Investigating the duality of Inpp4b function in the cellular transformation of mouse fibroblasts

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ABSTRACT

Inositol Polyphosphate 4-Phosphatase, Type II (INPP4B) is a tumour suppressor in breast, ovarian, prostate, thyroid and other cancers, attributed to its ability to reduce oncogenic Akt-signaling. However, emerging studies show that INPP4B also has tumour-promoting properties in cancers including acute myeloid leukemia, colon cancer, melanoma and breast cancer. Together these findings suggest that INPP4B may be a context dependent cancer gene. Whether INPP4B functions solely in a tumour suppressing or tumour promoting manner, or both in non-transformed cells is currently not clear. In this study, consequences of deficiency and overexpression of INPP4B on cellular transformation was investigated using a mouse embryonic fibroblast (MEF) model of cellular transformation. We observed that neither deficiency nor overexpression of INPP4B was sufficient to induce neoplastic transformation, alone or in combination with H-RasV12 or E1A overexpression. However, Inpp4b-deficiency did cooperate with SV40 T-Large-mediated cellular transformation, a finding which was associated with increased phosphorylated-Akt levels. Transformation and phosphorylated-Akt levels were dampened upon overexpression of INPP4B in SV40 T-Large-MEF. Together, our findings support a model where INPP4B function suppresses transformation mediated by SV40 T-Large, but is inconsequential for Ras and E1A mediated transformation.

INTRODUCTION

The phosphoinositide-3-kinase (PI3K) signaling pathway regulates cell survival, proliferation, metabolism and various other processes linked to cell growth. Human cancers frequently acquire mutations resulting in aberrant activation of PI3K signaling which is often associated with increased tumour progression and resistance to cancer therapies. The PI3K pathway is activated by growth-factor mediated activation of receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs) which transduce signals through class I PI3K via phosphorylation of the 3-hydroxyl group of the inositol ring of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) to generate phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P₃ [PIP₃]) [1, 2]. PIP₃ is subsequently dephosphorylated by either PTEN to form phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P₂] or by SHIP phosphatases to form phosphatidylinositol-3,4-bisphosphate [PtdIns(3,4)P₂]. The 3’phosphorylated phosphatidylinositolos like PIP, and PtdIns(3,4)P₂ recruit cytosolic proteins containing pleckstrin homology (PH) domains, such as the serine/threonine kinase Akt. Once recruited to membranes, the kinase activity of Akt is activated by phosphorylation to promote downstream signaling to cell survival, growth, proliferation, cell migration and angiogenesis, through phosphorylation of specific intracellular protein substrates [3].

Inositol Polyphosphate 4-Phosphatase, Type II (INPP4B), a lipid phosphatase that preferentially...
hydrolyzes PtdIns(3,4)P$_2$ to generate phosphatidylinositol-3-monophosphate [PtdIns(3)P], was characterized as a gene with a key role in PI3K pathway signaling [4]. Since both PtdIns(3,4)P$_2$ and PIP$_3$ promote Akt recruitment to the plasma membrane, INPP4B was predicted to act as a tumour suppressor by inhibiting Akt recruitment, activation and thus downstream PI3K pathway signaling [5–8]. The first evidence characterizing INPP4B as a gene with importance in cancer was from a RNA interference screen in immortalized human mammary epithelial cells (HMEC) designed to identify candidate tumour suppressors [9]. Several studies have subsequently validated a tumour suppressor role for INPP4B in breast, ovarian, skin, and prostate cancer among others [10, 4, 8, 11–23]. In these studies, INPP4B loss resulted in elevated Akt activation, increased cell survival and a more aggressive growth phenotypes associated with poor outcomes for cancer patients [13, 16, 24]. These findings for INPP4B contribute to the increasing role of phosphoinositide phosphatases other than PTEN in cancer; these include the INPP5-family members such as INPP5J/PIPP, INPP5D/SHIP1, INPP1L/SHIP2, and INPP5E [25–29]. Notably, despite the abundance of clinical data supporting a tumour suppressor role for INPP4B, there is no evidence that Inpp4b deletion alone in mouse models leads to tumour formation [17, 19, 30]. However when Inpp4b loss was combined with Pten heterozygosity, it altered the penetrance of the Pten-spectrum of tumours, and notably malignant thyroid cancer was observed [17, 19, 30]. Thus it has been suggested that INPP4B may be a tumour suppressor in the context of PTEN loss, and may have weak tumour suppressive function otherwise [31].

Conversely, emerging findings in malignancies including acute myeloid leukemia (AML), colon cancer, melanoma and breast cancer among others suggest that overexpression of INPP4B is also associated with promoting aggressive cancer phenotypes [32–36]. Signaling downstream of PtdIns(3)P has been explored as a possible mechanism. For instance, PtdIns(3)P mediated activation of Serum/Glucocorticoid Regulated Kinase Family Member 3 (SGK-3) was observed downstream of INPP4B overexpression in some cancers [34, 36–39]. Moreover, PtdIns(3)P has very important cellular roles, which include endosomal trafficking and autophagy which are currently unexplored in the context of INPP4B overexpression [40]. Moreover, INPP4B was reported to have both tumour promoting and tumour suppressing features in different subsets of the same cancer. For instance in melanoma and breast cancer, both INPP4B loss and INPP4B overexpression were associated with downstream oncogenic signaling through Akt and SGK3, respectively [8, 37, 38, 41]. Altogether, these findings point to a putative contextual role for INPP4B in cancer [42, 43]. Nevertheless, mechanisms underlying the context-dependent cancer functions of INPP4B remain to be elucidated.

A growing body of evidence links altered levels of INPP4B expression to the progression of cancer. However, a role for INPP4B in the transformation of primary cells remains unexplored. Herein, we sought to investigate the consequences of Inpp4b-deficiency and INPP4B-overexpression on the cellular transformation of primary MEF in combination with oncogenic drivers including H-Ras$^{V12}$, E1A or SV40 T-Large. Exploring a role for Inpp4b in MEF transformation may provide insight on whether co-operating driver mutational signaling will alter Inpp4b context dependent outcome in tumourigenesis.

RESULTS

Characterization of primary Inpp4b$^{+/+}$, Inpp4b$^{−/−}$ and Inpp4b$^{−/+}$ MEF

To investigate the role of Inpp4b loss on cellular transformation we generated E13.5 MEF from a constitutive Inpp4b$^{+/−}$ mouse model [30]. PCR genotyping of cultured MEFs derived from timed-matings of Inpp4b$^{−/−}$ mice was performed to determine Inpp4b$^{+/−}$, Inpp4b$^{−/−}$ or Inpp4b$^{−/+}$ genotypes (Figure 1A). RT-qPCR and immunobLOTS were performed to validate loss of both transcript and protein levels of Inpp4b in Inpp4b$^{−/−}$ MEF (Figure 1B). Growth characteristics of primary MEF was evaluated in short term growth assays where we observed no significant differences in the mean growth rates of Inpp4b$^{−/−}$, Inpp4b$^{+/-}$ and Inpp4b$^{+/-}$ MEF (Figure 1C). Similarly, long term clonogenic growth potential was tested in primary Inpp4b$^{+/-}$ and Inpp4b$^{−/−}$ MEF. After 11 days of growth, only sparse spontaneous clone formation was observed in both Inpp4b$^{+/-}$ and Inpp4b$^{−/−}$ MEF, with no measurable difference between genotypes (Figure 1D). Finally, neither Inpp4b$^{−/−}$ nor Inpp4b$^{−/+}$ MEF were observed to grow as anchorage independent colonies in soft agar (Figure 1E).

To investigate the necessity for Inpp4b in cellular transformation, we examined the consequences of Inpp4b deficiency on H-Ras$^{V12}$/E1A mediated MEF transformation. For these experiments we infected early passage Inpp4b$^{−/−}$ and Inpp4b$^{−/+}$ MEF with retrovirus expressing both H-Ras$^{V12}$ and E1A (12SLRC). Upon infection of cells, we observed no difference in the morphology in all H-Ras$^{V12}$/E1A transformed cells of either genotype. Moreover, we observed no difference between the transformed MEF from Inpp4b$^{−/−}$ and Inpp4b$^{−/+}$ backgrounds on foci formation in clonogenic assays (Figure 1F) and anchorage independent colonies in soft agar (Figure 1G, 1H). These data suggest that Inpp4b-deficiency does not lead to spontaneous transformation of MEF and that Inpp4b expression is dispensable for H-Ras$^{V12}$/E1A mediated MEF transformation.
Neither loss nor overexpression of \textit{INPP4B} cooperate with \textit{H-Ras}^{V12} in MEF transformation

To characterize the cooperativity of \textit{Inpp4b}-deficiency with \textit{H-Ras}^{V12} overexpression in cellular transformation, early passage \textit{Inpp4b}^{+/+} and \textit{Inpp4b}^{−/−} MEF were infected with retroviral particles generated from the \textit{pBabe-H-Ras}^{V12-puro} vector. Morphologically, both \textit{H-Ras}^{V12}; \textit{Inpp4b}^{+/+} and \textit{H-Ras}^{V12}; \textit{Inpp4b}^{−/−} MEF demonstrated the expected features of oncogene-induced senescence (OIS) including complete growth inhibition (Figure 2A) and large multinucleated senescent-like cells (Figure 2B). Neither \textit{H-Ras}^{V12}; \textit{Inpp4b}^{+/+} nor \textit{H-Ras}^{V12}; \textit{Inpp4b}^{−/−} MEF were able to form colonies in clonogenic assays (Figure 2C). Similarly, no anchorage-independent colonies were observed in soft agar (Figure 2D). Conversely, positive control MEF infected with \textit{H-Ras}^{V12} and \textit{E1A} retroviruses generated rapidly proliferating cells (Figure 2A), abundant foci (Figure 2E, top) and numerous anchorage independent colonies (Figure 2E, bottom).

**Figure 1**: Generation and characterization of primary \textit{Inpp4b}^{+/+}, \textit{Inpp4b}^{−/−} and \textit{Inpp4b}^{−/−} MEF. (A) Schematic illustrating breeding strategy for generation of primary MEF and a typical \textit{Inpp4b} genotyping PCR result is illustrated. (B) RT-qPCR and Immunoblot demonstrating \textit{Inpp4b} expression levels in \textit{Inpp4b}^{+/+} and \textit{Inpp4b}^{−/−} MEF. (C) Growth curve, (D) colony formation (E) and soft agar assay of primary \textit{Inpp4b}^{+/+} and \textit{Inpp4b}^{−/−} MEF. (F) Colony formation assay with \textit{Inpp4b}^{+/+} and \textit{Inpp4b}^{−/−} MEF empty vector and \textit{I2SLRC H-Ras}^{V12/E1A} infection. (G) Representative soft agar assay of \textit{I2SLRC H-Ras}^{V12/E1A} infected \textit{Inpp4b}^{+/+} and \textit{Inpp4b}^{−/−} MEF. Photos of representative colonies at 20X. (H) Quantitation of quantitation of foci formation assay.
Next, to examine the potential of INPP4B as an oncogene in MEF, we investigated whether INPP4B-overexpression could cooperate with H-Ras<sup>V12</sup> in cellular transformation. Retrovirus generated from pWZL-H-Ras<sup>V12</sup>-hygro vector was combined with either MSCV-Puro-IRE5-GFP (PIG)-empty or PIG-INPP4B retrovirus to co-infect wild-type MEF followed by selection with hygromycin B and puromycin. Morphologically both H-Ras<sup>V12</sup>; PIG or H-Ras<sup>V12</sup>; PIG-INPP4B infected MEF appeared large and multinucleated (Figure 2F) as expected with H-Ras<sup>V12</sup> infection. Foci were not observed in clonogenic assays (Figure 2G) and similarly, no anchorage-independent colonies were observed in soft agar assays (Figure 2H). Inpp4b<sup>+/+</sup> MEF infected with H-Ras<sup>V12</sup> and E1A retroviruses which generated abundant foci (Figure 2I, top) and anchorage independent colonies (Figure 2I, bottom) were used as positive controls. Overall, neither deficiency nor overexpression of INPP4B demonstrated cooperativity with H-Ras<sup>V12</sup>-overexpression in cellular transformation.

Neither loss nor overexpression of INPP4B cooperate with E1A in MEF transformation

To investigate whether loss of Inpp4b is sufficient to cooperate with E1A in promoting cellular transformation, early passage Inpp4b<sup>+/+</sup> and Inpp4b<sup>-/-</sup> MEF were infected with pWZL-E1A-hygro retrovirus and selected with hygromycin B. Immediately after selection, MEF were plated for growth analysis, clonogenic assay and soft agar assays. Inpp4b<sup>+/+</sup>, Inpp4b<sup>-/-</sup> and Inpp4b<sup>-/-</sup> MEF infected with E1A alone were growth inhibited compared to H-Ras<sup>V12</sup>; E1A controls (Figure 3A). Morphologically both Inpp4b<sup>+/+</sup> and Inpp4b<sup>-/-</sup> MEF appeared smaller and more dispersed compared to primary MEF (Figure 3B); clonal outgrowth on plastic observed after 21 days was similarly very minimal in both Inpp4b<sup>+/+</sup> and Inpp4b<sup>-/-</sup> MEF (Figure 3C) and no anchorage independent growth colonies formed in soft agar (Figure 3D). Conversely, Inpp4b<sup>+/+</sup> and Inpp4b<sup>-/-</sup> MEF overexpressing both H-Ras<sup>V12</sup> and E1A rapidly proliferated in 6-day growth curves (Figure 3A), grew many more foci in the clonogenic assay (Figure 3E, top) and formed large robust colonies in soft agar (Figure 3E, Bottom).

Next, INPP4B overexpression was tested for its ability to cooperate with E1A to induce cellular transformation in MEF. For these experiments, wild-type MEF were retrovirally infected with pWZL-E1A-hygro vector with either PIG or PIG-INPP4B and selected with hygromycin B and puromycin for 48 hours. Morphologically, E1A; PIG MEF appeared smaller than primary cells (Figure 3F, top) whereas E1A; PIG-INPP4B MEF appeared larger, more elongated with long spindle-like protrusions and multinucleated (Figure 3F, bottom). No significant differences in foci counts were observed between E1A; PIG and E1A; PIG-INPP4B in clonogenic assays (Figure 3G). Anchorage independent growth was not observed in either E1A; PIG and E1A; PIG-INPP4B MEF (Figure 3H), but both clones and foci were clearly observed in the control cells infected with H-Ras<sup>V12</sup> and E1A retroviruses (Figure 3I). Overall, as with H-Ras<sup>V12</sup>-overexpression, neither Inpp4b-deficiency nor INPP4B-overexpression were able to cooperate with E1A in cellular transformation.

Inpp4b deficiency increases colony forming potential in SV40 T-Large MEF

We next investigated cooperativity of Inpp4b-deficiency with SV40 T-Large overexpression. As before, SV40 T-Large retrovirus was used to infect early passage Inpp4b<sup>+/+</sup> and Inpp4b<sup>-/-</sup> MEF. Morphologically, no differences were observed between Inpp4b<sup>+/+</sup> and Inpp4b<sup>-/-</sup> MEF lines (Figure 4A). Immunoblot analysis of SV40 T-Large Inpp4b<sup>+/+</sup> and Inpp4b<sup>-/-</sup> demonstrated similar levels of p53 protein and no changes in PTEN expression (Figure 4C). Notably, unlike H-Ras<sup>V12</sup> and E1A, SV40 T-Large infected Inpp4b<sup>-/-</sup> MEF demonstrated a significant (P = 0.025) increase in foci formation (Figure 4C) and a significant (P = 0.0011) increase in anchorage independent colonies in soft agar (Figure 4D). These findings demonstrate cooperativity between SV40 T-Large overexpression and Inpp4b deficiency in MEF and suggest that Inpp4b may function as a tumour suppressor in the context of SV40 T-Large transduction.

Given this increase in SV40 T-Large transformation potential observed in Inpp4b<sup>-/-</sup> MEF, we reasoned that INPP4B overexpression would limit anchorage independent growth of SV40 T-Large MEF. To investigate this, early passage SV40 T-Large MEF were transduced with PIG and PIG-INPP4B retrovirus (Figure 5A). Although there were no gross morphological differences between PIG and PIG-INPP4B transduced SV40 T-Large MEF (Figure 5B), we did observe that INPP4B overexpression in MEF SV40 T-Large MEF displayed a significant reduction (P = 0.0018) in colony number as compared to control (Figure 5C). Immunoblot analysis of SV40 T-Large Inpp4b<sup>+/+</sup> and Inpp4b<sup>-/-</sup> demonstrated no changes Pten protein levels upon INPP4B overexpression (Figure 5A).

Inpp4b modulates EGF stimulated PI3K signaling in SV40 T-Large MEF

To explore the relationship of Inpp4b deficiency on PI3K signaling in MEF, low-passage Inpp4b<sup>+/+</sup> and Inpp4b<sup>-/-</sup> SV40T-Large MEF were serum starved overnight and stimulated with epidermal growth factor (EGF). We consistently observed that Inpp4b<sup>-/-</sup> MEF achieved higher peak levels of pSer<sup>473</sup>-Akt activation at 5–10 minutes compared to timepoints in Inpp4b<sup>+/+</sup> MEF. Inpp4b<sup>-/-</sup> MEF maintained prolonged peak levels of pSer<sup>473</sup>-Akt beyond
10 minutes of EGF and remained higher than Inpp4b<sup>+/+</sup> MEF up to 30 min (Figure 6A). Conversely, INPP4B overexpression in SV40 T-Large MEF led to markedly decreased levels of pSer<sup>473</sup>-Akt after EGF stimulation. This decrease was observed at the peak pSer<sup>473</sup>-Akt concentrations of 5 and 10 minutes (Figure 6B). Together these results indicate that INPP4B regulates Akt activation dynamics in stimulated SV40T-Large MEF.

**DISCUSSION**

Altered levels of INPP4B expression have been linked with cancer progression in various different human tumour types [42]. Previous works have demonstrated that INPP4B knockdown induced anchorage-independent cell growth, increased invasion and migration, and augmented Akt activation in immortalized HMECs [8, 9]. In contrast,

![Figure 2: Inpp4b loss or overexpression do not cooperate with H-Ras<sup>V12</sup> in MEF transformation.](image)

(A) 6-day growth curve of Inpp4b<sup>+/+</sup>, Inpp4b<sup>+/−</sup> and Inpp4b<sup>−/−</sup> MEF after HRas<sup>V12</sup> infection. 12SLRC H-Ras<sup>V12</sup>/E1A infected Inpp4b<sup>+/+</sup> MEF used as control. (B) Morphology of eGFP-expressing Inpp4b<sup>+/+</sup> and Inpp4b<sup>−/−</sup> MEF after H-Ras<sup>V12</sup> infection. (C) Colony formation and (D) Soft agar assay of primary Inpp4b<sup>+/+</sup> and Inpp4b<sup>−/−</sup> MEF after H-Ras<sup>V12</sup> infection. (E) 12SLRC H-Ras<sup>V12</sup>/E1A infected colony assay (top) and soft agar (bottom) controls. Full wells are depicted. (F) Morphology of eGFP-expressing Inpp4b<sup>−/−</sup> MEF after H-Ras<sup>V12</sup> and PIG or PIG-Inpp4b infection. (G) Colony formation and; (H) Soft agar assay of primary Inpp4b<sup>−/−</sup> MEF after H-Ras<sup>V12</sup> and PIG or PIG-Inpp4b infection (I) 12SLRC H-Ras<sup>V12</sup>/E1A and PIG or PIG-Inpp4b infected colony assay (top) and soft agar (bottom) controls.
other reports show that INPP4B overexpression enhanced proliferation and promotes anchorage-independent growth of HEMn-MP melanocytes and FHC normal colon epithelial cells [34, 38]. Herein, we present results which indicate that deficiency of Inpp4b in MEF can cooperate with very specific oncogenic alterations to induce cellular transformation. In our study, we observed that neither heterozygosity nor deficiency of Inpp4b were sufficient to alter growth characteristics of MEF on their own (Figure 1C). Spontaneous immortalization of MEF was also not observed in colony formation assays (Figure 1D, 1E). Similarly, Inpp4b deficiency was inconsequential for H-RasV12/E1A induced cellular transformation of MEF (Figure 1F, 1G). Inpp4b deficiency did not inhibit H-RasV12/E1A-induced cellular transformation as both Inpp4b−/− and Inpp4b+/+ MEF displayed long-term growth potential consistent with immortalization and in an anchorage independent manner in the soft agar assay (Figure 1G, 1H).

Figure 3: Inpp4b loss or overexpression do not cooperate with E1A in MEF. (A) 6-day growth curve of MEF after E1A infection. 12SLRC H-RasV12/E1A infected Inpp4b+/+ MEF used as control. (B) Morphology of eGFP-expressing Inpp4b+/+ and Inpp4b−/− MEF after E1A infection. (C) Colony formation and; (D) Soft agar assay of primary Inpp4b+/+ and Inpp4b−/− MEF after E1A infection and (E) 12SLRC H-RasV12/E1A infected colony assay (left) and soft agar (right) controls. Half wells are depicted. (F) Morphology of eGFP-expressing Inpp4b−/− MEF after E1A and PIG or PIG-Inpp4b infection. (G) Colony formation and; (H) Soft agar assay of primary Inpp4b+/+ MEF after E1A and PIG or PIG-Inpp4b infection (I) 12SLRC H-RasV12/E1A and PIG or PIG-Inpp4b infected colony assay (left) and soft agar (right) controls.
Figure 4: *Inpp4b* deficiency increases colony forming potential of *SV40 T-Large* in MEF. (A) Morphology of eGFP-expressing *Inpp4b*+/+ and *Inpp4b*−/− MEF after *SV40 T-Large* infection. (B) Immunoblot of *Inpp4b*+/+ and *Inpp4b*−/− *SV40 T-Large* MEF. (C) Representative clonogenic and (D) Soft agar assay plates with quantitation below. *P*-values were determined using the Student’s *t*-test.

Figure 5: *Inpp4b* overexpression decreases transformation potential in *SV40 T-Large* MEF. (A) Immunoblot measuring INPP4B overexpression and Pten levels in *SV40T-Large* MEF (*n* = 5). (B) Morphology of eGFP-expressing *Inpp4b*+/+ *SV40T-Large* MEF with PIG or PIG-Inpp4b transduction. (C) Representative soft agar assay plates and quantitation of *Inpp4b*+/+ *SV40T-Large* MEF with PIG or PIG-Inpp4b colonies. *P*-values were determined using the Student’s *t*-test.
When co-expressed in MEF, H-RasV12 and E1A potently induce cellular transformation due to the strong proliferative signaling through H-RasV12 concomitant with inhibition of the Rb tumour suppressor pathway by E1A which bypasses Ras-induced senescence [44, 45]. Inpp4b deficiency did not cooperate with H-RasV12 nor E1A oncogene overexpression to promote MEF transformation suggesting that Inpp4b may not play a role in E1A or Ras signaling pathways in MEF (Figures 2–3).

However, Inpp4b deficiency did promote SV40 T-Large mediated transformation of MEF, as demonstrated by an increase in anchorage independent growth and colony formation as compared to controls (Figure 4C–4D). Increased SV40 T-Large mediated cellular transformation was associated with elevated Akt activation as demonstrated by elevated pSer473-Akt after stimulation of starved MEF with EGF (Figure 6). Together, these findings are consistent with studies by Westbrook et al. [9] and Gewinner et al. [8] which demonstrated that shRNA knock-down of INPP4B in HMEC cells immortalized with SV40 T-Large and hTERT promoted cellular transformation. In addition, that Inpp4a–/– MEF also displayed elevated pAkt levels compared to wild type after SV40 T-Large immortalization [46] support the notion that 4-phosphatase function is tumour suppressive in part through regulation of Akt signaling. Other mechanisms which have been linked to INPP4B loss include loss of ATM and ATR, both upstream regulators of the p53 pathway [22, 47] and INPP4B was reported to downregulate PTEN protein through its protein phosphatase activity [34]. Our efforts in this study demonstrated that p53 and Pten expression levels were unchanged between Inpp4b+/+ and Inpp4b–/– SV40 T-Large MEF (Figure 4B) indicating that these mechanisms may not be associated with the phenotypes we observed in MEF.

**Figure 6:** Inpp4b modulates EGF stimulated Akt activation in SV40 T-Large MEF. SV40T-Large MEF were serum starved and stimulated with 100 ng/mL of EGF and immunoblotted with pSer473-Akt. (A) Immunoblot of pSer473-Akt of SV40 T-Large Inpp4b+/+ and Inpp4b–/– MEF with quantitation of pSer473-Akt activation status (n = 4) (B) Immunoblot of pSer473-Akt SV40T-Large; PIG or SV40T-Large; PIG -INPP4B MEF with quantitation plot of pSer473-Akt activation status (n = 3). P-values were determined using ANOVA. *P < 0.05, **P < 0.01, ***P < 0.005.
Conversely, emerging evidence suggests that INPP4B overexpression may also promote tumourigenesis and cancer progression [32–36]. Thus, we tested whether INPP4B overexpression in MEF drives or cooperates with other transforming oncogenes in cellular transformation. We observed that INPP4B overexpression was unable to promote transformation in combination with H-RasV12, E1A nor SV40 T-Large MEF. In fact, overexpression of INPP4B in SV40 T-Large MEF significantly decreased cellular transformation and concomitantly decreased pSer473-Akt levels (Figures 5, 6). These findings were reminiscent of those reported by Fedele et al. [10] in serum starved and EGF stimulated MCF7 at similar time points, where peak pAkt levels were lower after INPP4B knockdown, but at 30 minutes INPP4B overexpressing cells displayed slightly increased pAkt levels compared to the control [10]. Taken together, these findings highlight a tumour suppressive role for Inpp4b in the context of SV40 T-Large that coincides with elevated Akt activation and suggest that a functional alteration conferred by SV40 T-Large transduction cooperates with Inpp4b loss to promote cellular transformation in vitro.

In summary, our study aimed to address the role for Inpp4b in cellular transformation. Given the numerous contrasting models for INPP4B function in cancer, we investigated both deficiency and overexpression to elucidate predominant tumour suppressor or oncogenic roles, respectively in MEF. With respect to MEF transformation, we observed that INPP4B overexpression did not promote oncogenesis when combined H-RasV12, E1A or SV40-T-large transduction. Although Inpp4b deficiency did not cooperate with overexpression H-RasV12, nor E1A, we did observe that Inpp4b deficiency can increase the transformation potential of SV40-T-large transduction suggesting that Inpp4b may function in a tumour suppressive manner in this context, at least in part through its control of Akt activation. This suggests that an event specifically associated with suppressing transformation is relieved by INPP4B overexpression in MEF. In fact, overexpression of INPP4B in the context of SV40 T-Large decreased H-RasV12 transduction suggesting that INPP4B overexpression may also promote tumourigenesis (DMEM) supplemented with 10% FBS and 1% penicillin/ streptomycin) for 12–16 hours. Once the MEF had grown to confluency, the cells were frozen down and designated as Passage 1. Primary MEF were passaged every 3 days to pass cell cultures for less than 1.5 × 10^4 cells per 10 cm dish. MEF were considered primary from Passage 1-Passage 4. All experiments were performed with primary MEF unless otherwise indicated.

**Genotyping**

DNA was extracted by lysing the heads of the mouse embryos in DNA extraction buffer (1M Tris (pH8), 20% SDS, 0.5M EDTA, 5M NaCl made up in water) containing 10% proteinase K at 55°C shaking for approximately 16 hours. An equal volume of 100% ethanol was added and centrifuged at 21.9 × g for 10 minutes. The supernatant was discarded, and the DNA pellet was washed with 70% ethanol, dried completely, and re-suspended in 50 μL of sterile water. PCR was performed using 2X FrosaaMix (FroggaBio), to distinguish Inpp4b+/–, Inpp4b+–, and Inpp4b––. Genotyping primer pairs: wild type forward: 5’ GCTTCTGATAAAACATGGG 3’, wild type reverse: 5’ TGGGCAACATTATAAGCCTTTC 3’, mutant forward: 5’ GCTTCTGATAAAACATGGG 3’, and mutant reverse: 5’ TGGGCAACATTATAAGCCTTTC 3’. 

**Retroviral constructs**

The following plasmids were purchased from AddGene: pWZL hygro H-RasV12 (#18749), pBabe-puro H-RasV12 (#1768), pWZL hygro 12S E1A(#18748), and MSCV-puro-IRE-GFP (#21654). pWZL hygro SV40T-large was a kind gift from Pier Paolo Pandolfi, and 12SLRC H-RasV12 /E1A was a kind gift from Marisol Soengas. The MSCV-PIG-FLAG-Inpp4b was cloned by adding FLAG- Inpp4b to the MSCV-puro-IRE-GFP plasmid through Gibson Assembly (NEB).

**Retroviral transduction**

For transfection of retroviral constructs, 3.0 × 10^6 293T cells were seeded on a 10 cm dish 24 hours prior to calcium phosphate transfection. Media was changed 3–4 hours prior to transfection. For each 10 cm dish, 10 μg of retroviral plasmid and 5 μg of retroviral packaging vector, pCL-Eco, were combined with 2M CaCl₂ and made up to a final volume of 300 μL with sterile water. This DNA/ CaCl₂ mixture was vortexed while 300 μL of 2X HEPES-Buffered saline (HBS; 140 mM NaCl, 1.5 mM Na₂HP0₄) was slowly added to mixture. 600 μL of DNA/CaCl₂/HBS mixture was gently pipetted onto 293T cells. Media was changed 24 hours after transfection and supernatants was collected at 48- and 72-hours post-infection. MEF were seeded at 8.0 × 10^3 cells/10 cm dish 24 hours prior to...
infection. Fresh virus-rich media was collected and filtered using a 0.45-micron filter and added to respective plate along with 8 μg/ml protamine sulfate. Infections were repeated every 8 hours to increase infection efficiency. PWZL-hygro and PWZL- H-Ras<sup>V12</sup> -hygro were used as negative controls, and coinfection with PWZL-E1A-hygro and pBABE- H-Ras<sup>V12</sup> -puro were used as positive controls. 

Wild Type MEF cells were transfected with MSCV-PIG-Inpp4b and PWZL- H-Ras<sup>V12</sup>-hygro to investigate INPP4B overexpression in cooperation with H-Ras<sup>V12</sup> overexpression. Inpp4b<sup>−/−</sup> MEF cells were transfected with pBABE- H-Ras<sup>V12</sup> -puro to investigate the effects of inpp4b deficiency in cooperation with H-Ras<sup>V12</sup> overexpression on cellular transformation. Cells were selected for 4 days with hygromycin B at 75 μg/ml or 2 days of puromycin at 2 μg/ml selection, then cells recovered for 24 hours before plating for experiments. MEF infected with both puromycin and hygromycin B resistance plasmids were first selected for 4 days with hygromycin B at 75 μg/mL, allowed to recover for 24 hours and subsequently selected with 2 μg/ml puromycin; The cells were then allowed to recover for 24–48 hours before plating experiments.

Growth assays

MEF were seeded at a density of 2.5 × 10<sup>4</sup> cells per well in a 12-well plate for a 6-day growth curve. At each time point, cells were fixed with 10% formalin for 10 minutes and stored in phosphate-buffered saline (PBS) at 4°C. Once all time points were collected, cells were stained with 0.1% crystal violet, 20% methanol solution, then washed with water and dried for at least 4 hours. The crystal violet stain was solubilized by 10% acetic acid for 20 minutes and the absorbance was measured at 590 nm with Spectramax M3.

Clonogenic assays

MEF were plated at 1000 cells per well in a 6-well plate and allowed to grow for 10–20 days without media change. Cells were then washed with PBS, fixed with 10% formalin and stained with 0.1% crystal violet, 20% methanol solution, washed with water and allowed to dry. Colonies were imaged and counted using Image J. Cell counts were normalized to the control group.

Anchorage independence assays

200 mL of 6% Noble Agarose in sterile water was heated until liquefied and brought to 42°C. Soft agar plates were prepared by mixing 2 mL of melted 6% Noble Agarose in MilliQ water in 18 ml of complete DMEM. 3 mL of 0.06% Noble Agar was plated per well of a 6 well dish and placed at 4°C to solidify. 1 hour prior to plating the dish was preheated in a 37°C cell incubator. 20,000 infected MEF were plated in 7.2 ul of media at 37°C then mixed with 0.8 ml of 42°C, 3% Noble Agarose in MilliQ water to obtain a final concentration of 5000 cells per well in triplicate of a 6 well dish in 0.3% Agar in DMEM. Colonies were stained using 0.05% crystal violet in 40% methanol. Colonies were counted using OpenCFU colony counting software (http://opencfu.sourceforge.net).

qPCR

RNA was isolated using the Qiagen RNeasy Mini Kit, and subsequently treated with DNase. Reverse transcription was performed using the SuperScript IV VILO Master Mix (Invitrogen, 11766050). TaqMan Fast Advanced Master Mix (appliedbiosystems, 4444557). TaqMan probes for Inpp4b (Mm01247230_m1) and actin (Mm02619580_g1) were purchased from Thermo Fisher Scientific. All of the above conducted according to manufacturers’ instructions.

Akt signaling assay

Passage 3–5 SV40 T-Large MEFs were seeded at 200 000 cells per well in 6 wells of a 6 well dish. After the MEF adhered to the bottom of the dish (4–6 hours after plating) the cells were washed twice with serum free DMEM then plated in starvation media (DMEM + 0.01% FBS). 16 hours later each well was allocated to the indicated time point (minutes). 100 ng/mL EGF was used to activate PI3K signaling. Activation was terminated at the indicated time points with ice cold PBS, followed by drying of wells, and subsequently flash frozen with liquid nitrogen and placed at −80°C prior to immunoblot.

Western blots

Immunoblots were performed using standard conditions as previously published [32]. The following antibodies were used in this study. Anti-phospho-Ser<sup>473</sup>-Akt (NEB 14543); anti-pan Akt (NEB 29020); anti-β-Actin (NEB 4967); anti-FLAG (Abgent AP1013a-ev). Anti-INPP4B antibody detecting murine Inpp4b was a kind gift from Jean Vacher [30].

Statistics

To compare means of 2 groups p-values were calculated using unpaired two-tailed Student’s t-test. For colony counts of the clonogenic assay and soft agar assay two-way parametric analysis of variance (ANOVA) was used to determine the significance value. A P-value < 0.05 was considered significant.

Abbreviations

AML: Acute myeloid leukemia; E1A: Adenovirus early region 1A; EGF: Epidermal growth factor;
H-Ras: Harvey rat sarcoma viral oncogene homolog; HMEC: Human mammary epithelial cells; INPP4B: Inositol Polyphosphate 4-Phosphatase, Type II; MEF: Mouse embryonic fibroblast; PIG: MSCV-Puro-IRE- GFP; OIS: Oncogene-induced senescence; PtdIns: Phosphatidylinositol; pAKT: Phosphorylated Akt; Ras: Rat Sarcoma; SGK-3: Serum/Glucocorticoid Regulated Kinase Family Member 3; SV40: Simian Vacuolating Virus 40.

Author contributions

EEM and LS conceived and designed the study. EEM performed the bulk of experiments with assistance from KC and BS. JV contributed critical reagents. EEM and LS wrote the manuscript. All authors critically reviewed the manuscript and approved the final draft for submission.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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