From LPA signaling to polycomb: oncogenic cooperations revealed by genetic screens and dynamics and recruitment of polycomb group proteins
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Chapter 3

Search for Novel Oncogenes that Cooperate With Activated Ras in Cellular Transformation Using *In Vitro* Genetic Screens
Oncogenic Cooperation with RasV12

Search for Novel Oncogenes that Cooperate with Activated Ras in Cellular Transformation Using In Vitro Genetic Screens

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Mutational activation of Ras proteins is one of the most common oncogenic alterations and is considered to play a causal role in many human tumors. However, constitutively active mutants of Ras (RasV12) cannot transform primary cells in culture. Overexpression of RasV12 in primary mouse embryo fibroblasts (MEFs) induces premature senescence by upregulating the tumor suppressor pathways p19Arf/p53 and p16Ink4a/pRb. Inactivation of any of these tumor suppressors can prevent this growth arrest. Tbx2, a transcriptional repressor of p19Arf, rescues MEFs from RasV12-induced senescence. MEFs co-expressing RasV12 and Tbx2 are immortal but not transformed. To find genes that cooperate with RasV12 in oncogenesis, we performed two independent in vitro genetic screens in MEFs co-expressing RasV12 and Tbx2 using retroviral cDNA libraries. We found interleukin enhancer binding factor 1 (ILF1) to collaborate with RasV12 and Tbx2 to transform MEFs in vitro. Moreover, ILF1 can rescue MEFs from replicative- and Ras-induced senescence and is in addition capable of transforming MEFs in combination with either RasV12 or c-Myc alone. Unfortunately, the transforming ability of ILF1 is dependent on its fusion to the viral Gag protein. Our results underline the difficulties of finding new cooperating partners for RasV12 and the possible artifacts found using conventional retroviral cDNA libraries.

Introduction

Ras proteins are members of the small-GTPase super family and act as molecular switches that control many signaling pathways initiated by extracellular stimuli. In many tumors, the Ras pathway is overstimulated either by activating mutations of Ras itself or by activation of pathway members upstream or downstream of Ras. This overstimulation significantly contributes to tumor cell maintenance by promoting proliferation, metastasis and angiogenesis and inhibiting apoptosis (Downward, 2003; Downward, 2006; Malumbres and Barbacid, 2003). However, malignant transformation of primary cells by activated Ras requires the cooperation of an additional oncogene such as Myc or the viral oncogene E1A (Land et al., 1983; Ruley, 1983). Since tumor initiation and progression involves multiple steps requiring many genetic alterations, it remains extremely important to track cooperating partners in
oncogenic transformation.

The mitogenic signaling elicited by RasV12 in primary cells induces tumor suppressor proteins p19Arf/p53, p16Ink4a and p15INK4b. Hence, overexpression of RasV12 in primary MEFs induces a premature senescence response, which mimics replicative senescence (Serrano et al., 1997). This so called oncogene-induced senescence (OIS) serves as a fail safe mechanism which protects the cells against aberrant oncogenic signaling. The ablation of any involved tumor suppressor pathway, either by directly inactivating the tumor suppressor or by introducing cooperating oncogenes, rescues the cells from this premature growth arrest (Land et al., 1983; Ruley, 1983; Serrano et al., 1997). Cells can then either immortalize or transform depending on the cell type and cooperating oncogenes.

Tbx2, a transcriptional repressor of p19Arf and p21, collaborates with RasV12 to immortalize primary MEFs (Jacobs et al., 2000; Prince et al., 2004). In contrast to p19Arf-deficient MEFs overexpressing RasV12, wild type MEFs co-expressing RasV12 and Tbx2 are not transformed as measured by anchorage-independent growth in soft agar. Thus, Tbx2 overexpression in MEFs does not equal complete loss of p19Arf and the residual p19Arf level is still able to suppress transformation substantially (Jacobs et al., 2000). It therefore remains interesting to search for genes that do transform MEFs containing RasV12 and Tbx2. To identify such oncogenic collaborators, we aimed to screen retroviral cDNA libraries for genes capable of transforming MEFs which co-express RasV12 and Tbx2 using anchorage-independent growth assays in soft agar.

Of note, MEFs transduced with RasV12 and Tbx2 are more prone to transform than MEFs transduced with c-Myc and Tbx2 (Taghavi et al., 2008; chapter 2). For this reason, we expected to encounter potential complications during screening because of background transformation. Hence, we searched for distinct genetic backgrounds and predispositions which could be applicable in genetic screens that indentify genes cooperating with RasV12 in transformation. Thereby, we became interested in candidate of metastasis 1 (Com1/p8). p8 is a basic helix-loop-helix transcription factor which is induced by stress and implicated in both cell growth promotion (Mallo et al., 1997; Vasseur et al., 1999) and inhibition (Bratland et al., 2000; Malicet et al., 2003; Vasseur et al., 2002b) depending on cellular context. p8 has also been suggested to contain apoptotic and antiapoptotic activity (Carracedo et al., 2006; Malicet et al., 2006). The small size, lack of specific three-dimensional structure and flexible switching between nuclear and cytoplasmic localization allow p8 to interact with diverse signaling pathways, hence, achieving various functions (Malicet et al., 2006). Interestingly, while p8-deficient MEFs grow more rapidly than wild type MEFs, they seem to be resistant to transformation induced by RasV12 and E1A (Vasseur et al., 2002a). Although we were not able to reproduce this transformation resistance in p8-deficient MEFs when RasV12 and E1A were co-expressed, we did observe a significant reduction in transformation when RasV12 and c-Myc were co-expressed. Moreover, p8-
deficient MEFs co-expressing RasV12 and Tbx2 are, like wild type MEFs containing RasV12 and Tbx2, immortal but less prone to become transformed. Therefore, we hypothesize that p8 might be involved in downregulating a tumor suppressor pathway which inhibits RasV12 induced transformation.

In this study we screened for cooperating genes that enable RasV12-induced transformation in soft agar. We have set up two independent gain-of-function genetic screens in wild type MEF and p8-deficient MEFs both co-expressing RasV12 and Tbx2. Using p8-deficient MEFs, we wished to find genes and ultimately pathways that could provide insight into p8’s function.

Unfortunately, apart from c-Myc, we did not find any novel genes that cooperate with RasV12 and Tbx2 to transform p8-deficient MEFs. In wild type MEFs, we were able to identify ILF1, a forkhead box (Fox) transcription factor, to cooperate with RasV12 and Tbx2 to induce transformation in soft agar. However, this oncogenic collaboration could not be validated by additional experiments (see below).

Results

**Ras-induced transformation in MEFs investigated in different genetic backgrounds**

Since RasV12 induces a severe growth arrest in primary MEFs within a few passages after transduction, this “set up” context is too stringent to screen for cooperating genes. Tbx2 downregulates p19Arf to a specific threshold level, which permits bypass of Ras-induced senescence, and yet still restricts Ras-induced transformation (Jacobs et al., 2000). Therefore, co-expressing RasV12 and Tbx2 offers us the proper stringency to screen for cooperating genes in transformation.

To confirm and compare the transforming ability of RasV12 in combination with known cooperating oncogenes, we examined anchorage-independent growth of wild type MEFs vs. p19Arf-deficient and p8-deficient MEFs co-expressing RasV12/Control, RasV12/Tbx2, RasV12/E1A or RasV12/c-Myc. Therefore, primary MEFs were retrovirally transduced with different combinations of oncogenes and were then plated in soft agar. Wild type and p8-deficient MEFs transduced with RasV12 alone become senescent and do not grow in soft agar. RasV12/E1A transduced MEFs show the strongest transformation. As expected, complete loss of p19Arf significantly stimulates Ras-induced transformation (Table 1). Remarkably, p19Arf-deficient MEFs transduced with RasV12/Tbx2 or RasV12/Control are equally transformed, implying that the main function of Tbx2 in this context is the suppression of p19Arf. There is a significant reduction in transformation of p8-deficient MEFs transduced with RasV12/c-Myc compared to wild type MEFs. Likewise, p8-deficient MEFs co-expressing RasV12/Tbx2 are less prone to transformation than wild type MEFs. Contrary to what was previously reported (Vasseur et al., 2002a), we do not observe any differences in transformation of
wild type vs. p8-deficient MEFs that are transduced with RasV12/E1A retroviruses (Table 1 and Figure 1).

These experiments evaluate Ras-induced transformation in combination with E1A, c-Myc and Tbx2 and confirm the importance of p19Arf expression levels in inhibiting RasV12-induced transformation in MEFs. Moreover, we can conclude that RasV12/Tbx2 transduced wild type MEFs (hereafter called RasV12/Tbx2-MEFs) and RasV12/Tbx2 transduced p8-deficient MEFs (hereafter called RasV12/Tbx2-MEFs$^{p8}$) make suitable cell systems to screen for cooperating genes that will fully transform MEFs in conjunction with RasV12 and Tbx2.

Identification of genes that cooperate with RasV12 and Tbx2 in cellular transformation using in vitro genetic screens

As mentioned earlier, RasV12/Tbx2-MEFs and RasV12/Tbx2-MEFs$^{p8}$ are immortal but not transformed. Therefore, we designed transformation screens to select for
genes that transform these MEFs using retroviral cDNA expression libraries.

Early passage primary MEFs were transduced with retroviruses containing Tbx2 and RasV12 respectively and subsequently transduced with retroviral cDNA libraries derived from different human tumors and normal tissues (Taghavi et al., 2008; chapter 2). Cells were then plated in soft agar and screened for colony formation (Figure 2a). We observed no background colonies in RasV12/Tbx2-MEFs that were transduced with retroviruses containing no insert (negative control). However, we found some background colonies in RasV12/Tbx2-MEFs transduced with control vector as expected. Ninety-eight and 166 colonies were respectively picked and expanded as monolayers for genomic DNA isolation and further analysis. Given the apparent cooperation between Myc and Ras in cellular transformation and the abundance of Myc oncogenes in human cDNA libraries, we first tested for c-Myc and N-Myc integrations by southern blot analysis. Two out of 166 colonies from the RasV12/Tbx2-MEFs screen and 61 out of 98 colonies from the RasV12/Tbx2-MEFs screen contained inserts coding for c-Myc. None of the colonies contained integrations with N-Myc cDNA (Table 2). To enrich for relevant cDNA inserts and to “filter-out” the spontaneously transformed background colonies, we performed a secondary screen using wild type replication competent MoMuLV as described before (Jacobs et al., 2000).

Unfortunately, the secondary screen revealed no positive colonies amongst the remaining 37 colonies from the RasV12/Tbx2-MEFs screen. Because of the observed background transformation in RasV12/Tbx2-MEFs, we performed the secondary screen twice. Three colonies were consistently positive. Southern blot

<table>
<thead>
<tr>
<th>Screen</th>
<th>Total colonies</th>
<th>Positive after 2nd screen</th>
<th>Contained c-Myc</th>
<th>Contained N-Myc</th>
<th>Analyzed</th>
<th>Insert verified</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT MEFs RasV12/Tbx2</td>
<td>166</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>P8-/- MEFs RasV12/Tbx2</td>
<td>98</td>
<td>61</td>
<td>61</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. An overview of the validation analysis of colonies yielded from RasV12/Tbx2-MEFs and RasV12/Tbx2-MEFs screens. From 166 colonies that were picked in the RasV12/Tbx2-MEFs screen only 3 colonies (identical colonies, likely originated from the same colony) did not contain Myc and were positive after secondary screens and thus suitable for further analysis. From 98 colonies that were picked in the RasV12/Tbx2-MEFs screen, none proved to be positive for novel cooperating oncogenes after analysis.
Figure 2. Screening procedure and identification of relevant inserts. a) Schematic outline of the in vitro genetic transformation screen. Primary MEFs were transduced with retroviruses containing Tbx2 and RasV12 at passage 1 and 2, respectively. Thereafter, they were transduced with human retroviral cDNA libraries and subsequently plated in soft agar, allowing selection for anchorage-independent growth. Colonies were picked after 2-3 weeks and expanded for further analysis. b) Southern blot analysis revealing the number and pattern of integrations in one of the three identical positive primary colonies and its 5 secondary colonies, using a library specific probe (subF). Note that secondary colonies all contain a 4.5 kb insert which is efficiently selected for during secondary screen.
analysis, using a library specific probe, revealed that these colonies contained similar integrations (seemingly originated from the same colony). To facilitate cloning and testing the relevant insert, 5 secondary colonies were analyzed, which all had an approximately 4.5 kb insert in common (Figure 2b). The cDNA insert was cloned using a size-selected phage library, since we were not able to amplify the insert using different long range PCR protocols (Figure 2c). The insert coded for the complete human ILF1 (interleukin enhancer binding factor 1) cDNA (Figure 2d). ILF1 (Foxk1a) is a member of winged helix/forkhead transcription (Fox) factors and binds regulatory motifs in interleukin-2 (IL-2) promoter and human T-cell leukemia- and HIV virus LTRs. There are 3 different ILF proteins reported, which are generated by alternatively spliced transcripts (Figure 2d). Though Fox proteins have been implied in cell cycle regulation (Burgering, 2008; Carlsson and Mahlapuu, 2002), there are no reports so far associating ILFs in particular with cell cycle and transformation.

**ILF1-induced transformation and proliferation in RasV12/Tbx2-MEFs**

The ILF1 cDNA insert was recloned into the original pMXsubF retroviral library vector (ILF1-pMX) and was tested to validate its transforming ability in combination with RasV12 and Tbx2 in wild type MEFs. Therefore, RasV12/Tbx2-MEFs were retrovirally transduced with ILF1, c-Myc or control vector and subsequently plated in soft agar (Figure 3a, top panel). Additionally, we tested the effect of ILF1 on the proliferation rate of RasV12/Tbx2-MEFs in growth curves (Figure 3b). These experiments show that ILF1 increases the proliferation rate of RasV12/Tbx2-MEFs significantly and confirm the transforming ability of ILF1 in conjunction with RasV12 and Tbx2.

Having confirmed that ILF1 cooperates with RasV12 and Tbx2 in transformation, we next tested the contribution of each, RasV12 or Tbx2, in this context. We also examined if there is an oncogenic cooperation with other oncogenes such as c-Myc. MEFs were transduced with retroviruses containing ILF1 (ILF1-pMX) and subsequently transduced with retroviruses containing RasV12, Tbx2, c-Myc or control. Interestingly, ILF1 is able to transform MEFs in combination with RasV12 or c-Myc but not in combination with Tbx2 alone (Figure 3a, lower panel).

These results show that ILF1 is able to increase cellular proliferation and can efficiently cooperate with RasV12 or c-Myc to induce cellular transformation in
primary MEFs. We can also conclude that Tbx2 does not play an essential role in this context, since ILF1 is sufficient to cooperate with RasV12 or c-Myc by itself.

**ILF1 rescues MEFs from replicative and RasV12-induced senescence**

Primary MEFs undergo an irreversible growth arrest when continuously cultured (after 6-7 passages). This process is called replicative senescence and is characterized by upregulation of the p19Arf and p16Ink4a tumor suppressor pathways. RasV12-
induced senescence is mechanistically indistinguishable from replicative senescence. However, it is induced after about 3 passages and therefore is referred to as premature senescence (Palmero et al., 1998; Serrano et al., 1997). ILF1 increases cellular proliferation and cooperates with RasV12 to transform primary MEFs. Is ILF1 then able to rescue MEFs from RasV12-induced senescence? To answer this question, we studied the effect of ILF1 on replicative and RasV12-induced senescence in primary MEFs that are serially passaged on a 3T3 schedule.

**Figure 4.** ILF1 is capable of rescuing wild type MEFs from replicative- and RasV12-induced senescence. a) Replicative senescence; wild type MEFs retrovirally transduced with Tbx2 (positive control), ILF1-pMX or control vector were propagated on a 3T3 schedule. While control MEFs enter a senescent state after about 7 passages, MEFs overexpressing ILF1 are rescued from this replicative senescence. b) Ras-induced senescence; wild type MEFs retrovirally transduced with RasV12, ILF1, control vector, RasV12/ILF1 or RasV12/Tbx2 (positive control) were passaged on a 3T3 schedule. RasV12 transduced MEFs undergo premature senescence after about 3 passages. Similar to Tbx2, ILF1 is able to rescue MEFs from Ras-induced senescence.
Figure 4a illustrates the growth kinetics of MEFs transduced with ILF1, Tbx2 (positive control) or control vector for 18 passages. Whereas control MEFs undergo senescence after about 7 passages, ILF1 transduced MEFs seem to have bypassed senescence and are immortal. However the growth rate of MEFs overexpressing ILF1 is lower than MEFs overexpressing Tbx2.

Next, we examined if ILF1 can rescue MEFs from RasV12-induced senescence. Figure 4b shows that MEFs overexpressing RasV12 enter a proliferative arrest after about 3 passages. Tbx2, as previously described (Jacobs et al., 2000), is able to rescue RasV12-induced senescence. Likewise, ILF1 is capable of rescuing MEFs from RasV12-induced premature senescence. Interestingly, cells transduced with ILF1 and RasV12 are transformed based on their ability to grow anchorage-independent but grow slower than cells transduced with Tbx2 and RasV12, which are not transformed. These results show that ILF1 is not only able to rescue MEFs from replicative senescence but is also capable of bypassing RasV12-induced senescence.

**ILF1-induced proliferative advantage and transformation depends on the expression of the Gag-ILF1 fusion protein from the retroviral expression vector**

Once ILF1 cDNA was cloned into other retroviral vectors than the original library backbone (pMXsubF), we were not able to reproduce the effects of ILF1 in transformation and proliferation assays. ILF1-pMX is able to rescue MEFs from replicative senescence, whereas ILF1-pBP or ILF1-LZRS are clearly not able to do so (Figure 5a). Likewise ILF1-pBP and ILF1-LZRS are not capable of transforming RasV12/Tbx2-MEFs (Table 3).

Sequencing of ILF1 cDNA towards the 5’ viral Gag protein in the original retroviral backbone (ILF1-pMX) revealed that the ILF1 gene was fused in frame with the viral Gag gene (Figure 5b). The pMXsubF vector includes a ± 1 kb piece of retroviral Gag gene sequence for enhanced retroviral packaging. The two ATG’s within this extended Gag region are capable of generating “Gag-gene-of-interest” fusion proteins (Koh et al., 2002). We reasoned that either due to very high overexpression levels induced by the pMX vector or due to the fusion protein itself, the results were not reproducible when ILF1 was expressed from other vectors. To validate the effects of ILF1 in our previous experiments and to uncover the physiological relevance of our findings, we made an ILF1-pMX construct in which the Gag-ILF1 fusion was disrupted using an existing BamH1 site, called ILF1-pMX-out-of-frame (OF) (Figure 5b). In order to examine the expression levels of ILF1, the cDNA was additionally 3’ FLAG-tagged in both ILF1-pMX and ILF1-pMX-OF constructs. We also sub-cloned ILF1 in pcDNA3.1 both 5’- and 3’-FLAG-tagged to study the transforming ability of ILF1 in NIH3T3 fibroblasts upon strong overexpression.

Figure 5a clearly demonstrates that ILF1 is only able to rescue MEFs from replicative senescence when it is fused to viral Gag, since ILF1-pMX-OF transduced MEFs undergo senescence similar to control cells. Furthermore, ILF1-pMX-OF is not able to transform RasV12/Tbx2-MEFs or c-Myc-MEFs in soft agar (Table 3).
Figure 5. ILF1 is not able to immortalize MEFs unless it is fused to the viral Gag protein. a) 3T3 growth assays showing that only ILF1-pMX is able to rescue MEFs from replicative senescence. Wild type MEFs were retrovirally transduced with ILF1-LZRS, ILF1-pBP, ILF1-pMX-OF, ILF1-pMX, Tbx2 (positive control) or empty vector (negative control) and were then propagated on a 3T3 schedule. None of the constructs (ILF1-LZRS, ILF1-pBP or ILF1-pMX-OF) in which ILF1 is not fused with viral Gag, are able to induce bypass of replicative senescence. b) Scheme depicting ILF1 cDNA position in the pMXsubF retroviral backbone (ILF1-pMX). ILF1 is fused in frame with the viral Gag gene. c) Western blot analysis representing protein expression levels of FLAG-tagged ILF1 protein in different expression vectors. Note that Gag-ILF1 fusion protein is only expressed upon ILF1-pMX transduction (lane 3). This is the original construct in which the reading frame between viral Gag and ILF1 is not disrupted and also the only construct that is able to induce proliferation and transformation.
The same results were obtained when ILF1-3’FLAG-pMX and ILF1-3’FLAG-pMX-OF constructs were used (Table 3 and data not shown). This is not due to different expression levels, since both constructs (ILF1-3’FLAG-pMX and ILF1-3’FLAG-pMX-OF) induce comparable expression of ILF1 (Figure 5c).

Table 3. Transformation assays performed using different ILF1 constructs. MEFs were retrovirally transduced with different combination of genes after which anchorage-independent growth was monitored in soft agar. Soft agar colony formation was scored relatively to the amount of colonies induced in MEFs containing RasV12/Tbx2/c-Myc (positive control) and ILF1/control (negative control). ILF1-pMX-OF is not capable of transforming MEFs in combination with, RasV12/Tbx2, c-Myc/RasV12 or c-Myc alone.

<table>
<thead>
<tr>
<th>MEFs</th>
<th>Transformation</th>
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<tbody>
<tr>
<td>ILF1-pMX/Control</td>
<td>-</td>
</tr>
<tr>
<td>ILF1-pMX/RasV12</td>
<td>++</td>
</tr>
<tr>
<td>ILF1-pMX/c-Myc</td>
<td>+++</td>
</tr>
<tr>
<td>ILF1-pMX/Tbx2</td>
<td>-</td>
</tr>
<tr>
<td>c-Myc/RasV12</td>
<td>++</td>
</tr>
<tr>
<td>RasV12/Tbx2/Control</td>
<td>±</td>
</tr>
<tr>
<td>RasV12/Tbx2/ILF1-pMX</td>
<td>++</td>
</tr>
<tr>
<td>c-Myc/RasV12/ILF1-pMX</td>
<td>++++</td>
</tr>
<tr>
<td>RasV12/Tbx2/ILF1-pMX-OF</td>
<td>-</td>
</tr>
<tr>
<td>c-Myc/RasV12/ILF1-pMX-OF</td>
<td>++</td>
</tr>
<tr>
<td>c-Myc/ILF1-pMX-OF</td>
<td>-</td>
</tr>
<tr>
<td>RasV12/Tbx2/ILF1-3’FLAG-pMX</td>
<td>++</td>
</tr>
<tr>
<td>RasV12/Tbx2/ILF1-3’FLAG-pMX-OF</td>
<td>-</td>
</tr>
<tr>
<td>RasV12/Tbx2/ILF1-pBP</td>
<td>-</td>
</tr>
<tr>
<td>RasV12/Tbx2/ILF1-LZRS</td>
<td>-</td>
</tr>
<tr>
<td>RasV12/Tbx2/c-Myc</td>
<td>++++</td>
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</tbody>
</table>

The same results were obtained when ILF1-3’FLAG-pMX and ILF1-3’FLAG-pMX-OF constructs were used (Table 3 and data not shown). This is not due to different expression levels, since both constructs (ILF1-3’FLAG-pMX and ILF1-3’FLAG-pMX-OF) induce comparable expression of ILF1 (Figure 5c).

FLAG-tagged constructs of ILF1 in pcDNA3.1 (Figure 5c) were used to induce transformation in combination with RasV12 or c-Myc in NIH3T3 fibroblasts which are more prone to transformation than primary fibroblasts (susceptible to single-hit oncogenic transformation). ILF1 is not able to increase RasV12 induced transformation in these cells, even though the expression level is very high (Table 4 and Figure 5c). Similarly ILF1 is not able to induce transformation in combination with c-Myc in these cells (Table 4).
Table 4. Transforming ability of ILF1 tested in NIH3T3 fibroblasts. Soft agar experiments testing if ILF1 has any transforming ability, by itself or in combination with RasV12 or c-Myc, when strongly overexpressed. NIH3T3 fibroblasts were transfected with different ILF1-pcDNA3.1 constructs in combination with RasV12, c-Myc or control vector. Cells were then plated in soft agar. Transformation was scored relatively to the amount of colonies induced in cells containing RasV12/c-Myc (positive control) and c-Myc/control (negative control). ILF1 is clearly not able to induce transformation in NIH3T3 cells, not even when it is highly expressed or combined with other potent oncogenes. The same results were obtained using either ILF1-3’FLAG-pcDNA3.1 or ILF1-5’FLAG-pcDNA3.1 constructs.

These results unfortunately suggest that the proliferative advantage and cellular transformation induced by ILF1 are likely caused by the Gag-ILF1 fusion protein which is visualized in Figure 5c lane 3.

Discussion

In this study we have searched for genes that cooperate with activated Ras (RasV12) and reduced p19Arf levels to transform primary MEFs, using a genetic screen based on anchorage-independent growth in soft agar. Identification of such genes is of significant value since both alterations are frequently found in many tumors. We have taken advantage of the fact that RasV12/Tbx2-MEFs and RasV12/Tbx2-MEFs\textsuperscript{p8}, although immortal, are not transformed, which permitted us to search for genes that transform these cells using cDNA expression libraries. The p8-deficient background allows the search for genes biased towards p8 growth stimulatory/inhibitory effects, which could clarify the role of this small protein in tumorigenesis.

In contrast to an earlier report, we observe equal transformation in RasV12/E1A-MEFs and RasV12/E1A-MEFs\textsuperscript{p8} (Vasseur et al., 2002a). This difference could be caused by differences in expression levels of RasV12 and E1A proteins in these
cells. However, we do observe that RasV12/c-Myc-MEFs\textsuperscript{p8} are significantly less transformed than RasV12/c-Myc-MEFs implying an essential difference in RasV12 induced transformation between wild type and p8-deficient MEFs. Likewise, RasV12/Tbx2-MEFs\textsuperscript{p8} are less prone to transformation than RasV12/Tbx2-MEFs. Consequently there were less background colonies observed when we screened with p8-deficient MEFs.

Finding inserts containing c-Myc validates our screening method. However, the fact that we found considerably less c-Myc integrations in the RasV12/Tbx2-MEFs screen than in the RasV12/Tbx2-MEFs\textsuperscript{p8} screen hints towards a bigger background problem in wild type MEFs than initially expected. It also suggests that the screening set-up in p8-deficient MEFs was more stringent. Only three colonies, obtained from the RasV12/Tbx2-MEFs screen, proved to be positive for a potentially novel transforming cDNA integration after secondary screen. Although the secondary screen has allowed us to select for true positive colonies, it also shows that a vast majority of the colonies are transformed regardless of the cDNA integrations. This highlights the negative impact of background colonies on our screening read-out, which has greatly disturbed our ability to find transforming oncogenes.

Nevertheless, in wild type MEFs we identify ILF1, a Fox transcription factor, which can transform MEFs in combination with RasV12 and Tbx2. Forkhead proteins share a winged-helix DNA-binding domain, but are functionally very diverse. Whereas FoxO family members are inhibitors of cellular proliferation and transformation (Burgering, 2008), FoxM family members promote cell cycle progression (Laoukili et al., 2007).

ILF1 can rescue wild type MEFs from replicative and RasV12-induced senescence and promotes proliferation of RasV12/Tbx2-MEFs significantly. ILF1 is also capable of transforming primary MEFs in conjunction with either RasV12 or c-Myc and co-expression of Tbx2 does not seem to cooperate with ILF1. As mentioned earlier, Tbx2 is not able to completely abrogate p19Arf, which explains why RasV12/Tbx2-MEFs, in contrast to p19Arf-deficient MEFs overexpressing RasV12, are not transformed. This suggests that ILF1 can be a p19Arf suppressor, as highlighted by its collaboration with either c-Myc or RasV12 in transformation. ILF1 should therefore be capable of bypassing c-Myc-induced apoptosis or Ras-induced senescence through suppression of p19Arf. In mouse cells, the p19Arf/p53 pathway is more favored to become inactivated during immortalization than p16Ink4a/pRb pathway (Gil and Peters, 2006). However, to conclude that ILF1 might suppress p19Arf, it is necessary to examine the p19Arf and p53 status of cells from several time points in 3T3 growth assays representing replicative and RasV12-induced senescence-bypass. This will exclude the possibility of spontaneous immortalization through loss of the Ink4a-Arf or p53 locus and/or function (Lundberg and Weinberg 2000). Furthermore, RasV12 overexpressing MEFs, which are retrovirally transduced with ILF1, although transformed, grow slower than the same MEFs transduced with Tbx2, which are only immortal. Therefore, even if ILF1 is a p19Arf suppressor, it
must exert other functions that cooperate with RasV12 or c-Myc in transformation. This is underlined by the observation that p19Arf-deficient MEFs overexpressing c-Myc alone are not transformed (Taghavi et al., 2008; chapter 2).

Previous studies have shown a prominent role for forkhead transcription factors in cell cycle regulation (Burgering, 2008; Laoukili et al., 2007). Like many Fox proteins, ILF1 contains a forkhead transactivation domain. Site selection studies have shown that with this DNA binding domain, ILF1 is able to recognize the core sequence TGTGTAC in the IL2 promoter region and has been suggested to positively regulate IL2 expression (Nirula et al., 1997). Other forkhead transcription factors can bind to similar consensus sites containing a TGTGTAC core sequence known as the forkhead binding motif (Furuyama et al., 2000). Therefore, it remains interesting to test whether ILF1 is able to transactivate such a general forkhead responsive element.

Although ILF1 did make a good candidate to be involved in cell cycle regulation, proliferation and transformation, we were unfortunately not able to prove its transforming ability, when it was not fused to the viral Gag protein. The ILF1 protein is expressed to comparable levels when it is in frame or out of frame with Gag. Therefore we can conclude that the reason for observed differences in transforming ability likely comes from the differential expression of the Gag-ILF1 fusion protein rather than being caused by differences in overexpression levels from distinct vectors. Furthermore, we cannot exclude that the Gag-ILF1 fusion protein might exert dominant negative or dominant positive functions caused by non-physiological size or unusual interacting partners in this context. Another possible explanation could be aberrant localization of ILF1 protein when fused to the viral Gag protein. The very N-terminal MA sequence of the Gag protein is essential for myristoylation, a lipid based signal which is necessary for proper membrane localization and intracellular trafficking of the molecule (Yi and Rosenberg, 2007; Yi and Rosenberg, 2008). Some proto-oncogenes encoded by transforming retroviruses such as v-Abl are a product of the fusion of a cellular oncogene (c-Abl) with the retroviral Gag gene (Rosenberg and Witte, 1988). This fusion protein is a constitutively active tyrosine kinase whose localization is now dictated by the N-terminally fused Gag protein. Elegant studies have shown that deletions in the MA region abrogate the ability of v-Abl to transform cells. Gag sequences are thus important for normal signaling, cellular localization (nuclear exclusion) and trafficking of v-Abl, which underlie the transforming ability of this fusion protein (Yi and Rosenberg, 2007; Yi and Rosenberg, 2008). Likewise the Gag-ILF1 fusion protein could be targeted to the plasma membrane or interact with other subcellular compartments where it might exert its transforming function. However, ILF1 seems to be a bona fide transcription factor with no predicted enzymatic activity, which makes it difficult to speculate by which mechanism its interaction with cytoplasmic- or membrane-associated proteins can induce transformation. The only obvious possibility could be the recruitment of other proteins to unusual
locations that might interfere with cell cycle progression.

Finally, ILF3, an alternative splice variant of ILF1, does not contain a nuclear localization signal. It would be interesting to test whether this protein variant could induce transformation due to its nuclear exclusion and the consequent aberrant localization. Altogether, we were not able to identify novel proteins cooperating with RasV12 in oncogenesis. Nevertheless, our study emphasizes the negative impact of background problems during screening and the absolute necessity of proper and optimal screening “set-up” in order to minimize false positive hits.

Material and Methods

Cell culture, constructs, retroviral production and transduction
Wild type and p19Arf-deficient MEFs were isolated and cultured as previously described (Jacobs et al., 1999). p8-deficient MEFs were obtained from S. Vasseur (Vasseur et al., 2002a). Production of ecotropic retroviruses and retroviral transductions were performed as previously described (Taghavi et al., 2008; chapter 2). RasV12/Tbx2-MEFs were generated as follows: primary MEFs were transduced with retroviruses containing LZRS-Tbx2-iresGFP at passage 1, and subsequently transduced with retroviruses containing pBabePuro-RasV12 (Jacobs et al., 2000) at passage 2. Transduced cells were subsequently selected using 4 μg/ml puromycin (Sigma). Other constructs used were; LZRS-MychA-iresGFP (positive control) and LZRS-iresGFP or pLib empty vector (negative control). For pilot transformation experiments we also used LZRS-E1A12S-iresGFP. Human ILF1 cDNA (NM_004514) was cloned in the original library retroviral vector pMXsubF, pBabePuro (pBP), LZRS-iresGFP and pcDNA3.1 viral- and expression vectors. ILF1-pMX (ILF1 cDNA cloned in frame with viral Gag in pMXsubF) and ILF1-pMX-OF (ILF1 cDNA cloned out of frame with viral Gag in pMXsubF). The pMX constructs contain a 3'FLAG-tagged ILF1 cDNA and pcDNA3.1 constructs contain 3’ or 5’FLAG-tagged ILF1 cDNA. For all the transductions, empty vector control is used to equalize the number of transductions whenever necessary.

Soft agar assays
Per well of a six-well plate, 6 x 10⁴ cells were resuspended in Dulbecco’s Modified Eagle Medium (GIBCO), supplemented with 10% fetal bovine serum (PAA laboratories) and 0.4% low gelling temperature agarose (Sigma) and plated on top of a coating bottom-agar (DMEM supplemented with 10% FBS, containing 0.6% agarose). Cells were then allowed to grow under standard conditions for 2-3 weeks, after which plates were scored relative to the positive and negative controls. All experiments were performed at least twice in several independently isolated batches of MEFs.

Genetic transformation screen
Retroviral cDNA expression libraries used here are: human prostate, human leukocyte, human mammary gland, human placenta, human whole brain, all purchased from Clontech and JEG3(1-3), JEG3(>3) size fractionated human JEG3 choriocarcinoma-derived cDNA library, K562(1-3) and K562(>3) size-fractionated K562 human erythroleukemia cDNA library (Jacobs et al., 2000; Koh et al., 2002). High titer viral supernatants from libraries were freshly collected with 12 h intervals and directly used to transduce RasV12/Tbx2-MEFs or RasV12/Tbx2-MEFs⁺⁺ each in 2 consecutive rounds of transduction of 1-2 x 10⁶ cells plated per 10 cm culture dishes. Empty pMXsubF- or pLib-vectors are used as negative controls. Forth-eight hours after library transduction, cells were plated in soft agar in 10 cm culture dishes as described above. RasV12/Tbx2-MEFs and RasV12/Tbx2-MEFs⁺⁺ transduced with retroviruses containing c-Myc were used as positive control. After 2-3 weeks, colonies were
picked from cells that were transduced with cDNA libraries. Colonies were expanded as monolayers for genomic DNA isolation and secondary screen. Southern blot analysis was performed using a conventional protocol. DNA probes were prepared from c-Myc (full length human c-Myc cDNA) and N-Myc (786-1342 fragment from human N-Myc cDNA, NM_005378). Secondary screen using wild type replication competent MoMuLV was achieved as described before (Jacobs et al., 2000). Three colonies from the RasV12/Tbx2-MEFs screen proved to be positive from which the number and pattern of integrations were examined by southern blot analysis using a library specific probe (subF). The primary colonies proved to be identical and therefore only one was used for further testing. The relevant insert was approximately 4.5 kb, which was similar in all five secondary colonies. The cDNA insert was cloned using the ZAP express undigested vector kit (Stratagene) following standard Stratagene lambda ZAP protocols and the insert was identified after sequencing. ILF1 cDNA was then cloned back into the original construct (pMXsubF) and was re-tested in RasV12/Tbx2-MEFs in soft agar.

**Growth curves and 3T3 growth assays**

For growth curves, the infected cells were plated in 12-well culture dishes (2.5 x 10^4 cells per well), in triplicate for each time point. The cells were then left to grow and subsequently fixed at various time points using 4% formaldehyde (10% formalin) in PBS for 5-10 minutes at RT. Plates were then stained with crystal violet (Sigma). Dye extraction is performed using 10% acetic acid and optical density is quantified at OD 590 nm. Values are normalized to the density measured at day 0 (24 h after plating). For 3T3 growth assays, 4 x10^5 cells were passaged every 3 days in a 25 cm² culture flasks. The relative increase/population doubling is calculated and graphed (all performed in duplicate and in independently isolated batches of MEFs).

**Western blot analysis**

RasV12/Tbx2-MEFs were either transfected with ILF1-5’-FLAG-pcDNA3.1, ILF1-3’-FLAG-pcDNA3.1 or pcDNA3.1 empty vector using calcium-phosphate co-precipitation method or retrovirally transduced with ILF1-3’-FLAG-pMX, ILF1-3’-FLAG-pMX-OF or pMXsubF empty vector. Forthly-eight hours after transduction cells were harvested for protein lysate preparation. Cells were washed with phosphate buffered saline (PBS) and lysed using RIPA buffer (150 mM NaCl, 1% NP40, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA (pH 8.0), 0.5% DOC, 0.1% SDS) supplemented with 1 mM dithiothreitol (DTT), 1 mM phenyl-methyl-sulphonyl-fluoride (PMSF), 1 mM NaVO3, 10 mM NaF, 10 mM Pyrophosphate and 50 mM β-glycerophosphate). Protein expression was analyzed using a conventional Western-blot protocol. Primary antibodies were: goat anti-FLAG (Abcam 1:1000) and mouse anti-tubulin (1:500, Sigma). Secondary antibodies were: goat anti mouse (1:10000, Zymed) and swine anti goat (1:10000, Biosource), both conjugated to HRP.

**References**


Oncogenic Cooperation with RasV12


