

A genome-wide RNAi screen in mouse embryonic stem cells identifies Mp1 as a key mediator of differentiation

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Despite intense investigation of intrinsic and extrinsic factors that regulate pluripotency, the process of initial fate commitment of embryonic stem (ES) cells is still poorly understood. We used a genome-wide short hairpin RNA screen in mouse ES cells to identify genes that are essential for initiation of differentiation. Knockdown of the scaffolding protein Mek binding protein 1 (Mp1, also known as Lamtor3 or Map2k1ip1) stimulated self-renewal of ES cells, blocked differentiation, and promoted proliferation. Fibroblast growth factor 4 (FGF4) signaling is required for initial fate commitment of ES cells. Knockdown of Mp1 inhibited FGF4-induced differentiation but did not alter FGF4-driven proliferation. This uncoupling of differentiation and proliferation was also observed when oncogenic Ras isoforms were overexpressed in ES cells. Knockdown of Mp1 redirected FGF4 signaling from differentiation toward pluripotency and up-regulated the pluripotency-related genes *Esrrb*, *Rex1*, *Tcl1*, and *Sox2*. We also found that human germ cell tumors (GCTs) express low amounts of Mp1 in the invasive embryonic carcinoma and seminoma histologies and higher amounts of Mp1 in the noninvasive carcinoma in situ precursor and differentiated components. Knockdown of Mp1 in invasive GCT cells resulted in resistance to differentiation, thereby showing a functional role for Mp1 both in normal differentiation of ES cells and in germ cell cancer.

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Abbreviations used: AP, alkaline phosphatase; BRL, buffalo rat liver conditioned; CB, cytoskeleton buffer; CC, choriocarcinoma; CIS, carcinoma in situ; EC, embryonic carcinoma; EpiSC, epiblast-derived stem cell; Erk, extracellular signal-regulated kinase; ES, embryonic stem; FGF, fibroblast growth factor; GCT, germ cell tumor; LDM, LIF-depleted medium; LIF, leukemia inhibitory factor; MAPK, mitogen-activated protein kinase; MBD3, Methyl-CpG binding domain protein 3; Sem, seminoma; shRNA, short hairpin RNA; shRnd, random shRNA; Ter, teratoma; YST, yolk sac tumor.

Self-renewal of mouse embryonic stem (ES) cells has been studied extensively, which has resulted in the identification of growth factors that are able to keep ES cells undifferentiated when cultured in vitro (Smith and Hooper, 1987; Smith, 1991; Ying et al., 2003, 2008; Greber et al., 2010). Downstream of the pathways activated by these growth factors are the core pluripotency regulating transcription factors Oct3/4, Sox2, Klf4, and Nanog, which form a self-sustaining network (Niwa et al., 2000; Chambers et al., 2003; Mitsui et al., 2003; Boyer et al., 2005; Kuroda et al., 2005; Li et al., 2005; Ivanova et al., 2006; Loh et al., 2006;

Kim et al., 2008). Introduction of these factors in different combinations, including cMyc and lin28, into somatic cells leads to reprogramming and functional conversion into an induced pluripotent stem cell (Takahashi and Yamanaka 2006; Okita et al., 2007; Takahashi et al., 2007; Yamanaka 2008).

In vitro cultured mouse ES cells can be differentiated into any cell of the mouse body

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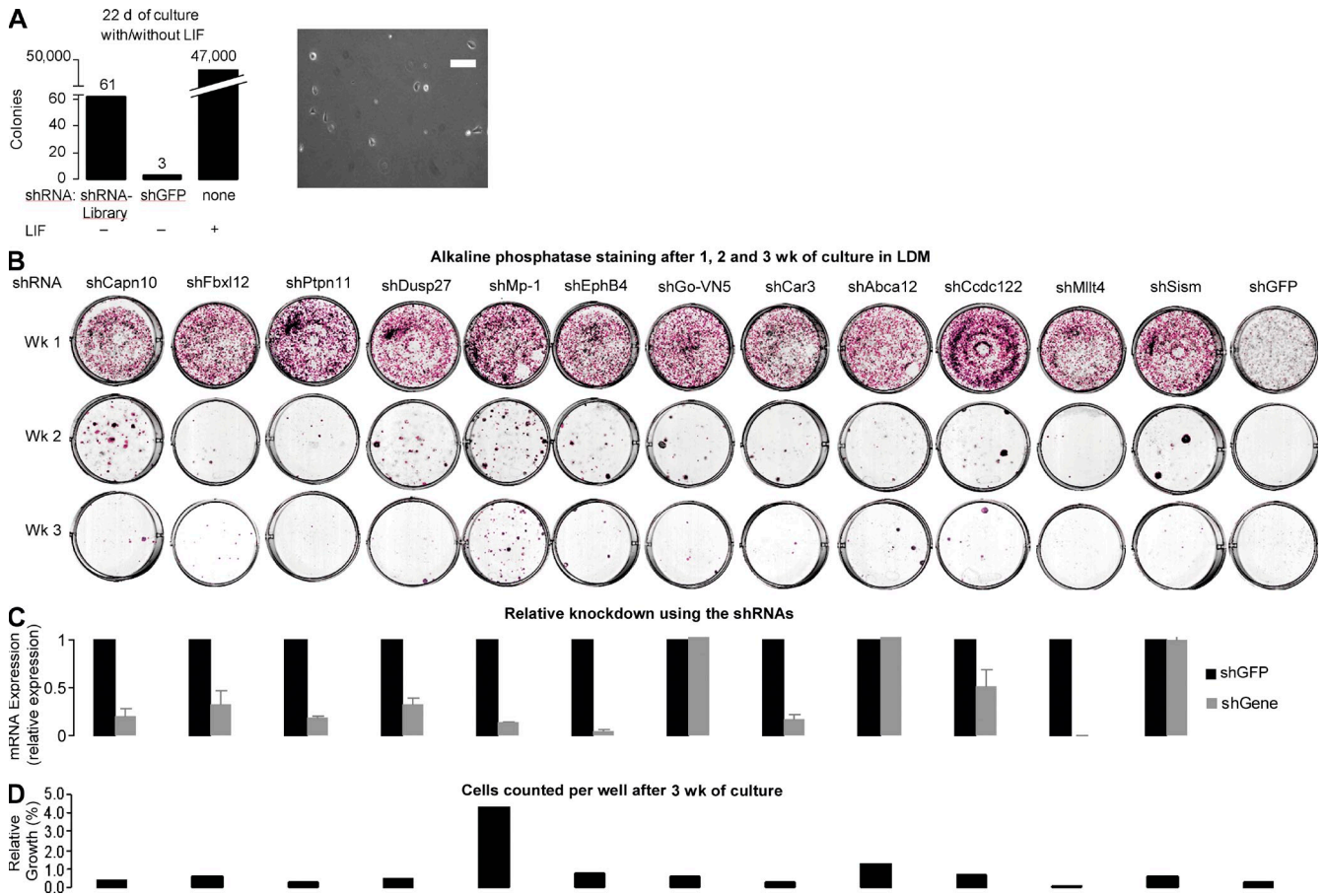


Figure 1. shRNA screen for genes influencing mouse ES cell differentiation. Mouse ES cells were retrovirally transduced with a shRNA library targeting 16,000 genes (or shGFP specific). Cells were grown in LDM for 1 wk, and this was repeated two times. Colonies remaining after 3 wk were pooled, and 300 recovered shRNAs were sequenced. (A) Bar graphs show the absolute number (above bars) of recovered ES colonies after 3 wk of culture. The seeding density of the ES cells 1 d after plating is shown on the right. (B–D) The identified shRNAs were reintroduced into mouse ES cells, which were grown for one, two, or three replatings. (B) At the indicated time points, cells were stained with AP. (C) Efficiency of target gene knockdown (relative to shGFP) was measured by qPCR. (D) Cells in each well were counted and compared with +LIF conditions (100%) after 3 wk of culture. Error bars, standard deviation. Data represent two independent experiments. Bar, 100 μ m.

when placed back into blastocysts (Beddington and Robertson, 1989) and, therefore, ES cells are named pluripotent. In the absence of mouse embryonic fibroblasts, ES cell pluripotency is maintained by the IL-6 family cytokine leukemia inhibitory factor (LIF; Smith and Hooper, 1987; Smith et al., 1988; Williams et al., 1988; Niwa et al., 2009). Stat3 is the key downstream target of the LIF pathway, and dominant-negative Stat3 induces differentiation of ES cells in the presence of LIF (Boeuf et al., 1997). Mouse ES cells can be maintained pluripotent in the absence of any cytokine signaling in medium that contains the fibroblast growth factor (FGF) receptor inhibitor SU5402, and the phospho-extracellular signal-regulated kinase (Erk) inhibitor PD184352 together with a pharmacological inhibitor of GSK3, CHIR99021 (Ying et al., 2008). This finding highlights the fact that inhibition of the Ras–Mek–Erk pathway is pivotal for prevention of differentiation of mouse ES cells (Kunath et al., 2007). Moreover, it was shown that Stat3-deficient ES cells remained undifferentiated using these three inhibitors, showing

that Stat3 is dispensable for self-renewal (Ying et al., 2008). When Stat3-deficient ES cells are grown in medium containing LIF, this leads to differentiation. Because LIF induces Ras–Mek–Erk signaling, this indicates that in Stat3-proficient cells, Stat3 overrules the differentiation cues given by activation of the Ras–Mek–Erk pathway.

In addition to the requirement of LIF/Stat3 signaling, it was found that serum is required to propagate ES cells to prevent neuroectodermal commitment. Serum can be substituted by Bmp4, showing that the Smad1,5,8 pathway inhibits neural commitment (Ying et al., 2003). Furthermore, Bmp4 has been shown to repress the p38 mitogen-activated protein kinase (MAPK) pathway (Qi et al., 2004).

To address which factors contribute to the initial commitment of ES cells to germ layer fates, we performed a genome-wide loss of function screen using a short hairpin RNA (shRNA) approach. We found that shRNA-mediated knockdown of the scaffolding protein Mp1 inhibits ES cell differentiation, whereas FGF4- or HrasV12-mediated proliferation is

Table 1. Summary of genes corresponding to the hairpins identified in the ES cell screen

Number	shRNA 19mer identified	Matching gene	Full gene name	Hairpin identity	Number of hairpins out of 300	Percentage of hairpins	Described before?	Reference
1	TAGCGATGTGGTACACAGC	Capn10	Calpain 10	19/19 (100%)	151	50	No	
2	TTTGCTTATAAGGTCACCTC	Fbxl12	F-box and leucine-rich repeat protein 12	19/19 (100%)	24	8.0	No	
3	TTGAGTGGCAATGTAACCTC	Ptpn11 (Shp2)	Protein tyrosine phosphatase, non-receptor type 11	19/19 (100%)	19	6.3	Yes	Burdon et al., 1999
4	TTGCTATCCATCTCGTTAC	Dusp27	Dual specificity phosphatase 27	19/19 (100%)	16	5.3	No	
5	TATCTGACACAACAATTGC	MP1	<i>Mus musculus</i> similar to mitogen-activated protein kinase 1-interacting protein 1 (MEK binding partner 1)	19/19 (100%)	8	2.6	No	
6	TTGACCAAGATGTTCCGAG	Ephb4	Eph receptor B4	19/19 (100%)	3	1.0	Yes	Wang et al., 2006
7	TTGAATAAACTTGGTAAAC	GOVN5	<i>Mus musculus</i> similar to putative pheromone receptor (Go-VN5)	19/19 (100%)	3	1.0	No	
8	CCCATTGAACTGCATACTA	Car3	Carbonic anhydrase 3	19/19 (100%)	2	0.7	No	
9	TACTCTCTATAAGCCAGGC	Abca12	ATP-binding cassette, sub-family A (ABC1), member 12, transcript variant 3	19/19 (100%)	2	0.7	No	
10	CATTATGACTGAACTCTA	Ccdc122	Coiled-coil domain containing 122	19/19 (100%)	1	0.3	No	
11	TACTAAGTAGGGCAGCTTC	Mllt4	Myeloid/lymphoid or mixed-lineage leukemia	19/19 (100%)	1	0.3	Yes	Ikeda et al., 1999
12	GGATTATATCCATATTCAA	Sism	Similar to suppressor of initiator codon mutations	19/19 (100%)	1	0.3	No	

The total number of hairpins was 234 (78%), with other hairpins numbering 66 (22%), for a grand total of 300 (100%).

not affected. Mp1 controls the branching downstream of FGF/Ras signaling and thereby regulates pluripotency gene transcription. Furthermore, we show that early invasive germ cell tumors (GCTs; seminoma [Sem] and embryonal carcinoma) have low expression levels of Mp1, compared with the preinvasive stage as well as differentiated components, and this low expression contributes to inhibition of differentiation.

RESULTS

A genome-wide screen identifies candidate genes that, upon knockdown, maintain pluripotency in the absence of LIF

To gain insight into the early events that regulate the first steps of ES cell differentiation, we performed an shRNA-mediated knockdown screen to inhibit differentiation of ES cells in the absence of LIF/Stat3 signaling. For this, we retrovirally transduced mouse ES cells with an shRNA library targeting 16,000 genes (Huang et al., 2009). Cells were subsequently plated at a density that allowed colony outgrowth from a single cell (plating density is shown in Fig. 1 A) and cells were grown in LIF-depleted medium (LDM) for 1 wk. This was repeated two times, allowing a three-fold clonal expansion. The colonies that were present after 3 wk ($n = 61$) were pooled and genomic DNA was recovered (Fig. 1 A,

bar graph). The control plates, containing ES cells infected with an shRNA against GFP (referred to as shGFP; Table S1), gave a low number of colonies ($n = 3$). The shRNAs were recovered by PCR, recloned into the pRetrosuper backbone, and sequenced. Sequencing of 300 individual plasmids led to identification of 12 putative target genes of these shRNAs. A 100% match of each 19-mer was observed with the corresponding mRNA. The relative representation of the shRNAs, given as the percentage of all hairpins that were recovered, is shown in Table 1. The shRNA sequence targeting the gene calpain 10 (Capn10) was identified in roughly half of the sequences, and for the other shRNAs the relative representation ranged from 8 to <1% of the clones. Three of the genes have previously been described to be involved in differentiation of ES cells, i.e., Ptpn11 (also called Shp-2; Burdon et al., 1999), Ephb4 (Wang et al., 2006), and Mllt4 (myeloid/lymphoid or mixed-lineage leukemia [trithorax homologue, *Drosophila*]; Ikeda et al., 1999), which validates our screening strategy. Recovered shRNA sequences are given in Tables S3 and S4.

The identified shRNAs were reintroduced into ES cells individually to validate the phenotype of each shRNA. Alkaline phosphatase (AP) staining was used to visualize the number of

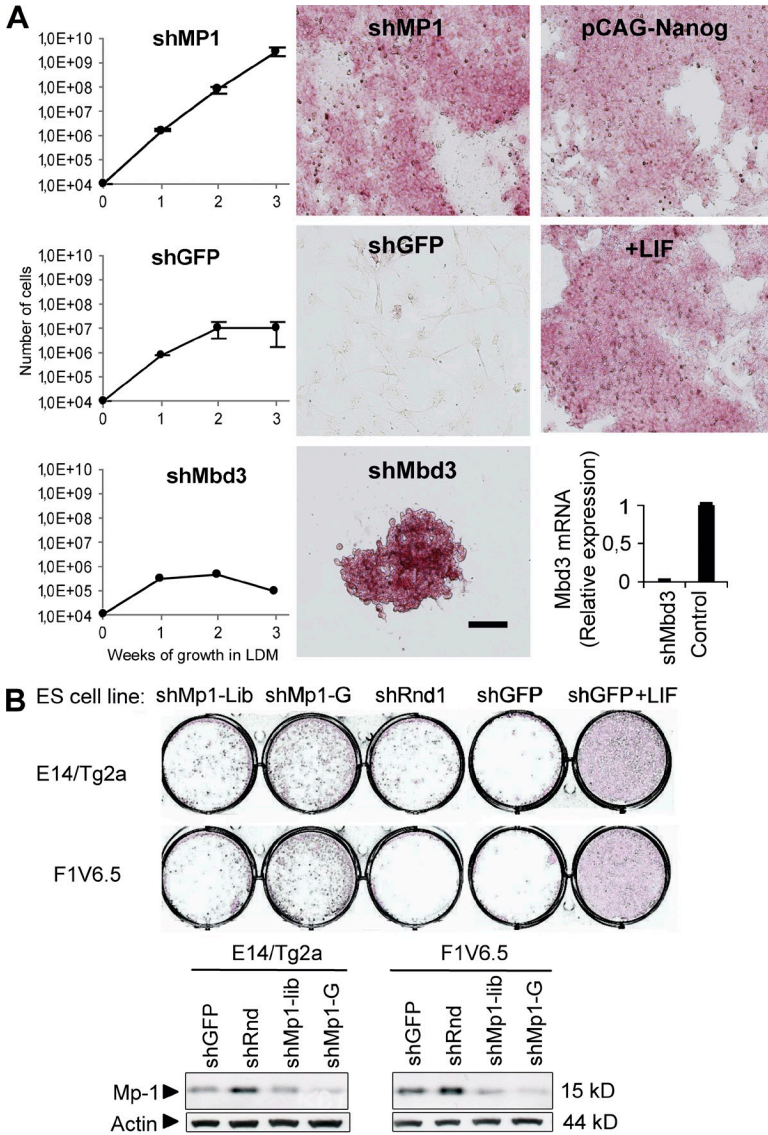


Figure 2. Knockdown of Mp1 in E14T ES cells resembles Nanog overexpression or growth in the presence of LIF. (A) Growth curves (left) of ES cells infected with shMp1, the negative control shGFP, or the positive control shMbd3 when grown in LDM for 3 wk. After 3 wk, colonies were stained for AP. Additional positive controls were transfection with pCAG-Nanog or addition of LIF. Knockdown of Mbd3, as measured with qPCR, is shown below the Mbd3 growth curve. (B) AP staining shows that knockdown of Mp1 in two other ES cell lines, E14/Tg2a and F1V6.5, inhibits differentiation when these cells were grown in LDM for 2 wk. Knockdown was performed using the shRNA identified from the library (shMp1-Lib) and an additional shRNA indicated by shMp1-G. Negative controls were shGFP and a shRNA containing a random sequence (shRnd1). Western blot analysis shows the knockdown levels of Mp1 in these cell lines (right). Data represent at least two independent experiments. Error bars, standard deviation. Bar, 100 μ m.

colonies after \sim 1 wk (Fig. 1 B). Mp1 knockdown resulted in a phenotype that was comparable to ES cells overexpressing Nanog as indicated by the ES morphology, colony size, and positive staining for AP (Fig. 2 A). In contrast, knockdown of the positive control Methyl-CpG binding domain protein 3 (Mbd3; Kaji et al., 2006; Liang et al., 2008; Fig. 2 A) and the previously described Ptpn11 (Burdon et al., 1999; Fig. S1) showed relatively small colonies, indicating that these and other hits of the screen, such as shFbx12 and shDusp27, block differentiation but do not necessarily contribute to proliferation. Because of the striking phenotype of Mp1 knockdown, we analyzed the function of this gene in further detail.

Mp1 knockdown results in a block in differentiation and a proliferation advantage in ES cells

To rule out cell line-specific artifacts, we confirmed the inhibitory effect of Mp1 knockdown on differentiation in F1V6.5 and E14/Tg2a ES cell lines (Fig. 2 B). To independently test whether Mp1 knockdown leads to a block in differentiation in the absence of LIF, we used Nanog-GFP reporter ES cells (Fig. 3 A). After knockdown of Mp1 with the shRNA recovered from the screen and another shRNA (shMp1-G), the Nanog reporter activity was stably maintained when compared with the control which showed a clear decline in reporter activity. Knockdowns are shown in Fig. 3 A.

To test whether Mp1 knockdown prevents neuroectodermal differentiation, we cultured ES cells in serum-free medium (N2B27) containing LIF, without Bmp4, according to Ying et al. (2003). After a week, cells were replated, the cells with Mp1 knockdown stained positive for AP, and immunofluorescence staining showed expression of Oct3/4 (Fig. 3 A and not depicted). In contrast, cells infected with the control shRNAs formed aggregates that resembled neurospheres.

ES cells after 1, 2, and 3 wk of consecutive replating at clonal density (1,000 cells/cm²), using shGFP as a negative control (Fig. 1 B). For each shRNA, the knockdown level was measured by quantitative PCR (qPCR) using shGFP as a reference (Fig. 1 C). Most shRNAs gave a considerable knockdown, except for shG0VN5, shSism, and shAbca12, which could indicate that these shRNAs act off-target.

Of the tested 12 putative hits, shFbx12, shPtpn11, and shMp1 stood out as the shRNAs that showed an ES cell morphology after 3 wk of culture without LIF, which resembled the morphology of cells expressing pCAG-Nanog or cells that were grown in the presence of LIF (Fig. 2 A and Fig. S1). Transduction of ES cells with the shRNA against the scaffolding protein Mek binding protein 1 (Mp1, Lamtor3, Map2k1ip1) resulted in higher cell numbers per well after 3 wk when compared with the other hits. (Fig. 1 D; and growth curves are shown in Fig. 2 A and Fig. S1). The control plates containing shGFP showed a rapid decline in AP-positive

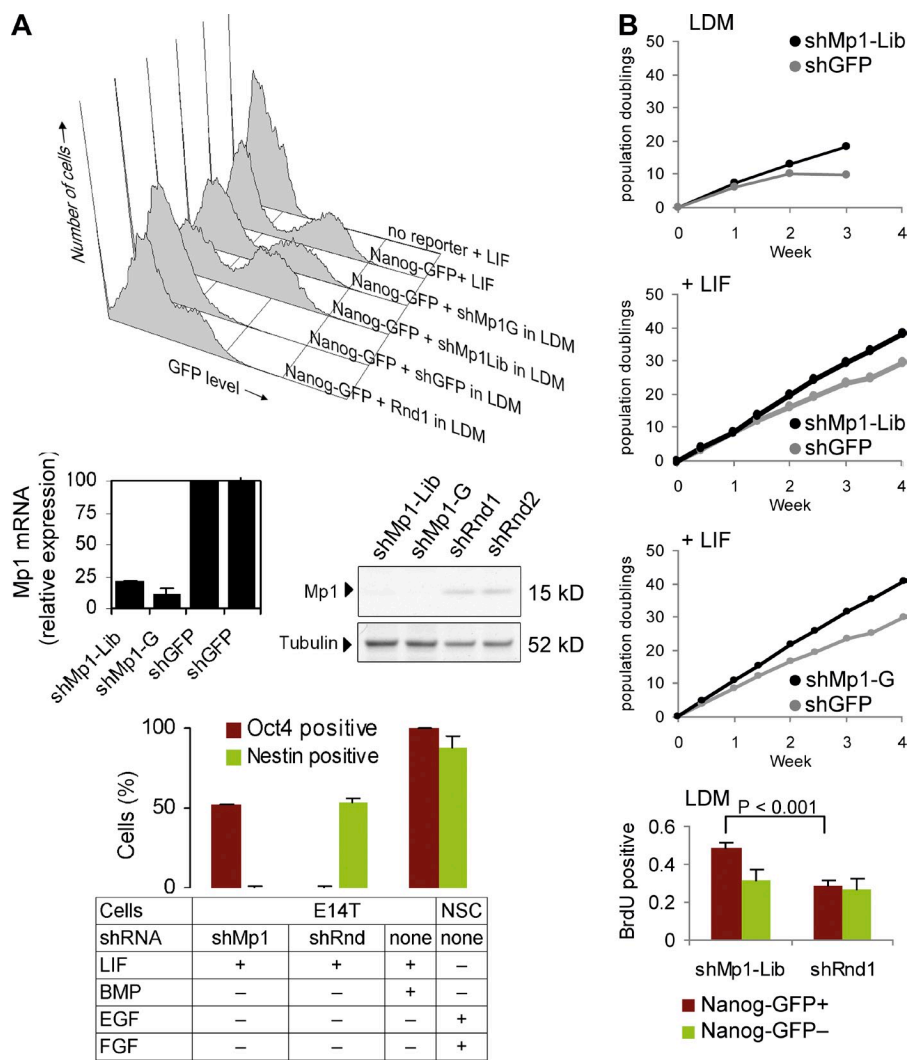


Figure 3. Knockdown of Mp1 inhibits differentiation and stimulates proliferation in ES cells.

(A) FACS plot of E14T-Nanog-GFP reporter ES cells containing a randomly targeted Nanog promoter fragment that drives GFP expression. Reporter activity was assayed after growing the cells in LDM for 4 d after knockdown of Mp1 or after using a control shRNA containing a random sequence (shRnd1). Knockdown was performed using the shRNA identified from the library (shMp1-Lib) and an additional shRNA indicated by shMp1-G. Positive control cells were grown in the presence of LIF for 4 d. Knockdown of Mp1 was confirmed by qPCR (bottom) and Western blotting (middle right). Knockdown of Mp1 in the absence of Bmp4 maintains OCT3/4 expression (positive fraction is shown in red) while suppressing Nestin expression (positive fraction is shown in green). Positive controls were: undifferentiated ES cells and subventricular zone neural stem cells. (B) Knockdown of Mp1 gives a proliferation advantage in the absence (top) and presence (middle) of LIF in the total population of seeded cells, but also in Nanog-GFP-positive ES cells that were cultured 4 d without LIF (bottom). Error bars, standard deviation. Data represent two independent experiments.

After attachment to laminin-coated plates, immunofluorescence microscopy showed that these cells had down-regulated OCT3/4 and expressed nestin, an early neural marker, confirming that the control cells had indeed gone into a neuroectodermal cell fate. However, because ~50% of the ES cells lose their expression of Oct4, we cannot rule out that knockdown of Mp1 inhibits neuroectodermal differentiation and does not necessarily depend only on Mp1-mediated pluripotency regulation.

We measured whether the knockdown of Mp1 led to a proliferation advantage. Mp1 knockdown showed exponential proliferation in the absence of LIF in contrast to the control shGFP, which revealed a rapid decrease in proliferation after 2 wk of culture (Fig. 3 B, top). A similar result was obtained in the presence of LIF, albeit to a lesser extent (Fig. 3 B, middle). To see whether this proliferation advantage was restricted to undifferentiated ES cells, we FACS sorted Nanog-GFP reporter ES cells that were differentiated for 4 d on LDM and analyzed BrdU incorporation in these sorted populations (Fig. 3 B, bottom). This showed that undifferentiated

reporter activity, showed no Mp1-dependent proliferation advantage. These data show that Mp1 knockdown inhibits differentiation and leads to a proliferation advantage restricted to ES cells.

Differentiation induced by constitutive forms of Ras and Raf can be inhibited by Mp1 knockdown

Ras-Mek-Erk signaling plays a pivotal role in the initiation of differentiation of ES cells. To analyze the role of activators of the FGF-Mek-Erk pathway in ES cells, we analyzed the function of different constitutive active isoforms of HRasV12, KRasV12, and Braf V600 in ES cells. The overexpression of HrasV12 in both ES cells and F9 mouse embryonic carcinoma (EC) cells has been shown to induce differentiation, in contrast to Eras, KrasV12, and NrasV12, which primarily control proliferation of ES cells (Eras) or their derived endosomal lineages (KrasV12 and NrasV12; Takahashi et al., 2003; Quinlan et al., 2008). We analyzed whether Mp1 could interfere with this differentiation (Fig. 4, A and B).

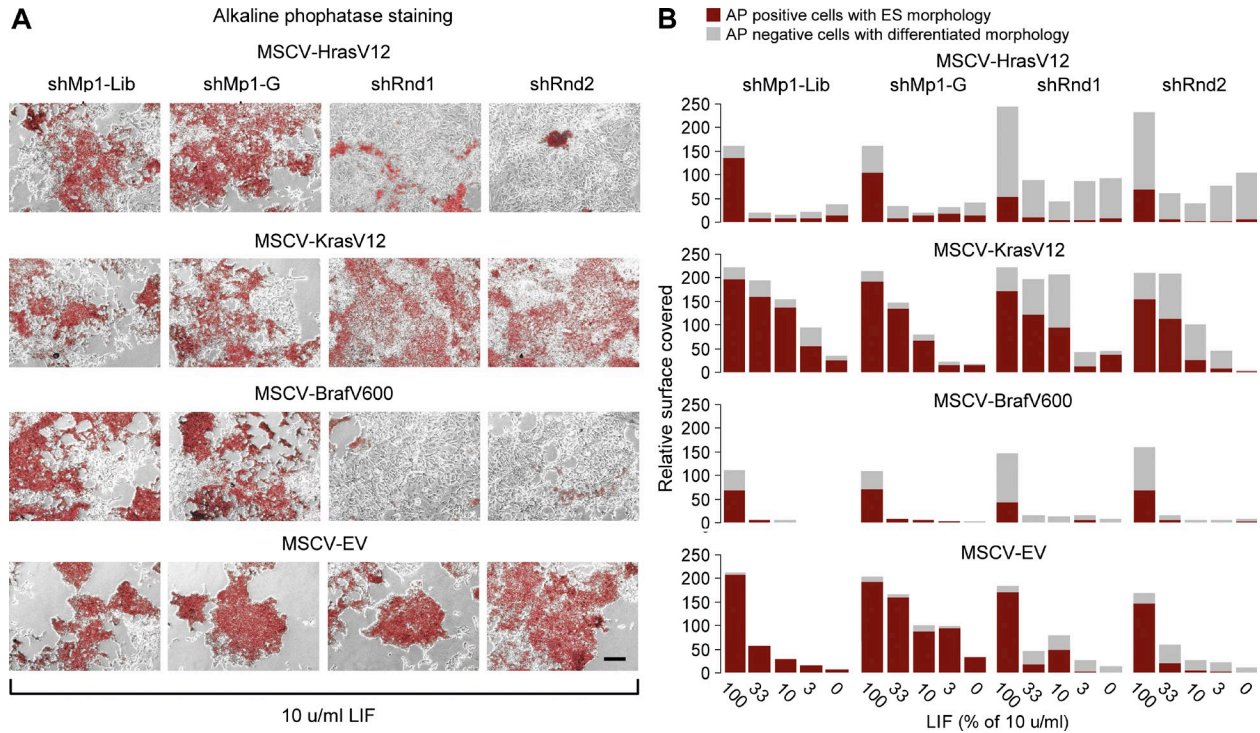


Figure 4. Ras/Raf-induced differentiation can be inhibited by Mp1 knockdown. (A) AP stainings show the effect of overexpression of constitutively active forms of Ras/Raf on ES cells combined with or without knockdown of Mp1. (B) The red bars in the histogram show the relative surface covered by AP-positive cells with an ES cell morphology and the gray bars show the surface covered by AP-negative cells with a differentiated morphology. When constitutively active Hras-V12 was overexpressed, this led to a strong induction of differentiation. Knockdown of Mp1 was, for the most part, able to inhibit this differentiation capacity of HrasV12. When constitutively active Kras-V12 was overexpressed, the inhibition of differentiation was less pronounced, and this weaker effect was inhibited by Mp1 knockdown. Overexpression of the constitutively active form of Braf, BrafV600, led to strong differentiation, which was comparable to HrasV12. This strong differentiation effect could be inhibited largely by Mp1 knockdown. When control cells were transduced with an empty vector, differentiation was observed only in the absence of LIF. Data represent more than two independent experiments. Bar, 100 μ m.

HrasV12 overexpression strongly induced differentiation both in the presence of high LIF and at lower concentrations of LIF within 5 d. Upon knockdown of Mp1, HrasV12-induced differentiation was largely inhibited, especially in the presence of LIF. When KrasV12 was overexpressed however, this led to major expansion of the ES cells at lower LIF concentrations. Besides this expansion of ES cells, a lower amount of differentiation was observed compared with HrasV12, and this differentiation was inhibited by Mp1 knockdown at lower concentrations of LIF. BrafV600-induced differentiation was inhibited by Mp1 knockdown, and BrafV600 overexpression did not show any proliferation phenotype comparable to that of KrasV12. This shows that overexpression of HrasV12 and BrafV600 lead primarily to differentiation which can be inhibited by Mp1 knockdown.

Mp1 knockdown uncouples FGF4-mediated proliferation and differentiation and results in activation of *Esrrb*, *Zfp42*, *Tcl1*, and *SOX2* and enhanced PI3K signaling

FGF4-deficient ES cells do not differentiate under serum-free conditions because of lack of endogenous sources for Ras-Mek-Erk activation (Kunath et al., 2007; Ying et al., 2008). To study whether Mp1 knockdown interferes with

FGF4-dependent differentiation and proliferation, we used FGF4-deficient ES cells (Wilder et al., 1997). These cells indeed maintained their pluripotency under differentiation-inducing conditions (LDM). Addition of FGF4 to these FGF4^{-/-} ES cells resulted in strong proliferation and differentiation (compare growth curves and AP stainings in Fig. 5 A), in agreement with the earlier findings of Kunath et al. (2007).

We analyzed the effect of FGF4 signaling onto Mp1-dependent differentiation. For this, FGF4^{-/-} ES cells with Mp1 knockdown (Fig. 5 B) were stimulated with increasing doses of FGF4 in the absence of LIF, which resulted in increasing numbers of undifferentiated AP-positive colonies (Fig. 5 C). In contrast, the control shRNA-infected cells did not show self-renewal upon stimulation with FGF4 and detached from the plate within 6 d of culture. This shows that FGF4 stimulates self-renewal upon knockdown of Mp1, instead of driving differentiation.

The previous data show that Mp1 levels control self-renewal versus differentiation in ES cells, and therefore we reasoned that Mp1 might be induced upon removal of LIF or BMP4, thereby enabling differentiation. However, when either of these growth factors was removed from the medium, no up-regulation of Mp1 was observed (unpublished data). A rather small decrease was seen when cells started differentiation

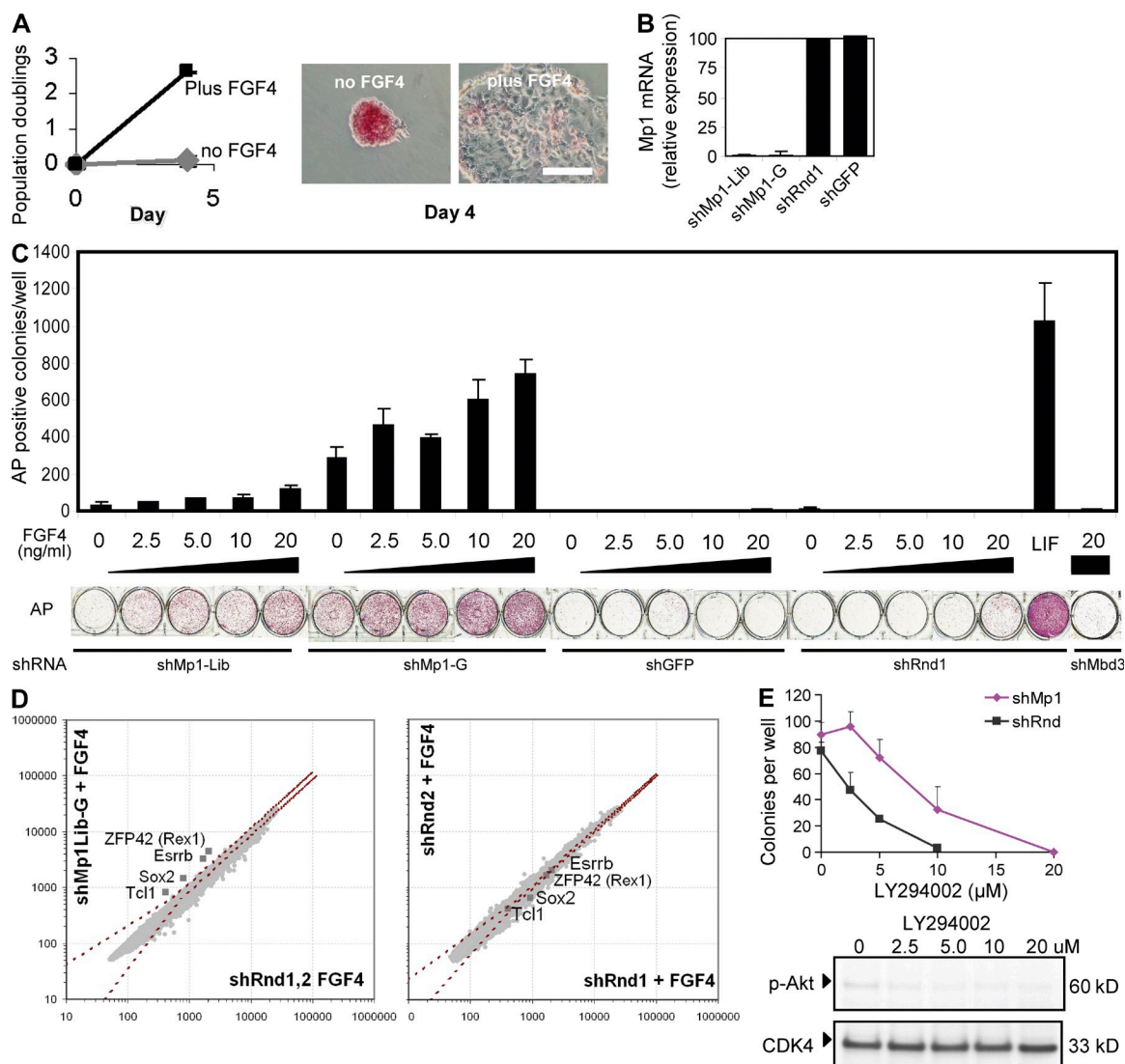


Figure 5. Mp1 knockdown uncouples proliferation and differentiation of ES cells in the absence of LIF. (A) Growth curve and graphs showing that addition of FGF4 to FGF4-deficient ES cells leads to both proliferation and differentiation. (B) qPCR showing Mp1 knockdown in FGF4-deficient ES cells. (C) FGF4-deficient ES cells were infected with Mp1 shRNAs or control shRNAs and cells were subsequently treated with increasing doses of FGF4 to induce differentiation in the absence of LIF. Knockdown of the positive control gene Mbd3 gave only a very minor increase in AP-positive colonies. (D) Microarray analysis shows that FGF4-deficient ES cells that have knockdown of Mp1 combined with FGF4 treatment have enhanced expression of the pluripotency genes Zfp42 (Rex1), Esrrb, Sox2, and Tcl1 when compared with cells that were infected with control shRNAs and were treated simultaneously with FGF4. The dashed red stripes show standard deviations of duplicate experiments. (E) Titration of the PI3K inhibitor LY294002 showed enhanced resistance to this drug by cells with Mp1 knockdown. Increasing the LY294002 dose led to inhibition of phosphorylated Akt. CDK4 was used as a loading control. Error bars, standard deviation. Data represent two independent experiments. Bar, 100 μ m.

in the absence of LIF. We argued that overexpression of Mp1 or its heterodimerization partner p14 (Wunderlich et al., 2001; Teis et al., 2002, 2006) might induce differentiation. When these genes were lentivirally expressed, no differentiation was observed in ES cells or F9 EC cells (unpublished data). Alternatively, knockdown of Mp1 might stimulate the activation of the JAK–STAT3 pathway; however, we did not observe enhanced p–STAT3 levels (unpublished data). These experiments show that Mp1 is not negatively controlled by LIF or BMP4, that Mp1 overexpression is insufficient to

induce differentiation, and that Mp1 does not repress LIF/STAT3 signals.

To identify which genes are important for the maintenance of self-renewal in ES cells with Mp1 knockdown after induction with FGF4, we performed microarray analysis on day 3 after induction with FGF4 (Fig. 5 D). We found that a panel of pluripotency determinators were regulated by FGF4 upon knockdown of Mp1, i.e., Esrrb, Zfp42, Tcl1, and Sox2. These genes were not activated by FGF4 when the control shRNAs were used (Fig. 5 D, compare left and right).

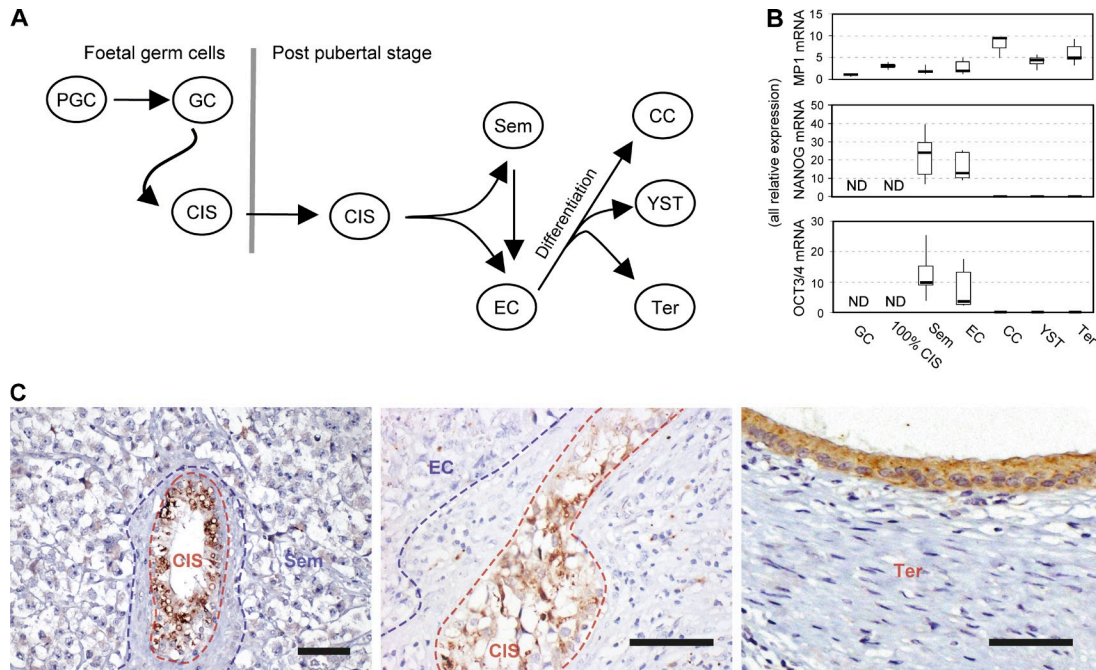


Figure 6. GCTs of invasive EC and Sem show low expression of MP1. (A) Schematic outline of GCT progression. Primordial germ cells (PGCs) can progress into CIS which can give rise to Sem and/or EC, of which the latter can progress to Ter, CC, and YST. (B) Bar plot showing the relative RNA expression levels of MP1, OCT3/4, and NANOG in different stages of human GCTs as measured by qPCR. Horizontal lines represent the median value, boxes the upper and lower quartiles, and lines the maximal and minimal measured value. PGCs ($n = 5$) have lower expression of MP1 than CIS ($n = 6$; $P < 0.05$). CC ($n = 3$) and Ter ($n = 6$) have higher expression of MP1 than EC ($n = 7$) or Sem ($n = 10$; $P < 0.05$), similar to YS ($n = 6$), which has higher expression of MP1 than Sem ($n = 12$; $P < 0.05$). (C) Immunohistochemistry shows that preinvasive CIS are positive for MP1, whereas both Sem and EC in the same specimen have low to undetectable levels of MP1. MP1 is expressed in differentiated components of Ter tumors in $\sim 15\%$ of the patients. Panel A was adapted from R. Lothe, with permission. Bars 100 μm .

Results were confirmed by qPCR. Enhanced expression of *Esrrb*, *Zfp42*, *Tcl1*, and *Sox2* is also observed when cells remain undifferentiated either by treatment of LIF or when treated without FGF4 (unpublished data).

Western blot detection of phosphorylated Akt shows that in the absence of PI3K-inducing ligands such as FGF4, LIF, and serum, the PI3K pathway is active (unpublished data). To analyze whether knockdown of Mp1 led to differences in PI3K signaling activity, we titrated the transduced cells with the PI3K inhibitor LY294002. This showed that knockdown of Mp1 was able to generate a stronger resistance to LY294002-induced proliferation inhibition, which suggests that PI3K signaling levels are enhanced upon knockdown of Mp1 (Fig. 5 E).

Knockdown of Mbd3 is able to inhibit the differentiation of ES cells, however, without showing the proliferation phenotype as seen with Mp1 knockdown. We used Mbd3 knockdown as a control for FGF4-mediated proliferation/differentiation experiments, and these cells were treated the same way as the Mp1 experiments and tested as to whether FGF4 stimulation resulted in enhanced self-renewal, similar to what was observed for Mp1. In this case, however, we noticed no, or a very minor, effect of FGF4 to enhance self-renewal (Fig. 5 C). Notably, phospho-Erk1,2 levels were markedly lower in cells with Mbd3 knockdown (unpublished

data) as compared with the shGFP control, suggesting that the cells lack the ability to respond positively to the FGF4 signal through the lack of phospho-Erk1/2 activation. In contrast, global phospho-Erk levels were only mildly affected by Mp1 knockdown, although reduced levels were observed in Lamp1-positive endosomes, as well as in cytoplasmic vesicles that lack Lamp1 expression (unpublished data). These data show that Mp1 knockdown does only mildly affect phospho-Erk levels in ES cells, which argues that the differentiation inhibitory effect of knockdown of Mp1 is therefore not likely to be a result of total ablation of Erk signaling but rather a result of modulation of the Erk signaling network that leads to differentiation, possibly through endosomal signaling because Mp1 localizes to late endosomes in ES cells (unpublished data).

In summary, our results indicate a bimodal action of Mp1 in differentiation and proliferation of ES cells. Mp1 knockdown directs FGF4 signaling toward self-renewal by transcriptional activation of the self-renewal regulators *Esrrb*, *Zfp42*, *Tcl1*, and *SOX2* and simultaneously enhances PI3K signaling without changing the responsiveness to FGF4-mediated Erk2 phosphorylation. Our data therefore implicate that the Mp1 knockdown rewires Ras-Mek-Erk signaling toward self-renewal without affecting the global Ras-Mek-Erk and PI3K signaling that contribute to the proliferative effect.

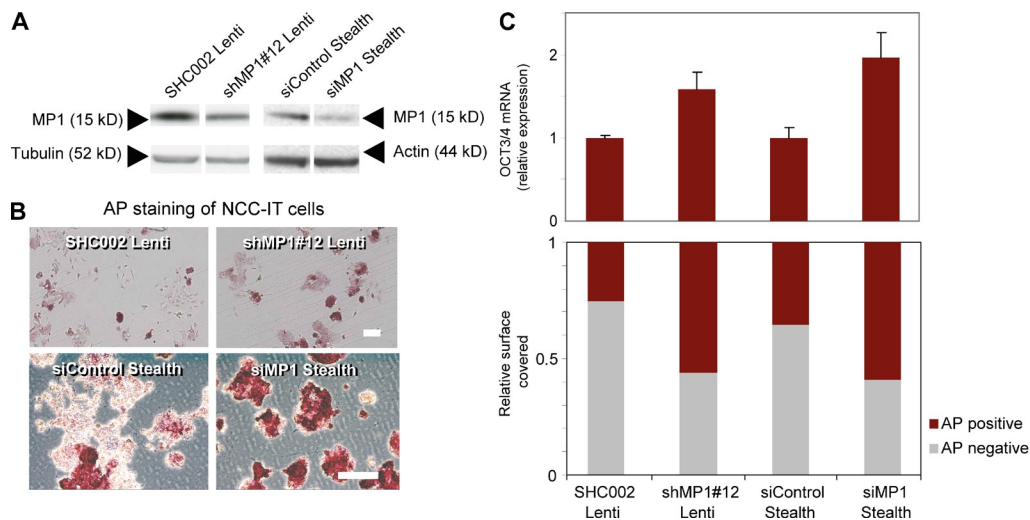


Figure 7. Knockdown of MP1 in NCC-IT cells prevents differentiation. Knockdown of MP1 in NCC-IT cells (A) prevents differentiation as a result of SU-5402 treatment at 30 μ M, as shown by AP staining (B), enhanced OCT3/4 mRNA expression, and enhanced numbers of cells AP-positive cells (C) with an undifferentiated morphology versus AP negative/weak cells with a differentiated morphology. Error bars, standard deviation. Bars, 100 μ m. Data represent two independent experiments.

EC and Sem GCTs show low expression of Mp1 and knockdown of Mp1 results in resistance to differentiation

GCTs are subdivided into Sem and non-Sem tumors, of which the latter can contain histologically undifferentiated elements composed of EC cells. A schematic representation of GCT progression is shown in Fig. 6 A. Because EC and Sem cells resemble ES cells (Andrews, 2002; Looijenga et al., 2003; de Jong et al., 2008; Gillis et al., 2011), we reasoned that low levels of MP1 might resist differentiation cues and can thereby contribute to the progression of this tumor. We analyzed the expression of MP1 in different histological elements of GCTs using qPCR (Fig. 6 B) and immunohistochemistry (Fig. 6 C; results are summarized in Table 2). The earliest developmental stage of GCT of the adult testis is carcinoma in situ (CIS; Oosterhuis and Looijenga, 2005). A set of CIS samples ($n = 56$) was analyzed and all showed expression of MP1. In all cases, strong expression of MP1 was seen in the tumor cells, whereas surrounding nontumorous tissue was negative. Note that mRNA levels underestimate the cellular levels in CIS samples because only a subfraction of the

specimen contains tumor cells. CIS cells can progress into Sem and EC. Strikingly, none of the Sem ($n = 48$) and the majority of EC ($n = 40$) showed detectable expression of MP1, which was especially clear in tissues that represent multiple tumor stages (Fig. 6 C). Mp1 expression levels inversely correlated with NANOG and OCT3/4 mRNA expression levels in the invasive components (Fig. 6 B).

EC can differentiate into choriocarcinoma (CC), yolk sac tumor (YST), and teratoma (Ter). Although CC and YST showed low expression of Mp1 on the protein level (not depicted), some of the Ter samples (15%) that were analyzed ($n = 124$) expressed high levels of MP1 in differentiated tumor elements (Fig. 6 C and not depicted). In summary, we observed expression of Mp1 in all CIS samples of GCTs, and this expression was lost in the early invasive components after tumor progression, i.e., Sem and EC. This shows a correlation between absence of expression of Mp1 and progression to invasive growth of GCTs.

EC and Sem have been shown to rely on autocrine FGF signaling for their self-renewal (Greber et al., 2007b). For early malignant cells to become invasive and less dependent on FGF ligands produced by the bulk of the tumor, we argued that low MP1 levels might, in analogy to the mouse ES cells, be able to direct FGF signaling toward self-renewal. To test this hypothesis, we inhibited autocrine FGF signaling by the FGFR inhibitor SU-5402 and we simultaneously knocked down MP1. For this we used NCC-IT cells, which are considered to reflect a tumor progression stage between EC and Sem.

Addition of 30 μ M SU-5402 to NCC-IT cells resulted in a clear induction of differentiation after 5 d of culture. Knockdown of MP1 (Fig. 7 A) under these conditions was able to rescue SU-5402-mediated differentiation, as was observed

Table 2. Summary of Mp1 stainings on human GCT samples

Stage	Number of samples	Mp1 positive	Fraction
			%
CIS	56	56	100
Sem	48	0	0
EC	49	0	0
YST	65	0	0
CC	18	0	0
Ter	124	19	15

by reduced levels of protruding differentiating cells from the EC colonies resulting in sharp edged colonies. In addition, we saw that these cells show up-regulated OCT3/4 levels upon MP1 knockdown (Fig. 7 C, top) and showed increased numbers of AP-positive colonies (Fig. 7 B, quantified in Fig. 7 C), showing that MP1 knockdown results in enhanced levels of self-renewal under differentiation inducing conditions.

In summary, we show that low MP1 levels, as observed in post-CIS-stage GCTs does not only correlate with undifferentiated and invasive components of GCTs but also contributes to prevention of differentiation of EC/Sem cells under conditions that induce differentiation of these cells. Together, our results show that knockdown of MP1 is able to inhibit differentiation of ES cells as well as GCTs.

DISCUSSION

In this paper, we describe the results of a knockdown screen in mouse ES cells to identify factors required for differentiation. Grouping of the identified genes into functional pathways shows that multiple hits are involved in Ras–Mek–Erk signaling. EphB4 receptors can regulate the activity of the Ras family of GTPases, including H-Ras and R-Ras (Zou et al., 1999; Miao et al., 2001; Wang et al., 2006). When Ptpn11 (also called Shp-2), another hit from our screen, was prevented from interacting with a mutated gp130 receptor that failed to activate ERKs, this led to self-renewal (Burdon et al., 1999). These data show that our unbiased, genome-wide knockdown approach identified several factors that were previously identified to be important in self-renewal of ES cells and validate our screening strategy. An shRNA against Capn10 was found in ~50% of the sequences and, when tested individually, this shRNA showed strong ES colony outgrowth during the first 2 wk after removal of LIF. During the 3rd wk however, this proliferation declined and most cells died. This indicates that knockdown of Capn10 gives a phenotype distinguishable from other hits such as shMp1 or shPtpn11, which gave prolonged pluripotency throughout the course of the experiment. Among the candidate hits, we identified MP1 as a mediator of differentiation of ES cells, and knockdown of this gene prevents differentiation of ES cells. The phenotype observed was the strongest among all other identified hits.

Mouse ES cells differentiate when LIF is removed from the medium, when Bmp4 is removed from the medium in the presence of LIF, when cells are stimulated with FGF4 in the absence of LIF, and when HrasV12 or BrafV600 are overexpressed. We show that all these differentiation-inducing cues can be inhibited by Mp1 knockdown.

Fgf4 signaling leads to differentiation of ES cells (Kunath et al., 2007) and it also enhances proliferation of the differentiating ES cells. We observed that knockdown of Mp1 resulted in increased proliferation upon stimulation with FGF4, but without induction of differentiation, showing that knockdown of Mp1 results in uncoupling between proliferation and differentiation in ES cells. We show that Sox2 and Esrrb are up-regulated by FGF4 in the absence of Mp1. These genes

directly control pluripotency (Boyer et al., 2005; Zhang et al., 2008) and are able to induce induced pluripotent stem cells in conjunction with OCT3/4 (Takahashi and Yamanaka, 2006; Feng et al., 2009). In addition, Tcf1 and Zfp42 (Rex1) are enhanced by FGF4 in the absence of Mp1 and these genes enhance ES cell proliferation (Matoba et al., 2006) and prevent germ layer commitment in ES cells (Scotland et al., 2009), respectively.

It has been described that HrasV12 overexpression in ES cells and mouse EC cells results in differentiation, even in the presence of LIF. Overexpression of KrasV12, Eras, and Nras primarily leads to a proliferation phenotype (Takahashi et al., 2003; Quinlan et al., 2008). We show that Mp1 knockdown primarily affected HrasV12-induced differentiation, which suggests that HrasV12 specifically triggers a portion of Ras/Mapk signaling that leads to differentiation. Indeed, HrasV12 induces a strong Mapk signal as seen from high phospho-Erk1/2 levels after infection of ES cells (unpublished data). Erk2 is the downstream target of the Mapk pathway in ES cells (Kunath et al., 2007). FGF4- and Erk2-deficient ES cells have been shown to be unable to commit to mesodermal and endodermal fates (Kunath et al., 2007). However, in both cases there is no activation of the Ras–Mapk–Erk pathway, resulting in a poor proliferation, consistent with data of Ying et al. (2008). Several scaffolds are known which regulate the activation of Mapk–Erk on distinct subcellular compartments (focal adhesions, Golgi, plasma membrane, and early endosomes) in mammalian cells (Dhanasekaran et al., 2007; Pullikuth and Catling, 2007). Study of the intrinsic differences of different Ras isoforms has revealed that posttranslational modifications of their C termini has functional consequences, leading to palmitoylation (Hras, Nras, and Kras4A) or only farnesylation and geranylation (Kras 4B; Konstantinopoulos et al., 2007). Palmitoylation of Hras, Nras, and Kras4A is believed to result in different trafficking within microdomains of the membranes (Hancock, 2003). These data indicate that the intracellular localization of the Ras–Mek–Erk signal might play a causal role in the differentiation-inducing ability of these Ras members. Hras has been shown to enter the endocytic compartment and its return to the membrane depends on the endocytic recycling pathway (Roy et al., 2002; Gomez and Daniotti, 2005). Ubiquitination of Hras stabilizes its association with endosomes (Jura et al., 2006). This is in contrast to Kras, which only minorly and transiently associates with the endosomes (Roy et al., 2002). Because Mp1 is localized to the late endosomes, the loss of the small fraction of late endosomal activated Erk in ES cells might not be able to change the global levels of phospho-Erk, although we noticed that the intracellular localization of phospho-Erk was affected by Mp1 knockdown after FGF4 stimulation, resulting in reduced levels of phospho-Erk in Lamp1-positive endosomes and lipid rafts throughout the cell.

There are at least two stages in early embryogenesis where FGF signaling plays a significant role, i.e., in the epiblast stem cells stage and during gastrulation. Mouse epiblast-derived stem cells (EpiSCs), i.e., cells derived from the epiblast of the

early postimplantation mouse embryo, share similarities with human ES (hES) cells (Brons et al., 2007; Tesar et al., 2007). These cells require FGF signaling for their self-renewal in addition to Activin A/Smad2–3 signaling (Vallier et al., 2005; Xu et al., 2005; Greber et al., 2007a, 2010; Xu et al., 2008). FGF signaling enhances Nanog expression in hES cells (Ludwig et al., 2006; Li et al., 2007) and prevents activation of neural differentiation genes in EpiSCs (Greber et al., 2010). This is consistent with a recent study showing that lowering FGF signaling in mouse ES cells after initial FGF-mediated differentiation leads to enhanced differentiation (Stavridis et al., 2010). These data indicate that low Mp1 levels might, together with Activin/Smad2–3 signaling, lead to a maturation arrest in the EpiSC stage. However, high expression of the inner cell mass marker Zfp42/Rex1 (Fig. 5 D; Rathjen and Rathjen, 2003), positive staining for AP, and the virtual absence of the epiblast marker FGF5 (not depicted; Rathjen et al., 1999) indicates that Mp1 knockdown does not result in an EpiSC maturation arrest.

The second step where FGF/Ras/Mapk signaling plays a role during early embryogenesis is during gastrulation. In this process, cells of the primitive streak undergo a Ras/MAPK governed epithelial to mesenchymal transition (EMT) and disruption of FGF8-, FGFR1-, or MAP4K4-mediated signaling results in inhibition of this EMT process and, therefore, mesoderm formation is inhibited (Yamaguchi et al., 1994; Sun et al., 1999; Ciruna and Rossant, 2001; Xue et al., 2001). Therefore, low Mp1 levels in combination with FGF signaling might create a nonpermissive state to adapt to a mesodermal program, possibly overlapping with processes that occur in the primitive streak, i.e., through an inability to enter a MAPK-governed EMT program. In this context, it is interesting to note that LIF/STAT3 treatment resulted in repression of many regulating genes of the Ras–Mapk pathway (unpublished data), indicating that LIF/STAT3 signaling could functionally overlap with the effect of Mp1 knockdown.

The phenotype observed with loss of Mp1 does partially overlap with loss of p14, the heterodimerizing partner of Mp1 (unpublished data). In short-term assays, knockdown of p14 resulted in a maturation arrest similar to Mp1 knockdown; however, secondary lethal effects prevent a long-term effect *in vitro* and *in vivo*. These data suggest that the functions of Mp1 and p14 have only a limited overlap in ES cells. This is consistent with earlier studies, which have shown that mice deficient for p14 and p18 die because of growth retardation with severe developmental defects (p14; Teis et al., 2006) and defects in the organelle organization of the visceral endoderm (p18; Nada et al., 2009).

Human EC cells have an FGF2-dependent self-renewal program (Greber et al., 2007b) and murine EC cells are blocked in their differentiation by activin A (van den Eijnden-van Raaij et al., 1991). Mp1 is absent or expressed on a low level in any of the invasive GCTs with the histology of Sem and EC (except some Ters, YST, and CC), whereas it is positive in all non invasive CIS samples. We hypothesized that knockdown of Mp1 could direct low levels of FGF signaling

toward self-renewal in the invasive GCT stages Sem and EC. We show that knockdown of Mp1 in NCC-IT cells results in inhibition of differentiation after treatment of the cells by SU-5402. Mouse ES cells can self-renew under high levels of FGF4 signaling (unpublished data), which indicates that the signaling pathways that determine differentiation and self-renewal might have a shifted balance in GCTs when compared with mouse ES cells. Our results indicate that inhibition of Mp1 could be part of a common mechanism that maintains self-renewal of GCTs. The data indicate that Mp1 down-regulation is not required to maintain an embryonic germ cell phenotype in the niche of Sertoli cells, although this is needed in the invasive tumors. This might elegantly explain why CIS cells are, in principle, able to differentiate to all lineages upon leaving their specific niche (i.e., interaction with the basal lamina and Sertoli cells). In fact, in spite of this intrinsic pluripotency, they do not differentiate in their pre-invasive niche.

In conclusion, by performing an unbiased genome-wide shRNA screen, we have identified the scaffolding protein Mp1 as a critical factor that can determine whether FGF4–Ras–Mek–Erk signaling results in differentiation or pluripotency in mouse ES cells while maintaining Erk2 and PI3K signaling. Our results show that knockdown of Mp1 can overrule the differentiation-inducing signal from FGF4/Ras signaling by activation of pluripotency regulators. Knockdown of MP1 in NCC-IT GCT cells is able to partially block the differentiation of these cells. Our results shed new light on the FGF signaling in the early stages of mouse embryogenesis and provide insight on the role of MAPK scaffolding proteins in cancer.

MATERIALS AND METHODS

ES cell culture. E14T (Li et al., 1998), F1V6.5, and E14/Tg2a ES cells were cultured on gelatin-coated tissue culture plastic. E14/Tg2a ES cells lack the constitutively expressed polyoma large T from an expression cassette pMGDneo20 (Gassmann et al., 1995) that is expressed in E14T cells to enable episomal replication of pPyCAGIP expression vectors. The E14T and FGF4^{-/-} cells, based on the R1 mESC line (Wilder et al., 1997), were obtained from the Austin Smith laboratory with permission of M. Capecchi (Howard Hughes Medical Institute, University of Utah, Salt Lake City, UT) for the latter cell line. Cells were grown in 60% buffalo rat liver conditioned (BRL) medium plus LIF (60% BRL, 40% LDM medium, including 2 mM L-glutamine [Invitrogen], 0.1 mM 2-mercaptoethanol, and 10 U/ml LIF [ESGRO LIF; Millipore]). LDM consists of GMEM (Glasgow Modified Eagle Medium), 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 10% FCS (PAA; Pasching), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). BRL was obtained by collecting LDM that was conditioned for 1 wk onto Buffalo Rat Liver cells. For serum-free culture of ES cells, N2B27 medium (Li et al., 1998; Ying et al., 2003), including 10 U/ml LIF and 10 ng/ml Bmp4 (R&D Systems), was used.

Ethics statement. All animal experiments were approved by the advisory board for animal experimentation of the Medical University of Innsbruck and by the Austrian ministry for Science and Research based on the Austrian Animal Testing Act of 1988. Use of the patient tissues is approved by an institutional review board (MEC 02.981). Samples are used according to the Code for Proper Secondary Use of Human Tissue in the Netherlands, from the Dutch Federation of Medical Scientific Societies (FMWV; version 2011).

Genome-wide knockdown screen. We used the NKI shRNA library targeting 16,000 mouse genes, which contains at least two specific hairpins for each gene (library construction is described by Huang et al., 2009). We based our protocol on the screen protocol by Chambers et al. (2003), which uses differentiation induction by removal of LIF from the medium. To ensure that the full library was represented, we used a 100-fold excess of the library complexity. Cells were transduced with the pooled shRNA library or with the pRS-shGFP negative control simultaneously (the shRNA sequences is shown in Table S1). Cells were also electroporated with pCAG-Nanog control (positive control) or with a pCAG-empty control (negative control). 3.2 million cells were replated at a clonal density of 200,000 cells per 180-cm² dish (17 plates). The next day, the cells were washed twice with LDM medium to remove LIF and the cells were cultured in the absence of LIF for 1 wk. Cells were replated at the same density every 7 d for a total of 22 d. Colonies were counted after 13 (second round) and 20 (third round) d of culturing. After 22 d of culture in the absence of LIF, the cells were harvested and genomic DNA was isolated.

Identification of shRNA cassettes. The shRNA cassettes were recovered from isolated genomic DNA or plasmid by PCR using the Expand Long Template polymerase kit (Roche) and the following primers using an annealing temperature of 62°C: 312-MSCVpuro-seq5MCS, 5'-CTTGAACCTCCTCGTTCGACC-3'; 374-MSCV, 5'-CTAAAGCGCATGCTCCAGACTG-3'. The inserts were checked for their lengths (643 bp). The inserts were then digested with the EcoRI-XhoI restriction enzymes obtaining a 301-bp fragment containing the H1 promoter and shRNA sequence. The obtained fragments were then cloned back into the pRetrosuper plasmid using ElectroMAX DH10β cells. Samples with genomic DNA from uninfected cells and an H₂O control were included to check for any contamination in the PCR reactions. 300 colonies were sequenced (shRNA sequences are shown in Tables S3 and S4).

ES cell knockdown and overexpression experiments. Retroviral delivery of pRS-shRNA was performed by performing four 5–6-h rounds of infection, followed by selection on 4 μg/ml puromycin. All knockdown experiments were done using these conditions. E14T cells were seeded at 40,000–80,000 per well in 6-well plates. The next day, retrovirus (collected in LDM) was mixed 1:1 with infection medium (100% BRL, 20 U/ml LIF, 4 mM L-glutamine, 0.2 mM 2-mercaptoethanol, and 1.6 μg/ml polybrene) and put on cells in duplicate. Medium was replaced by 60% BRL plus LIF and incubated overnight. All shRNAs were cloned into pRetrosuper according to standard procedures. As negative controls for the knockdowns, shGFP and random shRNA (shRnd) were designed (Table S1). Knockdown was measured by qPCR using SYBR Green PCR Master Mix (Applied Biosystems) using Actin or Hprt housekeeping primers as a reference. Primers are listed in Table S2.

For overexpression of HrasV12 and BrafV600, we used the MSCV-blast-HrasV12 and MSCV-Blast-BrafV600 constructs made by Chrysis Michaloglou (Department of Molecular Genetics, Netherlands Cancer Institute). MSCV-blast-Kras was cloned from the pBabe K-Ras V12 construct (Addgene) from BamHI into the BglII sites of MSCV-Blast. These overexpression constructs were used to infect ES cells twice. Infected cells were selected with 5 μg/ml blasticidin for 4–6 d and plated at 8,000 or 40,000 per 24- or 6-well plates, respectively. Cells were analyzed at days 5 and 6 and stained with AP (see ES/EC differentiation assays).

cdNAs encoding Mp1 and p14 were cloned into pLenti6V5/Dest vector by Gateway cloning from pENTR constructs, which were provided by M. De Araujo (Huber laboratory, Innsbruck Medical University, Innsbruck, Austria). ES cells were infected with a multiplicity of infection of ~1. Cells were analyzed at days 6 and 14 for differentiation.

ES/EC differentiation assays. After infection with shRNA viruses followed by 3 d of puromycin selection, E14T ES cells were seeded into 6-well plates at a density of 10,000/well. The next day, cells were cultured in LDM. At day 7, cells were replated at 10,000 per well and cultured for another week. This was repeated at day 14. At day 21, cells were stained for AP, a

marker of undifferentiated ES cells (Sigma-Aldrich). Alternatively, the protocol was reduced to a 2-wk differentiation protocol by refreshing the medium at days 5 and 12. The shMp1 phenotype was confirmed with two independent mouse ES cell lines: F1V6.5 and E14/Tg2a. In both cases, cells were seeded at 40,000 cells per well and treated as in the previous section.

To induce neural differentiation of ES cells, cells were grown in serum-free medium (N2B27; Li et al., 1998; Ying et al., 2003), including 10 U/ml LIF but excluding Bmp4 for 1 wk. Cells were then replated, and this resulted in neurosphere formation during this 2nd wk of culture. Cells were plated on poly-ornithine and laminin-coated plates to grow them as a monolayer. To measure the effect of FGF4 on differentiation, FGF4-deficient ES cells were grown overnight in serum-free medium (N2B27), including Bmp4 at 10 ng/ml, and treated with FGF4 at a concentration of 20 ng/ml for 5 d after. ES cells were visualized with the AP staining kit.

A Nanog-GFP reporter construct was made in a pACGFP1-1 vector (Takara Bio Inc.) based on Rodda et al. (2005). Primers for amplification of the mouse -289 to +177 Nanog fragment were (with restriction sites for cloning purposes indicated in lowercase): Nanog -289 forward, 5'-CGC-gtcgacTAAAGTAAAATGAGGTAAAGCC-3'; and Nanog +177 reverse 5'-CGCggatccGGAAAGATCATAGAAAGAAGAG-3'. An ES cell clone that had stably integrated the reporter construct was selected and used in subsequent reporter assays. For differentiation, cells were seeded at 40,000/well of a 6-well plate in LDM medium. Medium was refreshed every day. At day 4 after seeding, the GFP levels of the cells were analyzed using a FACSCalibur and plotted in graphs.

To analyze whether undifferentiated ES cells have a proliferation advantage upon knockdown of Mp1 versus the control hairpin shRnd1, Nanog-GFP reporter cells were treated in LDM for 4 d, as in the previous paragraph, pulsed with 10 μM BrdU for 1 h, and then FACS sorted. The populations with highest versus the lowest Nanog-GFP expression (both representing 10% of the viable population) were analyzed. Cells were fixed in 70% ethanol and permeabilized with 5 M HCl and 0.5% Triton X-100 for 20 min, followed by neutralization with 2/5-vol 0.1 M Na₂B₄O₇. Cells were treated with anti-BrdU antibody (Dako M074402) for 30 min and subsequently with anti-mouse IgG-Alexa Fluor 568 (Invitrogen) for 30 min. DNA was counterstained with TO-PRO3 (Invitrogen T3605) to exclude cell clumps from the analysis. Statistical analysis was done using a Student's *t* test.

EC cells use autocrine FGF signaling for their self-renewal program (Greber et al., 2007b). To differentiate NCC-IT cells (Damjanov et al., 1993), which are considered equivalent to a stage intermediate between Sem and embryonal carcinoma, cells were transfected with a stealth siRNA against MP1 or a mock siRNA using Lipofectamine, or cells were infected with an shRNA against MP1 (Sigma-Aldrich) or a mock shRNA vector (SHC002; Sigma-Aldrich). Sequences are shown in Table S1. After selection on puro (in case of the infection) at 2 μg/ml, cells were seeded in 6-well plates at a density of 25,000 cells/well, and cells were subsequently treated for 5 d with 30 μM of the FGFR inhibitor SU-5402, followed by AP staining and quantification as in the first paragraph of this section, as well as qPCR analysis for NANOG and OCT3/4 (primers listed in Table S2).

Immunostainings. Immunofluorescence was performed by fixing cells onto gelatin-coated permanox chambers in 4% paraformaldehyde. Antibodies used were anti-V5 (R960-25; Invitrogen), anti-OCT3/4 (C10; Santa Cruz Biotechnology, Inc.), anti-nestin (611658; BD), anti-tubulin-III (Sigma-Aldrich), anti-phospho-ERK1/2 (Cell Signaling Technology), and anti-mouse CD107a (lysosomal associated membrane protein 1 [LAMP1]; BD). Secondary antibodies were anti-mouse IgG Cy3 (Jackson Immuno-Research Laboratories), Alexa Fluor 568 anti-rabbit (Invitrogen), Alexa Fluor 568 anti-mouse (Invitrogen), or Alexa Fluor 647 anti-mouse (Invitrogen). DAPI was obtained from Sigma-Aldrich and Alexa Fluor 488-Phalloidin from Invitrogen. As a positive control for the nestin staining, we used postnatally derived subventricular zone neural stem cells.

For localization analysis of phospho-Erk, E14T shMP1 and E14T shRnd cells were kept in 60% BRL + β-mercaptoethanol + LIF and seeded on glass slides which were coated with 0.1% gelatin (Millipore) for 4 h.

Cells were either left untreated or stimulated with 20 ng/ml FGF4 after starvation for 14 h in N2B27 with 10 ng/ml Bmp4. Upon stimulation for various time points, cells were washed with PBS and immediately fixed in 4% paraformaldehyde supplemented with 2 mM Na₃VO₄ in cytoskeleton buffer (CB; 10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.8, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, and 5 mM MgCl₂) for 20 min. Next, cells were permeabilized in 0.3% Triton X-100 in CB for 30 min. After 1 h of blocking in gelatin containing blocking buffer, cells were incubated with the primary antibody overnight (blocking buffer supplemented with 0.1% Triton X-100). The next day, cells were washed five times in washing buffer (1 × CB and 50 mM NH₄Cl), incubated for 60 min with the secondary antibody (blocking buffer supplemented with 0.1% Triton X-100), and again washed five times. The last washing step included DAPI to counterstain the cells, which were then mounted in Fluoromount-G (SouthernBiotech). Z stacks of cells were acquired using a microscope (LAS AF 2.2.0, HCX PL APO 63×/Glyc Corr 37°C objective; Confocal SP5; Leica). Images were subjected to the Huygens Professional deconvolution software. Snapshots of single z planes were taken and exported as tiff files. Figures were arranged with Macromedia FreeHand MX 11.0 software (Adobe Systems) and exported as jpeg files.

Western blotting. To measure the effect of FGF4 on phospho-Erk, E14T- and FGF4-deficient ES cells were grown overnight in serum-free medium (N2B27), including Bmp4 at 10 ng/ml, and treated with FGF4 at a concentration of 20 ng/ml. Samples were harvested after the indicated time points in RIPA buffer containing Complete protease inhibitors (Roche) and phosphatase inhibitors (2 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate decahydrate, and 10 mM β-glycerophosphate) and subsequently run on precast SDS-PAGE gels (Invitrogen). The Mek1,2 inhibitor U0126 (Cell Signaling Technology) was used 2 h before FGF4 stimulation at a concentration of 10 μM.

The following antibodies were used: anti-Mp1 (generated at the Huber Laboratory), anti-phospho-Erk (p42/44; Cell Signaling Technology), anti-phospho-Stat3 (Cell Signaling Technology), anti-phospho-Akt (Cell Signaling Technology), anti-β-actin (Abcam), anti-CDK4 (Santa Cruz Biotechnology, Inc.), and anti-phospho-Mek1/2 (Cell Signaling Technology). Immunohistochemistry of EC tissues was performed using an antibody against human Mp1 (Epitomics) according to Hersmus et al. (2008).

Microarray analysis. To measure the effect of FGF4 combined with Mp1 knockdown on transcriptional targets, FGF4-deficient ES cells were grown overnight in serum-free medium (N2B27), including 10 ng/ml Bmp4, and treated with FGF4 at a concentration of 20 ng/ml for 3 d. RNA was isolated using TRIzol B (Invitrogen) and amplified using the Ambion Illumina Total-Prep RNA Amplification kit (Ambion) according to the manufacturer's procedure. Microarray analysis was performed using the MouseWG-6 v2 Expression BeadChip procedure of Illumina and normalized using R script. GNF profiles (www.biogps.gnf.org) were used to indicate during which stage of early embryogenesis genes are expressed. All samples were normalized to the FGF4-treated conditions. Microarray data are available under GEO accession no. GSE32595.

Online supplemental material. Fig. S1 shows validation of the shRNAs identified in the screen. Table S1 shows sequences of sh/siRNAs. Table S2 shows qPCR primer sequences. Table S3 shows sequencing of shRNA inserts with the corresponding blast results of genes that were functionally analyzed. Table S4 shows sequencing of shRNA inserts with the corresponding blast results of genes that were not functionally analyzed. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20102037/DC1>.

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