SUPPLEMENTAL DATA

Supplemental Figure 1. Characterization of stable LOX cell lines. **(A-B)** Cells were grown with doxycyline (2mg/ml) to suppress mutant ARF6 expression or without doxycyline for 24 hrs, to induced expression of ARF6 mutants. In A, equal amounts of cell lysates were subjected to SDS-PAGE followed by Western blotting for GFP, ARF6, HA to detect mutant ARF6 expression, or α-tubulin, as a loading control. Transgene expression in the presence and absence of doxycyline is shown in B. **(C)** Equal amounts of lysates were subjected to the MT2 pull-down assay and representative blots shows the levels of ARF6-GTP in each cell line. Total ARF6 and α-tubulin in cell lysates were examined by Western blotting. **(D)** $LOX^{ARF6\text{-}GTP}$ and $LOX^{ARF6\text{-}GDP}$ cells expressing HA-tagged ARF6 mutants were fixed and stained with mouse HA antibody. Representative confocal images of LOX^{GFP}, $LOX^{ARF6\text{-}GTP}$ and $LOX^{ARF6\text{-}GDP}$ cells are shown.

Supplementary Figure 2. Proliferation of LOX cell lines. 1 X 10^5 LOX^{GFP}, $LOX^{ARF6\text{-}GDP}$ and $LOX^{ARF6\text{-}GTP}$ cells were seeded in individual wells of 6-well tissue culture plates. Cells were trypsizined each day and counted on a hemacytometer. The graph shows the average proliferation rates of the cell lines from two independent experiments, each performed in duplicate.

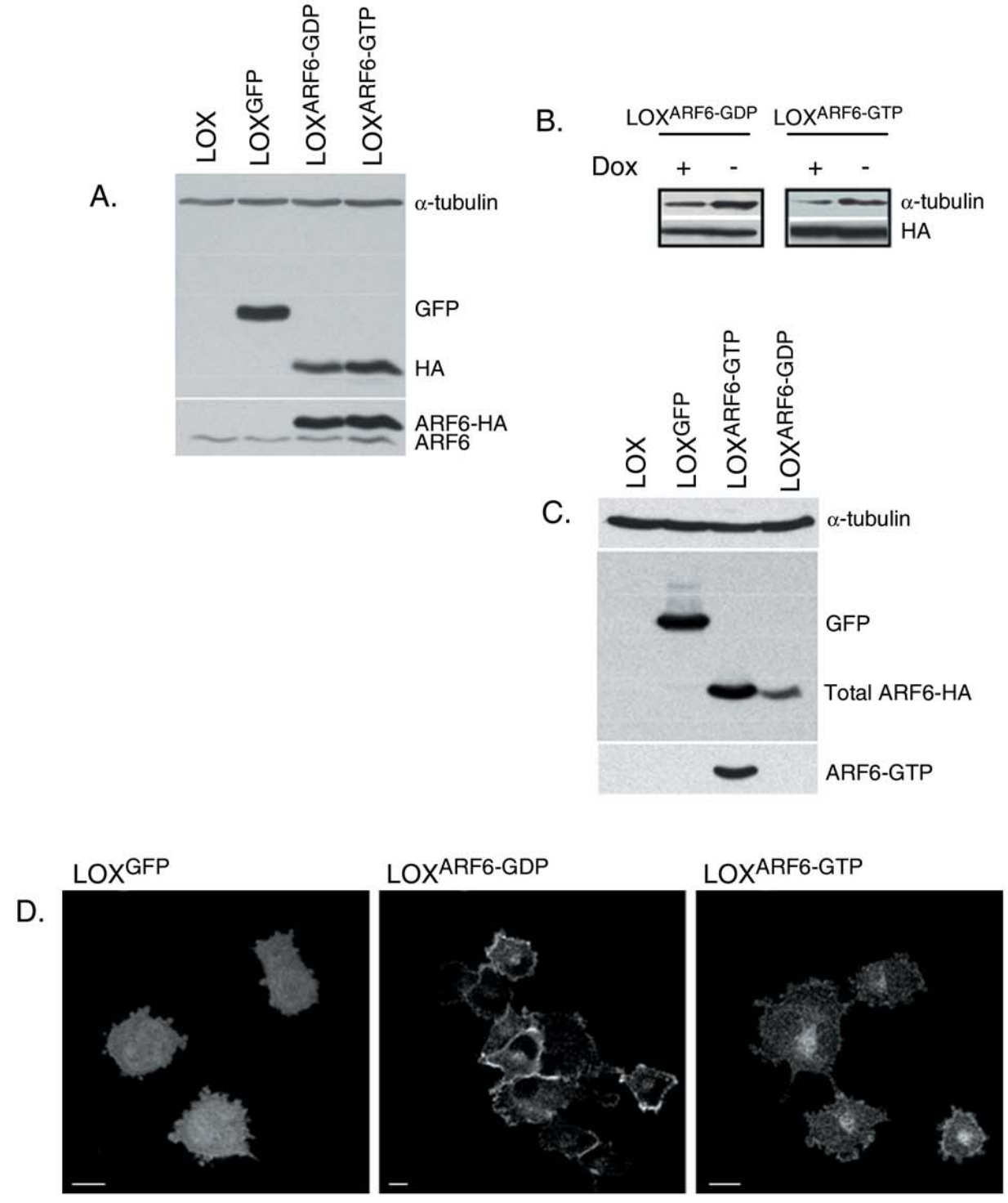
Supplemental Figure 3. Morphological analysis of LOX^{GFP} primary tumors. Primary tumors and the surrounding tissues were fixed at 2, 3, and 4 weeks post implantation, sectioned at 4 μm and dried onto glass slides. The sections were then processed for **(A)** immunofluorescense microscopy (Red= nuclear stain, ToPro-3; green=GFP) or **(B)** histological analysis (black=nuclei, red=muscle, blue=collagen). The images are representative cross

sections of tumors excised from mice at the skin and tumor interface. Note the progressive compromise to fat and muscle (arrows) layers by week 4.

Supplemental Figure 4. Subcultured cells from tumor explants. **(A)** LOX^{GFP} tumor cells were isolated and cultured in G418 as described. Image shows subcultured cells expressing GFP and co-stained with rhodamine phalloidin (right) isolated from corresponding tissue with LOX^{GFP} cells co-stained with ToPro-3 (left). **(B)** Representative phase contrast images of cells subcultured from LOX^{GFP} (upper panel) and $LOX^{ARF6\text{-}GDP}$ macrometastases from tissues as indicated.

Supplemental Figure 5. PLD is required for tumor cell invasion. **(A)**. $LOX^{ARF6\text{-}GDP}$ cells and sub-cultured $LOX^{ARF6\text{-}GDP}$ cells from metastatic tumors seeded on gelatin-coated coverslips were treated with 4-butanol or 1-butanol as indicated. Cells were scraped, lysed, probed for ERK and phospho-ERK by Western blotting. **(B and C)** Sub-cultured $LOX^{ARF6\text{-}GDP}$ cells were seeded on FITC-gelatin coated coverslips in serum free (SF) media at which point cells are quiescent. SF media was replaced with complete media alone or with 0.3% 1-butanol or 4-butanol. Cells were fixed and stained for actin using rhodamine-phalloidin (red). Sub-cultured $LOX^{ARF6\text{-}GDP}$ cells exhibiting matrix degradation underneath were scored and the percentage is shown (C).

Supplementary Figure 1



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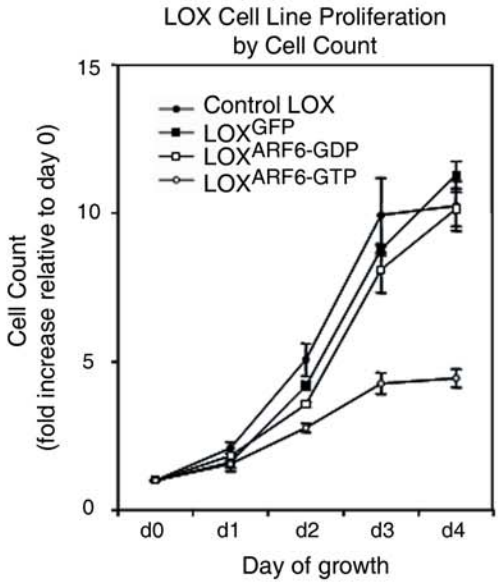
Supplementary Figure 2



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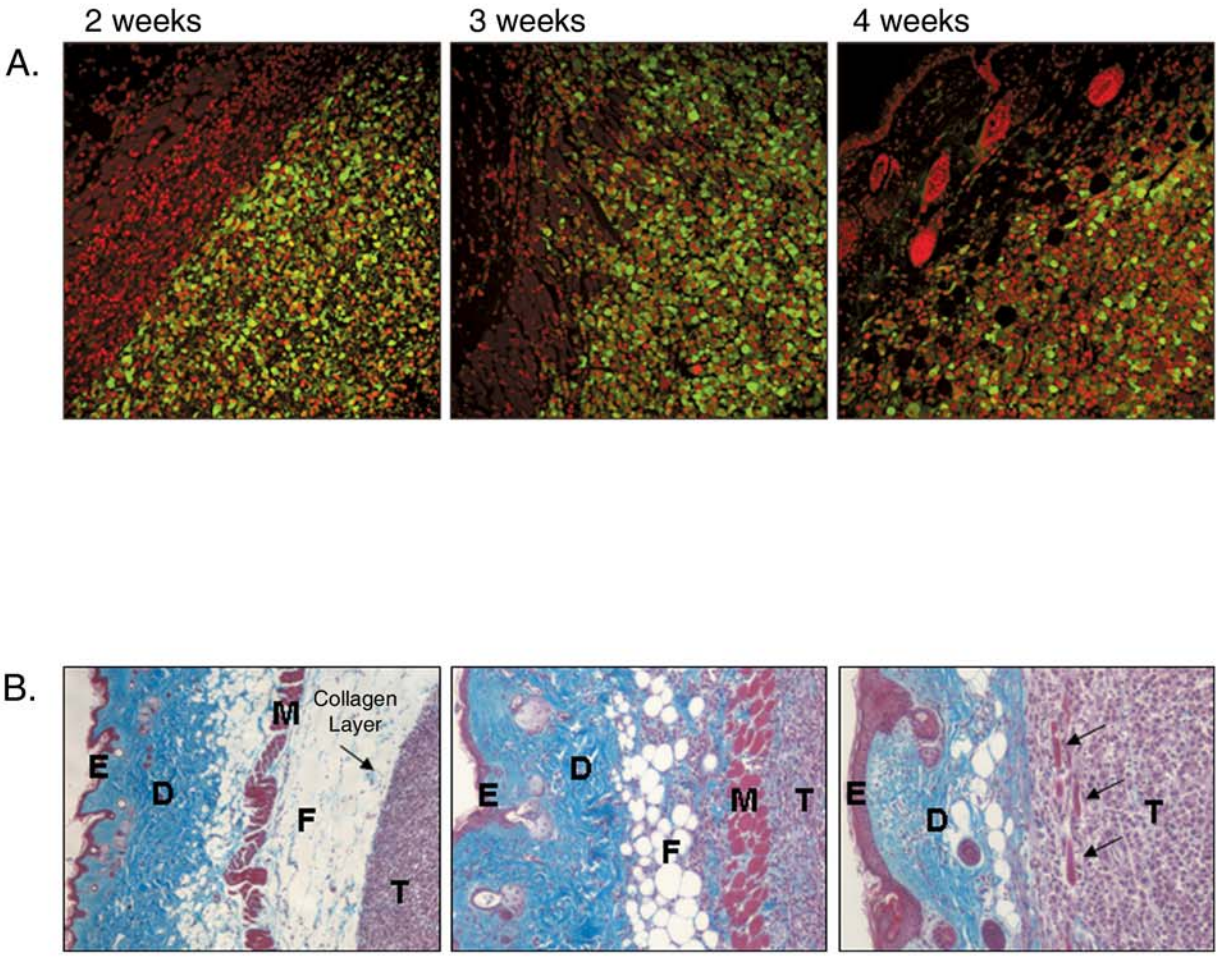
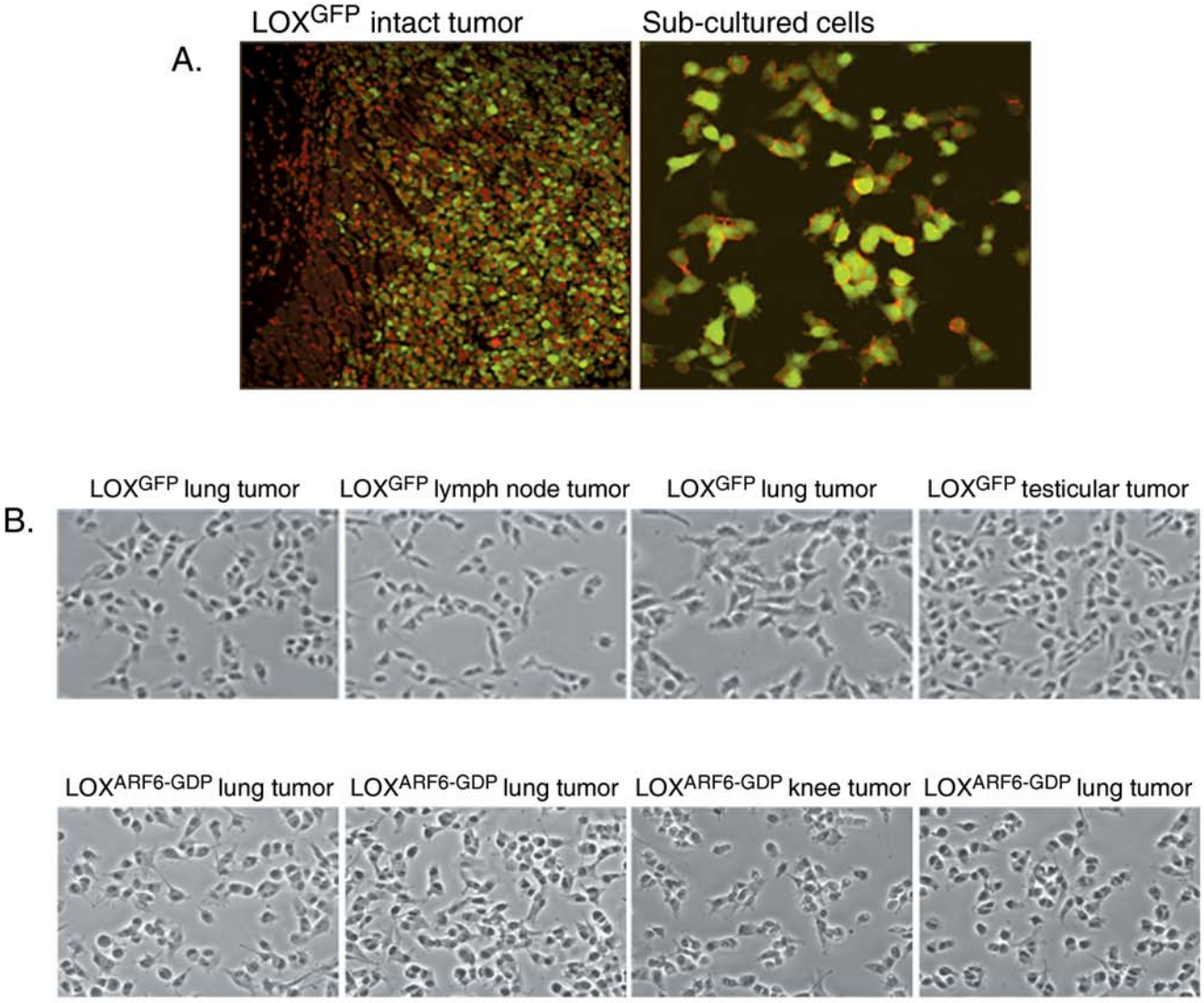
Supplementary Figure 3



Supplementary Figure 4



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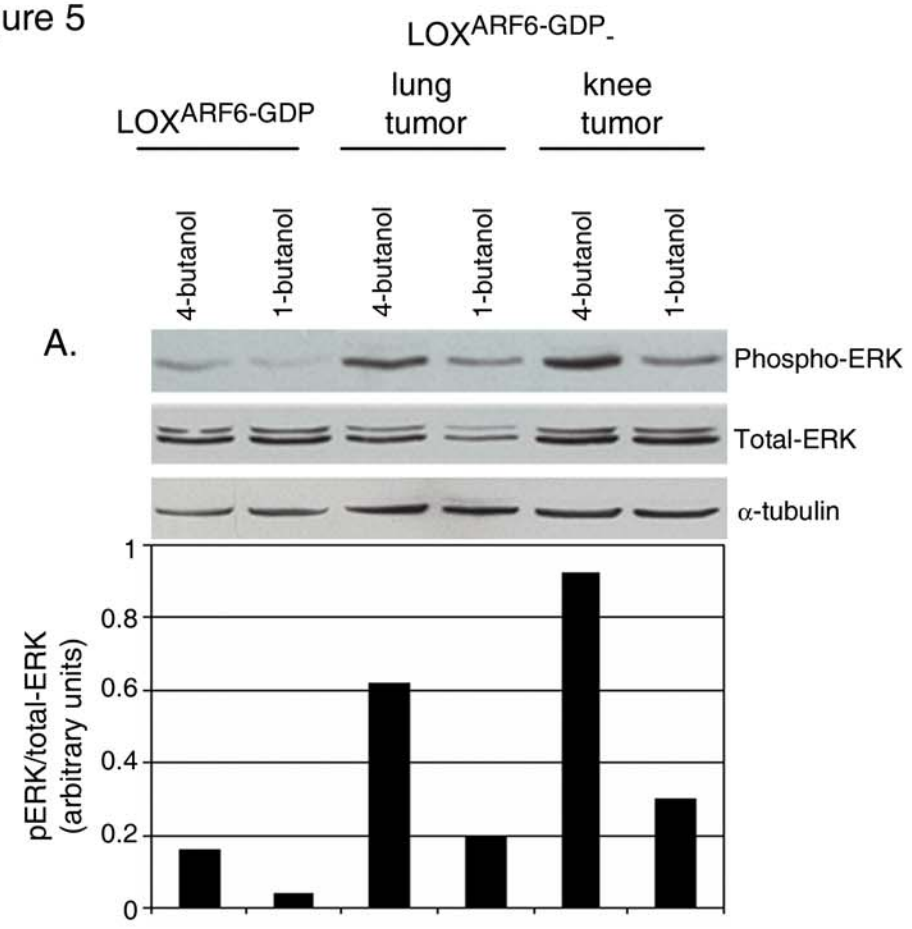
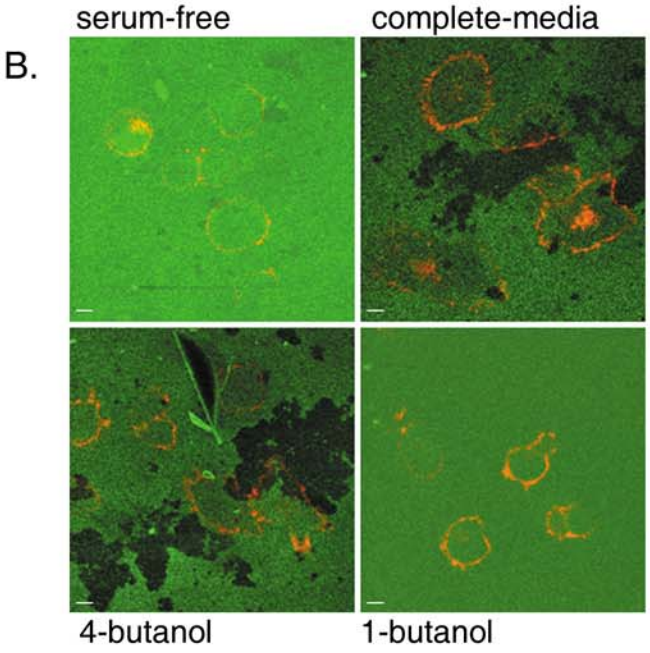
Supplementary Figure 5





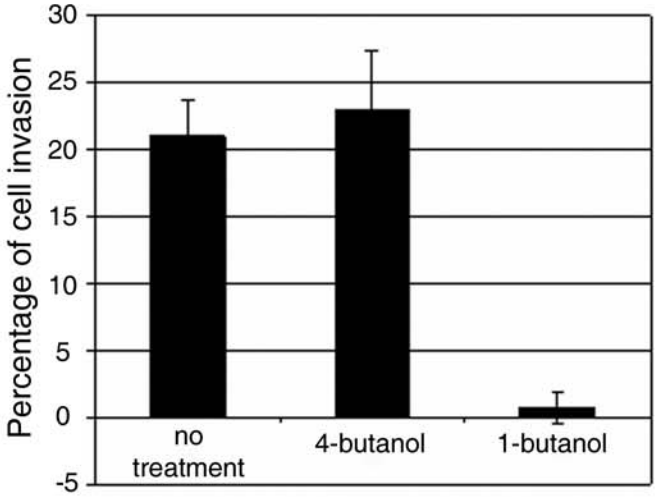


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Table I. Percentage of LOX cells in each stage of the cell cycle

LOX^{GFP}, $LOX^{ARF6\text{-}GDP}$, $LOX^{ARF6\text{-}GTP}$ cells isolated from the primary tumors were analyzed by flow cytometry using Cytomics FC-500 flow cytometer. The percentage of cells undergoing apoptosis and in each phase of the cell cycle was determined according to an internal protocol. Representaive data is shown.

	Cells in stages of the cell cycle (%)			
Cell line	Apoptosis	Go/G1	S	G2/M
LOX^{GFP} Prim. Tum.	4.3	60.6	18	17.2
$LOX^{ARF6\text{-}GDP}$ Prim. Tum.	2.7	59.4	13.3	25.6
$LOX^{ARF6\text{-}GTP}$ Prim. Tum.	3.7	65.6	3.3	28.3

SUPPLEMENTAL METHODS

Generation of LOX^{GFP}, LOX$^{ARF6\text{-}GDP}$, or LOX$^{ARF6\text{-}GTP}$ cell lines. Stable GFP-LOX cells were generated by transfection with pEGFP (Clontech) and selection with 800 μg/ml G418. Stable ARF6(T27N)-HA and ARF6(Q67L)-HA LOX cell lines were generated by sequential transfection with Metafectene Reagent (Biontex) first with the pTet-Off (Clontech) followed by selection of individual clones with G418 (800 μg/ml) and second, with pTRE2-ARF6 mutant plasmids and selected with hygromycin (500 μg/ml). One representative ARF6(T27N)-HA and ARF6(Q67L)-HA clonal population was characterized and used in the experiments. Stable cell lines expressing the ARF6 mutants were cultured under the same conditions as GFP-LOX except that tetracyclin-free FBS was used. After weeks of selection, cells were maintained in 200μg/ml hygromycin and/or 200 μg/ml G418. Integration of the plasmids was confirmed by sequencing the transgene from genomic DNA.

Generation of pTRE2-ARF6(Q67L)-HA and pTRE2-ARF6(T27N)-HA plasmids. pTRE2-ARF6(Q67L)-HA was generated from by PCR amplification from pcDNA3.1(-) ARF6(Q67L)-HA (9) with Sal I ends and inserted into the pTRE2 hygro vector (Clontech) at the Sal I site. The following primers were used for PCR amplification: [Fw: ACGCGTCGACATGGGGAAGGTGC], [Rev: ACGCGTCGACTTAGAGGCTAGCG]. ARF6(T27N)-HA was cut with Xba I from pcDNA3.1(-) and inserted into the pTRE hygro at the Nhe I site to create the pTRE2-ARF6(T27N)-HA plasmid.