SUPPLEMENTARY DATA LEGENDS

Supplementary Figure 1. Summary of experimental design. **(*A*)** Schematic of experimental protocol for single-cell inoculation into immunodeficient NCR nu/nu mice after FACS. **(*B*)** Representative picture of a single phase-bright cell after purification. **(*C*)** Demonstration of a single cell within microdroplet after cell retrieval.

Supplementary Figure 2. Evaluation of mouse melanomas. **(*A*)** Left panel, Pten wild-type gene band (left lane) from non-treated mouse toe tissue and biallelic gene recombination induced by 4-OHT treatment in tumor tissue from the same mouse (right lane). Middle panel, Cdkn2a wild-type gene (left lane) and band shift from the floxed gene in tumor (right lane). Right panel, Beta-catenin monoallelic gene recombination in melanoma 1445 and wild-type gene PCR product in melanomas 1118, 1111 and in normal melanocyte DNA. **(*B*)** Immunofluorescent staining for melanoma with melanoma markers Tyrp1 and S100 demonstrate intracellular positivity as represented here for culture 1445. Equivalent results were obtained with cultures 1118 and 1111. **(*C*)** Electron microscopy revealed perinuclear retention of melanosomes in a subset of neoplastic cells. Scale bars represent magnification level. Images are not of the same cell.

Supplementary Figure 3. Flow cytometry of mouse melanoma cells using a variety of cell surface markers and post-sorting validation of CD34 and p75 cell sorts. **(*A*)** CD44 staining revealed near 100% positivity in all three melanoma cultures analyzed, while CD117, CD20, CD202, and SSEA-1 surface proteins were absent. Epcam represented a

minor population in culture 1445, but not 1118 or 1111. **(*B*)** Flow cytometry for stem cell marker Prom1/CD133 showed rare positive populations in cultures 1445, 1118 and 1111. Red line = negative control. Green line = Prom1 signal. **(*C*)** *Left panels,* Prom1 post-sort validations demonstrated >98% purity in both $Prom1^+$ and $Prom1^-$ fractions. Panels shown are for culture 1445. *Right panel,* Single cell colony formation assays by FACS were variable among $Prom1^+$ versus $Prom1^-$ purified subsets (mean ± s.e.m., n = 9 for 1445; n = 5 for 1118 and 1111; *P < 0.01; **P < 0.001; NS = not significant; student's t-test). **(*D*)** Post-sort validations for $CD34^+$, $CD34^-p75^-$ and $p75^+$ subsets in culture 1118. Validations for cultures 1445 and 1111 were equivalent, demonstrating >99% purity in each sorted fraction. **(*E*)** Validation of $p75^+$ subset viability following cell sorting. *Top panels,* After sorting of the $p75^+$ subset, no significant increase in Annexin-V positivity is evident relative to the pooled $CD34^+$ and $CD34^-p75^-$ post-sort populations. *Bottom left panel,* Propidium iodide (PI) staining revealed a small increase in cell death in the $p75^+$ subset post-FACS; however, this increase was comparable to other cell fractions indicating that the FACS protocol did not significantly alter the viability of cellular subsets. Panels shown are from culture 1445. Other cultures showed equivalent results. *Bottom right panel,* Sorts without p75 antibody ($CD34^+$ versus $CD34^-$), revealed similar colony forming rates in the three short-term melanoma cultures. These findings suggest that incubation with anti-p75 antibody does not account for the relative lack of $p75^+$ colony formation. $CD34^-$ cells formed a low rate of colonies most likely because of a high probability for a $p75^+$ cell being sorted (**P<0.001, n=2 for each culture). **(*F*)** Cell-cycle analysis on purified $CD34^+$, $CD34^-p75^-$, and $p75^+$ cell subsets demonstrated that similar proportions of cells were in G1, S, and G2/M phases of the cell cycle, suggesting

that marker expression was stable throughout the cell cycle. Panels shown are for culture 1118. Other cultures were similar in their cell cycle signature.

Supplementary Figure 4. Confocal immunfluorescent analysis of marker staining in tumor cultures and original tumor sections. **(*A*)** Cultures 1445, 1118 and 1111 in vitro immunostaining: *top row*, CD44 (green) and DAPI nuclear stain (blue); *middle row*, dual-staining with CD34 (red) and p75 (green) and DAPI nuclear stain (blue); *bottom row*, secondary antibodies-only with DAPI counterstain for negative control. **(*B*) T**issue immunofluorescence on original tumor sections: *top row*, CD34 (red) and p75 (green) with DAPI counterstain (blue); *bottom row*, secondary antibodies-only with DAPI counterstain for negative control.

Supplementary Figure 5. Tumor formation from single-cell inoculations and validation experiments. **(*A*)** *Left panel,* Beginning tumor growths from a representative mouse injected at 2 flank sites with 1 $CD34^{+}p75^{-}$ cell each from culture 1118. Photograph was taken around 3 weeks. *Right panel*, Same mouse at around 5 weeks, demonstrating a dramatic increase in tumor volume. **(*B*)** PCR validation of representative grafted tumors 3297 and 3474 formed from single uncultured melanoma cells. Homologous Pten recombination confirms that a neoplastic cell was injected and not a non-neoplastic contaminating cell. **(*C*)** Hematoxylin and eosin-stained tumor sections reveal that the parental tumor (left) more closely resembles $CD34^{-}p75^{-}$ cell–derived tumors (middle), which also are focally edematous and have a heterogenous appearance. $CD34^{+}$ cell-derived tumors (right) could be distinguished histologically from $CD34^{-}p75^{-}$ cell–

derived tumors by their uniform epithelioid/rhabdoid cytology and increased pleomorphism, which resembled focal areas in the parental tumor as well.

Supplementary Table 1. **(*A*)** Summary chart of tumors grown from purified single cells per total sites injected. $CD34^+$ cells exhibited full tumor penetrance, while $CD34^-p75^-$ cells displayed intermediate capacity for tumor propagation. Cells from the $p75^+$ subset from cultures 1445 and 1111 did not form tumors; however a minority of single $p75^+$ cells from 1118 could grow tumors. **(*B*)** Summary chart of single-cell injections from uncultured melanomas. 3/3, 1/3 and 0/3 tumors formed from individual $CD34^+$, $CD34^-p75^-$ and $p75^+$ cells, respectively, from each freshly-dissociated tumor in keeping with tumor-propagating rates of short-term melanoma cultures. Additionally, rates of tumor volume growth were highest in $CD34^+$ cell-derived tumors, followed by $CD34^-p75^-$ cell-derived tumors.

Supplementary Table 2. Comprehensive summary of flow cytometry performed on clonal subsets for tumor cultures 1445, 1118 and 1111. Grayed rows indicate clones that displayed heterogeneity after cell culture passaging. $CD34^+$ clones were essentially self-renewing while $CD34^-p75^-$ clones generated heterogeneous progeny to varying degrees. Rare $p75^+$ clones were self-renewing in melanomas 1445 and 1111, however, clones from culture 1118 $p75^+$ cells generated substantial $p75^+$ and $CD34^-p75^-$progeny.