brigham. Marcel Decker Inc., New York.

8. Chiocca, S., Kurzbauer, R., Schaffner, G., Baker, A., Mautner, V., and Cotten, M. (1996). The complete DNA sequence and genomic organization of the avian adenovirus CELO. *J. of Virology* **70**, 2939-2949.

9. Cohen-Fix, O., Peters, J.-M., Kirschner, M.W., and Koshland, D. (1996). Anaphase initiation in *Saccharomyces cerevisiae* is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev.* **10**, 3077-3080.

10. Cotten, M., Baker, A., Saltik, M., Lehrmann, H., Panzenböck, B., and Chiocca, S. (1996). Receptor-mediated Gene Delivery. In: Falk Symposium no. 88, Molecular Diagnosis and Gene Therapy. Kluwer Academic Publishers, Lancaster, U.K.

11. Czerny, T. (1996). High primer concentration improves PCR amplification from random pools. *Nucl. Acids Res.* **24**, 985-986.

12. Dörfler, P., and Busslinger, M. (1996). C-terminal activating and inhibitory domains determine the transactivation potential of BSAP (Pax-5), Pax-2 and Pax-8. *EMBO J.* **15**, 1971-1982.

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7TH **IMP** SPRING CONFERENCE ON

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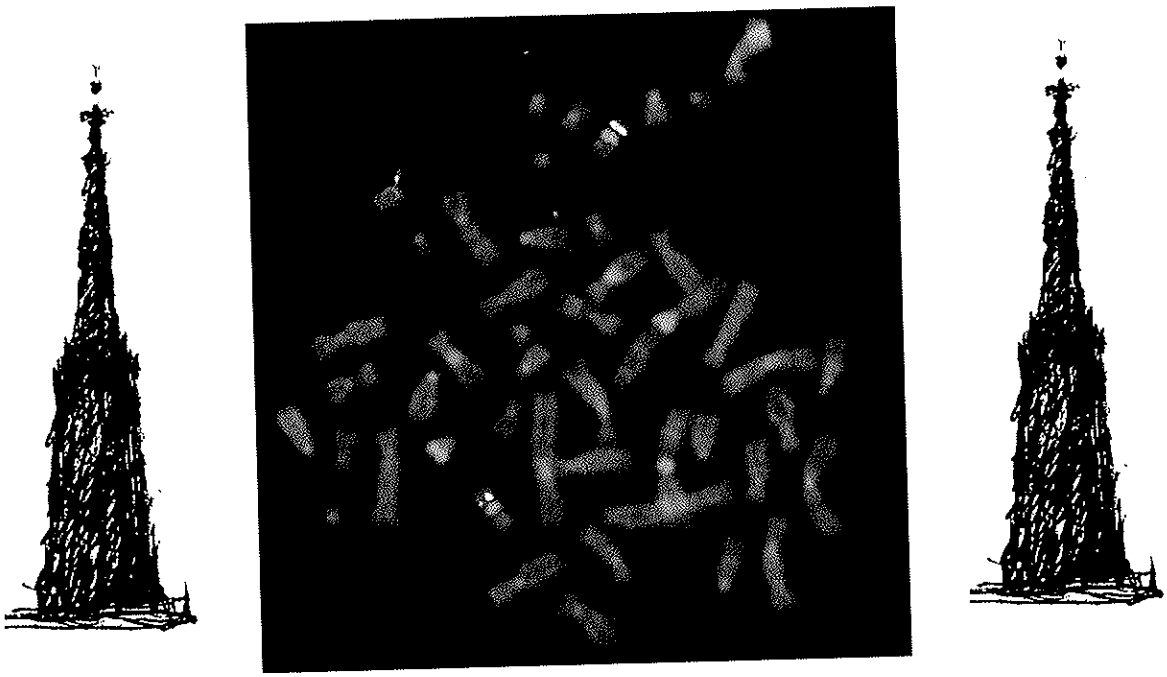

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Abstracts for poster presentations are invited; a few will be selected for oral presentations.

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