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

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Chapter 2

*In Vitro* Genetic Screen Identifies a Cooperative Role for LPA Signaling and c-Myc in Cell Transformation

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In Vitro Genetic Screen Identifies a Cooperative Role for LPA Signaling and c-Myc in Cell Transformation

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c-Myc drives uncontrolled cell proliferation in various human cancers. However, in mouse embryo fibroblasts (MEFs), c-Myc also induces apoptosis by activating the p19Arf tumor suppressor pathway. Tbx2, a transcriptional repressor of p19Arf, can collaborate with c-Myc by suppressing apoptosis. MEFs overexpressing c-Myc and Tbx2 are immortal but not transformed. We have performed an unbiased genetic screen, which identified 12 oncogenes that collaborate with c-Myc and Tbx2 to transform MEFs in vitro. One of them encodes the LPA2 receptor for the lipid growth factor lysophosphatidic acid (LPA). We find that LPA1 and LPA4, but not LPA3, can reproduce the transforming effect of LPA2. Using pharmacological inhibitors, we show that the in vitro cell transformation induced by LPA receptors is dependent on the Gi-linked Erk and PI3K signaling pathways. The transforming ability of LPA1, LPA2 and LPA4 was confirmed by tumor formation assays in vivo and correlated with prolonged Erk1/2 activation in response to LPA. Our results reveal a direct role for LPA receptor signaling in cell transformation and tumorigenesis in conjunction with c-Myc and reduced p19Arf expression.

Introduction

Almost all human cancers are the unfortunate consequence of accumulating mutations that lead to oncogene activation and tumor suppressor inactivation. These changes are ultimately subject to selection of collaborative pathways that promote proliferation, evasion from apoptosis and metastasis. The c-Myc transcriptional regulator is one of the most studied classical onco-proteins, the aberrant expression of which is implicated in many human cancers. Enhanced expression of Myc-family members is involved in nearly every feature of tumorigenesis. However, c-Myc overexpression by itself fails to induce cell transformation in primary human or rodent cells (Adhikary and Eilers, 2005; Land et al., 1983; Drayton et al., 2003). Therefore, additional mutations are necessary for cell transformation and tumor initiation. These mutations either activate oncogenes that collaborate with c-Myc, such as Ras, and/or inactivate tumor suppressor pathways that protect cells from transformation by c-Myc, such
as the p19Arf-Mdm2-p53 tumor suppressor pathway. Activated Ras cooperates with c-Myc to induce cell transformation and oncogenesis (Land et al., 1983). On the contrary, the p19Arf tumor suppressor is one of the fail safe mechanisms that protect cells against excessive mitogenic signals (Gil and Peters, 2006; Sherr, 2004).

In primary MEFs c-Myc induces apoptosis through p19Arf upregulation and subsequent p53 pathway activation (Zindy et al., 1998). In addition, p19Arf inhibits c-Myc’s function by direct interaction and inhibition of its transactivation ability independent from p53 (Datta et al., 2004; Qi et al., 2004; Sherr, 2006). These actions of p19Arf antagonize c-Myc and inhibit cellular proliferation. Hence overexpression of c-Myc in p19Arf deficient MEFs allows cells to proliferate rather than undergo apoptosis (Jacobs et al., 1999).

In a senescence bypass screen, we identified Tbx2 as a transcriptional repressor of p19Arf (Jacobs et al., 2000; Lingbeek et al., 2002). Tbx2 is able to downregulate p19Arf to immortalize c-Myc overexpressing MEFs, yet it is not sufficient to fully transform these cells to allow growth in soft agar. This interesting observation provided us with a base to screen for cooperative genes that enable cellular transformation as measured by anchorage-independent growth in soft agar. To identify genes that collaborate with c-Myc and Tbx2 in tumorigenesis, we have performed a genetic gain of function transformation screen in MEFs overexpressing both genes. We identified twelve genes belonging to diverse functional groups that cooperated with c-Myc and Tbx2 to transform MEFs. One of the inserted encoded LPA2, a G protein-coupled receptor (GPCR) for the lipid mediator lysophosphatidic acid (LPA).

LPA acts on several GPCRs triggering Ras-Erk, Rho, Rac and PI3K pathway activation which in turn stimulate the proliferation, migration and survival of many cell types, both normal and malignant (Ishii et al., 2004; Moolenaar et al., 2004). LPA signaling has been implicated in the progression of human cancers such as ovarian, prostate and colorectal cancer (Mills and Moolenaar, 2003). Given the emerging evidence for a role of LPA signaling in cancer, we sought to examine the transforming potential of the LPA2 receptor (and its relatives) in combination with c-Myc and Tbx2 both in vitro and in vivo. We demonstrate that enhanced expression of some, but not all, LPA receptors transforms cells in combination with c-Myc and Tbx2 in vitro and in vivo. In addition, we examine the contribution of downstream LPA signaling pathways to cell transformation.

Results

Identification of genes that cooperate with c-Myc and Tbx2 in cell transformation using a genetic screen

Tbx2 downregulates p19Arf levels to a specific threshold, just enough to
immortalize MEFs and counteract c-Myc induced apoptosis (Jacobs et al., 2000). The residual p19Arf level is however still capable of suppressing transformation. Therefore MEFs coexpressing c-Myc and Tbx2 are immortal but not transformed (Supplementary Figure 1). We have taken advantage of this cooperation to design an unbiased transformation screen to find genes that will transform MEFs in combination with c-Myc and Tbx2 using retroviral cDNA expression libraries (Jacobs et al., 2000). This genetic predisposition allows the appropriate stringency, since c-Myc overexpressing p19Arf-deficient MEFs transform more easily, regardless of the introduced cDNA, and cause high background (Supplementary Figure 1).

Early passage primary MEFs were transduced with retroviruses containing Tbx2 and c-Myc (hereafter called c-Myc/Tbx2-MEFs) and subsequently transduced with retroviral cDNA libraries derived from different human tumors and normal tissues (Jacobs et al., 2000). Infected c-Myc/Tbx2-MEFs were then plated in soft agar and screened for colony formation (Figure 1). Colonies were picked and expanded as monolayers. The colonies differed in adhesion properties, ranging from semiadherent to almost nonadherent floating spheres. As expected we observed no background colonies in c-Myc/Tbx2 cells that were transduced with retroviruses containing no insert (negative control).

Using long range PCR and sequencing analysis, 12 cDNA inserts were identified, subcloned and reintroduced in c-Myc/Tbx2-MEFs to verify their transforming ability. Most inserts contained a full length cDNA and belonged to different functional groups (Table 1).

![Figure 1. Schematic outline of the in vitro genetic transformation screen.](image_url)

Figure 1. Schematic outline of the in vitro genetic transformation screen. MEFs were transduced with retroviruses containing Tbx2 and c-Myc respectively at passages 1 and 2. Thereafter, they were transduced with human retroviral cDNA libraries and subsequently were plated in soft agar, allowing selection for anchorage-independent growth. Colonies were picked after 2-3 weeks and expanded for further analysis. To enrich for the relevant insert a secondary screen was performed. Southern blot, PCR and sequence analysis were used to indentify the inserts. Transforming ability of the inserts was validated in c-Myc/Tbx2-MEFs.
Twelve genes belonging to distinct functional groups (cytoskeletal remodeling, Ras-Raf-Erk pathway, RTK signaling, lipid signaling, extracellular matrix and G2-M cell cycle progression) listed here, were identified and verified to transform MEFs in combination with c-Myc and Tbx2 (reduced p19Arf levels). Five of the identified inserts contained 5’ truncated cDNAs.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Accession number</th>
<th>Gene name</th>
<th>Size insert</th>
<th>Functional group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actn4</td>
<td>NM_004924</td>
<td>Alpha-actinin-4</td>
<td>Full length</td>
<td>Cytoskeleton</td>
</tr>
<tr>
<td>Agrn</td>
<td>NM_198576</td>
<td>Agrin</td>
<td>5’ 4831 bp truncated</td>
<td>Extra cellular matrix</td>
</tr>
<tr>
<td>Dbs/Mcf2l</td>
<td>NM_024979</td>
<td>Guanine nucleotide exchange factor Dbs</td>
<td>5’ 1324 bp truncated</td>
<td>Signaling and cytoskeletal remodeling</td>
</tr>
<tr>
<td>Edg4/LPA2</td>
<td>NM_020028</td>
<td>Lysophosphatic acid receptor 2</td>
<td>Full length</td>
<td>Lipid signaling</td>
</tr>
<tr>
<td>Flnc</td>
<td>NM_001458</td>
<td>Filamin C, gamma</td>
<td>5’ 5074 bp truncated</td>
<td>Cytoskeleton</td>
</tr>
<tr>
<td>FosB</td>
<td>NM_006732</td>
<td>FBJ murine osteosarcoma viral oncogene homolog B</td>
<td>Full length</td>
<td>Ras-Raf-Erk pathway</td>
</tr>
<tr>
<td>Grhl1</td>
<td>NM_014552</td>
<td>Grainyhead-like protein 1 homolog (Lbp-32)</td>
<td>Full length</td>
<td>Cytoskeleton</td>
</tr>
<tr>
<td>Hrs</td>
<td>NM_004712</td>
<td>Hepatocyte growth factor-regulated tyrosine kinase substrate</td>
<td>5’ 1223 bp truncated</td>
<td>Receptor tyrosine kinase signaling</td>
</tr>
<tr>
<td>Map3k3</td>
<td>NM_203351</td>
<td>Mitogen-activated protein kinase kinase 3</td>
<td>Full length</td>
<td>Ras-Raf-Erk pathway</td>
</tr>
<tr>
<td>Nek6</td>
<td>NM_014397</td>
<td>Nima (never in mitosis gene a)-related kinase 6</td>
<td>Full length</td>
<td>Mitosis and cell cycle</td>
</tr>
<tr>
<td>Pdgfb</td>
<td>NM_002608</td>
<td>Platelet-derived growth factor beta polypeptide</td>
<td>Full length</td>
<td>Receptor tyrosine kinase signaling</td>
</tr>
<tr>
<td>Raf-1</td>
<td>NM_002880</td>
<td>Raf proto-oncogene serine/threonine-protein kinase (C-Raf)</td>
<td>5’ 964 bp truncated</td>
<td>Ras-Raf-Erk pathway</td>
</tr>
</tbody>
</table>
The first group contained the cytoskeletal proteins alpha-actinin-4 (Actn4), filamin C (FlnC) and laminin binding protein 32 (Lbp-32). We also found truncated Dbs, constitutively active mutant of a Rho-specific guanine nucleotide exchange factor, which is involved in cytoskeletal remodeling and cell cycle progression (Kostenko et al., 2005). The second group contained components of the Ras-Raf-Erk signaling pathway (Raf-1, Map3k3 and FosB) that is known to cooperate with c-Myc in cell transformation. The third group contained PdgfB and hrs, both involved in receptor tyrosine kinase (RTK) signaling (Benito and Lorenzo, 1993; Toyoshima et al., 2007). Among the remaining genes we identified were Nek6, a serine/threonine kinase implicated in mitotic G2-M progression (O’regan et al., 2007) and Agrn, a large proteoglycan involved in neuromuscular junction development (Bowe and Fallon, 1995). This last group also contained LPA2, a GPCR for LPA.

As mentioned, Tbx2 reduces p19Arf levels but does not completely abolish it. Since the p19Arf threshold levels play a decisive role in transformation, we examined the transforming ability of the identified genes in the complete absence of p19Arf and in combination with c-Myc (Figure 2). c-Myc/Tbx2 (wild type)-MEFs and p19Arf-deficient c-Myc-MEFs were transduced with retroviruses containing the identified genes and tested for soft agar growth. All genes but one (Map3k3) were able to induce significantly larger number of colonies in soft agar in combination with c-Myc and in the absence of p19Arf. This shows that the residual levels of p19Arf in c-Myc/Tbx2-MEFs play an important role in attenuating transformation.

We next asked if any of these genes can cooperate with c-Myc or Tbx2 alone.
Figure 3. Transforming ability of LPA receptors in vitro. a) Expression analysis of LPA receptors using quantitative RT-PCR. Absolute mRNA levels of exogenous LPA receptors were determined in c-Myc/Tbx2-MEFs overexpressing different LPA receptors and normalized to Gapdh. b) Transforming potential of LPA receptors in soft agar. c-Myc/Tbx2-MEFs were retrovirally transduced with LPA1-4, RasV12 or control vector and were plated in soft agar after selection with G418 or puromycin. Upper panel shows soft agar assays scanned per well of a six-well plate and lower panel shows photographs from soft agar colonies taken at 25-fold magnification. c) Graph depicting quantification of soft
to transform MEFs. As shown in Supplementary Table 1, Actn4, Agrn, Dbs, FlnC, FosB, Hrs and PdgfB can cooperate with c-Myc alone to transform MEFs to at least the same extent as RasV12 does. None of the genes caused transformation in combination with Tbx2 alone.

These results reveal novel and established cooperative pathways for c-Myc and Tbx2 in transformation and underline the strength of gain-of-function screens to identify new oncogenes and oncogenic cooperations.

### Transforming potential of LPA receptors in vitro and in vivo

LPA acts on at least five distinct GPCRs (LPA1-5), which show both overlapping and distinct signaling properties and tissue expression (Ishii et al., 2004; Moolenaar et al., 2004). The so-called ‘Edg-family’ LPA receptors (LPA1, LPA2 and LPA3) are the best studied, whereas LPA4 and LPA5 receptors have only recently been identified and are more closely relayed to the purinergic receptor family (Lee et al., 2006a; Noguchi et al., 2003). Growing evidence points to a role of LPA receptor signaling in tumor progression and metastasis (Mills and Moolenaar, 2003). LPA1 overexpression in breast carcinoma cells drives their metastatic spread to bone (Boucharaba et al., 2004), while the LPA-producing exo-enzyme autotaxin is found overexpressed in various cancers and promotes tumor progression in preclinical models (van Meeteren and Moolenaar, 2007). However, the role of LPA receptor signaling during the initiating steps of tumor formation has not been investigated so far.

Having found that LPA2 can transform Myc/Tbx2-MEFs, we next examined if three other GPCRs for LPA, namely LPA1, LPA3 and LPA4, share this ability. Myc/Tbx2-MEFs were transduced with retroviruses encoding LPA1-4, RasV12 or with control vector and examined for growth in soft agar. As expected, RasV12-expressing Myc/Tbx2-MEFs transformed easily (Figure 3). Both LPA1 and LPA4 reproduced the transforming capacity of LPA2 (Figure 3b, c). Strikingly, LPA3 failed to induce a transformed phenotype in Myc/Tbx2-MEFs even though LPA3 expression levels were significantly higher than those of the other LPA receptors.
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If overexpression of LPA receptors can transform Myc/Tbx2-MEFs, would overstimulation of endogenous LPA receptors by LPA be sufficient to trigger the same effect? To address this question, we plated Myc/Tbx2-MEFs in soft agar in the presence or absence of 5 or 10 μM LPA. Figure 3d shows that addition of excess LPA (up to 10 μM) does not induce transformation of Myc/Tbx2-MEFs. This is consistent with the amount of LPA in serum-containing medium being saturating, and it suggests that the number of endogenous LPA receptors and, hence, signal strength determines the extent of transformation.

In addition, we analyzed how p19Arf may affect LPA receptor-mediated transformation. We therefore compared c-Myc/Tbx2-MEFs with p19Arf-deficient c-Myc-MEFs expressing LPA1-4 (Figure 3e). Similar to what is observed with LPA2 (Figure 2), the absence of p19Arf strongly potentiated the LPA1- and LPA4-

Figure 4. Overexpression of LPA1, LPA2 and LPA4 receptors is sufficient to induce tumor formation in combination with c-Myc and Tbx2 in nude mice. 1 x 10^6 c-Myc/Tbx2-MEFs overexpressing LPA1-4, RasV12 or control vector were s.c. injected in each flank of the mice (n=4 mice, eight flanks per condition). Tumor formation and growth was monitored during 75 days. Survival plots are shown here, (flanks with tumors/total flanks injected). Mice injected with Myc/Tbx2-MEFs overexpressing LPA3 (grey) or control vector (green) do not develop any tumors.

(Figure 3a and Supplementary Figure 2). If overexpression of LPA receptors can transform Myc/Tbx2-MEFs, would overstimulation of endogenous LPA receptors by LPA be sufficient to trigger the same effect? To address this question, we plated Myc/Tbx2-MEFs in soft agar in the presence or absence of 5 or 10 μM LPA. Figure 3d shows that addition of excess LPA (up to 10 μM) does not induce transformation of Myc/Tbx2-MEFs. This is consistent with the amount of LPA in serum-containing medium being saturating, and it suggests that the number of endogenous LPA receptors and, hence, signal strength determines the extent of transformation.

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induced transformation (Figure 3e). Again, LPA3 was incapable of transforming MEFs even in the absence of p19Arf. None of the LPA receptors tested could induce transformation in combination with either Tbx2 or c-Myc alone (data not shown), consistent with c-Myc and Tbx2 being cooperating partners in cell transformation. These results indicate a direct role for LPA receptor signaling in oncogenic transformation in cooperation with c-Myc and Tbx2. Furthermore, they reveal that LPA3 mitogenic signaling differs fundamentally from that induced by LPA1, LPA2 and LPA4.

We next examined the ability of LPA receptors to induce tumor formation in vivo. To this end, 10⁶ Myc/Tbx2-MEFs overexpressing distinct LPA receptors, RasV12 or control vector were subcutaneously injected into nude mice and tumor formation was monitored up to 75 days. As shown in Figure 4, control c-Myc/Tbx2-MEFs failed to form tumors, whereas RasV12 MEFs produced sizeable tumors within 10 days. Overexpression of LPA1, LPA2 or LPA4 in Myc/Tbx2-MEFs induced tumor formation very efficiently. In contrast, LPA3 overexpression did not lead to detectable tumor formation over a 3-month period. These results are fully consistent with the in vitro (soft agar) data and show that overexpression of LPA1, LPA2 or LPA4 is sufficient to initiate tumor formation in combination with c-Myc and Tbx2.

\textbf{LPA receptor-induced transformation depends on Gi, Erk and PI3K activity}

Which LPA signaling pathways are responsible for mediating cell transformation? LPA receptors couple to multiple G proteins, particularly Gi/o, Gq/11 and G12/13, to activate various downstream effectors. LPA-induced cell proliferation and survival are critically dependent on the Gi-linked Ras-Map kinase and PI3K pathways (Moolenaar et al., 2004). We tested the effect of pertussis toxin (PTX), which specifically ADP-ribosylates Gi/o proteins and thereby prevents their activation. PTX treatment (50 ng/ml) completely abolished cell transformation induced by LPA1, LPA2 and LPA4 in c-Myc/Tbx2-MEFs, but not the transformation induced by RasV12 (Figure 5a). Thus, Gi signaling is essential for LPA receptors to transform MEFs. Figure 5a also shows that PD-98059 (10 μM), a specific inhibitor of Erk1/2, strongly inhibited LPA receptor-induced transformation of Myc/Tbx2-MEFs. In addition, we examined the involvement of the PI3K pathway using the PI3K inhibitor LY-294002 and its inactive analog LY-303511. As shown in Figure 5b, transformation of Myc/Tbx2-MEFs expressing distinct LPA receptors is severely inhibited by LY-294002 (5 μM), whereas LY-303511 (up to 10 μM) had no effect. From these results, we conclude that LPA receptor-induced transformation is dependent on both the Gi-linked Erk1/2 and PI3K pathways.

RasV12-transduced cells showed only a partially reduced transforming capacity (reduced number of soft agar colonies) in the presence of Erk or PI3K inhibitors (Figures 5a and 5b). Although concentrations used in these experiments may not be sufficient to inhibit RasV12-induced transformation more efficiently, it should
be emphasized that Ras-induced transformation does not solely depend on either Erk1/2 or PI3K activation (Khosravi-Far et al., 1996; Rodriguez-Viciana et al., 1994; Shields et al., 2000; Zuber et al., 2000).

**Different kinetics of Erk activation by distinct LPA receptors**

The Erk activation by growth factors usually shows biphasic kinetics. An initial, very transient phase of Erk activation (lasting 5-20 min.) is followed by a second phase of activation that is much more sustained. The latter phase is necessary for S-phase entry and cell proliferation (Jones and Kazlauskas, 2001). We examined the kinetics of Erk activation in Myc/Tbx2-MEFs expressing the respective LPA receptors. Myc/Tbx2-MEFs overexpressing LPA1-4 receptors showed very low basal Erk1/2 activity, similar to control MEFs (Figure 6a). Upon LPA stimulation for 5 min, all cells showed enhanced Erk1/2 activity (Figure 6a). The LPA1-4 overexpressing cells initiated rapid Map kinase activation similar to control cells. We then monitored the prolonged phase of LPA-induced Erk1/2 phosphorylation
in LPA2-expressing MEFs versus control MEFs (Supplementary Figure 3). The LPA2 overexpressing cells showed stronger and more sustained Erk1/2 activation (up to 2 h) when compared to control MEFs. Next we tested Erk1/2 activation for the other three LPA receptors. Figure 6b shows that LPA1 and LPA4, similar to LPA2, maintained prolonged Erk activation (up to 2 h after LPA stimulation). In marked contrast, LPA3-overexpressing cells showed only the early transient Map kinase activity phase but not the second prolonged phase, similar to what was observed for the control cells. In conclusion, the transforming ability of LPA receptors is closely linked with their ability to sustain prolonged Erk1/2 activation.

**Figure 6. Transient and prolonged Erk activation by distinct LPA receptors.** Western blot analysis detecting activated Map kinase, using Erk1/2 phospho-specific antibodies. a) Transient Erk1/2 phosphorylation and b) prolonged Erk1/2 phosphorylation in LPA-stimulated versus non-stimulated MEFs. c-Myc/Tbx2-MEFs retrovirally transduced with LPA1-4, RasV12 or control vector were serum starved for 16 h and subsequently stimulated with 1 μM LPA for the indicated time points (5, 30, 60, 120 min), after which cell lysates were prepared. Anti-tubulin was used to show equal loading. c) LPA signaling pathways that may collaborate with c-Myc and Tbx2 (reduced p19Arf levels) in transformation. LPA receptors can trigger G protein-mediated Ras-Erk, PI3K-Akt and Rho pathway activation to regulate cell proliferation, survival and motility. When p19Arf levels are suppressed, c-Myc is able to collaborate with LPA signaling, notably Erk1/2 and/or PI3K activation, to fully transform cells.
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Discussion

In the present study, we have explored pathways that cooperate with c-Myc in tumorigenesis in the context of reduced p19Arf expression. Anchorage-independent growth is one of the hallmarks of transformation and correlates with tumorigenicity *in vivo*. Therefore, we screened for genes and ultimately pathways that cooperate with c-Myc in transformation using selection for anchorage-independent growth in soft agar as read-out. With this, we not only confirmed already established cooperative pathways for c-Myc in transformation, but also identified new oncogenic collaborators, underlining the use of gain-of-function screens.

In response to prolonged mitogenic signals from the c-Myc onco-protein, primary MEFs upregulate p19Arf levels and consequently undergo apoptosis (Zindy *et al.*, 1998). In contrast, primary cells lacking functional p19Arf efficiently bypass apoptosis and occasionally transform under high selection pressure in soft agar (Jacobs *et al.*, 1999). Therefore using c-Myc overexpressing wild type- or p19Arf-deficient MEFs does not offer the proper stringency to screen for oncogenic collaborations. However, downmodulating p19Arf by Tbx2 to a threshold level which bypasses apoptosis and yet not easily predisposes cells for transformation allowed us the opportunity to screen for cooperating genes (Supplementary Figure 1). In addition to p19Arf, Tbx2 represses p21 which is also repressed by c-Myc (Prince *et al.*, 2004; Seoane *et al.*, 2002). Since c-Myc/Tbx2 overexpression in p19Arf-deficient MEFs does not cause more soft agar colonies than c-Myc overexpression alone in p19Arf-deficient MEFs (Supplementary Figure 1), we can conclude that the main function of Tbx2 in these MEFs is to repress p19Arf.

With this screen, we identified twelve genes that induce transformation in MEFs in cooperation with c-Myc and Tbx2 (reduced p19Arf expression). It is known that c-Myc can cooperate with activated Ras and oncogenes that function in the Ras pathway to induce transformation (Drayton *et al.*, 2003; Land *et al.*, 1983; Land *et al.*, 1986). Corroborating this, we find truncated Raf-1, which has been shown to be the constitutively activated form of Raf-1 (Heidecker *et al.*, 1990). In addition, we find Map3k3 and FosB which are also players in the Ras-Raf-Erk pathway. Identification of genes that are already known to directly cooperate with c-Myc validates our screen.

More upstream we found the proto-oncogene PdgfB, which stimulates cellular proliferation and motility through its RTKs and we identified Hrs which is an important downstream regulator of RTK signaling. Both genes have been implicated in tumorigenesis (Pietras *et al.*, 2003; Toyoshima *et al.*, 2007). RTK signaling can activate the Ras and PI3K pathways (Ramjaun and Downward, 2007), thereby explaining their collaboration with c-Myc and Tbx2 in transformation.

However, we also find genes that are involved in cytoskeletal remodeling, such as Actn4, truncated Dbs, FlnC and Grhl1. Regulation of cytoskeletal changes play
a crucial role in cell motility, anchorage-independent growth and metastasis. For example, Actn4, an actinin bundling protein, has been implicated in breast cancer, and progressed stages of esophageal squamous cell carcinoma and lung cancer (Fu et al., 2007; Honda et al., 1998; Honda et al., 2005). Moreover, recently Actn4 was found to be one of the binding partners of Akt1, thereby having a crucial function in activation and translocation of Akt1 to the cell membrane (Ding et al., 2006).

Interestingly, we find Actn4, Agrn, Dbs, Flnc, Fosb, Hrs and Pdgfb capable of cooperating with c-Myc alone independent from p19Arf. This transforming ability is as efficient as the transforming ability of c-Myc and RasV12. These genes may be important players up- or downstream of the Ras-Raf-Erk pathway contributing to oncogenic transformation. They might also directly or indirectly suppress the p19Arf/p53 pathway.

p19Arf-deficient c-Myc-MEFs overexpressing Map3k3 are less transformed than c-Myc/Tbx2-MEFs overexpressing Map3k3. This observation is rather surprising since p19Arf deficiency was expected to facilitate a stronger transformation as was observed for the other identified genes. This however suggests that other tumor suppressors (such as p16Ink4a or p15Ink4b) could be activated in p19Arf-deficient cells overexpressing the specific combination of c-Myc and Map3k3 (Krimpenfort et al., 2007). It is also possible that Tbx2 exerts additional functions which cooperate with Map3k3 in wild type MEFs.

Furthermore, in addition to truncated Raf-1 and Dbs, which are known to be the constitutively active mutants, we also found Agrn, Flnc and Hrs inserts to be 5’-truncated. Therefore, we can not exclude any dominant negative or dominant active roles of these latter mutants.

LPA2 is one of the novel cDNA inserts that cooperates with c-Myc and Tbx2 in our screen. We show that LPA1 and LPA4, but not LPA3, can mimic the transforming action of LPA2 in c-Myc/Tbx2-MEFs in vitro and in vivo. This transforming ability is dependent on Gi, Erk and PI3K activity (Figure 6c). Strikingly, MEFs overexpressing LPA1, LPA2 and LPA4 retain prolonged Erk1/2 activation when stimulated with LPA, whereas LPA3 is incapable of sustaining Erk1/2 activation, which may provide a mechanistic explanation for LPA3 lacking transforming potential. This suggests that sustained Erk1/2 activation may underlie the cooperation with c-Myc and Tbx2 in transformation, in particular because activated Ras can cooperate with c-Myc in tumorigenesis. Here, we show not only a direct role for LPA1, LPA2 and LPA4 in transformation, but also a vital difference amongst LPA receptors regarding their signaling and contribution to transformation and tumorigenesis.

It should be noted that overexpression of LPA receptors in MEFs by itself does not lead to ‘spontaneous’ receptor activation as inferred from Map kinase activity assays. Instead, LPA present in serum is necessary to activate LPA receptor signaling.

Overexpression of LPA2 has been implicated in ovarian cancer (Erickson et al.,
Even though LPA2 transgenic mice do not develop ovarian malignancies, there is a significant increase in the expression of angiogenesis and metastasis inducing factors such as VEGF, VEGF receptor and uPA in the ovaries of these mice (Huang et al., 2004). Therefore, LPA signaling is suggested to contribute to tumor progression and metastasis rather than to promote tumor formation by itself. Here, we demonstrate that additional alterations such as enhanced c-Myc activity and inactivation of the p19Arf tumor suppressor (both frequently found in tumors) can nevertheless cooperate with enhanced LPA signaling and lead to tumor initiation. LPA receptors are not able to induce transformation in combination with c-Myc alone. The specific requirement of p19Arf repression (brought by Tbx2) for LPA receptor-induced transformation suggests that prolonged LPA signaling might trigger a p19Arf tumor suppressor response by itself.

Besides the differences in sustained Map kinase activation downstream of LPA receptors, there may also be additional explanations underlying their distinct transforming potential. Deregulated expression of Edg-family LPA receptors in different combinations has been reported in several cancers. Whereas enhanced expression of LPA2 has been implicated in ovarian, prostate and colon cancer, LPA1 and LPA3 expression patterns are more diverse. LPA1 and LPA2 share many downstream signaling pathways, whereas LPA3 seems to act differently. Previous analysis of LPA receptor signaling (Ishii et al., 2000) suggested that LPA3 fails to couple to the G12/13-linked RhoA pathway. RhoA not only regulates the actin cytoskeleton but also can contribute to cell cycle progression and cell transformation (Jaffe and Hall, 2005). To what extent G12/13-Rho signaling pathways contribute to LPA-induced cell transformation remains a challenge for future studies.

Consistent with the divergence of downstream signaling pathways of LPA1 and LPA2 versus LPA3, targeted deletion studies of LPA receptors in mice have revealed functional redundant roles for LPA1 and LPA2 in neuronal and craniofacial development (Ishii et al., 2004), whereas LPA3 deletion leads to impaired female reproduction (Ye et al., 2005).

Downstream signaling pathways of LPA4 have not yet been delineated as extensive as those of the Edg-family members. LPA4 mediates calcium signaling and Rho activation through stimulation of Gq/Gi and G12/13, respectively and in contrast to LPA1-3, LPA4 can raise cAMP levels by coupling to Gs (Lee et al., 2007). Here, we report that LPA4 induces PI3K and prolonged Erk1/2 activity through Gi signaling and we demonstrate a role for LPA4 (a non-Edg LPA receptor) in cell transformation and tumorigenesis.

Our data connect LPA1, LPA2 and LPA4 overexpression to transformation and tumorigenesis with no apparent role for LPA3 in transformation, which correlates with their capability to sustain Map kinase activation. Although the exact mechanism by which LPA signaling promotes tumorigenesis needs to be elucidated, our study shows that LPA receptor signaling contributes not only to tumor progression but also to tumor initiation. Since the LPA receptor family is still expanding (Pasternack
et al., 2008), it remains interesting to examine the importance of those newly identified LPA receptors in tumor formation.

Materials 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). Ecotropic retroviruses were produced by transient transfection of phoenix-packaging cells with retroviral expression vectors, using calcium-phosphate co-precipitation method. Supernatant collection started 48 h after transfection. Retroviral transduction was performed as described before (Jacobs et al., 1999). c-Myc/Tbx2-MEFs were generated as follows: primary MEFs were transduced with retroviruses containing LZRS-Tbx2-iresGFP (Jacobs et al., 2000) at passage 1, and subsequently were transduced with retroviruses containing LZRS-MycHA-iresGFP (Jacobs et al., 1999) at passage 2. For LPA receptor overexpression experiments we made use of the following human constructs: LZRS-HA-LPA1-iresNeo, LZRS-HA-LPA2-iresNeo, LZRS-HA-LPA3-iresNeo and LZRS-HA-LPA4-iresNeo. LZRS-iresNeo empty vector is used as negative control and pBabePuro-RasV12 is used as positive control. 48 h after transduction cells were selected using 1 mg/ml G418 (Invitrogen) or 4 μg/ml puromycin (Sigma). The proper membrane localization of LPA receptors using the overexpression constructs has been verified by anti-HA immunofluorescence experiments (data not shown).

Soft agar assays

Cells (6 x 10⁴) were per well of a six-well plate resuspended in Dulbecco’s Modified Eagle Medium (GIBCO) supplemented with 10% fetal bovine serum (PAA laboratories) and 0.35% 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 scanned (Epson perfection 4900 photo scanner) and the amount of colonies were quantified using ImagePro-Plus 5.1 software (Media Cybernetics). All the experiments were performed in triplicate in several independently isolated batches of MEFs.

Genetic transformation screen

Retroviral cDNA expression libraries used here are: human prostate, human leukocyte, human mammary gland (clontech), 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 c-Myc/Tbx2-MEFs in two consecutive rounds of transduction of 1-2 x 10⁶ cells plated per 10 cm culture dishes. Empty pMXsubF- and pLIB-vectors are used as the negative control. 48 h after library transduction, cells were plated in soft agar in 10cm culture dishes as described above. c-Myc/Tbx2-MEFs transduced with retroviruses containing RasV12 were used as positive control. After 2-3 weeks, from cells that were transduced with cDNA libraries, 175 colonies were picked and expanded as monolayers for genomic DNA isolation and secondary screen. Southern blot analysis revealed multiple integrations (1-8) per colony. To facilitate cloning and testing of the relevant inserts, a secondary screen using wild type replication competent MoMuLV was performed as described before (Jacobs et al., 2000). For each primary colony, 3-5 secondary colonies were picked. From 175 original colonies, 164 were positive after the secondary screen. Hereby true positive colonies were selected for further analysis. Using long range PCR techniques (Expand long template PCR system, Roche) and retroviral specific primers, the transforming inserts were amplified and subsequently subcloned (TOPO TA cloning, Invitrogen) and finally identified by sequencing. Identified inserts were either cloned back into the original
constructs (pLIB and pMX) or in pBabePuro and were re-tested in c-Myc/Tbx2-MEFs in soft agar.

**Quantitative RT-PCR expression analysis**
Total RNA isolation was performed using TRIZOL reagent (Invitrogen). First strand cDNA was prepared using Superscript II reverse transcriptase and oligo(dT) primers (Invitrogen). qRT-PCR is performed using an ABI PRISM 7000 Real Time PCR system and SYBR green PCR master mix (Applied Biosystems). Primer sequences are: LPA1-F 5'-AATCGGGATACCATGATGAGTCTT-3', LPA1-R 5'-CCAGGAGTCCAGCAGATGATAAA-3', LPA2-F 5'-CGCTCAGCCTGGTGTCAGACT-3', LPA2-R 5'-TTGCAGGACTCACAGCCTAAAC-3', LPA3-F 5'-AGGACACCCCATGAAGCTAAATGAA-3', LPA3-R 5'-GCGGTCAGGGAGCAGAAC-3', LPA4-F 5'-CCTAGTCCTAGTGCGGTATT-3', LPA4-R 5'-CTCTAAAGCAGTGTTGTTT-3'. The amount of target mRNA was calculated using a standard curve based on serial dilutions with known quantities (absolute quantification protocol, Applied Biosystems), and normalized to glyceraldehyde-3-phosphate dehydrogenase (Gapdh).

**Pharmacological inhibition experiments**
c-Myc/Tbx2-MEFs were retrovirally transduced with LZRS-HA-LPA1-4-iresNeo, pBabePuro-RasV12 or LZRS-iresNeo empty vector. Cells were selected with 1mg/ml G418 or 4 μg/ml puromycin starting 48 h after transduction. Hereafter cells were plated in the presence or absence of inhibitors, namely 50 ng/ml PTX (List Biological Laboratories), 5 and 10 μM LY-294002/inactive control LY-303511 (Calbiochem) and 10 μM PD-98059 (Calbiochem) in soft agar as mentioned above.

**Erk phosphorylation Western blot analysis**
c-Myc/Tbx2-MEFs were retrovirally transduced with LZRS-HA-LPA1-4-iresNeo, pBabePuro-RasV12 or LZRS-iresNeo empty vector. After selection 3 x 10^5 cells were plated per well of a six-well plate. Next day the cells were washed with phosphate buffered saline (PBS) and grown in DMEM containing 0.1% FBS for at least 16 h. Thereafter cells were stimulated with DMEM containing 1 μM LPA (Avanti Polar Lipids Inc. Alabaster, AL) for indicated time points (5, 10, 30, 60, 120 or 240 minutes). Cells were 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: monoclonal anti-phospho Map kinase antibody (Sigma 1:1000), anti-tubulin (Sigma), polyclonal anti-CDK4 (Santa Cruz. 1:1000). Secondary antibodies were: goat anti mouse (Zymed) or goat anti rabbit (Biosource) both conjugated to HRP.

**In vivo tumor formation assays**
Male BALB/c nude mice (7-8 weeks of age), were injected subcutaneously with 1 x 10^6 cells (resuspended in PBS) in each flank. For each condition a total of 8 flanks (four mice) were injected. Tumor size was monitored every 2-3 days by measuring the length (L) and the width (W) of the developing tumor using a caliper. Tumor volume was calculated following the formula (W^2 x L)/2. Mice were sacrificed when tumors reached a maximum size of 1 cm in the largest diameter.
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References


Chapter 2


LPA Signaling and Cell Transformation


Chapter 2


Supplementary Information

**Supplementary Figure 1.** Graph depicting absolute number of soft agar colonies (from $6 \times 10^4$ cells plated) from wild type c-Myc/Tbx2-MEFs versus p19Arf-/-MEFs transduced either with c-Myc or with c-Myc/Tbx2. P19Arf-/- c-Myc-MEFs are more prone to transformation than wild type c-Myc/Tbx2-MEFs. P19Arf-/- c-Myc/Tbx2-MEFs and p19Arf-/- c-Myc-MEFs are equally transformed. P19Arf-/- c-Myc/RasV12-MEFs are shown for comparison.

**Supplementary Figure 2.** Western blot analysis detecting hA-tagged LPA1-3. c-Myc/Tbx2-MEFs were transduced with retroviruses encoding HA-tagged-LPA1-3 and control vector. Protein expression analysis was performed after G418 selection. Ponceau S-staining (PS) is shown for equal loading.
Supplementary Figure 3. Western blot analysis detecting Erk1/2 phosphorylation in LPA-stimulated versus nonstimulated MEFs. c-Myc/Tbx2-MEFs transduced with retroviruses containing LPA2, RasV12 or control vector were serum starved for 16 h and subsequently stimulated with 1 μM LPA for indicated time points (3, 10, 30, 60, 120 and 240 minutes), after which cell lysates were prepared. Anti-tubulin is used as loading control.

**Supplementary Table 1.** The cooperation potential of identified genes with either Tbx2 or c-Myc in cell transformation. Identified genes were co-expressed with c-Myc/Tbx2, c-Myc or Tbx2 in MEFs and were subsequently tested for soft agar growth. Colony formation was scored relatively to transformation observed in the positive and negative controls (RasV12 and empty vector).

<table>
<thead>
<tr>
<th>Gene</th>
<th>c-Myc/Tbx2</th>
<th>Tbx2</th>
<th>c-Myc</th>
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<tbody>
<tr>
<td>Actn4</td>
<td>+++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Agrn</td>
<td>++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Dbs/Mcf2L</td>
<td>++++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Edg4/LPA2</td>
<td>++++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FlnC</td>
<td>++++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>FosB</td>
<td>++++++</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td>Grhl1</td>
<td>++</td>
<td>-</td>
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<tr>
<td>RasV12</td>
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<td>Control</td>
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