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In vivo detection of the BCR/ABL1 protein: towards a new therapeutic strategy for fusion protein associated leukemias

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In vivo Detektion des BCR/ABL1 Fusionsproteins: Enwicklung einer neuen Therapiestrategie für Fusionsprotein-assoziierte Leukämien

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Abstract

The BCR/ABL1 fusion protein is found in virtually all cases chronic myeloid leukemia (CML) and a large proportion of acute lymphoblastic leukemia (ALL). The fact that the BCR/ABL1 fusion protein is crucial for the development of leukemia makes this fusion protein an attractive target for therapy development. We have developed a strategy for the *in vivo* detection of the BCR/ABL1 fusion protein, in which the presence of the BCR/ABL1 fusion protein is detected intracellularly and if the fusion protein is present an arbitrary action is initiated in the cell (e.g. mark the cells or selectively kill the cells).

Our BCR/ABL1 detection strategy is based on protein-protein interactions. Two detection proteins are expressed in the cells: 1) protein A, a GAL4-DNA binding domain/BCR interacting protein fusion protein (GAL4DBD-BAP-1) and 2) protein B, a GAL4-activation domain/ABL interacting protein fusion protein (GAL4AD-CRKL). Only when BCR/ABL1 is present in the cell, do protein A, protein B, and BCR/ABL1 form a trimeric complex which activates the transcription of reporter genes under the control of GAL4-upstream activating sequence (UAS).

A proof of principle for the strategy was implemented in the yeast system. We did not use full length BAP-1 or CRKL but only those portions of the proteins that directly interacted with BCR or ABL, respectively. We showed in the yeast two hybrid system, that the C-terminus of BAP-1(amino acids 617-879) binds to full length BCR. The site of interaction of CRKL and ABL was confirmed to be the N-terminal SH3 domain (SH3n) of CRKL as described in the literature. Yeast cells (strain CG1945) transformed with a protein A expressing plasmid (pGBT9-BAP), a protein B expressing plasmid (pGAD424-CRKLSH3n), and a BCR/ABL expressing plasmid (pES1/BCR-ABL) showed expression of the reporter genes HIS3 and LACZ. The expression of the HIS3 reporter gene was assayed by growth of the yeast cells on

medium lacking histidine. The expression of the LACZ gene was verified by a beta-galactosidase filter assay. Yeast cells that were transformed with the pES1 plasmid without the BCR/ABL1 coding region did not show activation of the reporter genes. Several other negative controls demonstrated the specificity of the assay. Thus the method was able to clearly distinguish between BCR/ABL expressing cells and cells did not express BCR/ABL1.

We then adapted this system for use in mammalian cells. The open-reading frames encoding the proteins A and B were recloned into mammalian expression vectors. The human embryonal kidney cell line HEK293 and the murine myeloid progenitor cell line 32D which had been stably transfected with a BCR/ABL expressing plasmid were tested. The firefly luciferase gene and the yellow fluorescent protein (eYFP) were used to evaluate the whole cell population and single cell, respectively. Unfortunately, the system failed to work in the mammalian cell lines tested. Even though the detection system did not work in mammalian cells, most likely due to the cytoplasmic localization of the BCR/ABL1 fusion protein, it should still be a viable strategy for the detection of leukemia-associated fusion protein, which localize to the nucleus (i.e AML-ETO). This strategy could be adapted for purging the bone marrow of leukemia patients using therapeutically more useful effector genes like suicide genes, which encode pro-drug converting enzymes (e.g. HSV thymidine kinase), or markers that can easily be assayed (e.g. YFP).

Zusammenfassung

Man findet das BCR/ABL Fusionsprotein bei fast allen Fällen von chronisch myeloischer Leukämie (CML) und bei einem großen Anteil von akuten lymphoblastischen Leukämien (ALL). Die Tatsache, dass das BCR/ABL Fusionsprotein für die Entwicklung der Leukämie notwendig ist, macht dieses Fusionsprotein zu einem idealen Ziel für Therapieentwicklungen. Wir haben eine Strategie zur in vivo Detektion des BCR/ABL1 Fusionsproteins entwickelt, mit der die Anwesenheit von BCR/ABL1 in der lebenen Zelle nachgewiesen werden kann und mit der abhängig von der Anwesenheit des Fusionsproteins eine frei-wählbare Aktion angestoßen werden kann (z.B. die Zellen können mit einem Protein markiert werden oder die Zellen können spezifisch abgetötet werden).

Unsere BCR/ABL1 Detektionsstrategie basiert auf Protein-Protein Interatkionen. Zwei Detektionsproteine werden in den Zellen exprimiert: 1) Protein A, ein Fusionsprotein bestehend aus einer GAL4-DNA-Bindungsdomäne und einer BCR-Interaktionsdomäne (GAL4DBD-BAP-1) und 2) Protein B, ein Fusionsprotein, das aus einer GAL4-Aktivierungsdomäne und einer ABL-Interaktionsdomäne besteht (GAL4AD-CRKL). Nur wenn BCR/ABL1 in der Zelle vorhanden ist, kann sich aus Protein A, Protein B und BCR/ABL ein trimerer Komplex bilden, der die Transkription von Reportergenen, die von einer GAL4-Upstream Activating Sequence (UAS) reguliert werden, aktiviert.

Um die prinzipielle Durchführbarkeit dieser Detektionsstragie zu beweisen, wurde das System zunächst in Hefe etabliert. Hierzu wurden nicht das gesamte BAP-1 bzw. CRKL Protein verwendet, sondern nur die Domänen dieser Proteine, die direkt mit BCR bzw. ABL interagieren. Wir konnten im Hefesystem zeigen, daß der C-Terminus von BAP-1 (Aminosäuren 617-879) in der Lage ist, mit dem kompletten BCR-Protein

zu interagieren. Die N-terminale SH3-Domäne von CRKL konnte als ABL1 Interaktionsdomäne bestätigt werden, wie es bereits in der Literatur beschrieben wurde. Hefezellen (Stamm CG1945), die mit einem Protein A exprimierenden Plasmid (pGBT9-BAP), einem Protein B exprimierenden Plasmid (pGAD424-CRKLSH3n) und einem BCR/ABL1 exprimierenden Plasmid transformiert wurden, zeigten Expression der Reportergene HIS3 und LACZ. Die Expression des HIS3 Reportergens konnte dadurch gezeigt werden, daß die Hefezellen auf Histidin-freiem Medium wuchsen. Die Expression des LACZ-Gens wurde in einem beta-Galactosidase Filterassay nachgewiesen. Hefezellen, die mit dem pES1 Plasmid, das keine BCR/ABL kodierende Region enthielt, transformiert worden waren, zeigten keine Reportergenexpression. Durch zahlreiche negative Kontrollen konnte die Spezifität des Assays gezeigt werden. Somit war diese Strategie in der Lage, sehr klar zwischen Hefezellen, die das BCR/ABL1 Fusionsprotein exprimierten, und solchen, die BCR/ABL nicht exprimierten, zu unterscheiden.

Daraufhin adaptierten wir das System, so dass es auch in Säugetierzellen funktionierte. Hierzu wurden die für die Detektionsproteine A und B kodierenden Sequenzen in Säugeexpressionsvektoren umkloniert. Die menschliche embryonale Nierenzellinie HEK293 und die murine myeloische Vorläuferzellinie 32D, die beide stabil mit einem BCR/ABL1 exprimierenden Plasmid transfiziert worden waren, wurden benutzt, um das Detektionsystem zu testen. Als Reportergene wurde Firefly Luciferase und das “enhanced yellow fluorescent” Protein (eYFP) verwendet. Mit der Luciferase konnte die durchschnittliche Reportergenaktivierung gemittelt über die gesamte Zellpopulation ermittelt werden, während mit eYFP die Reportergenaktivität in einzelnen Zellen nachgewiesen werden konnte. Unglücklicherweise funktionierte das Detektionssystem in den verwendeten Zellinien nicht. Obwohl der Nachweis von BCR/ABL1 in Säugertierzellen nicht gelang, was mit großer Wahrscheinlichkeit auf die zytoplasmatische Lokalisation des BCR/ABL1 Fusionsproteins zurückzuführen ist, sollte die Strategie zur Detektion von nukleären Fusionsproteinen (eg. AML1-ETO)

geeignet sein. Diese Strategie könnte durch die Verwendung von geeigneten Reportergenen, wie Suizidgene, die ein Pro-drug Converting Enzym kodieren (e.g. HSV Thymidinkinase) oder Genen, die Markerproteine kodieren (e.g. YFP), für den therapeutischen Einsatz, wie z.B. dem Purgen von leukämischen Knochenmark, modifiziert werden.

Introduction

Brief overview of genetics of leukemia and rationale for the development of new therapeutic strategies.

Recent advances in molecular genetics have greatly increased our understanding of the essential clinical, biological and molecular features of leukemia. Leukemia is a very heterogeneous disease on the molecular level. A common biologic feature, shared by genetically heterogeneous acute myeloid leukemias (AML), is a block of hematopoietic differentiation caused by fusion proteins which result from chromosomal translocations (Burmeister and Thiel 2001; Moe-Behrens and Pandolfi 2003). AML-associated fusion proteins function as aberrant transcriptional regulators that interfere with the process of myeloid differentiation, result in a stage-specific arrest of maturation and enhance cell survival in a cell-type specific manner. The abnormal regulation of transcription networks occurs through common mechanisms that include, for example, disruption of common signaling pathway (Alcalay, Orleth et al. 2001). Considering the existence of common mechanism underlying leukemogenesis, the development of therapeutic strategies that target the pathways common to more than one fusion protein might be feasible, as well as the development of strategies which are specific for a given subtype. However, the development of therapeutic strategies that are specific for a leukemia-causing genetic aberration is still in its infancy. Current strategies for the treatment of leukemia are mainly based on conventional chemotherapeutic agents which do not differentiate between normal and

malignant hematopoietic cells.

Detecting leukemic fusion proteins *in vivo*

To take advantage of leukemia-specific genetic alterations to differentiate between normal and malignant cells for a therapeutic approach we have devised a strategy to detect leukemia-specific fusion proteins *in vivo*. The capability to detect leukemia-specific fusion proteins *in vivo* would be highly desirable because it would open up new approaches to study leukemia and might lead to novel treatment strategies. We chose the BCR/ABL1 fusion protein as a paradigm to develop our strategy because this fusion protein is the key factor in the development of chronic myelogenous leukemia (CML).

CML as a test disease for the development of targeted strategies

CML is a clonal proliferative disorder of hematopoeitic stem cells. Myeloid, erythroid, megakaryocyte, and B-lymphoid cells are involved in the process of this clonal proliferation and differentiation. Characteristically, CML has a biphasic course evolving from a chronic phase (CP) with a median duration of 3-4 years to an accelerated phase and finally to a blast crisis which is usually fatal within 3-6 month (Faderl, Talpaz et al. 1999; Faderl, Talpaz et al. 1999; Sawyers 1999). On the genetic level, in more than 95% of patients with CML, a reciprocal translocation between chromosomes 9 and 22 is present (t(9;22)(q34;11)) (Faderl, Talpaz et al. 1999). The der(22) chromosome is also known as Philadelphia chromosome (Ph+). The translocation causes the fusion of the ABL1 gene from 9q34 to the BCR gene from 22q11 (Bartram, de Klein et al. 1983; Pane, Intrieri et al. 2002). The resulting BCR/ABL1 fusion gene encodes the BCR/ABL1 fusion protein which is a constitutively active tyrosine kinase. Animal models have been instrumental to understand the role of the BCR/ABL1 fusion protein in inducing and sustaining the leukemic phenotype of CML (Daley, Van Etten et al. 1990; Heisterkamp, Jenster et al.

1990; Kelliher, McLaughlin et al. 1990). The fact that the BCR/ABL1 fusion protein is the main causative factor of CML pathology made this protein an ideal target for the development of new targeted therapies.

Imatinib mesylate (STI571), a potent and relatively selective tyrosine kinase inhibitor of ABL1 and BCR/ABL1, which represents a highly effective therapy for CML, has recently been approved by Federal Drug Administration (FDA) in the United States (Kantarjian, Cortes et al. 2002). However, clinical resistance against Imatinib due in part to point mutations in the BCR/ABL1 fusion gene occurs frequently thus making the development of alternative strategies for the treatment of this disease desirable (Gorre, Mohammed et al. 2001; Hochhaus, Kreil et al. 2001; Shah, Nicoll et al. 2002; Shah and Sawyers 2003). Recently, it could be shown that siRNAs (small interfering RNA), which are specific against the breakpoint sequences of the BCR/AL1 fusion gene can silence BCR/ABL1 expression and sensitize the cells against Imatinib mesylate (Scherr, Battmer et al. 2003). But, siRNAs are difficult to administer and cannot be used in patients yet. Furthermore, neither Imatinib nor siRNA lead to the apoptosis of all leukemic cells. These treatment strategies (tyrosine kinase inhibitor and repression via siRNA) either interfere with the function or expression of BCR/ABL1, but do not eliminate or selectively kill BCR/ABL positive cells. Therefore, the development of a new strategy which is able to selectively kill BCR/ABL positive cells would be advantageous.

Principle of BCR/ABL1 detection strategy

We have started to develop such a strategy which employs two steps (Fig1). The first step is to detect the presence of BCR/ABL1 fusion protein *in vivo*. If a fusion protein is detected in a cell, an action can be initiated in the second step. This action may be the expression of a marker protein,

which would allow selection of the BCR/ABL positive cells, or the expression of a pro-drug converting enzyme, which could permit the selective killing of the leukemic cells after adminstration of the appropriate pro-drug.

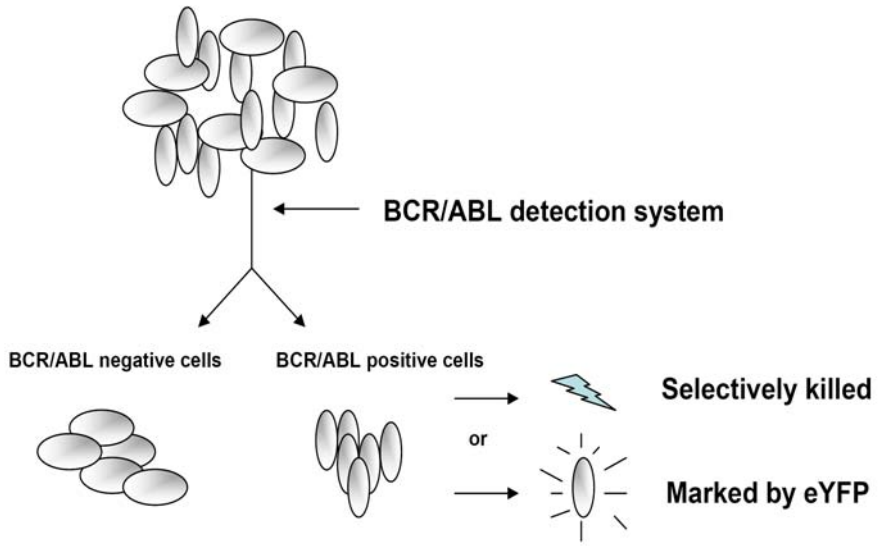


Figure 1 Principle of BCR/ABL1 detection strategy. The strategy consists of two steps. The first step is to detect the BCR/ABL1 fusion protein. If the BCR/ABL1 fusion protein is present, then the BCR/ABL1 positive cells will be marked by e.g. eYFP or selectively killed.

The first step in our strategy is the *in vivo* detection of the BCR/ABL1 fusion protein. To achieve this *in vivo* detection, we used protein-protein interactions. This strategy relies on two detection proteins (protein A and protein B) which are expressed in the cells: 1) protein A, which is a fusion of the yeast GAL4-DNA binding domain and an BCR-interacting protein, and 2) protein B, which is a fusion of the yeast GAL4 transcriptional activation domain and an ABL1 interacting protein. Only when BCR/ABL1 is present, do protein A, protein B, and BCR/ABL1 fusion protein form a trimeric complex which activates the transcription of a reporter gene under the control of GAL4 upstream activating sequence (GAL-UAS). It should be noted that in this setting the reporter gene can

be freely chosen. As mentioned above, this reporter gene can code for a marker protein (e.g. the yellow fluorescent protein) which would help to recognize BCR/ABL1 positive cells, or the reporter gene can be a pro-drug converting enzyme (e.g. thymidine kinase). In this case the BCR/ABL1 positive cells would be sensitive to the pro-drug.

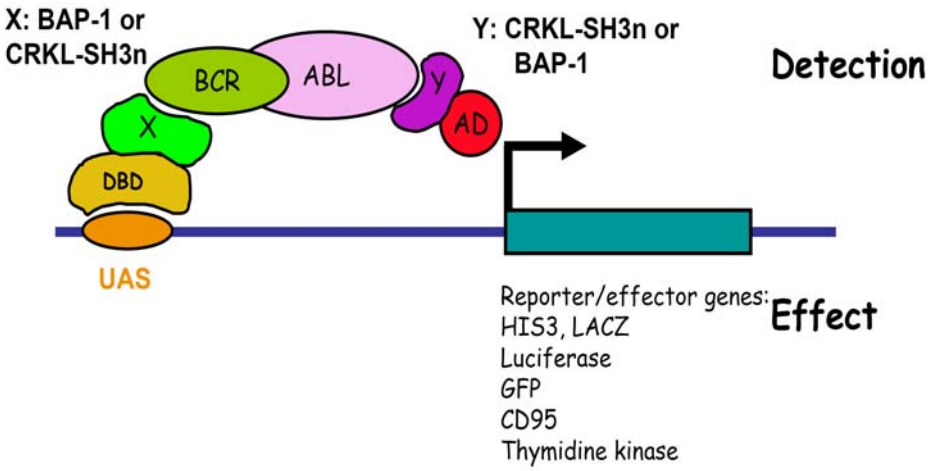


Figure 2 Diagram of the BCR/ABL1 fusion protein detection strategy. Two proteins are expressed in this system: one is fusion protein of BCR interacting protein BAP-1 (X) and the GAL4-DNA binding domain (protein A), the other is a fusion of the ABL interacting protein CRKL(Y) and the GAL4-activation domain (protein B). If the BCR/ABL1 fusion protein is present, the BCR/ABL1 protein, protein A and protein B form a trimeric complex which transactivates the reporter gene. The reporter gene is then only expressed in the presence of the BCR/ABL1 fusion protein. The reporter gene can be freely chosen.

It was the aim of this work to improve the efficiency of the BCR/ABL1 detection strategy and use it in mammalian cells.

Background

CML and the BCR/ABL1 fusion gene

CML is a clonal myeloproliferative disorder of primitive hematopoietic stem cells. It involves the myeloid, erythroid, megakaryocytic, B-lymphoid and occasionally the T-lymphoid lineages. CML has an incidence of 2 cases per 100,000 people per year and accounts for 15 % of leukemias in adults. CML typically is a biphasic disease that is characterized by a chronic phase followed by a blast crisis. Most cases are diagnosed in the chronic phase. Approximately 50 % of patients in the chronic phase have no symptoms and are diagnosed by routine testing. Signs and symptoms can include fatigue, weight loss, abdominal fullness, bleeding, sweating, purpura, splenomegaly, anemia and hepatomegaly. The white blood cell count is usually higher than 20,000/ul, in some cases more than 80.000/ul. The mean duration of the chronic phase is 3-4 years. Prior to entering the blast crisis, 75 % of patients develop an intervening accelerated phase, which is characterized by worsening of the blood counts and symptoms. The accelerated phase progresses to blast crisis within 3-18 months. One-third of blast crisis cases are acute lymphocytic leukemia (ALL), while two-thirds are acute undifferentiated leukemia (AUL) or acute myelogenous leukemia (AML).

Molecular pathogenesis of CML

The hallmark of CML is the Philadelphia chromosome, a der(22)t(9;22)(q34,q11) chromosome which carries the BCR/ABL1 fusion gene. In 1960, Nowell and Hungerford (Nowell P 1960) described an abnormally shortened G-group chromosome, later termed the Philadelphia chromosome (Ph+) in the leukemic cells of a patient with CML. 13 years later, in a

landmark paper, Janet Rowley using the newly developed technique of fluorescence chromosome banding (Quinacrine banding) demonstrated that Ph+ was in fact a der(22)t(9;22)(q34,q11) chromosome and the result of a reciprocal translocation of chromosome 9 and 22 (Rowley 1973). At the molecular level, the Ph+ chromosome results in the juxtaposing of the 5' portion of the *BCR* gene on chromosome 22 to the 3' portion of the *ABL1* gene on chromosome 9. The BCR/ABL1 fusion gene on the Ph+ chromosome can code for three variants of the BCR/ABL1 fusion protein which differ slightly in their molecular weight: the p190, the p210, and the p230. These three variants come about because there are slight differences in the breakpoint location on chromosome 22. These three main variants of BCR/ABL1 fusion protein are associated with distinct clinical types of leukemia. The p190 is typically associated with ALL, the p230 with chronic neutrophilic leukemia (CNL) and the p210 with CML. However, there is some overlap. The p210 occurs in 40% of Ph+ ALLs, the p190 in 2-3% of CML and the p230 in some cases of CML (Melo 1996).

Expression of the BCR/ABL1 fusion protein increases cell proliferation, decreases apoptosis, leads to cytokine independent growth, decreases adhesion to the bone marrow stroma and produces cytoskeletal abnormalities. Animal models of CML have been central to the understanding of the role of the BCR/ABL1 and served as models to evaluate the consequences of inhibiting BCR/ABL1 function. The following studies supported a role for the BCR/ABL1 fusion protein in the induction of leukemia: transgenic mice expressing p190 developed myeloid or lymphoblastic leukemia (Heisterkamp, Jenster et al. 1990); murine bone marrow cells infected with a retrovirus expressing p210 induced a CML-like pathology in 100% of recipient mice (Daley, Van Etten et al. 1990). The disease found in these mice had many features in common with human CML. The p230 also resulted in a CML-like

myeloproliferative disease in a murine bone marrow transplantation model (Li, Ilaria et al. 1999). In another study, using an inducible transgenic mouse model, p210 was expressed constitutively but could be rapidly down regulated upon administration of doxycycline to the animals. Most of the mice had an increased leukocyte count, and died from leukemia. Administration of doxycycline which caused repression of p210 expression induced leukemic cell apoptosis and normalization of the peripheral white cell count within 3 days (Huettner, Zhang et al. 2000). This study demonstrated that the fusion protein was required for the maintenance of the leukemia. These studies clearly showed that the BCR/ABL1 fusion protein would be an ideal target for treating CML.

Treatment of CML and targeted therapy

Three treatment options have been used in CML: chemotherapy, biological response modifier and bone marrow transplantation.

Chemotherapy: the first chemotherapeutic agent used to treat CML was busulfan, an excellent agent for controlling the chronic phase of the disease. Busulfan leads to hematological remission so that chemotherapy with this agent has been the mainstay of CML treatment even though it has serious side effects like pulmonary fibrosis, secondary leukemias, weakness and skin hyperpigmentation. Because of the toxic effects of busulfan, hydroxyurea has been used to initiate therapy in patients with CML. Hydroxyurea does not have the side effects observed with busulfan and for that reason some therapists prefer it (Hehlmann, Heimpel et al. 1993). Several other chemotherapeutic agents are also used to control the disease, however they are largely inferior to busulfan or hydroxyurea. With conventional treatment busulfan, hydroxyurea, or other cytotoxic drugs, it is usually possible to control the leukocyte count and to reduce the size of the spleen during the chronic phase of the disease, but complete

cytogenetic remissions (that is absence of the Ph+ from bone marrow cell metaphases) are only rarely observed. Conventional treatment, at best, has only a marginal effect on improving survival.

Biological response modifiers: in an effort to improve survival and to obtain complete hematologic and cytogenetic remission, IFN-α was introduced in 1983 (Talpaz, McCredie et al. 1983) and has since become the standard therapy for patients with CML, who are not candidates for bone marrow transplantation. 20-25% of patients achieve a complete cytogenetic remission and 10-15% a partial cytogenetic remission. However, the toxicities of IFN-α are significant and as many as one-quarter of CML patients stop treatment with IFN-α because of adverse reactions. Clinical trials of combination of IFN-α with chemotherapy have been shown to be superior to IFN-α alone (Lindauer and Fischer 2001). The precise mechanism of action of IFN-α in CML treatment remains unknown. Some studies show that the effect of IFN-α is related to its ability to modulate the immune system (Molldrem, Lee et al. 2000). Many other immunotherapeutics like dendritic cells, cytotoxic T lymphocyte and donor lymphocyte infusion are being used in the clinical trials. However, it is not yet clear whether they are able to improve the outcome. Longer periods of observation are necessary (Garcia-Manero, Faderl et al. 2003).

Bone marrow transplantation: Currently, allogeneic bone marrow transplantation (BMT) is the only curative treatment available for patients with CML. Allo-BMT achieves a 70% cure rate and an 80% five-year survival (Thijsen, Schuurhuis et al. 1999; Hehlmann, Hochhaus et al. 2000). However, only a minority of patients can be transplanted because of age or lack of an HLA-matched bone marrow donor (Thijsen, Schuurhuis et al. 1999).

Targeted therapy: Advances in the understanding of the molecular mechanisms which sustain leukemic cells in CML have made possible the development of selective therapies for this disease. STI571 or Imatinib, a potent and relatively selective tyrosine kinase inhibitor of ABL1, BCR/ABL1, and c-KIT has been of particular interest because of its efficacy in treating CML (Carroll, Ohno-Jones et al. 1997). STI571 treatment induces complete hematologic response in 90 % and a major cytogenetic response in 60% of patients with CML in chronic phase. Patients responding to STI571 have a 1000 to 100 000 fold reduction in BCR/ABL transcript levels compared to starting values (Hughes, Kaeda et al. 2003). However, the majority of patients who responded well to STI571 still have measurable of BCR/ABL1 transcript in their blood --- at least during the first two years of follow-up (Lahaye, Riehm et al. 2005). There is also evidence that CML patients in complete cytogenetic remission still have Ph+ myeloid progenitors and stem cells in their marrow. Another problem is that resistance to STI571 occurs frequently and it is not known how this can be prevented or delayed (Kaeda, Chase et al. 2002). The effect of STI571 on the long-term survival of CML patients remains unknown as well. Despite these problems, targetet therapy using STI571 has ushered in a new era of leukemia therapy. The success of STI571 therapy clearly shows that the BCR/ABL1 fusion protein is the correct target for CML therapy and using BCR/ABL1 as a target is the way to develop a more effective therapy, the aim of which should be the selective killing and elimination of Ph+ cells.

In vivo detection of BCR/ABL1 fusion protein

As mentioned above, we have developed a strategy to detect the BCR/ABL1 fusion protein *in vivo*, which is based on protein-protein interactions. This strategy is derived from the yeast two-hybrid assay which is briefly explained in the following paragraphs.

The yeast two-hybrid assay, developed by Stanley Fields and coworkers, is a genetic assay in yeast to detect protein-protein interaction *in vivo* (Fields and Song 1989). It is based on the fact that many eukaryotic transcriptional activators consist of two protein domains which can be physically separated: one acts as the DNA binding domain, while the other functions as the transcriptional activation domain. The DNA binding domain recognizes specific DNA sequences which are present in the upstream regions of the genes that are regulated by the factor, while the activation domain contacts and recruits components of the basal transcription machinery which are required to initiate transcription. Both domains are necessary for specific gene activation, and the two domains can be either part of the same protein or they can be on separated proteins and be assembled *in vivo* at the promoter. In the original yeast two hybrid system, which is based on the yeast GAL4 transcription factor, two hybrid proteins are expressed in the yeast cell: one consists of the GAL4-DNA binding domain fused to protein X, and the other consists of the GAL4-activation domain fused to a second protein Y. Interaction between protein X and protein Y leads to the transcriptional activation of a reporter gene driven by the specific UAS (upstream activation sequence) for the GAL4 DNA-binding domain, (Fig3)

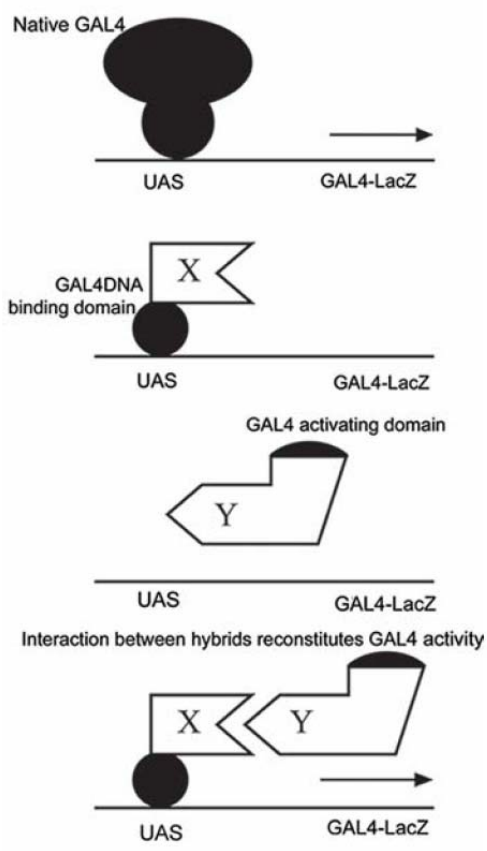


Figure 3 Schematic diagram showing the principle of the yeast two hybrid system. In the upper part of the figure, the native GAL4 protein containing both DNA-binding and activating domain induces GAL4-lacZ transcription. In the middle part of the figure, hybrids containing either the GAL4 DNA-binding with protein X or the GAL4 activating domain fused to protein Y are incapable of inducing transcription on their own. In the lower part of the figure, protein-protein interaction between protein X and protein Y brings the GAL4 activation domain into close proximity to the promoter, which results in the activation of transcription. Adapted from Fields and Song, Nature 1989, 340 (20): 245.

Based on the yeast two hybrid system we developed a strategy so that the expression of the BCR/ABL1 fusion protein could be detected in yeast. This was done in the following way: instead of protein X and protein Y interacting directly with each other to induce the expression of the reporter genes, we chose a protein X that would interact with BCR and a protein Y which is capable of interacting with ABL1. Protein X and Y are now no longer able to directly interact with each other and expressing

protein A (GAL4-DBD + BCR interactor) and protein B (GAL4-AD + ABL1 interactor) will not turn on the expression of the reporter gene. However, if the BCR/ABL1 fusion protein is expressed in the cells a trimeric complex consisting of protein A, BCR/ABL1fusion protein, and protein B will form which is able to turn on transcription of the reporter gene (Fig 2). In our experiments we chose as the BCR interacting protein the BAP-1 protein, which is a member of the 14-3-3 proteins (Reuther, Fu et al. 1994). As the ABL1 interacting protein the CRKL protein was chosen (Oda, Heaney et al. 1994).

The BAP-1 protein, BCR-associated protein, was first described in 1994 (Reuther, Fu et al. 1994). It was isolated from a mouse embryo cDNA expression library screened by the recombinant ^{32}P-labelled BCR kinase domain as a probe. Its sequence is identical to a member of the 14-3-3 family proteins, the 14-3-3 τ isoform. 14-3-3 proteins are expressed in all mammalian tissue and widely conserved in other eukaryotic organism including plants, insets and yeast. In some cell lines, i.e. Jurkat human T-cell lymphoma and the HeLa human carcinoma cell line, 14-3-3 proteins are also expressed. In *in vivo* and *in vitro* binding assays, the BAP-1 protein interacts directly with the full-length BCR protein and the BCR/ABL1 protein, but not with c-ABL. The region of the BCR protein that mediates the BAP-1 protein binding is located at the N-terminal serine-threoine kinase domain, which is capable of autophosphorylation (Reuther, Fu et al. 1994; Michaud, Fabian et al. 1995). The three dimensional structure of some 14-3-3 proteins have been solved and show a high proportion of α-helices. 14-3-3 proteins form homo-dimers which allows them to be adapter proteins between different signaling proteins (Petosa, Masters et al. 1998). The precise role of BAP-1 in CML remains

unclear.

As an ABL1 interactor we chose the CRKL protein. In contrast to the BAP-1 protein, which is the substrate of the BCR serine-threoine kinase, the CRKL protein is the substrate of the ABL1 tyrosine kinase (Oda, Heaney et al. 1994). The CRKL protein belongs to the CRK protein family and is located on human chromosome 22, band q11, at least 500 kb to 1000 kb proximal to the BCR gene (ten Hoeve, Morris et al. 1993). Lacking a catalytic domain, CRKL consists of an N-terminal SH2 domain, followed by two SH3 domains. Through its N-terminal SH3 domain, CRKL interacts directly with ABL1 and BCR/ABL1 in the proline rich region of ABL (ten Hoeve, Morris et al. 1993; Feller, Ren et al. 1994; Heaney, Kolibaba et al. 1997). This protein was observed to be tyrosine phosphorylated in all CML cell lines and CML patient samples (Nichols, Raines et al. 1994; Oda, Heaney et al. 1994). Many studies provide direct and indirect evidence that the CRKL protein plays an important role in signal transduction pathway that originate from the BCR/ABL1 fusion protein (Uemura, Salgia et al. 1997; Sattler and Salgia 1998; Rhodes, York et al. 2000; Grumbach, Mayer et al. 2001).

The BCR/ABL1 fusion protein physically or functionally interacts with a wide range of other proteins. The choice of BAP-1 and CRKL as the interacting partners for the BCR part and the ABL part, respectively, for the BCR/ABL1 detection system was shown to be correct since in yeast the system was shown to work properly. In her doctoral thesis, Nicole Froehlich could show that a trimeric complex consisting of BAP-1, BCR/ABL1, and CRKL formed, leading to the transcriptional activation of the reporter genes LacZ and His3 in yeast (Fröhlich 2000).

Although the detection of the BCR/ABL1 fusion protein was quite successful in yeast, we wanted to detect BCR/ABL1 in mammalian hematopoietic cells.

The yeast two-hybrid system had already been successfully adapted by Dang et al. (Dang, Barrett et al. 1991) for use in mammalian cells.

This so-called mammalian two-hybrid assay relies on the functional reconstitution of GAL4-VP16, an artificial transcription factor containing the GAL4-DNA binding domain fused to the acidic transactivation domain of the herpes simplex virus VP16 protein. If interaction between proteins X and Y occurs in the mammalian cell the transcription of a reporter gene, which is under the control of the UAS, the recognition motif of the GAL4 DNA-binding domain, is switched on (Fig 4). In general, to test a given protein-protein interaction in the mammalian two-hybrid system one has to clone the cDNAs of the two proteins to be tested into the appropriate mammalian expression vectors.

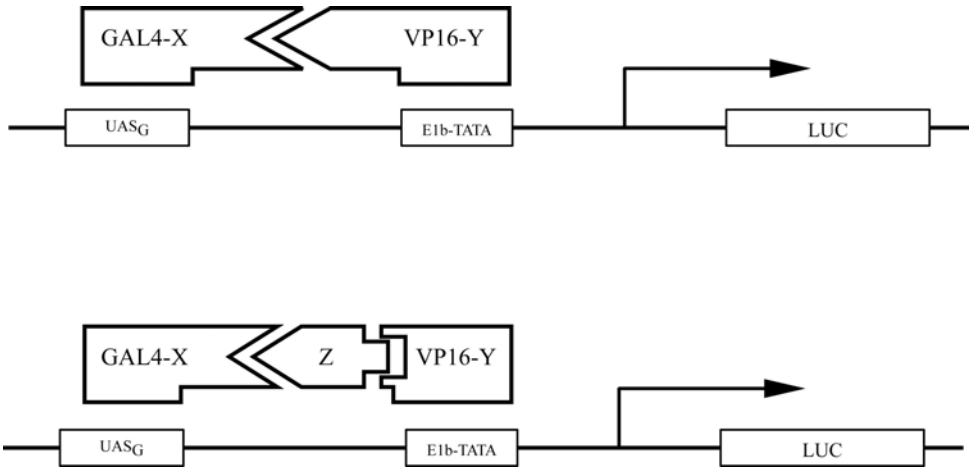


Figure 4 Mammalian Two-hybrid system. Upper panel: the conventional mammalian two-hybrid assay for the detection of protein–protein interactions. Cells are co-transfected with the reporter plasmid and expression vectors encoding the GAL4DBD-X and VP-16-Yfusion protein. *In vivo* association between the X and Y proteins induces transcription of the reporter gene. Lower panel: the bridge mammalian two hybrid assay to detect the formation of a multi-protein complex. Cells are co-transfected with the reporter gene, the expression vector for GAL4DBD-X, VP16-Y and an expression vector encoding a third protein (Z), which interacts with both the X and Y proteins. The expression of the reporter gene is induced by the formation of a stable trimeric complex involving proteins GAL4DBD-X, VP16-Y and Z. Adapted from Bartel P.L & Stanley Fieds et al, The Yeast Two Hybrid System Oxford University, 1997, 219

As an extension of the mammalian two-hybrid assay Wadman and Valge-Archer (Valge-Archer, Osada et al. 1994; Wadman, Li et al. 1994) developed the so-called mammalian bridge two-hybrid assay (Fig 4). This assay is capable of detecting the *in vivo* formation of multi-protein complexes in mammalian cells. The bridge two-hybrid system resembles closely the strategy that we developed to detect the BCR/ABL1 fusion proteins and shows that such a strategy can work in principle. However, it has to be kept in mind that both the mammalian two hybrid assay and especially the mammalian bridge two hybrid assay are much more stringent assays for detecting protein-protein interactions than the yeast two hybrid assay or other methods to detect protein-protein interactions like co-immunoprecipitations or glutathion-S transferase pull-downs. This means that interactions that can easily be detected in the yeast two-hybrid system are not necessarily amenable to analysis with the mammalian two-hybrid assay.

As stated above, one goal of this thesis work was to modify the detection of the BCR/ABL1 fusion protein from the yeast system so that it would work in mammalian cells (Fig 4). Nicole Fröhlich had already performed the first experiments to try to detect the BCR/ABL1 fusion protein in mammalian cells (murine NIH3T3 fibroblasts). However, these experiments had not been very successful, i.e. the detection of BCR/ABL1 fusion protein could not be achieved reliably. It was thus necessary to test different deletion mutants of interactor proteins and different cell lines and transfection methods to try to achieve a reliable detection of the BCR/ABL1 fusion protein. A further goal of this work was to test different reporter genes that would be more useful if the detection system was to be used in a therapeutic setting. This also implied that the detection of the BCR/ABL1 fusion

protein should be reliable at the single cell level.

Using our detection system, we would strive to eliminate the BCR/ABL positive cells from the bone marrow from patients with BCR/ABL positive leukemia. In the mean time the patient would have received high dosage myeloablative chemotherapy. AFter this myeloablative therpy, the patient would be given back his own purged bone marrow. It is our hope that such a therapy would be curative.

Material and Method

1. Chemicals and lab ware

1.1 Chemicals and reagents

Agarose	Roth, Karlsruhe
Agar	Roth, Karlsruhe
Bromophenol blue	Merck, Darmstadt
Calf intestine alkaline phosphatase	NEB, England
DMEM	PAN, Nürnberg
DMSO	Merck, Darmstadt
DNA-polymerase I (klenow fragment)	NEB, England
Ethanol	Merck, Darmstadt
Ethidimbromid	Amresco, Solon, USA
Fetal calf serum	Gibco-BRL, Karlsruhe
Isopropanol	Merck, Damstad
β-mercaptoethanol	Sigma-Aldrich, Hamburg
PBS	PAN, Nürnberg
Penicillin/Streptomycine	PAN, Nürnberg
Pepton	Merck, Damstadt
Restriction endonucleases	MBI-Fermentas, St. Leon-Rot
RNase	PAN, Nürnberg

RPMI-1640	PAN, Nürnberg
SDS	Merck, Damstadt
Tag DNA polymerase	PAN, Nürnberg
T4 DNA ligase (400 U/ µl and 2000 U/ µl)	NEB, England
0.4 % Trypan blue	Gibcol-BRL, Karlsruhe
Trypsin/EDTA	PAN, Nürnberg
X-Gal	Biomol, Hamburg
Xylene cyanol	Serva, Heidelberg
Yeast extract	PAN, Nürnberg
Yeast nitrogen base without amino acids	Difco, Detroit, USA

1.2 Reagents and Kits

Plasmid mini-, midi, -maxi preparation Kit	Qiagen, Hilden
Gel Extraction Kit	Qiagen, Hilden
Roti-Fect transfection reagent	Roth, Karlsruhe
Dual Luciferase Kit	Promega, Madision, USA
Sequencing Kit	ABI, Buckinghamshire,UK

1.3 Lab ware

Culture flasks and dishes	Nunc, Roskilde, Danmark
Electroporation Cuvettes	Peqlab, Erlangen
Filter (Whatman #5)	Whatman, Maidstone, England
All disposal staff	PAN, Nürnberg

2. Plasmids and primers

2.1 Plasmids

GAL4-Luc	Richard Bear, University of Texas
GAL4-STAT2	Gerhard Behre, LMU, Munich
pBluscript II SK (pBSK II)	Stratagene, La Jolla, USA
pBSKII/5'BCR	Nicole Froehlich, Göttingen
pcDNA3	Invitrogen, Groningen, Netherlands
pcDNA3/BCR-ABL (pcDNA3/B-A)	Michael Hallek, LMU,Munich
pGBT9	Clontech, Heidelberg
pGBT9/BAP	Nicole Froehlich, Göttingen
pGBT9/CRKLSH3n	Nicole Froehlich, Göttingen
pGAD424	Clontech,Heidelberg
pGAD424/BAP	Nicole Froehlich, Göttingen
pGAD424/CRKL-SH3n	Nicole Froehlich, Göttingen
pES1/BCR	Nicole Froehlich, Göttingen
pM1	[Sadowski et al. 1992]
pM1/BR304	Richard Bear, University of Texas
pRL-null	Promega, Mannheim
pVP-FLAG	[Tsan et al. 1997]
pVP-HA/B202-NB	Richard Bear, University of Texas

2.2 Primers

2.2.1 Sequencing primers:

Y2H1: 5′-TCATCATCGGAAGAGAGTAG-3′

Y2H2: 5′-AATACCACTACAATGGATG -3′

2.2.2 Primers used for the cloning of the constructs:

BAPATG Eco: 5′-CATGGAATTCATGGAGAAGACTGAG-3′

BAPB879 Sal: 5′-CAGTGTCGACGACACCCTGTATGGA-3′

BAPT441Eco: 5′-CATCGAATTCATAGCCAATGCAACTAAT-3′

BAPB613Sal: 5’-CATGTCGACGTGGGTTGCATCTCTTTC-3

BAPT617Eco: 5’-CTGAATTCCCAATCCGCCTGGGGC-3’

BAPB681Sal: 5’-CATGTCGACCTGGGTTATTAAGAATCT-3

3. Solutions and medium

3.1 Solutions

2 x BES-buffered saline

50 mM BES (N,N-bis [2-hydroxyethyl]-2-aminoethanesulfonic acid)

280 mM NaCl

1.5 mM $Na_2HPO_4 \cdot 2H_2O$

pH 6.96, filtered with 0.22 µM filter

10X T4 DNA Ligase Reaction Buffer

500 mM Tris-HCl

100 mM MgCl2

100 mM dithiothreitol

10 mM ATP

250 µg/ml BSA

pH 7.5

E1-buffer

100 µg/ml RNase
50 mM Tris-HCl
10 mM EDTA
pH8.0

E2-buffer

200 mM NaOH
1 % SDS

E3-buffer

3.1 M K-acetat, pH5.5

PBS

137 mM NaCl
2.7 mM KCl
4.3 mM NaH_2PO_4
1.47 mM KH_2PO_4
pH 7.4

10 × PCR Reaction Buffer

100 mM Tris-HCl
500 mM KCl
15 mM MgCl2
pH 8.3

PEG/LiAc solution (polyethylene glycol/lithium acetate)

40 % PEG-4000

0.1 M lithium acetate

10 mM Tris-HCl

1mM EDTA

6 × Loading buffer for agarose-gel electrophoroswas

0.25 % bromophenol

0.25 % xylene cyanol

45 % sucrose

5 × TBE buffer

0.45 M Tris

0.45 M boric acid

10 mM EDTA

pH 8.0

TE buffer

10 mM Tris-HCl

1 mM EDTA

TE/LiAc solution (lithium acetate)

10 mM Tris-HCl

1 mM EDTA

0.1 M LiAc

TFB I

15 % glycerol
30 mM KAc
50 mM $MnCl_2$
100 mM RbCl
10 mM $CaCl_2$ $2H_2O$
pH 5.8

TFB II

15 % glycerol
20 mM MOPS
75 mM $CaCl_2$ $2H_2O$
10 mM RbCl
pH 6,9

Trypsin/EDTA solution

0.05 % trypsin
0.02 % EDTA
in PBS

X-gal stock solution

20 mg X-Gal/ml N, N´-Dimethylformamid

Z-buffer

16.1 g/l $Na_2HPO_4 \cdot 7H_2O$

5.5 g/l $NaH_2PO_4 \cdot H_2O$

0.75 g/l KCl

0.246 g/l $mgSO_4 \cdot 7H_2O$

pH 7.0

Z-buffer/X-gal solution

100 ml Z-buffer

0.27 ml β-mercaptoethanol

1.67 ml X-gal stock solution

3.2 Growth Media

3.2.1 Bacterial Growth Medium:

LB medium:

10 g/l trypton

5 g/l yeast extract

10 g/l NaCl

15 g/l agar (for plates only)

pH 7.0

when used as selective medium, 50µg /l ampicillin was added after autoclaving.

3.2.2 Yeast Media:

YPD medium:

20 g/l pepton
10 g/l yeast extract
2 % glucose
20 g/l agar (for plates only)
pH 5.8

SD-medium:

6.7 g yeast nitrogen base without amino acids
2 % glucose
100 ml of the appropriate sterile 10 × Dropout Solution
20g agar (for plates only)
pH 5.8

10 ×Dropout solution:

200 mg/ml L-Adenine hemisulfate
200 mg/ml L-Arginine HCL
200 mg/ml L-Histidine HCL monohydrate
300 mg/ml L-Isoleucine
1000 mg/ml L-Leucine
300 mg/ml L-Lysine HCL
200 mg/ml L-Methionine
500 mg/ml L-Phenylalanine
2000 mg/ml L-Threonine
200 mg/ml L-Tryptophan
300 mg/ml L-Tyrosine

200 mg/ml L-Uracil
1500 mg/ml L-Valine

3.2.3 Mammalian Cell Culture Media:

Complete DMEM medium:

10 % fetal serum
2 mM glutamine
100 µg/ml streptomycine
100 I.E./ ml ampicillin
in DMEM

Complete RPMI-1640 medium:

10% fetal serum
2 mM glutamine
100 µg/ml streptomycine
100 I.E./ ml ampicillin
in RPMI-1640

4. Cloning into plasmid vectors

The appropriate plasmid vector was cleaved with one or more restriction enzymes and ligated to the insert DNA fragment bearing compatible termini. The products of ligation were then transformed into *E. coli* which were plated on appropriate selection medium. The transformed colonies were screened by PCR or by restriction enzymes to identify the recombinant plasmids.

4.1 Preparation of plasmid DNA:

Plasmid DNA can be isolated from bacterial cultures using the alkaline lysis method. The resulting DNA preparation from small-scale bacterial culture can be analyzed by restriction endonuclease digestion and further used for cloning. However, the yields from small scale cultures were generally too low for the transfection of mammalian cells. Yields from large-scale bacteria culture range from 20-500 µg. After column purification, the plasmid DNA can be used to transfect cultured mammalian cells.

4.1.1 Mini preparation of plasmid DNA

1.5 ml aliquots from 4 ml overnight bacterial cultures were pelleted for 1 min in an Eppendorf microcentrifuge. The bacterial pellet was then resuspended in 200 µl of E1 buffer by pipetting up and down. Then 300 µl of freshly prepared E2 buffer was added, mixed by inversion. After incubation on ice for 5 min the solution was neutralized by adding 300 µl of E3 buffer, mixed and incubated on ice for 5 min. The debris was pelleted and 600 µl of the supernatant were removed. The plasmid DNA in the supernatant was precipitated by adding 0.7 volumes of isopropanol, incubating the solution on ice for 20 min and then pelleting by centrifugation for 15 min at 4°C. The DNA pellet was washed with 500 µl of 70% ethanol, air-dried and dissolved in 20 µl of TE buffer.

4.1.2 Maxi preparation of plasmid DNA:

1 ml of a bacterial overnight culture was diluted into 100 ml LB selective medium and incubated at 37 °C for 12-16 hours. The cells were harvest by

centrifugation at 6000 x g for 15 min at 4 °C. The cell pellet was well resuspended in 10ml Buffer P1 by vortexing. Then 10 ml of Buffer P2 was added and mixed by inversion. After 15 min of incubation on ice, 10 ml of Buffer P3 was added and mixed well. After incubation on ice for 5 min and centrifugation at 15 000 x g for 30 min at 4 °C the supernatant was loaded onto a column equilibrated with Buffer QBT. After loading, the column was washed twice with 30 ml of Buffer QC and eluted with 15 ml of Buffer QN. DNA was precipated by adding 0.7 volumes of isopropanol to the eluted DNA and centrifuged at 15 000 x g for 30 min at 4 °C. The DNA pellet was washed with 5 ml of 70 % ethanol, air-dried and dissolved in 500-600 µl TE buffer.

4.2 Measuring DNA concentrations

Two methods were used to measure the amount of DNA. By spectrophotometry, reading should be taken at the wavelength of 260 nm and 280 nm. One corresponds to ~50 µg/ml double stranded DNA. The ratio between the OD260 and OD280 (OD260/OD280) provides an estimate of the purity of DNA. Pure preparations of DNA have OD260/OD280 ratios of 1.8. Contaminations with protein, RNA or other impurity will affect the reading and /or the ratio. If impurities were present accurate measurements were not possible. The alternative way was Ethidium bromide fluorescent quantification. The same volume of standard DNA and different diluted sample DNA were loaded onto an agarose gel and an electrophoresis was carried out. After Ethidium bromid staining, the gel was photographed. The quantity of DNA was estimated by comparing the fluorescence intensity of the DNA standard and the sample DNA.

4.3 Digestion of DNA with restriction endonucleases:

Restriction endonucleases II recognize and cleave 4-6 base pairs specific sequences within double strand DNA. Digestion of DNA with restriction enzymes creates fragments of DNA with sticky or blunt termini.

In the reaction system, 1 U of enzyme was sufficient for 1.0 µg DNA. The total volume was kept to a minimum, usually between 10 to 25 µl and the enzyme should comprise not more than 1/10 of the final reaction volume. For the restriction digest, buffers supplied by the manufacturers of the restriction enyzmes were used. Reaction was kept at the recommended temperature (usually 37°C) for a minimum of 1 hour to overnight. The reactions were stopped by adding EDTA or incubating at 65 °C for 20 min.

4.4 Generating blunt-ended DNA fragments

Klenow fragment was a proteolytic product of E.coli DNA Polymerase I. It exhibits 5′ → 3′polymerase and 3′ →5′exonuclease activity, but lacks 5′ →3′exonuclease activity. For generating blunt ends of DNA by 3′ overhang removal or fill-in of 3′ recessed (5′overhang), 1 µg of DNA was dissolved in restriction enzyme buffer and incubated with 33 µM of each dNTP and 1U Klenow for 15 min at 25°C. The reaction was stopped by adding EDTA to a final concentration of 10 mM and heating to 75 °C for 20 min.

4.5 Dephosphorylation of linearized DNA

When both ends of linearized vector DNA had the same restriction sites or were blunt ends, it was necessary to remove the 5′-phosphate group to reduce the frequencies of circularization and self-oligomerization. Since calf intestinal alkaline phosphatase (CIP)-treated fragments lack the

5’-phosphate termini required by ligase to self-ligate, it can be used to decrease the vector background in cloning experiments. CIP was active in all restriction enzyme buffers. When digestion was complete, 0.5 U/µg DNA CIP was added to the reaction, and incubated at 37 °C for 1 hour. The dephosphorylated vector was then purified by gel extraction.

4.6 Gel purification of DNA

QIAquick gel extraction kit was used to extract DNA fragment (70 bp-10 kb) from standard agarose gels in TBE or TAE buffer. Such extraction can be used whenever it was necessary to inactive and remove the enzyme, or to generate the expected DNA fragment.

After electrophoresis, the DNA fragment was cut from the agarose gel under UV light. 1 volume of gel was incubated with 3 volumes of Buffer QG (100 mg~ 100 µl) at 50 °C for 10 min to dissolve the gel. For example, 300 µl of Buffer QG was added to 100 mg of gel slice. The maximal weight of gel slice was 400 mg per column. After the gel slice has complete dissolved, the color of the mixture should be yellow, similar to the Buffer QG without dissolved agarose. For DNA fragments smaller than 500 bp or larger than 4kb, 1 volume of isopropanol was added to the sample and mixed well. This step increases the yield. For the DNA fragments between 500 bp and 4 kb additional isopropanol has no effect on yield. The sample was then applied to the QIAquick column and centrifuged at 13,000 rpm for 1 min. To wash away impurities, 0.75 ml Buffer PE was applied to the column, and centrifuged for 1 min at 13,000 rpm, the flow-through was discarded and the the QIAquick column was centrifuged at 13000 rpm for an additional 1 min. For elution of bound DNA, 50 µl Buffer EB was added to the column and the column was centrifuged 1 min at 13000 rpm. Alternatively, for

increased DNA concentration, add 30 µl Buffer EB to the center of the QIAquick membrane, let the column stand for 1 min, and centrifuge. The extracted DNA can be used directly for cloning or sequencing.

4.7 Ligation of vector and insert

T4 DNA ligase was used to catalyze the formation of a phosphodiester bond between 5′ phosphate and 3′ hydroxyl termini in double stranded DNA. It will join blunt and cohesive termini.

For protruding termini, the ligation reaction was set up as follows:

50 ng vector DNA

X ng insert DNA

1 µl T4 DNA ligase

2 µl ligation buffer (10×)

H_2O to 20 µl

The ligation was incubated at room temperature for 2 hours or at 4˚C overnight.

For blunt-ended DNA ligation, more DNA and more T4 DNA ligase were used (high concentration T4 DNA ligase was used in this reaction, 20000U/ml).

The optimal molar ratio of vector and insert was 1:1 to 1:10 with 1:3 being used most often. The following formula was used to calculate the amount of insert and vector DNA for the ligation reactions:

$$\frac{3 \times \text{vector concentration } (\mu g/\mu l) \times \text{insert size (bp)}}{\text{insert concentration } (\mu g/\mu l) \times \text{vector size (bp)}} = \frac{\text{x } \mu g \text{ of insert}}{50 \text{ ng of vector}}$$

4.8 Preparation and transformation of competent E coli.

4.8.1 Preparation of competent E.coli using calcium chloride

Treatment of E.coli with ice-cold Cacl2 induces a transient state of "competency" in the recipient bacteria, during which they were able to take up DNAs from a variety of sources.

1 ml of LB-broth overnight culture from E.coli XL-1 blue was diluted into 100 ml of LB broth. After incubation of the culture at 37˚C with vigorous shaking until the OD600 reached between 0.5 and 0.7, the cells were recovered by centrifugation at 4000rpm for 10 min at 4 ˚C. The cell pellet was then resuspended in 40 ml of ice-cold TFBI and incubated on ice for 10-15min. The cells were pelleted as before, and resuspended in 4 ml of ice-cold TFBII. Aliquots of 200 µl of competent cells for transformation can be used immediately or kept at −80 ˚C until use.

4.8.2 Transformation of E.coli

5-20 µl of ligation reaction was added to the 200 µl competent cells (no more than 50 ng DNA in a volume of 10 µl or less) and incubated on ice for 30 min. Then a heat-shock was carried out at 42 ˚C for exactly 90 sec. The cells were then chilled on ice for 2 min, and incubated with 900 µl LB broth at 37 ˚C for 1 hour. After a brief centrifugation (15000 × g, RT), the cells pellet was resuspended in 100 µl of LB broth and spread

onto the selective plates. Colonies usually appeared after 12-16 hours at 37° C.

5. The polymerase chain reaction (PCR)

PCR was used to amplify a segment of DNA that lies between two regions of known sequences. PCR was used for a variety of tasks in molecular cloning and DNA analysis. For example, generation of specific segment of DNA for subcloning.

Two oligonucleotides were used as primers for a series of synthetic reactions that were catalyzed by DNA polymerase. The template DNA was first denatured by heating in the presence of primers and four dNTPs. The reaction mixture was then cooled to a temperature that allows the primers to anneal to their target sequence, after which the annealed primers were extended in 5′ → 3′direction with Taq DNA polymerase (heat-stable polymerase purified from Thermus aquaticus). The cycle of denaturation, annealing and extension were repeated many times. The product of one round of amplification serves as template for the next. The major products will then be 2^n copies of the target DNA segments where n was the number of the cycles.

When using plasmid DNA as template, each of the following components was mixed in PCR thin-wall tubes:

10 fg –50 ng template DNA

10 pmoles primer 1 (top)

10 pmoles primer 2 (bottom)

0.1 µl Taq DNA polymerase

4 nmoles of each dNTPs

2 µl PCR buffer (10 x)

H_2O to 20 µl.

The PCR was carried out in Perkins –Elmer thermal cycler with the standard program.

94˚C 5min

94˚C 50 s
56˚C 50 s } 25cycles
72˚C 50 s

72˚C final extension

The annealing temperature can vary according to the primer and template properties and the extension time was normally 1 min/1 kb target DNA segment.

6. Sequencing amplified DNA by DYEnamic ET Terminator Cycle Sequencing Kits

DYEnamic ET Terminator Cycle Sequencing Kit was used for sequencing of subcloned DNA. It was based on a modification dideoxynucleotide chain termination chemistry in which terminators were labeled with fluorescent dyes for automated detection. In this case, however, each of the four dideoxy terminators—ddGTP, ddATP, ddTTP, ddCTP— was labeled with two dyes—fluroescein and one of four different rhodamine dyes. Acting as the donor dye, fluorescein absorbs energy from incident light and transfer it to the rhodamine acceptor dye on the same terminator molecule. Each

acceptor dye then emits light at its characteristic wavelength for detection that identifies the nucleotide that terminated extension of the DNA chain.

Each sequencing reaction was assembled as follows:

Template DNA (0.1 –0.2 pmol)

Primer (5 pmol)

Sequencing reagent premix 8 µl (supplied by the manufacturer and containing the Taq DNA polymerase enzyme, dNTPs, labeled ddNTPs in the appropriate buffer).

Water was added to a total volume 20 µl

The programm for the sequencing was:

96°C 2 min	
96°C 30 s 50° C 15s 60° C 4 min	25 cycles

After thermocycling, 2 µl (1/10 volume) of sodium acetate and 100 µl of 95 % ethanol were added to the tube. Then the tube was centrifuged at room temperature for 15 min at 12000 rpm. The DNA pellet was recovered, washed with 70 % ethanol and dissolved in 10 µl water.

7. Two-hybrid assay in yeast

7.1 Yeast strain

Strain: Saccharomyces CG-1945, from Clontech, Heidelberg

Genotype: MTA, ura3-52, his3-200, ade2-101, lys2-80, try-901, leu 2-3,

112, GAL4-542, gal80-538, cyhr2, lys::GAL1

Reporter genes: HIS3, LacZ

Transformation markers: trp1, leu2, cyhr2

7.2 Lithium acetate (LiAc) mediated yeast transformation

7.2.1 Small-scale LiAc-mediated yeast transformation

To introduce plasmid DNA into yeast cells (yeast transformation) yeast cells were made chemically competent using lithium acetate. 15 ml of an overnight culture of CG-1945 in YPD at 30°C was diluted into 300 ml YPD to bring the OD600 to 0.2-0.3. After incubation at 30°C for about 3 hours with shaking (230 rpm), an OD600 of 0.4-0.6 was reached. The cells were collected by centrifugation at 1000 × g for 5 min at room temperature. The cells were then washed with sterile water once and resuspended in 1.5 ml of freshly prepared, sterile 1×TE/ 1×LiAc to obtain competent yeast cells. 1.0 µg of plasmid DNA (if more than one plasmid was co-transformed 1 µg of each plasmid was used) and 0.1 mg of herring tested carrier DNA were added to 0.1 µl of competent yeast cells and the solution was mixed well. Then 0.6 ml of sterile PEG/LiAc solution was added. After 30 min of incubation at 30°C with shaking at 200 rpm, 70 µl of DMSO was added and cells were heat shocked at 42°C for 15 min. The yeast cells were then concentrated, resuspended in 100 µl TE buffer and plated on the appropriate medium to select for the transformants containing the introduced DNA. Colony growth was observed after 3-4 days.

7.2.2 Sequential yeast transformation

When three plasmids had to be introduced into the yeast strain, one of them was first transformed and then the transformed colony was picked, made competent according to the above-described procedure and then co-transformed with the other two plasmids.

7.3 β-Galactosidase assay

β-Galactosidase assay was a reporter assay to detemine the expression of the lacZ reporter gene. The colony-lift filter assay was a convenient, fast and relatively sensitive assay.

A dry filer was placed over the surface of an agar plate with yeast colonies so that the colonies will stick to the filter. When the filter had been evenly wetted, it was transfered to a pool of liquid nitrogen (colonies facing up). Using forceps, the filter was completely submerged for 10 sec. After the filter had frozen completely (~10 sec), it was removed from the liquid nitrogen and let thaw at room temperature. Then the filter was carefully placed, colonies side up, on a Whatman #5 filter which was presoaked in 2.5-5 ml of Z buffer/ X-gal solution and incubated at 30°C until the appearance of a blue color reaction.

8. Mammalian two hybrid assay

8.1 Cell line

The following cells were used

•HEK293 cell line: Human embryonic kidney cancer cells (ATCC), were grown in complete DMEM medium.

•32D cell line: Murine IL-3 dependent myeloblastic cells (ATCC), were grown in complete RPMI-1640 medium and 10% conditioned medium from the WEHI-3B cell line, as a source of IL-3.

•WT210 cell line: BCR/ABL expressing 32D cells, kindly provide by Karin Forster, University of Munich, were grown in complete RPMI-1640 medium.

8.2 Transfection of 293 cells with the calcium phosphate method

Transfection was used to transfer DNA into mammalian cells. In transient transfection, recombinant DNA was introduced into a recipient cell line in order to obtain a temporary but high level of expression of a target gene on the recombinant plasmid.

The uptake of DNA by cells in culture was markedly enhanced when the nucleic acid was present as a coprecipitate of calcium phosphate and DNA. A modification of the classical calcium phosphate transfection method that greatly enhances the efficiency of transfection differs from the classical method in that the calcium phosphate–DNA co-precipitatate was allowed to form in the tissue culture medium during prolonged incubation (15-24 hours) under controlled conditions of pH (6.96) and reduced CO_2 tension (2-4 %).

A total of 2.0 µg DNA (0.5 µg of GAL4-Luc reporter plasmid, 0.5 µg of the GAL4-X expression plasmid, 0.5 µg of the VP16-Y expression plasmid, 0.5 µg pcDNA3/BCR-ABL or 0.5 µg pcDNA3 empty vector, and 0.01 µg of control plasmid pRL-null) was diluted into 60 µl of water. 10 µl of 2.5 M $CaCl_2$ and 100 µl of 2 × BBS buffer were added to the DNA solution. After mixing, the solution was incubated at room temperature for 20 min to let the DNA-$CaCl_2$ precipitate form. Then the mixture was added drop-wise onto each 35 mm dish containing 3 × 10^4 HEK293 cells seeded one day before. After incubation of the transfected cells at 37°C in 2-4 % CO_2 for 15-24 h, the

medium was changed to fresh medium. After another 15-24 h of incubation at 37˚C in an atmosphere of 5 % CO_2, the cells were harvested and lysed for the luciferase assay. All the transfections were performed in duplicates and repeated three times.

8.3 Electroporation of 32D cells and WT210 cells

Pulsed electrical fields can be used to introduce DNA into cells. This technique is used when other transfection methods fail.

8.3.1 Electroporation with convention device

Cells were spun down and resuspended in growth medium at a concentration of 2.5 x10^7 cells/ml. 400 µl aliquots of the cell suspension (10^7 cells) were transferred into electroporation cuvettes. A total of 20 µg DNA (5.0 µg of GAL4-Luc reporter plasmid, 5.0 µg of the GAL4-X expression plasmid, 5.0 µg of the VP16-Y expression plasmid, 5.0 µg of pcDNA3/BCR-ABL or 5.0 µg of pcDNA3 empty vector, and 0.1 µg of control plasmid pRL-null) was added to the cell suspension in the cuvette and incubated at room temperature for 10 min. Then the cells were electroporated with 1500 µF, 250 V for 32D cells or 1500 µF, 220 V for WT210. The cells were then transferred immediately to a flask containing 10 ml RPMI-1640 complete medium. After incubation at 37˚C in an atmosphere of 5 % CO_2 for 48 hours, cells were harvested and lysed for the luciferase assay. All the transfection were performed in duplicates and repeated three times.

8.3.2 Electroporation with the Amaxa Nucleofector Device:

1 ×10^6 cells were sususpended in 100 µl in room temperature Nucleofector

SolutionV (component unknown) and mixed with total 3µg DNA (1.0 µg of GAL4-YFP reporter plasmid, 1.0 µg of the GAL4-X expression plasmid, 1.0 µg of the VP16-Y expression plasmid, 1.0 µg of pcDNA3/BCR-ABL or 1.0 µg of pcDNA3 empty vector). Then the nucleofection sample was transferred into an Amaxa certificated cuvette and inserted into the cuvette holder. The cells were then electroporated with program E-32. After the program had finished (display showing "ok"), 500 µl culture medium was added to the cuvette. The cells then were transferred immediately to the 6-well plates pre-prepared with 1.5 ml culture medium in each well. After incubation at 37°C in an atmosphere of 5 % CO_2 for 48 hours, cells were harvested and lysed for the luciferase assay. All the transfections were performed in duplicates and repeated three times.

9. Dual- luciferase assay (DLR)

The luciferase assay was a genetic reporter system for studying gene expression and cellular physiology. In the dual-luciferase system, there were two reporters, one was the firefly luciferase gene the expression of which was correlated to the effect of the specific experimental condition, and the other one was the *Renilla* luciferase which was used as an internal control. Normalizing the activity of the firefly luciferase to the activity of the *Renilla* luciferase minimizes experimental variability. The assays for firefly luciferase and *Renilla* luciferase activity were performed sequentially in the same reaction tube. 48 hours after transfection, the lysates were prepwered from the transfected cells by the addition of 200 µl lysis buffer (per 35 mm dish) and scraping the cells from the dishes. 20 µl of cell lysate was added to each luminometer tube containing 100 µl Luciferase Assay Reagent II to measure the luciferase activity. The luminometer was programmed to perform a 3-second

pre-measurement delay, followed by a 10 seconds measurement period for each reporter assay. After the measurement of the luciferase activity, the sample tube was removed from the luminometer, 100 µl of Stop& Glo Reagent was added and vortexed briefly to mix the solution. Then the sample was placed in the luminometer again to initiate the measurement of the Renilla luciferase activity. The ratio of Firefly luciferase activity to Renilla luciferase activity was calculated. Results were shown as mean±S.D and expressed as the ratio of relative Firefly luciferase avtivity to Renilla luciferase activity.

Results

Nicole Fröhlich had shown that the detection of BCR/ABL1 in mammalian cells was not very robust and reliable even though the same system had worked very well in yeast cells. One possible reason for this discrepancy was thought to be the fact that in mammalian cells there are many other proteins that are able to interact with BCR/ABL1 and the two protein interactors of BCR/ABL1 (BAP-1 and CRKL) used in the detection system. We therefore reasoned that by only using the protein domains of the interactors that were necessary for the interaction with BCR/ABL1 one would be able to minimize the interference from other protein interactions. To accomplish this, a series of deletion mutants of the BCR interactor BAP-1 was constructed to map the BCR interaction domain of this protein.

1 Mapping of the interaction domain of BAP-1 and BCR

To minimize the disturbances to the domain structure of BAP-1, we designed the different deletion mutants of BAP-1 according to the 3 dimensional structure of the protein. There is no 3 dimensional protein structure of BAP1 itself available. However, the 3D structure of the closely related 14-3-3ζ protein is deposited in the protein structure databases. Using the 3D structure of the 14-3-3 ζ protein as a guide, we made a series of deletion mutants of BAP1 to map the BCR interaction domain of BAP1 with the aim that this isolated BCR interaction domain would function better for the detection of BCR/ABL1 in mammalian cells.

1.1 Construction of BAP-1 deletion mutant

In order to identify the minimal region within BAP-1 that is able to

interact with BCR, we made various BAP-1 deletion mutants. Deletion mutant 1, Bap1-114 (amino acid 1-114), contained the N-terminal 4 α-helices of BAP-1. Mutants 2-5 various portions of the C-terminal half of BAP-1. Mutant 2, Bap106-246 (amino acid 106-246), contained the C-terminal half (helices 5-9); mutant 3, Bap106-163 (amino acid 106-163), encompassed helices 5 and 6; mutant 4, Bap164-246 (amino acid 164-246), contained helices 7, 8 and 9; and mutant 5, Bap164-187 (amino acid 164-187), contained the single helix α7. As 14-3-3ζ is composed of nine anti-parallel α-helices (A-I), the mutants were constructed in such a way that the α-helices would not be disrupted (Fig 5).

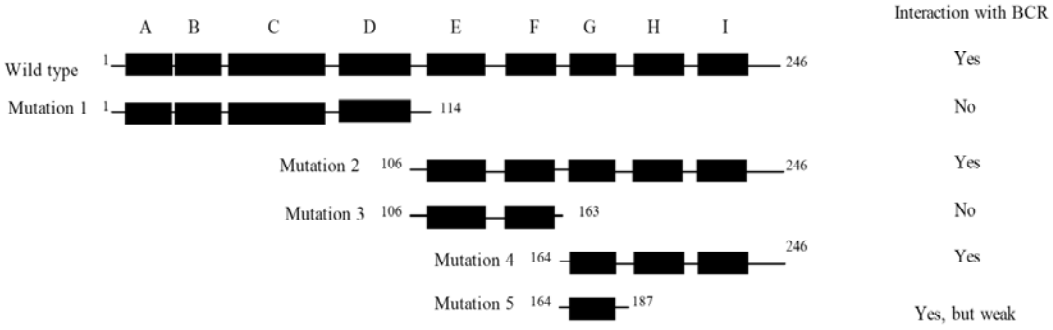


Figure 5 Schematic of deletion mutants of BAP-1 constructs. The 14-3-3ζ protein, which is very homologous to BAP-1, contains 246 amino acid residues and is composed of 9α-helices named A to I starting from the N-terminus. Each α-helix, as defined in 14-3-3ζ, is represented by a box and non-helical regions are shown as lines in this diagram of BAP1 and its mutants. Full length and deletion mutants of BAP-1 were subcloned into pGBT9 for mapping the interaction site with BCR using the yeast two-hybrid system. The numbers indicates the BAP1 amino acids included in each mutant. The deletion mutants used in this study are as follows: full length (wild type); mutant1: aa 1-114 (α1-4); mutant2: aa 106-246 (α5-9); mutant3: aa 106-163 (α5-6); mutant4: aa 164-245 (α7-9); mutant5: aa 164-187 (α7). On the right hand side, the results of the yeast two hybrid interaction assays with BCR are shown.

Except for mutant 1, the coding region for BAP-1 and its deletion mutants were generated by PCR with *EcoR*I and *Sal*I sites incorporated in the forward and reverse primers. PCR products were digested with *EcoR*I and *Sal*I, and

inserted into corresponding site of pGBT9 or pGAD424. The resulting constructs were named as: pGBT9/BAP or pGAD424/BAP (full length), pGBT9/BAP106-246 or pGAD424/BAP106-246 (mutant 2), pGBT9/BAP106-163 or pGAD424/BAP106-163 (mutant 3), pGBT9/BAP164-246 or pGAD424/BAP164-246 (mutant 4), pGBT9/BAP164-187 or pGAD424/BAP164-187 (mutant 5). Mutant 1 was generated by removing the C-terminal fragment of BAP-1. For this, the pGBT9/BAP or pGAD424/BAP construct was digested with *Kpn*I and *Sal*I, blunt ended and re-ligated (Table 1). The resulting construct was named pGBT9/BAP1-114 or pGAD424/BAP1-114.

All of the above constructs were sequenced to verify that no mutations had been introduced during PCR amplification. Fig 6 shows the restriction digests of the various BAP1 deletion constructs.

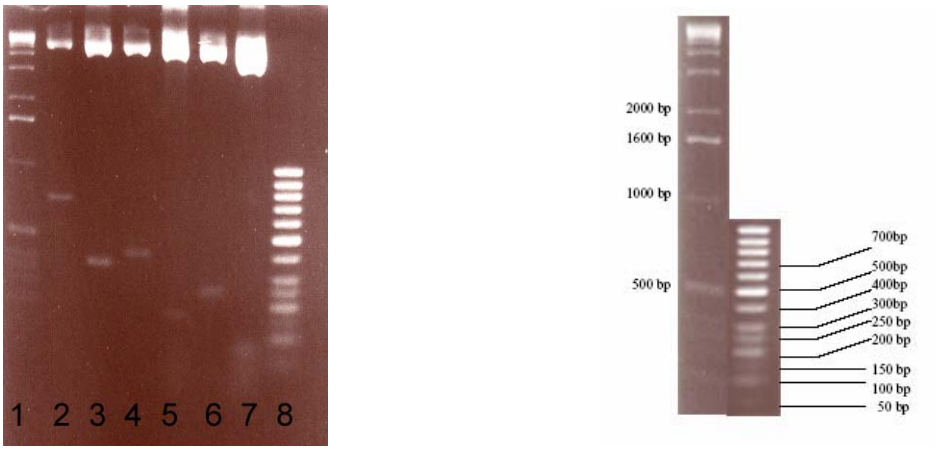


Figure 6 Restriction digest of pGBT9/BAP and the deletion mutants. Ethidium bromide stained 1.5% agarose gel after digestion of the pGBT9/BAP clones with EcoR1 and Sal1 to release the insert.

Table 1 Assignment of plasmids in Fig6

Lane	Plasmid	Insert	PCR primer for amplifying the insert	Cloning site	Size of insert (bp)	Enzymes used
1	1kb ladder					
2	pGBT9/BAP	BAP1 Full length	BAPATG*EcoR* BAPB879*Sal*	*EcoR*I and *Sal*I	740	*EcoR*I and *Sal*I
3	pGBT9/BAP1-114	¤ 1-4	_	*EcoR*I and *Kpn*I (blunt-ended)	342	*EcoR*I and *Pst*I
4	pGBT9/BAP106-246	¤ 5-9	BAPT441*EcoR* BAPB879*Sal*	*EcoR*I and *Sal*I	414	*EcoR*I and *Sal*I
5	pGBT9/BAP106-163	¤ 5-6	BAPT441*EcoR* BAPB613*Sal*	*EcoR*I and *Sal*I	174	*EcoR*I and *Sal*I
6	pGBT9/BAP164-246	¤ 7-9	BAPT617*EcoR* BAPB879*Sal*	*EcoR*I and *Sal*I	240	*EcoR*I and *Sal*I
7	pGBT9/BAP164-187	¤ 7	BAPT617*EcoR* BAPB879*Sal*	*EcoR*I and *Sal*I	60	*EcoR*I and *Sal*I
8	50 bp ladder					

All the deletion mutants were cloned into the yeast two-hybrid vectors pGBT9 and pGAD424. pGBT9 is a 5.5 kb yeast / E. coli shuttle vector that contains the sequence coding for the GAL4 DNA-binding domain (GAL4 1-147). It will express proteins as fusions with the GAL4DBD in yeast. pGBT9 carries the TRP1 gene that allows yeast cells of strains that are auxotroph for tryptophan carrying pGBT9 to grow on synthetic medium lacking tryptophan. pGBT9 also carries an Ampicillin resistance gene for selection in bacteria.

The shuttle vector pGAD424 is used to express a GAL4 activation domain fusion protein in yeast. It carries the LEU2 gene that allows yeast leucine auxotrophs carrying pGAD424 to grow on synthetic medium lacking leucine. pGAD424 also carries an Ampicillin resistance gene for selection in bacteria (Table 2).

Table 2: Yeast-two hybrid vectors

Vector	Description	Selection on SD medium	Size (kb)
pGBT9	GAL4-DNA binding domain TRP1, ampr	-Trp	5.5
pGAD424	GAL4-DNA activation domain LEU2, ampr	-Leu	6.6

1.2 Construction of pGAD424/BCR1-928

The interaction of the various BAP-1 mutants with BCR had to be tested with the yeast two hybrid system first. To simplify these experiments, we used only the BCR portion of the BCR/ABL1 fusion protein to map the BAP interaction domain with BCR. The BCR portion (aa 1 to 928 of BCR) of the BCR/ABL1 fusion gene was cloned into pGAD424. For this, the BCR/ABL coding region was released by *Xho*I and *Eco*RI digestion from the pCDNA3/BCR-ABL plasmid and subcloned into pBluescript II SK, resulting in pBSKII B-A/5'BCR which had two KpnI restriction enzyme sites, one was in the vector 5' of BCR and the other was at very beginning of the ABL portion. Then the BCR fragment was released from the plasmid pBSKII B-A/5'BCR by KpnI (blunt ended before *Eco*RI digestion) and *Eco*RI digestion. This fragment of BCR fragment was cloned into the *Eco*RI and *Sma*I sites of pGAD424. The resulting plasmid was called pGAD424/BCR1-928.

1.3 Mapping of the interaction domain of BAP-1 and BCR

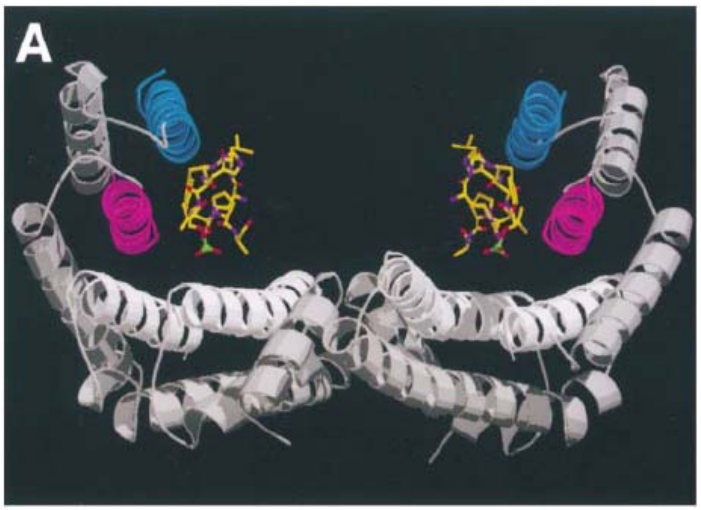
The deletion mutants of BAP-1 were used to map the BCR interaction domain of BAP-1 in the yeast two hybrid system. pGBT9/BAP or the deletion mutants and pGAD424/BCR1-928 were co-transformed into yeast stain CG1945. After transformation, the cells were plated on SD plates lacking tryptophan and leucine (SD –W, -L) to select for co-transformants, which carry both plasmids. The colonies were then restreaked onto SD plates lacking tryptophan, leucine and histidine (SD –W, -L, -H plates) to assay for the expression of the HIS3 reporter gene. Only those yeast cells in which the GAL4DBD fusion protein and the GAL4AD fusion protein interact will grow on SD –W, -L, -H plates. This analysis revealed that aa 109-264 of BAP-1 (c-terminus) and aa 164-264 of BAP-1 (α 7-9) bind to BCR, while aa 1-114 of BAP-1 (N-terminus) and aa 106-163 of BAP-1 (α 5-6) showed no interaction with BCR portion (aa 1 to 928 of BCR) of the BCR/ABL1 fusion gene. aa 164-187 of BAP-1 (the single α helix 7) showed week interaction with BCR portion. These experiments demonstrated that the C-terminus of BAP1 and helices α 7-9 of the C-terminus are sufficient for interaction with BCR. The results are shown in Table 3, Fig 7 and Fig 8. The results of the appropriate negative control experiments are also shown.

Table3 Mapping the BCR 1-928 interaction domain of BAP-1

Plasmids transformed into yeast	Selection SD plates	Growth on selection plates
PGBT9	-W, -H	–
PGBT9/BAP	-W, -H	–
pGBT9/BAP1-114	-W, -H	–
pGBT9/BAP106-246	-W, -H	–
pGBT9/BAP106-163	-W, -H	–
pGBT9/BAP164-246	-W, -H	–
pGBT9/BAP164-187	-W, -H	–
PGAD424	-L, -H	–
PGAD424/BCR	-L, -H	–
PGBT9 + pGAD424	-W, -L, -H	–
PGBT9/BAP+pGAD424/BCR	-W, -L, -H	+
pGBT9/BAP1-114+pGAD424/BCR	-W, -L, -H	–
pGBT9/BAP106-246+pGAD424/BCR	-W, -L, -H	+
pGBT9/BAP106-163+pGAD424/BCR	-W, -L, -H	–
pGBT9/BAP164-246+pGAD424/BCR	-W, -L, -H	+
pGBT9/BAP164-187+pGAD424/BCR	-W, -L, -H	Few colonies

Figure7 pGBT9/BAP or its deletion mutants and pGAD424/BCR1-928 were transformed into yeast cells as follows: 1. pGBT9/BAP + pGAG424/BCR 2. pGBT9/BAP1-114 + pGAG424/BCR 3. pGBT9/BAP 106-246 + pGAG424/BCR 4. pGBT9/BAP106-163 + pGAG424/BCR 5. pGBT9/BAP164-246 + pGAG424/BCR 6. pGBT9/BAP164-187 + pGAG424/BCR 7. pGBT9+ pGAG424. Full length, aa 106-246 (c-terminus) and aa 164-246 (α7-9) of BAP-1 interacted with BCR1-928, as shown by the growth of the yeast colonies on SD –W, -L, -H plates. aa 1-114 (α1-4) and aa 106-163 (α 5-6) of BAP-1 showed no interaction with BCR. Few colonies were found on the selection plates indicating weak interaction between aa 164-187 (α7) of BAP-1 and BCR1-928.

These results corresponded quite well with data from the 3D structure of the BAP-1 related protein 14-3-3ζ. As shown in Fig 8, this molecule consists of a bundle of nine α-helices organized in an antiparallel fashion, with α-helices participating in dimer formation. The dimeric molecule has a cup-like shape with a conserved inner surface and a variable outer surface. The inner surface is a groove roughly 25Å long formed by the four parallel helices α 3, α 5, α 7, and α 9 (Petosa, Masters et al. 1998; Rittinger, Budman et al. 1999). Based on the crystal structure, we predicted that this amphipathic groove would form the principal binding site for 14-3-3 ligands. In this respect, our results were consistent with the assumptions made from the crystal structure and further emphasized the importance of the α helices 7-9 in the protein interactions of 14-3-3 related proteins.



B

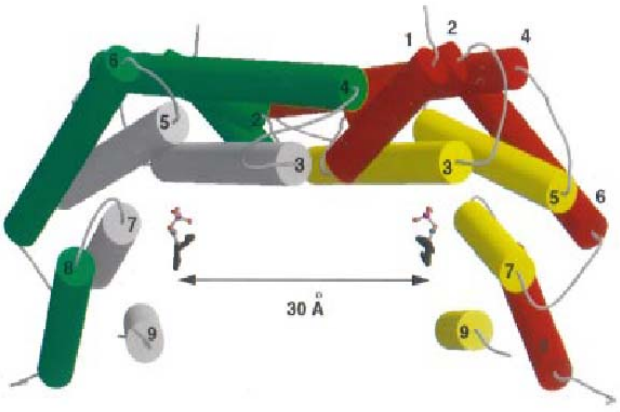


Figure 8. 3 dimensional structure of 14-3-3. The 14-3-3 proteins are dimeric. Each monomer is composed of nine anti-parallel α helices forming a large ligand binding groove as revealed by crystal structure analysis. α7-9 is located near the edge, which is the common structural element for ligand binding/interaction. B is A rotated by 180° around the horizontal axis. A adapted from Rittinger et al., Molecular Cell 1999, 4 (2): 153 and B adapted from Petosa et al, The Journal of Biological Chemistry 1998, 273(26): 16305

2 Detection of the BCR/ABL1 fusion protein in yeast

We first tested our BCR/ABL1 detection system in yeast. In her doctoral

thesis, Nicole Fröhlich had already shown that the minimal interaction of domain of CRKL which is required for making contact with ABL is the N-terminal SH3 domain of this protein. A series of sequential transformation of pGBT9/BAP or its deletion mutants, pGAD424/CRKL-SH3n and pES1/BCR-ABL was performed for this purpose. The pES1 vector carries a LYS gene that allows yeast auxotrophs for lysine carrying pES1 to grow on synthetic medium lacking lysine (K).

After plating the yeast cells on the plates lacking trytophan (-W), leucine (-L) and lysine (-K) to select for transformants containing all three plasmids, colonies were restreaked onto selection plates (SD –W, -L, -K, -H) which also lacked histidine to assay for the reporter gene HIS3. This would indicate the formation of a trimeric transcriptional activation complex of which the BCR/ABL1 fusion protein is the central component (see Fig2 Page 10 and the diagramm in Fig 9 C). These experiments were also performed using BAP-1 or its deletion mutant fused to pGAD424 and CRKLSH3n fused to pGBT9. If any one of the components of the trimeric complex was absent, no transcription would occur.

However, in the absence of a GAL4 based transcriptional activation complex, the expression of the HIS3 reporter gene, which encodes imidazoleglycerol-phosphate dehydratase (His3p), is slightly leaky and cells show weak growth on medium lacking histidine. 3-amino-1, 2, 4-triazole (3-AT) is a competitive inhibitor of the His3p and therefore only cells expressing higher amounts of His3p can survive in medium with 3-AT. All the His $^{+}$ colonies were also able to grow on the SD- W, L, K plates with 10 mM 3-AT (Fig 9 A-C).

We further tested the expression of the second reporter gene, LacZ, which is also under the control of a GAL4-promoter. The expression of LacZ can

be determined by measuring the β-galactosidase activity. Using a colony-lift filter assay, we found that the His^+ transformants were positive for β-galactosidase activity (they showed a blue color reaction within 6 hours, Fig 9 D), which further confirmed the interaction between BAP-1, CRKL-SH3n and BCR/ABL1. All of these results showed that in the yeast system the expression of reporter genes could be made strictly dependent on the presence of the BCR/ABL1 fusion protein.

We also included several other negative controls in our experiment to confirm that the activation of the reporter gene was caused by the complex of BCR/ABL1, BAP-1 and CRKL-SH3n. A summary of all experiments including the negative controls is shown in Table 5.

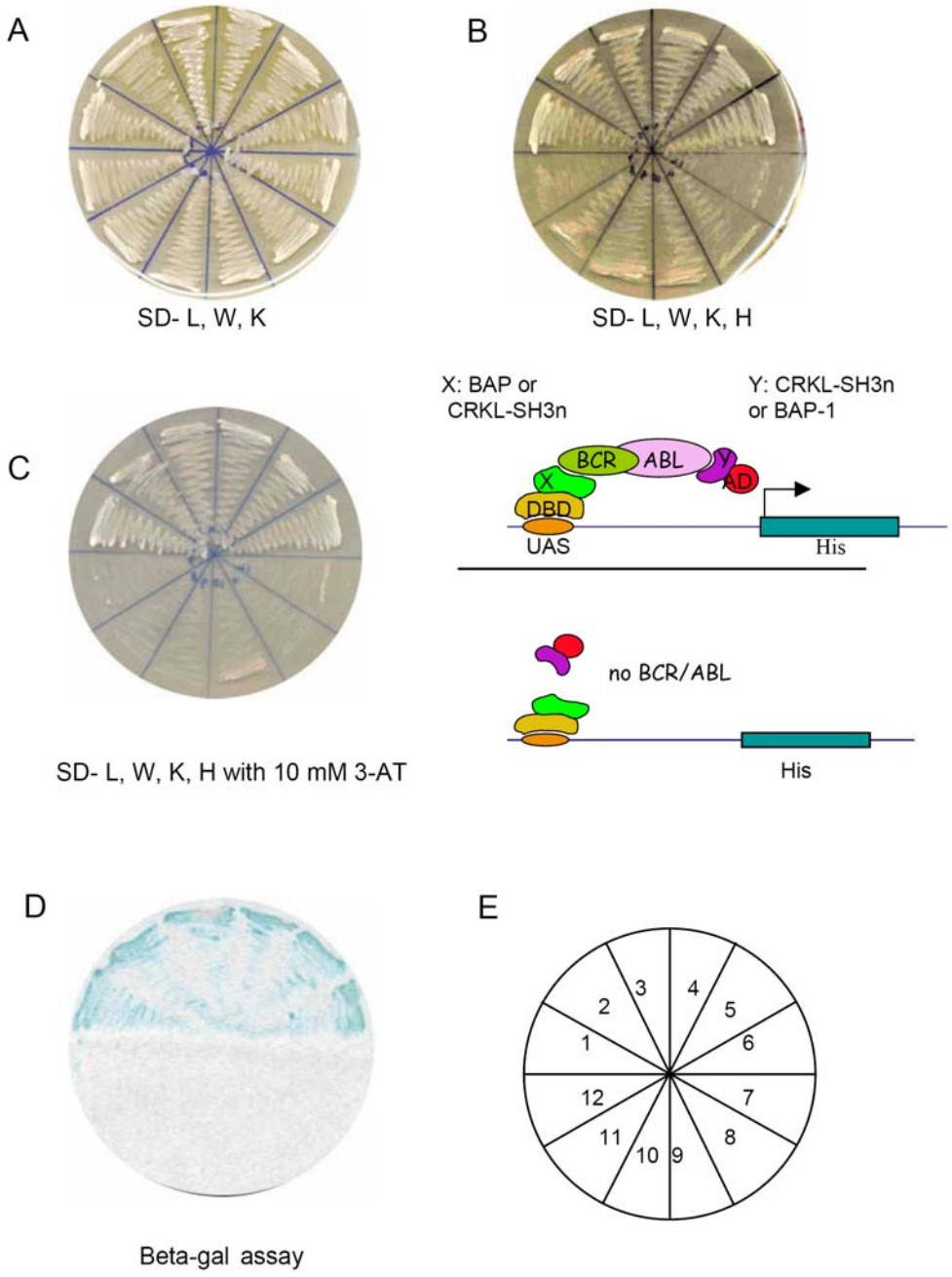


Figure 9. BCR/ABL1 fusion protein detection system in yeast. The three expression plasmids for DBD-X and AD-Y protein as well as for the expression of the BCR/ABL1 fusion protein were serially transformed into yeast strain CG1945 as explained in the table 4. The growth on SD –W, -L, -K indicates the presence of all of three plasmids (A). Expression of the His3 reporter constitutes evidence for the formation of the trimeric complex which is detected as growth in the absence of histidine. The trimeric complex consisting of BCR/ABL1, BAP-1 (or aa 106-246 and aa 164-246 of BAP) and CRKL-SH3n could form only in the presence of BCR/ABL1, resulting in the growth of the yeast cells on SD –W, -L,

-K, -H plates (B, Nr. 1-6) and SD –W, -L, -K, -H plates with 10 mM 3–AT(C, Nr. 1-6). If the BCR/ABL1 fusion is absent, the trimeric complex can not form, no transactivation of reporter occurs, resulting in no (or very little) growth of yeast cells on SD –W, -L, -K, -H plates (B, Nr. 7-12) and SD –W, -L, -K, -H plates with 10 mM 3–AT(C, Nr. 7-12). The His^+ transformant colonies also showed a positive β-galactosidase reaction (blue color) in a filter assay indicating the expression of the second reporter gene LacZ (D, Nr. 1-6). E denotes the nurmbering of transformation, shown in Table 4.

Table 4 Assignment of plasmids in Fig 9

Number	Transformed plasmids
1	pES1/BCR-ABL + pGBT9/BAP + pGAD424/CRKL-SH3n
2	pES1/BCR-ABL + pGBT9/BAP106-246 + pGAD424/CRKL-SH3n
3	pES1/BCR-ABL + pGBT9/BAP164-246 + pGAD424/CRKL-SH3n
4	pES1/BCR-ABL + pGAD424/BAP + pGBT9/CRKL-SH3n
5	pES1/BCR-ABL + pGAD424/BAP106-246 + pGBT9/CRKL-SH3n
6	pES1/BCR-ABL + pGAD424/BAP164-246+ pGBT9/CRKL-SH3n
7	pES1 + pGBT9/BAP-1 + pGAD424/CRKL-SH3n
8	pES1 + pGBT9/BAP106-246+ pGAD424/CRKL-SH3n
9	pES1 + pGBT9/BAP164-246 + pGAD424/CRKL-SH3n
10	pES1 + pGAD424/BAP + pGBT9/CRKL-SH3n
11	pES1 + pGAD424/BAP106-246 + pGBT9/CRKL-SH3n
12	pES1 + pGAD424/BAP164-246+ pGBT9/CRKL-SH3n

Table 5: Summary of experiments performed for the detection of the BCR/ABL1 fusion protein in yeast.

Plasmids transformed into yeast			Growth on selection plates			β -galactosidase assay (colony-lift filter assay)
BCR-ABL1	DBD-fusion	AD-fusion	SD-L,K,W	SD-L,K,W,H	SD-L,K,W,H (10mM 3-AT)	
pES1	pGBT9	pGAD424	+	-	-	-
	pGBT9/BAP	pGAD424/CRKL-SH3n	+	-	-	-
	pGBT9/BAP1-114	pGAD424/CRKL-SH3n	+	-	-	-
	pGBT9/BAP106-246	pGAD424/CRKL-SH3n	+	+	-	-
	pGBT9/BAP106-163	pGAD424/CRKL-SH3n	+	-	-	-
	pGBT9/BAP164-246	pGAD424/CRKL-SH3n	+	+	-	-
	pGAD424/BAP	pGBT9/CRKL-SH3n	+	+	-	-
	pGAD424/BAP1-114	pGBT9/CRKL-SH3n	+	-	-	-
	pGAD424/BAP106-264	pGBT9/CRKL-SH3n	+	+	-	-
	pGAD424/BAP106-163	pGBT9/CRKL-SH3n	+	-	-	-
	pGAD424/BAP164-246	pGBT9/CRKL-SH3n	+	+	-	-
pES1/ BCR-ABL	pGBT9	pGAD424	+	-	-	-
	pGBT9/BAP	pGAD424/CRKL-SH3n	+	+	+	+
	pGBT9/BAP1-114	pGAD424/CRKL-SH3n	+	-	-	-
	pGBT9/BAP106-246	pGAD424/CRKL-Sh3n	+	+	+	+
	pGBT9/BAP106-163	pGAD424/CRKL-SH3n	+	-	-	-
	pGBT9/BAP164-246	pGAD424/CRKL-SH3n	+	+	+	+
	pGAD424/BAP	pGBT9/CRKL-SH3n	+	+	+	+
	pGAD424/BAP1-114	pGBT9/CRKL-SH3n	+	-	-	-
	pGAD424/BAP106-264	pGBT9/CRKL-SH3n	+	+	+	+
	pGAD424/BAP106-163	pGBT9/CRKL-SH3n	+	-	-	-
	pGAD424/BAP164-246	pGBT9	+	weak	+	+

3 Detection of BCR-/fusion protein in Mammalian Cells

3.1 Construction of DBD fusion and AD fusion protein for the mammalian detection system

The rationale for the mapping of the BCR interaction domain of BAP1 was to improve the detection of the BCR/ABL1 fusion protein in mammalian cells. Nicole Fröhlich had shown in her doctoral thesis that using the N-terminal SH3 domain of CRKL (CRKL-SH3n), instead of the full length CRKL protein fused to the GAL4-DBD, the detection of the BCR/ABL1 fusion protein was possible in mammalian cells. However, the detection efficiency, even when using GAL4-DBD/CRKL-SH3n, was not very high and far too low to be used in a therapeutic setting.

After the encouraging results with the BAP1 deletion mutants in the yeast system, we recloned these mutants into mammalian expression vectors to be used for the BCR/ABL1 detection system in mammalian cells. These mammalian expression vectors had been used in the mammalian two hybrid system before (Dang, Barrett et al. 1991). The pM1 vector was designed for mammalian expression of a fusion protein that contains the DNA-binding domain of the yeast GAL4 protein (Sadowski, Bell et al. 1992). The pVP-FLAG5 plasmid was constructed for the expression of VP16AD fusion proteins. VP16AD is the acidic transactivation domain of herpes simplex virus VP16 protein (amino acids 411-455) (Dang, Barrett et al. 1991). The coding sequences of BAP-1 deletion mutants (full length, aa 106-246 and aa 164-246 of BAP-1) that interacted with the BCR portion of the BCR/ABL1 fusion protein were recloned to be expressed either as a fusion with the GAL4DBD or with the VP16AD.

The coding region for BAP-1 and its deletion mutants were generated by PCR with *Eco*RI and *Sal*I sites incorporated in the forward and reverse

primers. PCR products were digested with *Eco*RI and *Sal*I, and inserted in frame into corresponding sites of pM1 or pVP-FLAG5.1. The resulting constructs were named: pM1/BAP or pVP-FLAG5.1/BAP, pM1/BAP106-246 or pVP-FLAG5.1/BAP106-246 and pM1/BAP164-246 or pVP-FLAG5.1/BAP164-246. A modified (pVP-FLAG5.1) vector was used. It was generated by removing the *Nco*I site (digested, blunt-ended and then relegated) in the multiple cloning site, which put the *Eco*RI site into the same reading frame as pM1.

All of the above constructs were sequenced to verify that no mutations had been introduced during PCR amplification. Table 6 shows the constructs used for the detection system in mammalian cells.

Table 6 A summary of the constructs used in the mammalian BCR/ABL1 detection system

Plasmid		Insert	PCR primer	Cloning site	Remark
GAL4-DBD fusion protein	pM1/BAP	Full length BAP-1	BAPATG Eco BAPB879 Sal	*Eco*RI *Sal*I	
	pM1/BAP106-246	C-terminal of BAP-1	BAPT441Eco BAPB879 Sal	*Eco*RI *Sal*I	
	pM1/BAP164-246	Helices 7-9 of BAP-1	BAPT617Eco BAPB879 Sal	*Eco*RI *Sal*I	
	pM1/CRKl-SH3n	N-terminal of SH3 domain of CRKL		*Eco*RI *Sal*I	Provided by N. Fröhlich
GAL4-VP16AD fusion protein	pvp-FLAG5.1/BAP	Full length BAP-1	BAPATG Eco BAPB879 Sal	*Eco*RI *Sal*I	
	pVP-FLAG5.1/ BAP106-246	C-terminal of BAP-1	BAPT441Eco BAPB879 Sal	*Eco*RI *Sal*I	
	pVP-FLAG5.1/ BAP 164-246	Helices 7-9 of BAP-1	BAPT617Eco BAPB879 Sal	*Eco*RI *Sal*I	
	pvp-FLAG5/ CRKL-SH3n	N-terminal of SH3 domain of CRKL		*Eco*RI *Hindl*II	Provided by N.Fröhlich
BCR/ABL1 fusion protein	pcDNA3/ BCR-ABL	BCR/ABL1 fusion gene		*Eco*RI	Provided by M. Hallek

3.2 Detection of BCR/ABL1 fusion protein in mammalian cells with a luciferase reporter gene

In the mammalian version of the two-hybrid system, the transcriptional

activity of a GAL4p-responsive reporter gene provides a quantitative measure of the *in vivo* protein-protein interaction. The GAL4p-responsitive reporter GAL4-Luc contains the TATA element as a minimal promoter and five tandem copies of the GAL4 upstream activating sequence (UAS_G: GTACTGTCCTCCGAGCGGA) immediately upstream of the coding sequences of the luciferase gene. Using luciferase as a reporter gene provides the advantages commonly attributed to luciferase, including high sensitivity and abroad range of linear response. The successful detection of the BCR/ABL1 fusion protein in these cells involves the formation of a trimeric complex containing GAL4-BAP, VP16-CRKL-SH3n and BCR/ABL fusion protein, which should lead to the expression of the luciferase reporter gene. Absence of any of the three components does not result in the transactivation of the reporter gene (Fig10). In the mammalian detection system, we also included positive controls and negative control. One of the positive controls is plasmid expressing the strong transcriptional activator STAT2 fused directly to the GAL4DBD (GAL4STA2) which leads to a very high expression of the luciferase reporter gene. The other positive controls were two proteins (BRCA1 and BRCA1-associated ring domain protein) which were known to interact and result in a positive luciferase read-out in this system(Wu, Wang et al. 1996). As an internal control for transfection efficiency, the pRL-null construct containing a *Renilla* luciferase gene was used.

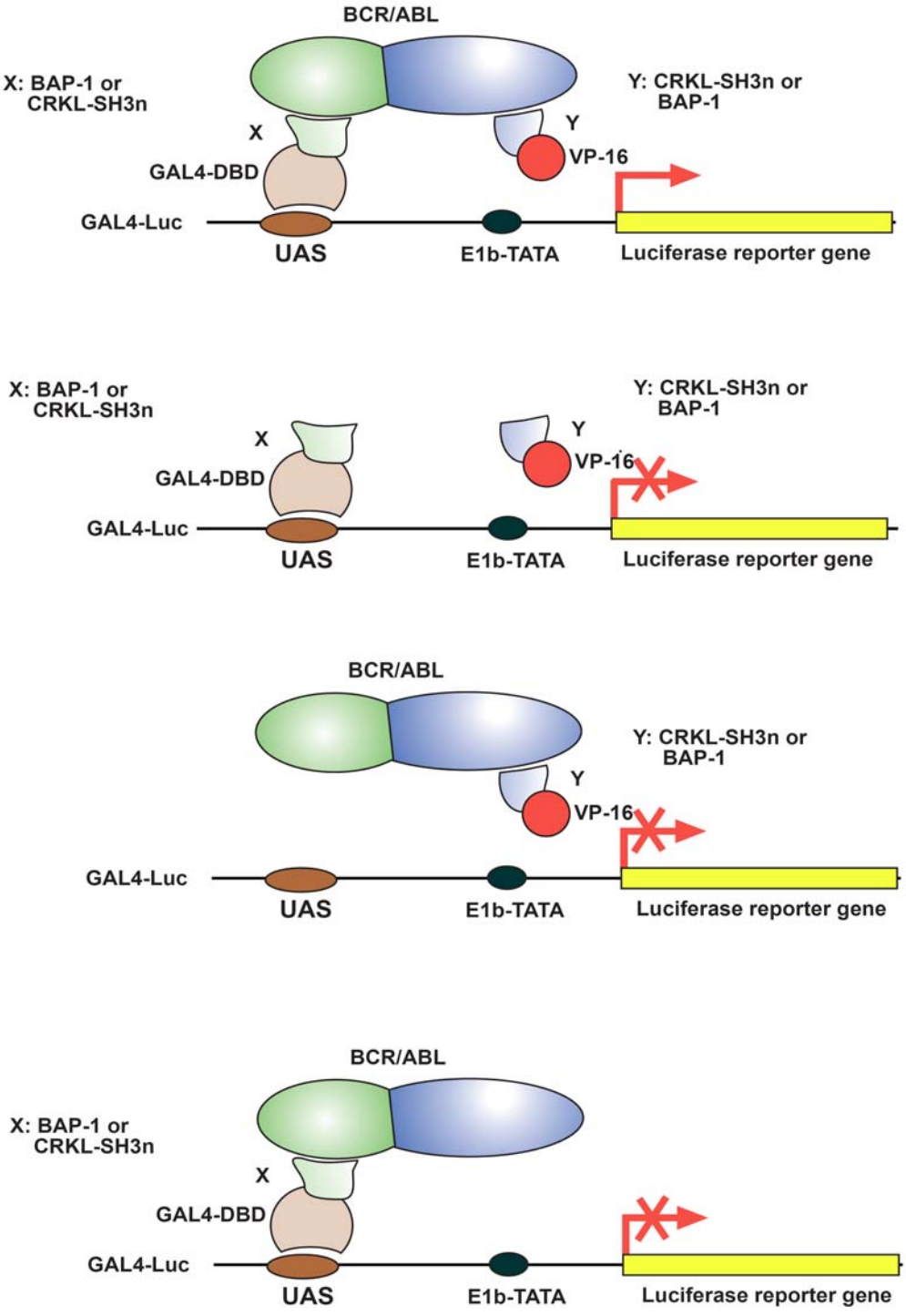


Figure 10 Mammalian detection system of BCR/ABL1 fusion protein. The successful detection of the BCR/ABL1 fusion protein in the cells is in the form of a trimeric complex involving GAL4DBD-X, AD-Y and the BCR/ABL1 fusion protein, which would lead to the expression of the luciferase reporter gene. If the BCR/ABL1 fusion protein or either protein A or protein B is absent, no reporter gene activation should occur.

3.2.1 Detection system of BCR/ABL 1 fusion protein in HEK293 cell

HEK293 cells were transiently co-transfected with GAL4-Luc, pM1/BAP-1 (or pM1/BAP106-246 or pM1/BAP164-246), pvp-FLAG/CRKL-SH3n and pcDNA3/BCR-ABL. As a transfection control, a *Renilla* luciferase reporter was included in each sample. Transfections were performed in duplicates and repeated at least three times. 48 hours after transfection, cells were lysed and assayed for luciferase assay. Detection was determined by comparing the relative activity of firefly luciferase to *Renilla* luciferase. Results were shown as mean±S.D and expressed as the ratio of firefly luciferase avtivity to *Renilla* activity.

As shown in Table 7 (Appendix 1) and Fig 10, the mammalian two hybrid positive control and the GAL4/STAT2 positive control (columns 13 and 15, respectively) showed a high induction of luciferase activity compared to the negative control (column 14), indicating that this detection system worked well to detect the protein-protein interactions. In Nicole Fröhlich's work, the SH3n domain of CRKL worked better in the detection of BCR/ABL1, in our experiment therefore only CRKL-SH3n was used. We first used the full length BAP-1 as interactor protein to detect BCR/ABL1. As described in the Introduction (Fig2 Page 10) and above, only when the BCR/ABL1 fusion protein was present, a trimeric complex will be formed consisting of BCR/ABL1, GAL4DBD-BAP (or VP16AD/BAP) and VP16AD/CRKL-SH3n (or GAL4DBD/CRKL-SH3n) which will lead to the transactivation of the luciferase reporter gene. However, the cells which were transfected with pcDNA3/BCR-ABL, pM1/BAP and pVP-FLAG/CRKL-SH3n did not show induction of luciferase activity (column 1) compared to the cells transfected only with pM1/BAP and pVP-FLAG/CRKL-SH3n, but without BCR/ABL1 expression (empty pCDNA3) (column 2). In the other setting, in which BAP was fused to the VP16AD and CRKL-SH3n was fused to the GAL4DBD (columns 3 and 4), the

presence of BCR/ABL1 also failed to produce a significant increase of luciferase activity compared to the negative controls. We then used the two deletion mutants of BAP, BAP106-264 and BAP164-246, which worked well in the BCR/ABL1 detection in the yeast system as interactor proteins. Unfortunately, the results were similar to the results achieved with the full length BAP, indicating that the BCR/ABL1 detection system did not work in HEK293 cells.

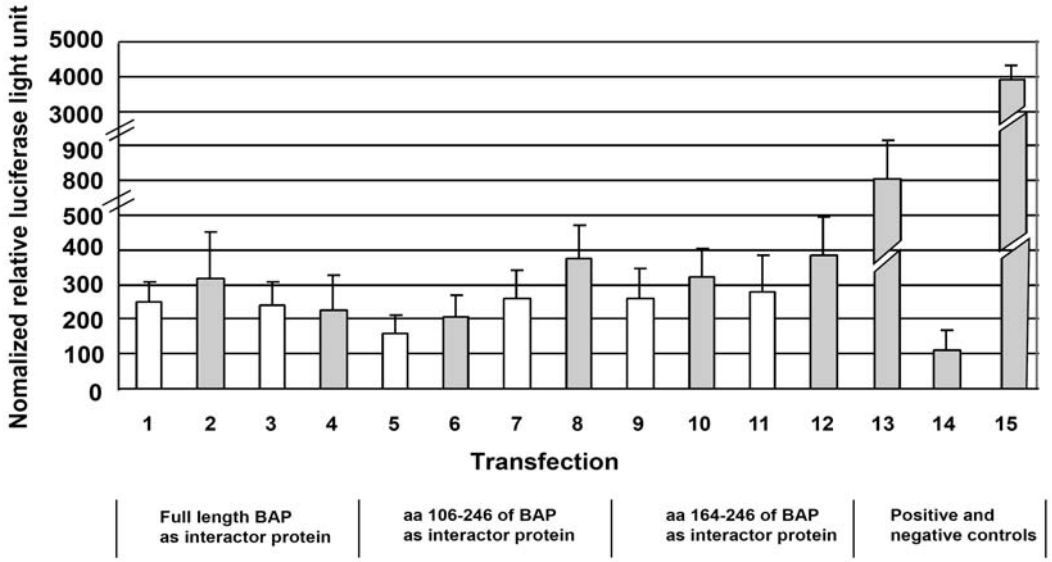


Figure 11 BCR/ABL1 detection system in 293 cells. 293 cells were transiently transfected with different plasmids shown in Table 7. Transfections were performed in duplicates and repeated three times.48 hours after transfection, cells were harvested for luciferase assay. Transfection results are shown as mean±S.D and expressed as the ratio of relative firefly luciferase avtivity compared to *Renilla* luciferase activity. The presence of BCR/ABL1 did not alter the luciferase activity. The standard deviations are indicated by error bars. The white columns denote the sample transfected with pcDNA3/BCR-ABL, and grey columns denote the samples transfected with pcDNA3 (no BCR/ABL1 insert).

3.2.2 Detection system of BCR/ABL1 fusion protein in BCR/ABL expressing 32D cells

After the relatively disappointing results that the BCR/ABL1 detection system achieved in the non-hematopoietic cell line HEK293, we decided to test whether this system would behave similarly in the hematopoietic cell

line 32D. 32D cells are derived from murine progenitors. Stably expressing BCR/ABL 32D cells, WT210, were used as a model for the detection of the BCR/ABL1 fusion protein. The BCR/ABL expressing WT210 cells were transiently transfected with the different interactor constructs shown in Table 8 (Appendix 2) and in Fig12. 32D parental cells, which do not express BCR/ABL1, were used as negative controls.

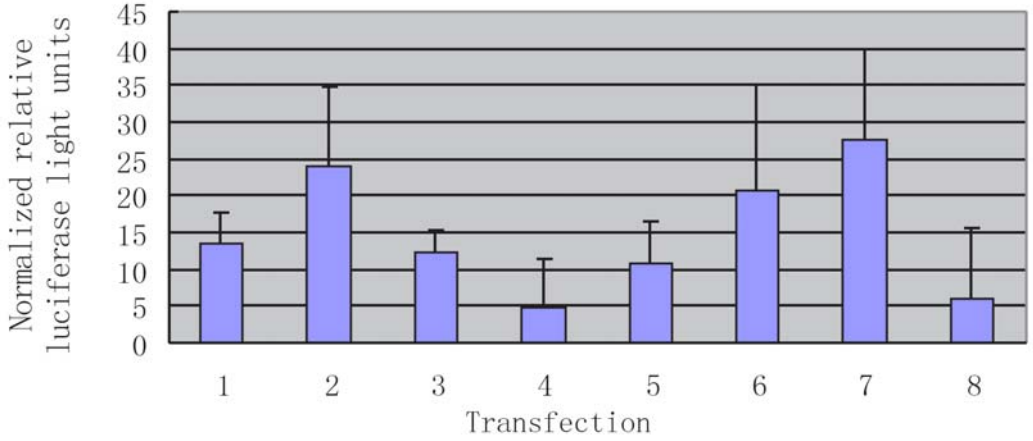
First, we used full length BAP-1 and CRKL-SH3n as interactor proteins in WT210 cells shown in Fig 12 transfection 1, the induction of luciferase activity is about 4 fold compared to the 32D cells (transfection 4), which indicated that the presence of the BCR/ABL1 fusion protein induced transactivation of the reporter. To further confirm that the induction of luciferase activity was the result of the formation of the trimeric complex, we replaced pM1/BAP with the empty pM1 vector or pVP-FLAG/CRKL-SH3n with the empty pVP-FLAG vector. Here no luciferase transactivation should occur. However, in WT210 cells, the transfections which included pM1/BAP, pvp-FLAG/CRKL-SH3n and BCR/ABL1 fusion protein (transfection 1) showed lower luciferase activity compared to the transfections in which the pM1 or the pVP-FLAG empty vector was used as negative control (transfection 2 and 3). In the other setting, in which BAP was fused to the VP16AD and CRKL-SH3n fused to the GAL4DBD (transfection 5), the induction of luciferase activity in WT210 cells was about 3 fold compared to the 32D cells (transfection 8). Similarly, compared to the sample in which pM1 instead of pM1/CRKL-SH3n or pVP-FLAG instead of pVP-FLAG/BAP1 was used (transfections 6 and 7), the sample which included pM1/CRKl-SH3n and pVP-FLAG/BAP1 showed lower luicferase activity (transfection 5).

We then used aa 106-246 of BAP1 and CRKL-SH3n as interactor proteins. We found that compared to 32D cells, the luciferase activity in WT210 cells

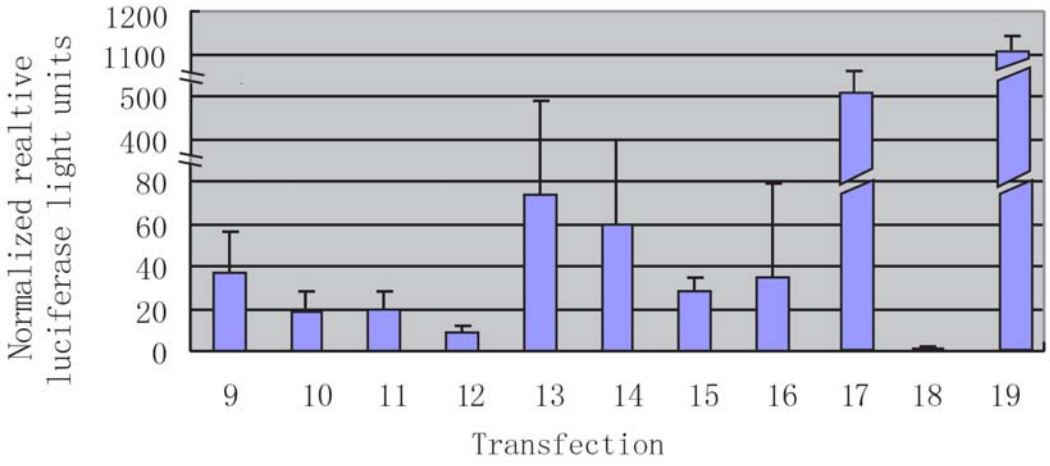
was about 2-4 fold higher than in 32D cells (transfections 9 and 12 or 13 and 16). Using empty vector as negative control, the transfections which included all the three components, the two interactors and the BCR/ABL1 fusion protein (transfections 9 and 13), showed a 1.3 - 2 fold higher luciferase activity compared to the transfections in which one of the interactors was absent (transfection 10, 11 and 14, 15). This indicated that the detection of BCR/ABL1 could be achieved in mammalian cell if the C terminus of BAP1 (aa 106-246) was used instead of full length BAP1. However, the fold change in luciferase activity in the cells expressing BCR/ABL1 was rather modest.

Finally, we used the smallest deletion mutant of BAP1, aa 163-264 of BAP1, as interactor protein, which worked well in yeast BCR/ABL1 detection system (transfections 20 to 27). Unfortunately, the results in this series of transfection were rather similar to the transfections 1 to 8, in which the full length BAP1 was used as the BCR interactor.

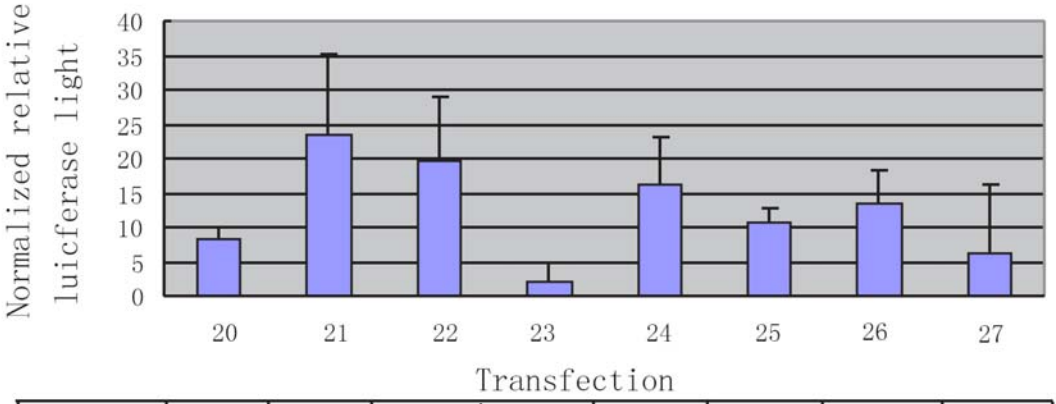
From these three experiments, it was concluded that the detection of the BCR/ABL1 fusion protein using BAP1 or deletion mutants and CRKL or deletion mutants of these proteins cannot be achieved reliably in the setting where luciferase is used as an assay system.



BCR/ABL	+	+	+	-	+	+	+	-
DBD/BAP	+	-	+	+				
AD/ CRKL-SH3n	+	+	-	+				
AD/BAP					+	-	+	+
DBD/ CRKL-SH3n					+	+	-	+



BCR/ABL	+	+	+	-	+	+	+	-	positive control and negative control, detail shown in Table 7
DBD/ BAP106-246	+	-	+	+					
AD/ CRKL-SH3n	+	+	-	+					
AD/ BAP106-246					+	-	+	+	
DBD/ CRKL-SH3n					+	+	-	+	



BCR/ABL	+	+	+	-	+	+	+	-
DBD/ BAP164-246	+	-	+	+				
AD/ CRKL-SH3n	+	+	-	+				
AD/ BAP164-246					+	-	+	+
DBD/ CRKL-SH3n					+	+	-	+

Figure 12 BCR/ABL1 detection system in BCR/ABL expressing 32D cells. BCR/ABL expressing 32D cells, WT210 cells, or 32D parental cells were transiently transfected with different plasmids shown in Table 8. In transfection 4, 8, 12, 16, 17, 18, 19, 23 and 27, 32D cells were used, WT210 were transfected in all other transfections. The table under each sub-Fig showed the different components of transfection briefly. '+' or '-'denotes 'yes' or 'no'. Transfections were performed in duplicates and repeated three times. 48 hours after transfection, cells were harvested for luciferase assay. The results were shown as mean±S.D and expressed as the ratio of firefly luciferase avtivity compared to Renilla luciferase activity. The standard deviations are indicated by error bars.

3.3 Detection of BCR/ABL1 fusion protein in mammalian cells with YFP as the reporter

The detection experiments described in the previous paragraphs using luciferase as a reporter gene represented measurements of the reporter gene activity averaged over a whole cell population. However, to use the BCR/ABL1 detection system in a therapeutic setting it would become necessary to determine the presence or the absence of the BCR/ABL1 fusion protein at the single cell level. Even though we were not able to see a

good detection of BCR/ABL1 when it was averaged over a whole cell population (luciferase experiments), the situation might be different if cell were examined individually. We chose the yellow fluorescent protein (eYFP) as a reporter gene to detect BCR/ABL1 in individual cells. The eYFP protein is derived from the green fluorescent protein found in certain jelly fish species. eYFP has a absorption maximum at 513 nm and an emission maxima at 527 nm (yellow light). When excited at 513 nm, eYFP is able to emit a bright fluorescent signal for flow cytometry or fluorescent microscopy. Because it requires no additional substrates for its fluorescence, it is ideal for use in living cell assays. For this, a new reporter plasmid, GAL4-YFP, containing eYFP under the control of TATA element and UAS_G was generated. We replaced the firefly luciferase coding region in the GAL4-luc plasmid with the coding region of the eYFP protein. When BCR/ABL1 fusion protein was present, CRKL, BAP and BCR/ABL1 would form the trimeric complex through protein protein interaction, which should result in the transactivation of eYFP (shown in Fig 13).

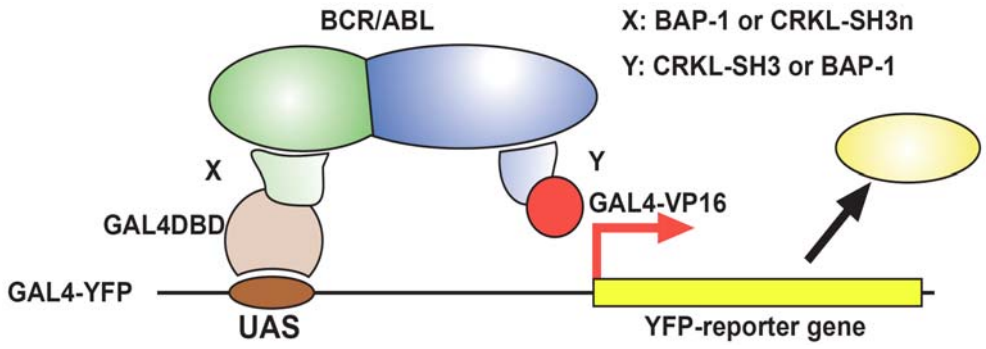


Figure 13 BCR/ABL1 detection system in mammalian cells using eYFP as reporter gene. eYFP is under the control of UAS. Protein X is BCR interactor, BAP-1 or its deletion mutant and protein Y is the ABL interactor, CRKL-SH3n (or vice versa). The reporter YFP gene is induced by the formation of the trimeric complex involving BCR/ABL1, GAL4DBD-X and GAL4VP16-Y.

As a transfection control, we planned to use a second fluorescent protein,

eCFP, to monitor the successfully transfected cells. The eCFP protein is an enhanced cyan fluorescent variant of the green fluorescent protein gene (GFP). eCFP produces an intense and stable cyan fluorescence noncatalytically by absorbing light maximally at 433 nm and emitting blue light with a peak at 475 nm. eYFP and eCFP were chosen because their emission spectra overlap minimally, so they can be distinguished when used simultaneously.

In this way, if we co-transfected the cells with eCFP, BCR/ABL1 detection proteins and GAL4-YFP reporter plasmid, a blue and yellow cell (CFP and YFP double positive) would be a successfully transfected cell with the BCR/ABL1 protein. A blue cell but not yellow (CFP single positive) would be a successfully transfected cell without BCR/ABL1. Cells without fluorescence (CFP and YFP double negative) would be non-transfected cells which cannot be evaluated for the presence of absence of the BCR/ABL1 fusion protein (Table 9).

Table 9 Predicted results using eYFP as reporter

	Transfected cells	Not-transfected cells
BCR/ABL1 positive	blue and yellow (CFP and YFP double positive)	no fluorescence
BCR/ABL1 negative	blue (CFP single positive)	no fluorescence

Unfortunately, the flow cytometer available to our group was not able to detect eCFP fluorescent properly because of weakness of the laser and improper filter sets. We then used eYFP empty vector as transfection

efficiency control. We assumed that all the transfections were under similar transfection efficiency. Based on these assumptions we calculated how many BCR/ABL1 positive cells could be detected.

3.3.1 Construction of GAL4-YFP

Since the luciferase reporter gene plasmid GAL4-LUC contains the TATA element and five tandem copies of UAS_G (GTACTGTCCTCCGAGCGGA) immediately upstream of the coding sequences of luciferase gene, we simply removed the firefly luciferase gene by cutting GAL4-LUC with *Hin*dIII and *Cla*I and inserted the eYFP coding region, which was generated by PCR from eYFP-C1 plasmid with *Hin*dIIII and *Cla*I sites incorporated in the primers (YFPTHindIII and YFPBClaI). The resulting reproter construct was named GAL4-YFP.

3.3.2 Electroporation of 32D cells using Amaxa Nucleofector device

Since the Amaxa nucleofector device has higher transfection efficiency than conventional electroporation devices, we used it in this experiment. Because of technical problems with the flow cytometer as mentioned in paragraph 4.2.3, we were not able to use eCFP as a transfection control in the same cells with the GAL4-YFP reporter plasmid and the detection protein constructs. We used as a control for the transfection efficiency cells which were singly tranfected with the eYFP empty vector. First we did a series of positive control experiments. 1 $\times 10^6$ 32D cells were elctroporated with different plasmids and the total amount of DNA transfected was kept constant adding varying amounts of pBSKII as a filler, as shown in Table 10. Transfections were performed in duplicates and repeated three times. 48 hours after transfection cells were harvested for flow cytometry to check the intensity of the fluorescence in the cells. Forward and side scatter signals were used to restrict the analysis to

viable cells. YFP fluorescence intensity (FL1, x-axis) was plotted on a log scale (Figure 14).

Table 10 Assignment of plasmids in Fig13

Sample	Type of transfection	Mainly transfected plasmids
A	**YFP transfection efficiency control**	**1 µg pE**YFP-C1
B	**STAT2 positive control**	**1 µg GAL4-STAT2** **1** µg GAL4-YFP
C	**Mammalian two hybrid positive control**	1 µg **pM1/ BR304** 1 µ**g pvp-HA**/B202-NB 1 µg GAL4-YFP
D	**Mammalian two hybrid negative control**	**1 µg pM1** 1 µg **pvp-HA**/B202-NB 1 µg GAL4-YFP

As shown in Fig 14, in the transfection efficiency control sample, 78.2% cells were YFP positive, indicating that 78.2 % of cells were transfected (Fig 14 A). We assumed that all the transfections were under similar condition; therefore these 78 % cells would be the "transfected cells" in all the transfection samples. In the GAL4-STAT2 positive control sample, 37.7 % of all the 32D cells were YFP positive cells (Fig 14, B). We interpreted this to mean that about 50 % of the "transfected cells" (37.7 % divided by 78 %) showed transactivation of the YFP reporter, indicating that the new GAL4-YFP repoter was able to be successfully transactivated by the strong transcriptional activator GAL4-STAT2. In the mammalian two hybrid positive control sample, which should give a positive read-out in this system because the two proteins have been shown to give positive read-outs in the luciferase based mammalian two hybrid assay, only 1.82 % of the cells were YFP positive, suggesting that in only 2.6 % of "transfected cells" transactivation of the YFP reporter gene occurred (Fig 14 C) when compared to the negative control (Fig14 D). The mammalian

two hybrid positive control experiment indicated that the percentage of false negatives in transfected cells would be extremely high (100-2.6=97.4%). This meant that using the GAL4-YFP as reporter the vast majority of successfully transfected BCR/ABL1 positive cells would go undetected and could not be sorted out. However, these conclusions can not really be taken at face value because we did not use a transfection control that would indicate the cells that had taken up all three plasmids required for the mammalian two hybrid assay.

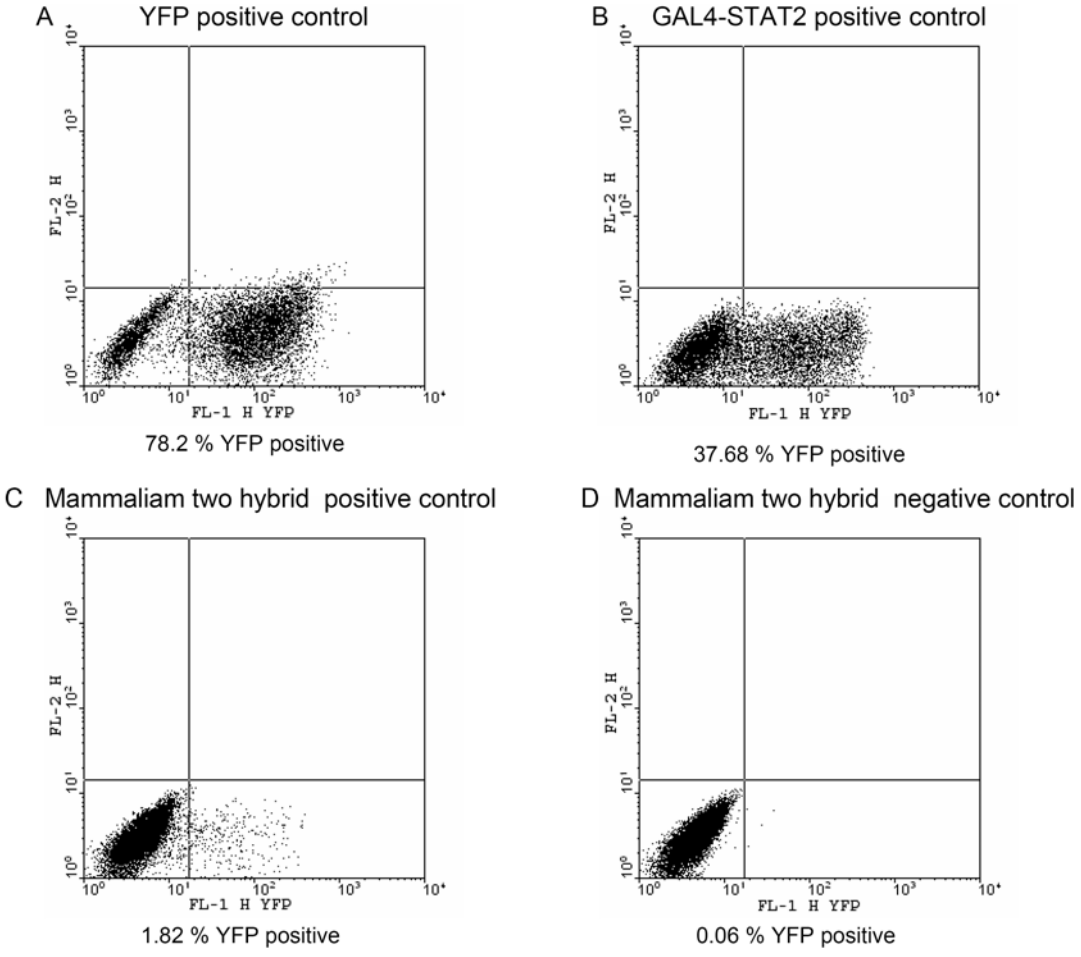


Figure 14 Positive controls of mammalian two hybrid system in 32D cells analyzed by flow cytometry. 32D cells were electroporated with different plasmids as shown in Table 9. Electroporations were performed in duplicate and repeated at three times. 48 hours after electroporation, cells were harvested for flow cytometric analysis. YFP fluorescent cells are

detected in the lower right quadrant. The percentage of positive cells is indicated.

Discussion

The BCR/ABL1 fusion is found in virtually all cases CML and in a large proportion of ALLs. The fact that the BCR/ABL1 fusion protein is necessary to sustain the malignant phenotype (Huettner, Zhang et al. 2000) makes the fusion protein the ideal target for therapy. We are in the process of developing a novel strategy for the treatment of BCR/ABL1 positive leukemia, which is based on the capability to detect the BCR/ABL1 fusion protein *in vivo* in the individual cell. After this first detection step an action can be initiated in a second step such as the induction of the expression of a marker protein or the expression of a pro-drug converting enzyme. After this second step, the BCR/ABL1 positive cells can be selectively sorted out (in the case of the expression of a marker protein) or they can be made susceptible to the action of a pro-drug (in the case of the expression of a pro-drug converting enzyme).

The main focus of the work presented here was the improvement of the first detection step of this strategy. The initial detection step is based on protein-protein interactions. A protein-protein interaction was chosen because most cellular processes are governed by very specfic protein-protein interactions. The yeast two-hybrid system is capable of detecting *in vivo* protein-protein interactions as a function of the transcriptional activation of a reporter gene. In the standard yeast two hybrid system, a protein-protein interaction is detected as the transcriptional activation of a reporter gene. One of the protein interaction partners is fused to a specific DNA binding domain (e.g. GAL4DBD) while the other interaction partner is fused to a transactivation domain (e.g. the trancriptional activation domain of the yeast transcription factor GAL4, GAL4AD). For the *in vivo* detection of the BCR/ABL1

fusion protein we used a modification of the standard yeast two hybrid assay, in which the GAL4DBD-X and the GAL4AD-Y fusion proteins can not interact directly with each other but are dependent on the presence of a third protein which acts as a bridge between these two proteins. This means that transcriptioanl activation of the reporter genes can only occur in the presence of this bridging protein. In our case we chose proteins X and Y of the GAL4DBD and the GAL4AD fusion, respectively, so that they would require BCR/ABL1 as this bridging protein (Fig 15). For this strategy to work with BCR/ABL1 as the bridge, it is required that X interacts with the BCR moiety of BCR/ABL1 while Y interacts with the ABL moiety of BCR/ABL1. We chose BAP-1 as the BCR interacting protein and used CRKL as the ABL interacting protein.

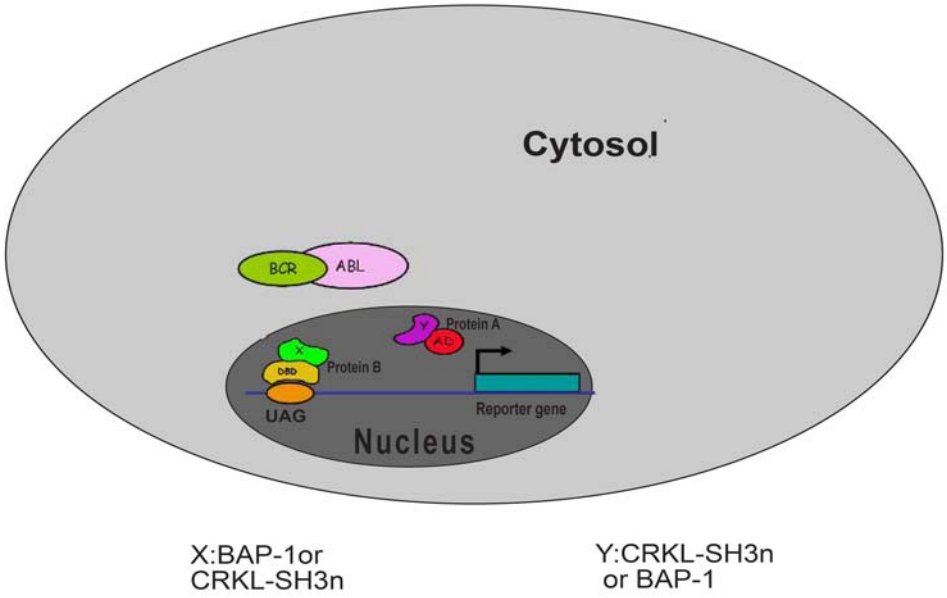


Figure 15 Diagram of the BCR/ABL1 detection strategy. Two proteins are expressed in this system: one is fusion protein of BCR interacting protein Bap-1 (X) and the GAL4-DNA binding domain (protein A), the other is a fusion of the ABL interacting protein CRKL (Y) and the GAL4-activation domain (protein B). If the BCR/ABL1 fusion protein is present, the BCR/ABL1 protein, protein A and protein B form a trimeric complex which transactivates the reporter gene. This system requires that the corresponding hybrid proteins be translocated to the nucleus, where reporter gene transcription occurs. The two detection proteins A and B localize in the nucleus. The BCR/ABL1 fusion protein localizes in the cytoplasma and presumably fails to be translocated into

nucleus.

Nicole Fröhlich had shown in her MD thesis work that the detection of the BCR/ABL1 fusion protein using this strategy worked very well in the yeast system but that the implementation of the same strategy in mammalian cells resulted in only a very weak detection signal for the BCR/ABL1 fusion protein. The signal to noise ratio that could be achieved in Dr. Fröhlich's work for the mammalian BCR/ABL1 detection system was so low that it would not be possible to use this strategy in a therapeutic setting (e.g. for the elimination of BCR/ABL1 positive cells from a bone marrow sample).

The reason for the poor performance of the detection system in mammalian cells was thought to be due to the fact that the BCR/ABL1 protein resides in the cytoplasm and that due to its many interactions with cytoplasmic proteins it will not readily translocate into the nucleus. However, nuclear translocaton of the BCR/ABL1 fusion protein is required for the transcriptional activation of the reporter genes which serve as a readouts for the presence of the BCR/ABL1 fusion protein. We also hypothesized that the two detection proteins A and B might engage in various other protein-protein interactions in mammalian cells preventing these proteins from interacting with the BCR/ABL fusion protein and/or from translocating into the nucleus. While the subcellular localization of BCR/ABL1 cannot be changed easily, using deletion mutants of the BCR and ABL interacting proteins which just contain the BCR and ABL interacting domains of these proteins might improve the performance of the detection system in mammalian cells by preventing these proteins from engaging in unnecessary or disruptive protein-protein interactions. In the yeast cells the cognate protein interaction of BCR/ABL1 would be largely absent which would result in a much weaker cytoplasmic localization of the BCR/ABL1 protein in the yeast cells. This in turn might explain why the detection system functions

well in yeast cell but fails in the more complex environment of the mammalian cells.

Nicole Fröhlich had already shown that using the N-terminal SH3 domain of the ABL interacting protein CRKL instead of full length CRKL markedly improved the perfomance of the BCR/ABL1 detection system in mammalian cells. The N-terminal SH3 domain of CRKL is the ABL interacting domain of CRKL (ten Hoeve, Morris et al. 1993).

We followed a similar strategy for the BCR interactor BAP-1. In the yeast two-hybrid system, we could demonstrated that the C-terminal half of BAP-1 (amino acid 107-245), and within this C-terminal half the helices α 7-9 (amino acid 165-245), are capable of interacting with BCR.

BAP-1, BCR-associated protein-1, is a member of 14-3-3 protein family and virtually identical to the 14-3-3ζ protein. BAP-1 is a substrate of the BCR serine-threonine kinase and is also phosphorylated on tyrosine by BCR/ABL1 but not by c-ABL. BAP-1 interacts with full length BCR and with the BCR/ABL1 fusion protein. As mentioned above, we could show that c-terminus, in particular α helices 7-9 of BAP-1 are sufficient for BCR interaction. These results correspond well to the data from the 3-dimensional structure of the BAP-1 related protein 14-3-3ζ. 14-3-3 proteins form dimers. In each 14-3-3 monomer, there are 9 anti-parallel α-helices. The dimer contains a large negatively charged groove. In the crystal structure, α helices 7-9 are located near the edge of this groove. These helices have a highly conserved amino acid sequence among the members of the 14-3-3 protein family and they are the common structural element for ligand binding.

After demonstrating that the detection system using the BAP-1 deletion mutants worked well in yeast, the open-reading frames of the BAP-1 deletions mutants were cloned into mammalian expression vectors. Unfortunately, when the BAP-1 deletion mutants were tested in mammalian cells we were not able to improve markedly the

detection efficiency of BCR/ABL1 in the cell lines used. These were the human embryonal kidney cell line HEK293 and the murine myeloid progenitor cell line 32D which had been stably transfected with a BCR/ABL expressing plasmid.

It is known that the mammalian two-hybrid system is a very stringent method for detecting protein-protein interaction *in vivo*. There are many protein interactions which can be verified by other methods (e.g. coimmunoprecipitation) which can not be detected in the mammalian two hybrid system. While the great majority of proteins that gave positive read-outs in the mammalian two hybrid system were nuclear factors, positive interactions could also be seen for some proteins which normally reside in the cytoplasma (Fearon, Finkel et al. 1992; Takacs, Das et al. 1993). These results imply that these cytoplasmic proteins were translocated to the nucleus, where reporter gene transcription occurs. This nuclear translocation is probably due to the action of nuclear localization signals which are a part of the Gal4-DNA binding domain or the VP16 activation domain. However, in the few reports of the mammalian bridge two-hybrid assay, which is in principle very similar to the BCR/ABL1 detection system, the bridging proteins that gave a positive readout all had a nuclear localization (Wadman, Li et al. 1994; Osada, Grutz et al. 1995). In both normal and leukemic human hematopoietic cells, ABL is found predominantly in the cytoplasm. ABL can also be detected in the nucleus, albeit at low levels. In contrast, normal endogenous BCR protein, as well as the aberrant p210 BCR/ABL1 or p190BCR/ABL protein consistently localize to the cytoplasma in both cell lines and primary cells (Wetzler, Talpaz et al. 1993). This argues again for the hypothesis that the weak performance of the BCR/ABL1 detection system is due to the strong cytoplasmic localization of the BCR/ABL1 fusion protein.

Recently, it was shown that BCR/ABL1 can be found in the nucleus after treating the cells with the ABL kinase inhibitor STI571 and the nuclear export inhibitor, leptomycin B (LMB) (Vigneri and Wang 2001). Inhibiting the tyrosine kinase activity of BCR/ABL1 with STI571 forces BCR/ABL1 to enter the nucleus and then

BCR/ABL1 can be trapped in the nucleus by the administration of LMB. Using the combination of STI571 and LMB to coax BCR/ABL1 into the nucleus could conceivably improve the performance of the BCR/ABL1 detection system. We are currently trying to combine these two drugs with the detection system experiments. However, preliminary experiments have shown no improvement of the detection efficiency but showed a considerable cellular toxicity.

In the BCR/ABL1 detection experiments using luciferase as the reporter gene the activity of the luciferase was measured from the whole cell population. In order to measure detection of BCR/ABL1 at the single cell level we used eYFP as the reporter gene. When we tested the induction of the YFP reporter with GAL4-STAT2, only about 50 % of the transfected cells showed transactivation of YFP. However, it has to be kept in mind that we did not have a reliable method to estimate transfection efficiency in this setting and that the percentage of transfected cells had to be inferred from a separate experiment. When we used the YFP reporter system to assay a known mammalian two hybrid interaction only very few cells became YFP positive. Also in these experiments, due to technical limitations, we did not have a proper control for transfection efficiency. When we extrapolated the results from this known mammalian two hybrid interaction to the BCR/ABL1 detection we expected a very high proprotion of false negative detections. This implied that the majority of BCR/ABL1 positive cells would not be detected. Therefore, we did not test GAL4-YFP reporter in our BCR/ABL1 detection system.

To overcome the difficulties encountered in developing a detection system for BCR/ABL1 we will persue the following strategy. Instead of relying on the transcriptional activation of a reporter gene for the detection of BCR/ABL1, which requires a nuclear localization of the trimeric detection complex, it should also be possible to induce a specific proteolytic cleavage as the readout of the trimeric detection complex. Since a proteolytic cleavage event can happen in the cytoplasm, the cytoplasmic localization of BCR/ABL1 would not be a problem. In the

split-ubiquitin system developed by Varshasky and colleagues (Johnsson and Varshavsky 1994), a protein-protein interaction results in a specific proteolytic cleavage event. This system might be adapted for the detection of BCR/ABL1 and other leukemic fusion proteins.

Even though our detection system did not work mainly because of the localization of BCR/ABL1 fusion protein, it should still be a viable strategy for the detection of leukemia-associated fusion protein, which localize to the nucleus (i.e AML-ETO).

In a clinical setting we envision that the detection system can be used to recognize and sort out (or selectively) kill leukemic cells. One would be able to purge the bone marrow of leukemia patients efficiently from leukemic cells and rescue the patients after a high dose chemotherapy treatment with an autologous bone marrow transplantation. In principle, this strategy should be superior to using small inhibitor molecules such as STI571, because STI571 treatment does not kill all leukemic cells but leads to a reduction in their number through growth inhibition and induction of apoptosis of leukemia cells.

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Appendix 1

Table 7 Transfection of HEK293 cells

Transfection	1	2	3
Type of Transfection	BCR/ABL detection 1	Negtive control 1 (without BCR/ABL)	BCR/ABL detection 2
Transfected plasmids	0.5 µg pM1/BAP 0.5 µg pvp-FLAG5/ CRKL-SH3n 0.5µg pcDNA3/ BCR-ABL 0.5 µg GAL4-Luc 0.01µg pGL-Null	0.5 µg pM1/BAP 0.5 µg pvp-FLAG5/ CRKL-SH3n 0.5 µg pcDNA3 0.5 µg GAL4-Luc 0.01µg pGL-Null	0.5 µg pM1/ CRKL-SH3n 0.5 µg pvp-FLAG5/ BAP 0.5 µg pcDNA3/ BCR-ABL 0.5 µg GAL4-Luc 0.01µg pGL-Null
Mean of normalized luciferase activity	251.34	317.28	239.45
Standard deviation	56.12	133.46	69.67

Transfection	4	5	6
Type of transfection	Negative control 2 (without BCR/ABL)	BCR/ABL detection 3	Negtive control 3 (without BCR/ABL)
Transfected plasmids	0.5 µg pM1/ CRKL-SH3n 0. 5µg pvp-FLAG/ BAP 0.5 µg pcDNA3 0.5 µg GAL4-Luc 0.01 µg pGL-Null	0.5 µg pM1/ BAP106-246 0.5 µg pvp-FLAG5/ CRKL-SH3n 0.5 µg pcDNA3 BCR/ABL 0.5 µg GAL4-Luc 0.01µg pGL-Null	0.5 µg pM1/ BAP106-246 0.5 µg pvp-FLAG5/ CRKL-SH3n 0.5 µg pcDNA3 0.5 µg GAL4-Luc 0.01µg pGL-Null
Mean of normalized luciferase activity	227.02	160.67	209.54
Standard deviation	99.12	53.92	61.37

Table 7 continued

Transfection	7	8	9
Type of transfection	BCR/ABL detection 4	Negtive control 4 (without BCR/ABL)	BCR/ABL detection 5
Transfected plasmids	0.5 µg pM1/ CRKL-SH3n 0.5µg pvp-FLAG5.1/ BAP106-246 0.5 µg pcDNA3/ BCR-ABL 0.5 µg GAL4-Luc 0.01 µg pGL-Null	0.5 µg pM1/ CRKL-SH3n 0.5 µg pvp-FLAG5.1/ BAP106-246 0.5 µg pcDNA3 0.5 µg GAL4-Luc 0.01 µg pGL-Null	0.5 µg pM1/ BAP164-246 0.5 µg pvp-FLAG5/ CRKL-SH3n 0.5 µg pcDNA3/ BCR-ABL 0.5 µg GAL4-Luc 0.01 µg pGL-Null
Mean of normalized luciferase activity	259.14	376.62	261.37
Standard deviation	81.25	96.71	86.58

Transfection	10	11	12
Type of transfection	Negtive control 4 (without BCR/ABL)	BCR/ABL detection 6	Negtive control 6 (without BCR/ABL)
Transfected plasmids	0.5µg pM1/ BAP164-246 0.5µg pvp-FLAG5/ CRKL-SH3n 0.5 µg pcDNA3 0.5 µg GAL4-Luc 0.01µg pGL-Null	0.5 µg pM1/ CRKL-SH3n 0.5 µg pvp-FLAG5.1/ BAP164-246 0.5 µg pcDNA3/ BCR-ABL 0.5 µg GAL4-Luc 0.01 µg pGL-Null	0.5 µg pM1/ CRKL-SH3n 0.5 µg pvp-FLAG5.1/ BAP164-246 0.5 µg pcDNA3 0.5 µg GAL4-Luc 0.01 µg pGL-Null
Mean of normalized luciferase activity	324.27	281.34	384.89
Standard deviation	78.29	105.78	112.45

Table 7 continued

Transfection:	13	14	15
Type of transfection	Mammalian two hybrid positive control	Mammalian two hybrid negative control	STAT2 activation control
Transfected plasmids	0.5 µg pM1/ BR304 0.5 µg pvp-HA/ B202-NB 0.5 µg PBSKII 0.5 µg GAL4-Luc 0.01 µg pGL-Null	0.5 µg pM1 0.5 µg pvp-HA/ B202-NB 0.5 µg PBSKII 0.5 µg GAL4-Luc 0.01 µg pGL-Null	0.5 µg Gal4-STAT2 1.0 µg PBSK 0.5 µg GAL4-Luc 0.01 µg pGL-Null
Mean of normalized luciferase activity	800.02	111.23	3915.667
Standard deviation	126.34	59.93	594.29

Appendix 2

Table 8 Trnasfection of WT210 and 32D cells

Transfection	1	2	3	4
Cells	WT210	WT210	WT210	32D
Type of experiment	BCR/ABL detection	Negative control (without BAP)	Negative control (without CRKL-SH3n)	Negative control (without BCR-ABL fusion
Transfected plasmids	5μg pM1/BAP 5μg pvp-FLAG5/ CRKL-SH3n 5μg GAL4-Luc 0.1μg pGL-Null	5 μg pM1 5 μg pvp-FLAG5/ CRKL-SH3n 5 μg GAL4-Luc 0.1μg pGL-Null	5 μg pM1/ BAP 5 μg pvp-FLAG5 5 μg GAL4-Luc 0.1 μg pGL-Null	5 μg pM1/BAP 5 μg pvp-FLAG5/ CRKL-SH3n 5 μg GAL4-Luc 0.1 μg pGL-Null
Mean of Normalized luciferase activity	13.43	24.60	12.18	4.72
Standard deviation	4.41	10.76	3.20	6.70

Transfection	5	6	7	8
Cells	WT210	WT210	WT210	32D
Type of experiment	BCR/ABL detection	Negative control (without BAP)	Negative control (without CRKL-SH3n)	Negative control (Without BCR-ABL fusion
Transfected plasmids	5 μg pvp-FLAG/ BAP 5 μg pM1/ CRKL-SH3n 5 μg GAL4-Luc 0.1 μg pGL-Null	5 μg pvp-FLAG 5 μg pM1/ CRKL-SH3n 5 μg GAL4-Luc 0.1 μg pGL-Null	5μg pvp-FLAG/ BAP 5 μg pM1 5 μg GAL4-luc 0.1 μg pGL-Null	5 μg pvp-FLAG/ BAP 5 μg pM1/ CRKL-SH3n 5 μg GAL4-Luc 0.1 μg pGL-Null
Mean of normalized luciferase activity	10.80	20.72	27.56	6.12
Standard deviation	5.71	14.43	12.46	9.38

Table 8 continued

Transfection	9	10	11	12
Cells	WT210	WT210	WT210	32D
Type of experiment	BCR/ABL detection	Negative control (without BAP106-246)	Negative control (without CRKL-SH3n)	Negative control (Without BCR-ABL fusion
Transfected plasmids	5μg pM1/ BAP106-246 5μg pvp-FLAG5/ CRKL-SH3n 5μg GAL4-Luc 0.1μg pGL-Null	5 μg pM1 5 μg pvp-FLAG5/ CRKL-SH3n 5 μg GAL4-Luc 0.1μg pGL-Null	5μg pM1/ BAP106-246 5 μg pvp-FLAG5 5 μg GAL4-Luc 0.1 μg pGL-Null	5μg pM1/ BAP106-246 5μg pvp-FLAG5/ CRKL-SH3n 5 μg GAL4-Luc 0.1μg pGL-Null
Mean of the Normalized luciferase activity	36.30	18.43	19.04	8.17
Standard deviation	20.41	10.05	9.54	3.96

Transfection	13	14	15	16
Cells	WT210	WT210	WT210	32D
Type of experiment	BCR/ABL detection	Negative control (without BAP106-246)	Negative control (without CRKL-SH3n)	Negative control (Without BCR-ABL fusion
Transfected plasmids	5μgpvp-FLAG5.1/ BAP106-246 5 μg pM1/ CRKL-SH3n 5 μg GAL4-Luc 0.1 μg pGL-Null	5 μg pvp-FLAG5 5 μg pM1/ CRKL-SH3n 5 μg GAL4-Luc 0.1 μg pGL-Null	5 μg pvp-FLAG5.1/ BAP106-246 5 μg pM1 5 μg GAL4-Luc 0.1 μg pGL-Null	5μg pvp-FLAG5.1/ BAP106-246 5 μg pM1/ CRKL-SH3n 5 μg GAL4-Luc 0.1 μg pGL-Null
Mean of the Normalized luciferase activity	74.00	59.91	28.36	34.92
Standard deviation	44.71	39.52	6.00	43.60

Table 8 continued

Transfection	17	18	19
Cells	WT210	WT210	WT210
Type of experiment	Mammalian two hybrid positive control	Mammalian two hybrid negative control	STAT2 activation control
Transfected plasmids	5 µg pM1/ BR304 5 µg pvp-HA/B202-NB 5 µg GAL4-Luc 0.1µg pGL-Null	5 µg pM1 5 µg pvp-HA/ B202-NB 5 µg GAL4-Luc 0.1µg pGL-Null	5 µg Gal4-STAT2 10 µg PBSK 5 µg GAL4-Luc 0.1µg pGL-Null
Mean of the Normalized luciferase activity	521.82	1.36	1110.04
Standard deviation	328.96	0.9	420.67

Transfection	20	21	22	23
Cells	WT210	WT210	WT210	32D
Type of experiment	BCR/ABL detection	Negative control (without BAP163-246)	Negative control (without CRKL-SH3n)	Negative control (without BCR-ABL fusion
Transfected plasmids	5µg pM1/ BAP163-246 5 µg pvp-FLAG5/ CRKL-SH3n 5 µg GAL4-Luc 0.1 µg pGL-Null	5 µg pM1 5 µg pvp-FLAG5/ CRKL-SH3n 5 µg GAL4-Luc 0.1 µg pGL-Null	5µg pM1/ BAP163-246 5 µg pvp-FLAG5 5 µg GAL4-Luc 0.1 µg pGL-Null	5 µg pM1/ BAP163-246 5µg pvp-FLAG5/ CRKL-SH3n 5 µg GAL4-Luc 0.1 µg pGL-Null
Mean of the Normalized luciferase activity	8.31	23.47	19.49	2.15
Standard deviation	1.68	11.66	9.49	2.67

Table 8 continued

Transfection	24	25	26	27
Cells	WT210	WT210	WT210	32D
Type of experiment	BCR/ABL detection	Negative control (without BAP163-246)	Negative control (without CRKL-SH3n)	Negative control (without BCR-ABL fusion)
Transfected plasmids	5μg pvp-FLAG5.1/ BAP163-246 5 μg pM1/ CRKL-SH3n 5 μg GAL4-Luc 0.1 μg pGL-Null	5 μg pvp-FLAG 5 μg pM1/ CRKL-SH3n 5 μg GAL4-Luc 0.1 μg pGL-Null	5 μg pvp-FLAG5.1/ BAP163-246 5 μg pM1 5 μg GAL4-Luc 0.1 μg pGL-Null	5μg pvp-FLAG5.1/ BAP163-246 5 μg pM1/ CRKL-SH3n 5 μg GAL4-Luc 0.1 μg pGL-Null
Mean of normalized luciferase activity	16.11	10.64	13.56	6.10
Standard deviation	7.08	2.23	4.72	10.23

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