Oncogenic Nras has bimodal effects on stem cells that sustainably increase competitiveness

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‘Pre-leukaemic’ mutations are thought to promote clonal expansion of haematopoietic stem cells (HSCs) by increasing self-renewal and competitiveness; however, mutations that increase HSC proliferation tend to reduce competitiveness and self-renewal potential, raising the question of how a mutant HSC can sustainably outcompete wild-type HSCs. Activating mutations in NRAS are prevalent in human myeloproliferative neoplasms and leukaemia1. Here we show that a single allele of oncogenic NrasG12D increases HSC proliferation but also increases reconstituting and self-renewal potential upon serial transplantation in irradiated mice, all prior to leukaemia initiation. NrasG12D also confers long-term self-renewal potential to multipotent progenitors. To explore the mechanism by which NrasG12D promotes HSC proliferation and self-renewal, we assessed cell-cycle kinetics using H2B–GFP label retention and 5-bromo-deoxyuridine (BrdU) incorporation. NrasG12D had a bimodal effect on HSCs, increasing the frequency with which some HSCs divide and reducing the frequency with which others divide. This mirrored bimodal effects on reconstituting potential, as rarely dividing NrasG12D HSCs outcompeted wild-type HSCs, whereas frequently dividing NrasG12D HSCs did not. NrasG12D caused these effects by promoting STAT5 signalling, inducing different transcriptional responses in different subsets of HSCs. One signal can therefore increase HSC proliferation, competitiveness and self-renewal through bimodal effects on HSC gene expression, cycling and reconstituting potential.

To gain a durable competitive advantage, mutant HSCs must sustainably self-renew more frequently than wild-type HSCs. Yet increased HSC division is almost always associated with reduced self-renewal potential and HSC depletion1–3. Many oncogenic mutations increase HSC proliferation but deplete HSCs, preventing clonal expansion4. Some oncogenic mutations do increase HSC self-renewal, including overexpression of Ezh2 (ref. 7) or Csf3r truncation5, and deletion of p18INK4C (ref. 9), Tcf2 (ref. 10), Dnmt3a6 or Lnk2,3,4. However, it remains uncertain whether these mutations can account for pre-leukaemic expansion.

Human leukaemias commonly have mutations that increase Ras signalling, including NRAS or KRAS point mutations2. Mouse models with conditional expression of oncogenic KrasG12D develop a rapid onset, aggressive myeloproliferative neoplasm (MPN)4,15. KrasG12D drives HSCs into cycle and reduces HSC frequency4,13, NrasG12D knock-in mice, on the other hand, develop an indolent MPN with delayed onset and prolonged survival14,17. NFI inactivation18 or NrasG12D expression19,20 allow bone marrow cells to outcompete wild-type cells in transplantation assays, but it remains unclear whether they promote sustained pre-leukaemic expansion, or how that might occur.

To conditionally activate a single allele of NrasG12D in HSCs we generated Mx1-cre; NrasG12D/+ mice in which the oncogenic G12D mutation was knocked into the endogenous Nras locus along with a floxed stop cassette21. To induce NrasG12D expression, mice were administered poly-insine/poly-cytosine (pIpC) at 6–10 weeks after birth (Extended Data Fig. 1). At 2 weeks and 3 months after pIpC treatment, more than twice as many NrasG12D+/CD150+/CD48+ Lineage–Sca-1+c-kit+(CD150+ CD48+ LSK) HSCs21 incorporated a 24-h pulse of BrdU as compared to control HSCs (P < 0.01; Fig. 1a). Consistent with this, twice as many NrasG12D+ HSCs were in G1 phase of the cell cycle as compared to control HSCs (Extended Data Fig. 1b). This increase in HSC proliferation did not significantly affect the number of HSCs or multipotent progenitors (MPPs) 2 weeks after NrasG12D activation (Fig. 1c). However, Mx1-cre; NrasG12D/+ mice had significantly more LSK cells in the bone marrow and spleen (Fig. 1c). We also observed a twofold increase in BrdU incorporation in HSCs, as well as an expansion of LSK cells in Vav1-cre; NrasG12D/+ mice as compared to controls (Fig. 1b; Extended Data Fig. 2a). Thus NrasG12D increased HSC division and expanded the pool of primitive haematopoietic progenitors.

To test competitiveness we transplanted 5 × 106 whole bone marrow cells from Mx1-cre; NrasG12D/+ or control donors into irradiated wild-type recipients along with 5 × 105 recipient bone marrow cells. The NrasG12D+ cells gave significantly higher levels of reconstitution than control cells in all lineages for at least 20 weeks after transplantation (Fig. 1d). In recipients of control donor cells, 69 ± 13% of HSCs, 47 ± 12% of MPPs and 44 ± 12% of LSK cells were donor-derived (Fig. 1e); however, in recipients of NrasG12D+ donor cells, 93 ± 8% of HSCs, 90 ± 8% of MPPs, and 85 ± 15% of LSK cells were donor-derived (Fig. 1e). NrasG12D+ HSCs therefore outcompeted wild-type HSCs.

To further test whether NrasG12D+ HSCs could outcompete wild-type HSCs we transplanted 10 CD150+/CD48– LSK donor HSCs from the bone marrow of Mx1-cre; NrasG12D/+ or littermate control mice (2 weeks after finishing pIpC) into irradiated wild-type recipients along with 3 × 104 recipient bone marrow cells. The NrasG12D+ HSCs gave significantly higher levels of reconstitution compared to control donor HSCs in all lineages for at least 20 weeks after transplantation (Fig. 1f).

To assess self-renewal potential we serially transplanted 3 × 106 whole bone marrow cells from three or four recipients per treatment into 2 to 5 irradiated mice per recipient (depending on the number of bone marrow cells we recovered) during each round of transplantation. In secondary, tertiary and quaternary recipient mice we continued to observe significantly higher levels of reconstitution from the NrasG12D+ donor cells than from control donor cells in all lineages (Fig. 2a–c). In tertiary recipient mice, the control cells gave only transient multilineage reconstitution as they appeared to exhaust their self-renewal potential. In contrast, the NrasG12D+ HSCs gave high levels of long-term multilineage reconstitution in all 9 tertiary recipients, suggesting increased self-renewal potential. In quaternary recipient mice, NrasG12D+ donor cells continued to give long-term multilineage reconstitution in most recipients whereas control donor cells gave only low levels of transient lymphoid reconstitution (Fig. 2c). NrasG12D+ thus increased the self-renewal potential of HSCs in addition to increasing their rate of division (Fig. 1a) and their ability to compete with wild-type HSCs (Fig. 1d, f).

A fifth round of transplantation from four quaternary recipients of NrasG12D+ cells did not yield any multilineage reconstitution by donor...
cells. Donor cell reconstitution in the myeloid (Mac-1) recipient mice (compared to recipients of control bone marrow cells). Treatment of 3 × 10^6 bone marrow cells from secondary recipient mice in Fig. 2a (n = 3 donors per genotype) into tertiary recipient mice (n = 8 recipients for control and 9 recipients for Nras^-G12D/+), c. Transplantation of 3 × 10^6 bone marrow cells from tertiary recipient mice (n = 3 donors for control and 4 donors for Nras^-G12D/+), d. Each serial transplant was performed at 20 weeks after the prior round of transplantation. Data represent mean ± s.d. Two-tailed Student’s t-tests were used to assess statistical significance. *P < 0.05, **P < 0.01, ***P < 0.001.

3 × 10^6 recipient bone marrow cells. Only one of 14 recipients of control MPPs exhibited long-term multilineage reconstitution by donor cells (Extended Data Fig. 4a). In contrast, 8 of 17 recipients of Nras^-G12D/+ MPPs were long-term multilineage reconstituted by donor cells. Nras^-G12D/+ MPPs were thus significantly (P < 0.01 across three independent experiments) more likely to give long-term multilineage reconstitution than control MPPs.

Nras^-G12D/+ did not detectably affect the reconstituting potential of 25 CD150^-/CD48^- LSK cells or 100 CD150^-/CD48^- LSK cells (which contain restricted myeloid progenitors) upon transplantation into irradiated mice (Extended Data Fig. 4b, c). Nras^-G12D/+ thus increases the self-renewal potentials of HSCs and MPPs but not necessarily other progenitors.

We did not detect any evidence of leukaemia or MPN in any of the recipient mice from the first, second, third or fourth rounds of serial transplantation in terms of blood cell counts (Extended Data Fig. 5) or histology (data not shown). Only two recipients of Nras^-G12D/+ cells and two recipients of control cells died spontaneously in these experiments. The effects of Nras^-G12D/+ on HSC function therefore occurred in the absence of leukaemogenesis.

To assess the effect of Nras^-G12D/+ on HSC cycling over time we mated the Mx1-cre, Nras^-G12D/+ mice with Col1A1-H2B–GFP; Rosa26-M2-rtTA double transgenic mice. These mice allowed us to label HSCs with H2B–GFP during a 6-week period of doxycycline administration and then to follow the division history of all cells in the HSC pool with H2B–GFP fluorescence (Fig. 3a). After treating the mice with doxycycline for 6 weeks, all HSCs were strongly GFP labelled.
The median level of GFP fluorescence in H2B–GFP− HSCs was significantly lower in NrasG12D+/− as compared to control mice (Extended Data Fig. 6b), suggesting that H2B–GFP− NrasG12D+/− HSCs underwent more rounds of division on average. In contrast, the median level of GFP fluorescence in H2B–GFP+ HSCs was significantly higher in NrasG12D+/− as compared to control mice (Extended Data Fig. 6b), suggesting that H2B–GFP+ NrasG12D+/− HSCs tended to divide less than control H2B–GFP+ HSCs on average. NrasG12D+/− thus had a bimodal effect, increasing the division of some HSCs and reducing the division of other HSCs.

We followed another cohort of age and sex-matched pairs of Mx1-cre; NrasG12D+/− and control mice for 15 weeks after doxycycline removal. In five independent experiments, NrasG12D+/− significantly increased the frequency of H2B–GFP+ HSCs in every pair of mice we examined (n = 7; P < 0.05) (Fig. 3c). We observed increased frequencies of H2B–GFP− HSCs in the NrasG12D+/− mice from some pairs but not others, and overall the effect was not statistically significant (Fig. 3c). As the rapidly dividing subset of NrasG12D+/− HSCs differentiates more quickly than control HSCs (Fig. 3d), prolonged periods of chase after H2B–GFP labelling may not be appropriate to quantify the frequency of these cells. NrasG12D+/− significantly increased the rate at which MPPs divided (Extended Data Fig. 6c).

To test the relationship between division history and competitiveness we transplanted 15 CD150+− CD48− LSK H2B–GFP+ HSCs, 50 H2B–GFP− moderately cycling HSCs, or 75 H2B–GFP− frequently cycling HSCs from NrasG12D+/− or control donor mice after 12 weeks of chase into irradiated wild-type recipients along with 3 × 10^6 recipient bone marrow cells. The NrasG12D+/− H2B–GFP− frequently cycling HSCs gave significantly lower levels of donor cell reconstitution, at least in the myeloid lineages, as compared to control H2B–GFP− HSCs (Fig. 3d). In contrast, the NrasG12D+/− H2B–GFP− HSCs gave significantly (P < 0.05) higher levels of donor cell reconstitution in all lineages than the control H2B–GFP+ and H2B–GFP− HSCs (Fig. 3d). NrasG12D+/− thus reduced the division and increased the competitiveness of some HSCs while increasing the division and reducing the competitiveness of other HSCs.

We continuously administered BrdU to Mx1-cre; NrasG12D+/− versus control mice beginning 2 weeks after pIpC treatment. We assessed the frequency of BrdU+ HSCs after 4, 10, 20 and 30 days of BrdU treatment (Extended Data Fig. 6d). Relative to control HSCs, significantly more Mx1-cre; NrasG12D+/− HSCs incorporated BrdU after 4 days (32 ± 0.1% versus 24 ± 1.2%, P < 0.01) and 10 days (64 ± 5.9% versus 45 ± 3.6%, P < 0.02) of BrdU administration. In contrast, significantly fewer Mx1-cre; NrasG12D+/− HSCs incorporated BrdU after 20 days (78 ± 4.7% versus 86 ± 0.7%, P < 0.05) and 30 days (86 ± 3.4% versus 92 ± 4.8%, P < 0.02) of BrdU administration. These data are consistent with the H2B–GFP label retention data in demonstrating that some NrasG12D+/− HSCs divide more frequently while other NrasG12D+/− HSCs divide less frequently than control HSCs.

We detected the activation of the canonical Ras effector, ERK, in bone marrow cells from Mx1-cre; NrasG12D+/− and control mice with the MEK inhibitors, PD0325901 (5 mg per kg per day) or AZD6244 (25 mg per kg per day), and assessed the effects on BrdU incorporation in CD150+− CD48− LSK H2B–GFP+ HSCs. After eight days of treatment, splenocytes from PD0325901–treated mice of both genotypes showed reduced pERK levels (Extended Data Fig. 8a), but this did not affect the increased rate of BrdU incorporation by NrasG12D+/− HSCs (Extended Data Fig. 8c). In contrast, when we performed the same experiments with AZD6244, pERK activation was completely blocked in bone marrow and spleen (Extended Data Fig. 8d) and the increased cycling of NrasG12D+/− HSCs was abolished (Extended Data Fig. 8e). These data suggest that the more stringent inhibition of pERK activation by AZD6244 blocks the effect of NrasG12D+/− on HSC cycling.
We did not detect increased Akt (Extended Data Fig. 8f), S6, or p38 phosphorylation (Extended Data Fig. 8e) as compared to control cells by microarray and quantitative real-time PCR. Only two genes showed a similar change in expression with NrasG12D/+ in HSCs as compared to H2B–GFPhi mice. We performed gene expression profiling of H2B–GFP HSCs from 3 pairs of Mx1-cre; NrasG12D/+ mice, Mx1-cre; NrasG12D/+; Stat5ab–/+, and Mx1-cre; NrasG12D/+; Stat5ab–/+, Stat5abfl/+ control mice, and littermate controls after finishing pIpC treatment, the western blot of flow cytometrically detected phosphorylated STAT5, and BrdU incorporation by the myeloid progenitors we examined (Extended Data Fig. 8i).

We next transplanted 5 × 10^6 whole bone marrow cells from control, Mx1-cre; NrasG12D/+, Mx1-cre; Stat5abf/-, or Mx1-cre; NrasG12D/+ (double mutant) donors (2 weeks after finishing plpC treatment) into irradiated wild-type recipients along with 5 × 10^6 recipient bone marrow cells. NrasG12D/+ cells gave significantly higher levels of reconstitution than control cells in all lineages for at least 20 weeks after transplantation (Fig. 4d). Loss of one Stat5ab allele, reduced the level of reconstitution by donor cells relative to control cells but the difference was not statistically significant. In contrast, loss of a single allele of Stat5ab in the NrasG12D/+ background completely blocked the increased reconstitution by NrasG12D/+ cells such that levels of donor cell reconstitution were indistinguishable from control cells (Fig. 4d). An increase in STAT5 signalling is therefore required for increased competitiveness by NrasG12D HSCs.

NrasG12D is probably an early mutation in some leukaemias as it is widely observed in both MPN and myeloid leukaemias, and NrasG12D mutations in mice lead only to a late onset MPN with prolonged survival.16,17. Nras and Kras mutations are frequently among the first mutations observed in pre-leukaemic clones that precede chronic myelomonocytic leukaemia (CMMI).26. Some juvenile myelomonocytic leukaemia (JMML) patients undergo remission, with or without therapy, yet continue to carry Nras mutations in their haematopoietic cells.17,28. Germline Nras mutations have also been reported in JMML patients,29, or patients with Noonan syndrome that develop JMML.30. The evidence that Nras mutations can be found in normal haematopoietic cells, despite predisposing for the development of neoplasms, is consistent with our conclusion that they promote pre-leukaemic clonal expansion.

Our data provide a molecular explanation for how pre-leukaemic clonal expansion may occur. NrasG12D has a bimodal effect on HSCs, increasing self-renewal potential and reducing division in one subset of HSCs while increasing division and reducing self-renewal in another subset of HSCs. Short-lived but rapidly dividing NrasG12D HSCs presumably outcompete wild-type HSCs and are replenished over time by quiescent NrasG12D HSCs that are slowly recruited into cycle. It will be interesting to determine whether the ability to induce bimodal responses in stem cell pools is a common feature of mutations that promote pre-malignant clonal expansion.

**METHODS SUMMARY**

**Mice.** All mice were housed in the Unit for Laboratory Animal Medicine at the University of Michigan and protocols were approved by the University of Michigan Committee on the Use and Care of Animals. NrasG12D/+ mice (ref. 20), Stat5abf/- (ref. 25), Col1A1-H2B-GFP; Rosa26-M2-rtTA, Vav1-cre, and Mx1-cre mice were backcrossed for at least 10 generations onto a C57BL/6j-Cd45.2-Thy1.1 background. Recipients in reconstitution assays were adult C57BL/6j-Cd45.1-Thy1-12 mice, at least 8-week-old at the time of irradiation. plpC (Amersham) was reconstituted in PBS and administered at 0.5 μg per gram body mass per day by intraperitoneal injection. BrdU (Sigma) was administered as a single dose of 200 mg per kg body mass by intraperitoneal injection followed by 1 mg ml⁻¹ BrdU in the drinking water. For long term BrdU administration, BrdU water was changed every 3 days. Doxycycline (Research Products International) was added to the water at a concentration of 0.2% (m/v) along with 1% sucrose (Fisher). Both male and female mice were used in experiments and no randomization or blinding was performed. For all experiments, either littermates or age- and gender-matched mutants and control mice at 6-10-weeks of age were used.

**Flow cytometry and HSC isolation.** Bone marrow cells were harvested and CD190CD48 Lin Sca1 c-kit+ HSCs and CD150 CD48 Lin Sca1 c-kit- MPPs sorted CD48− LSK cells confirmed that deletion of one allele of Stat5ab in NrasG12D+ HSCs reduced the levels of pSTAT5 and total STAT5 (Extended Data Fig. 8g). Stat5ab+/− HSCs showed normal BrdU incorporation and NrasG12D+ HSCs showed increased proliferation relative to control HSCs (Fig. 4c). Deletion of one allele of Stat5ab in NrasG12D+ HSCs significantly reduced the rate of BrdU incorporation (P < 0.05). A reduction in STAT5 levels thus rescued the effects of NrasG12D on HSC cycling. Neither deletion of Stat5ab nor activation of NrasG12D+ significantly affected BrdU incorporation by the myeloid progenitors we examined (Extended Data Fig. 8j).

Figure 4 | Increased STAT5 activation mediates the effect of NrasG12D+ on HSCs. a, b Western blots for pSTAT3, pp38, and β-actin (a) and pSTAT5, total STAT5 and β-actin (b) Two additional experiments are shown in Extended Data Fig. 8j. Cells were stimulated in culture with stem cell factor and thrombopoietin for 30 min before protein extraction. The frequency of BrdU− CD150− CD48− LSK HSCs after a 24-h pulse of BrdU to Mx1-cre; Col1A1-H2B-GFP; Rosa26-M2-rtTA mice and littermate controls

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were isolated as previously described. BrdU incorporation in vivo was measured by flow cytometry using the APC BrdU Flow Kit (BD Biosciences). To perform pyronin Y and DAPI staining, CD150⁺CD48⁻LSK HSCs were sorted into 100% ethanol and placed in the cold room overnight. The cells were then washed with PBS and stained with pyronin Y (1 μg ml⁻¹) and DAPI (10 μg ml⁻¹) for 30 min before flow cytometric analysis.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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1. Rossi, D. J., Jamieson, C. H. & Weissman, I. L. Stems cells and the pathways to aging and cancer. Cell 132, 681–696 (2008).
2. Ward, A. F., Braun, B. S. & Shannon, K. M. Targeting oncogenic Ras signaling in hematologic malignancies. Blood 120, 3397–3406 (2012).
3. Essers, M. A. et al. IFNα activates dormant haematopoietic stem cells in vivo. Nature 458, 904–908 (2009).
4. Foudi, A. et al. Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. Nature Biotechnol. 27, 84–90 (2009).
5. Wilson, A. M., et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell 135, 1118–1129 (2008).
6. Ross, L. et al. Less is more: unveiling the functional core of hematopoietic stem cells through knockout mice. Cell Stem Cell 11, 302–317 (2012).
7. Kammenga, L. M. et al. The Polycomb group gene Ezh2 prevents hematopoietic stem cell exhaustion. Blood 107, 2170–2179 (2006).
8. Liu, F. et al. Csf3r mutations in mice confer a strong clonal HSC advantage via activation of Stat5. J. Clin. Invest. 118, 946–955 (2006).
9. Yuan, Y., Shen, H., Franklin, D. S., Scadden, D. T. & Cheng, T. In vivo self-renewing divisions of hematopoietic stem cells are increased in the absence of the early G1-phase inhibitor, p18Ink4c. Nature Cell Biol. 6, 436–442 (2004).
10. Moran-Crusio, K. et al. Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. Cancer Cell 20, 11–24 (2011).
11. Challen, G. a. et al. Dnmt3a is essential for hematopoietic stem cell differentiation. Nature Genet. 44, 23–31 (2012).
12. Takizawa, H. et al. Enhanced engraftment of hematopoietic stem/progenitor cells by the transient inhibition of an adaptor protein, Lnk. Blood 107, 2968–2975 (2006).
13. Buza-Vidas, N. et al. Cytokines regulate postnatal hematopoietic stem cell expansion: opposing roles of thrombopoietin and LNK. Genes Dev. 20, 2018–2023 (2006).
14. Braun, B. S. et al. Somatic activation of oncogenic Kras in hematopoietic cells initiates a rapidly fatal myeloproliferative disorder. Proc. Natl Acad. Sci. USA 101, 597–602 (2004).
15. Sabnis, A. J. et al. Oncogenic Kras initiates leukemia in hematopoietic stem cells. PLoS Biol. 7, e59 (2009).
16. Li, Q. et al. Hematopoiesis and leukemogenesis in mice expressing oncogenic NrasV12 (2012) from the endogenous locus. Blood 117, 2022–2032 (2011).
17. Wang, J. et al. Endogenous oncogenic Nras mutation promotes aberrant GM-CSF signaling in granulocytic/monocytic precursors in a murine model of chronic myelomonocytic leukemia. Blood 116, 5991–6002 (2010).
18. Zhang, Y., Taylor, B. R., Shannon, K. & Clapp, D. W. Quantitative effects of Nf1 inactivation on in vivo hematopoiesis. J. Clin. Invest. 108, 709–715 (2001).
19. Wang, J. et al. NrasV12 (2012) promotes leukemogenesis by aberrantly regulating hematopoietic stem cell functions. Blood 121, 5203–5207 (2013).
20. Haigis, K. M. et al. Differential effects of oncogenic K-Ras and N-Ras on proliferation, differentiation and tumor progression in the colon. Nature Genet. 40, 600–608 (2008).
21. Kiel, M. J., Yilmaz, O. H., Washita, T., Tserhorst, C. & Morrison, S. J. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 121, 1109–1121 (2005).
22. Oguro, H., Ding, L. & Morrison, S. J. SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. Cell Stem Cell 13, 102–116 (2013).
23. Krebs, D. L. & Hiltan, D. J. SOCS proteins: negative regulators of cytokine signaling. Stem Cells 19, 378–387 (2001).
24. Li, L. X., Goetz, C. A., Katerndahl, C. D., Sakuguchi, N. & Farrar, M. A. A. Flt3- and Ras-dependent pathway primes B cell development by inducing a state of IL-7 responsiveness. J. Immunol. 184, 1728–1736 (2010).
25. Cui, Y. et al. Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. Mol. Cell Biol. 24, 8037–8047 (2004).
26. Itzykson, R. et al. Clonal architecture of chronic myelomonocytic leukemias. Blood 121, 2186–2198 (2013).
27. Kotecha, N. et al. Single-cell profiling identifies aberrant STAT5 activation in myeloid malignancies with specific clinical and biologic correlates. Cancer Cell 14, 335–343 (2008).
28. Matsuda, K. et al. Spontaneous improvement of hematologic abnormalities in patients having juvenile myelomonocytic leukemia with specific RAS mutations. Blood 109, 5477–5480 (2007).
29. De Filippi, P. et al. Germ-line mutation of the NRAS gene may be responsible for the development of juvenile myelomonocytic leukemia. Br. J. Haematol. 147, 706–709 (2009).
30. Kraoua, L. et al. Constitutional NRAS mutations are rare among patients with Noonan syndrome or juvenile myelomonocytic leukemia. Am. J. Med. Genet. A 158A, 2407–2411 (2012).

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Author Contributions Q.L. performed most of the experiments. N.B., T.W. and V.N. performed some of the experiments with help from Q.L. J.M. performed the western blot analysis of Pten mutant cells. S.C. performed statistical analysis of microarrays. Q.L., K.S., and S.J.M. conceived the project, designed experiments, interpreted results and wrote the manuscript.

Author Information Gene expression data have been deposited to the Gene Expression Omnibus with accession code number GSE45194. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to Q.L. (Iqing@umich.edu) or S.M. (Sean.Morrison@UTSouthwestern.edu).
METHODS

Mice. All mice were housed in the Unit for Laboratory Animal Medicine at the University of Michigan and protocols were approved by the University of Michigan Committee on the Use and Care of Animals. Nrg1G222+/- (ref. 20), Stat5ab+/+ (ref. 25), Col1A1-H2B-GFP; Rosa26-M2-RTA (ref. 4), Vav1-cre and Mx1-cre mice were backcrossed for at least 10 generations onto a C57BL/Ka-CD45.2-Thy1-1 background. Recipients in reconstitution assays were adult C57BL/Ka-CD45.1-Thy1-2 mice, at least 8-weeks-old at the time of irradiation. plpC (Amerham) was reconstituted in PBS and administered at 0.5 μg per g of body mass per day by intraperitoneal (i.p.) injection. The 5-bromodeoxyuridine (Brdu, Sigma) was administered as a single dose of 200 μg per kg of body mass by i.p. injection followed by 1 mg/ml −1 BrdU in the drinking water. For long term BrdU administration, BrdU water was changed every 3 days. Doxycycline (Research Products International) was added to the water at a concentration of 0.2% (m/v) along with 1% sucrose (Fisher).

Statistical methods. Multiple independent experiments were performed to verify the reproducibility of all experimental findings. Group data always represents mean ± standard deviation. Unless otherwise indicated, two-tailed Student’s t-tests were used to assess statistical significance. Randomization or blinding was used in any experiments. Experimental mice were not excluded in any experiments. In the case of measurements in which variation among experiments tends to be lower (for example, HSC frequency) we generally examined between 3 to 6 mice. In the case of measurements in which variation among experiments tends to be higher (for example, reconstitution assays) we examined larger numbers of mice (7–20).

PCR of genomic DNA for genotyping. To assess the degree of Nrg1G222Δ recombination in Hscs from Mx1-cre; Nrg1G222Δ mice after plpC treatment, bone marrow cells were harvested and stained for surface markers as described above. Single Hscs (CD150+ CD48− LSK cells) were sorted into 96-well plates containing methylcellulose medium (M3434, Stem Cell Technologies) and incubated for 14 days at 37 °C. Cells from each colony were resuspended in PBS then incubated with alkaline lysis buffer (25 mM NaOH, 0.2 mM EDTA), boiled, then neutralized by addition of an equal volume of neutralizing buffer (40 mM Tris–HCl). The neutralized extract was used for PCR with the following primers: F2, 5'-AGACGG GGAAGCTTGGCCAG-3'; R1, 5'-GCTGGATCGTCAAGGCGCTTTTCC-3'. To genotype mouse tail DNA for the presence of the Nrg1G222Δ allele, primers R1 and F2 were used in addition to primer SD5', 5'-AGCTAGCCACCATGTCGTTGAGTAAGTCTGCA-3'. To genotype for the presence of the Mx1-cre transgene, primers F1 and R1 were used: F1' 5'-ATTGTCGTCTCAAGTGGTGTCG-3'; R1, 5'-GAAAATGCTTGGCTGTCGGTG-3'. To check the presence of the Rosa26-M2-RTA transgene, the following primers were used: 5'-AAAGTGCGCTTGACGTTGTTAT-3'; 5'-GGAAAGGAGGTTGTCGAC-3'; and 5'-GGAAGGGGA AAAAACTATG-3'. To genotype mice for the presence of the Col1A1-H2B-GFP transgene, the following primers were used: 5'-CTGAGATTCTTCATGCAC-3'; 5'-GAATGTTGCACACTGAGTGC-3'; and 5'-AATGTATCTCGGATGATGGA-3'.

Flow cytometry and HSC isolation. Bone marrow cells were flushed from the long bones (tibiae and femurs) with Hank’s buffered salt solution without calcium or magnesium, supplemented with 2% heat-inactivated calf serum (HBSS, Invitrogen). Cells were triturated and filtered through a nylon screen (70 μm) or magnesium, supplemented with 2% heat-inactivated calf serum (HBSS; Invitrogen). EDTA (0.06%) was added to the cells, and the cells were gently passed through a 25-gauge needle. The cell suspension was then incubated with 1 mg/ml −1 BrdU in the drinking water. For long term BrdU administration, BrdU water was changed every 3 days. Doxycycline (Research Products International) was added to the water at a concentration of 0.2% (m/v) along with 1% sucrose (Fisher).

Western blotting. The same number of cells (30,000 cells for CD48− LSK cells or CD48+ LSK cells; 100,000 cells for LSK or Lineage− ckit− Sca1− cells) from each population to be analysed were sorted into HBSS with 2% FCS. The cells were then washed and incubated with 100 μg/ml −1 thrombopoietin and 100 ng/ml −1 stem cell factor at 37 °C for 30 min. The cells were then washed with PBS and precipitated with trichloroacetic acid (TCA) at a final concentration of 10% TCA. Extracts were incubated on ice for 15 min and spun down for 10 min at 16,100g at 4 °C. The supernatant was removed and the pellets were washed with acetone twice and then dried. The protein pellets were solubilized with solubilization buffer (9 M urea, 2% Triton X-100, 1% DTT) before adding lithium dodecyl sulphate loading buffer (Invitrogen). Proteins were separated on a Bis-Tris polyacrylamide gel (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). All antibodies were purchased from Cell Signaling Technology. These include anti-Stat3 (Y705), anti-ERK (137F5), anti-Stat5 (3H7), anti-pp38 (T180/Y182), anti-Mx1 (24G2), anti-Stat5ab, and anti-Tnf (D43) (2B8) conjugated to APC, and anti-Sca1 antibody (D7) conjugated to PerCP/Cy5.5 (all antibodies were purchased from BioLegend unless otherwise stated). BrdU incorporation was measured by flow cytometry using the APC BrdU Flow Kit (BD Biosciences).

Gene expression profiling. CD150+ CD48− LSK HSCs and CD150− CD48− LSK MPPs were isolated by flow cytometry. Total RNA was isolated using Trizol (Invitrogen) followed by Qiagen RNaseq microkit purification according to the manufacturer’s protocols. For microarray analysis, reverse transcription and linear amplification was performed on total RNA using the NuGen Ovation pico WTA system version 2 and then purified with the Qiaquick PCR purification kit (Qiagen). Six micrograms of amplified cDNAs were labelled with biotin using the Enco Bioin Module (NuGen) and submitted to the Microarray Core Facility of the University of Michigan Comprehensive Cancer Center for hybridization to Affymetrix Mouse Genome 430 2.0 Arrays. Statistical analyses were performed using R (ref. 31) version 2.15.2 and Bioconductor version 2.11 (ref. 32). Gene expression signals were normalized to the trimmed average of 500 using the Affymetrix MAS 5.0 algorithm. MASS signals less than 2 were set to 2 before log transformation. Probe sets with MASS absent calls for all samples were excluded. Differential expression analysis was performed by limma with estimation of fold change. Probe sets with limma P-value <0.05 and fold change >2 were considered differentially expressed. Gene Set Enrichment Analysis was used to assess pathway enrichment. Gene expression data have been deposited to the Gene Expression Omnibus with accession number GSE45194 (http://www.ncbi.nlm.nih.gov/geo/).

Quantitative RT–PCR. Total RNA was collected as described above and reverse transcription was performed with the High Capacity cDNA reverse transcription kit (Applied Biosystems). Real time PCR was performed with Absolute SYBR Green Rox mix (Thermo Scientific) using an ABI 7300 PCR machine. RNA from 100 cells was used for each reaction. Transcript levels were normalized to β-actin.

Long-term competitive repopulation assay. Adult recipient mice (CD45.1) were irradiated with an Orthovoltage X-ray source delivering approximately 300 rad min −1 in two equal doses of 540 rad, delivered at least 2 h apart. Cells were injected into the retro-orbital venous sinus of anaesthetized recipients. Beginning 4 weeks after transplantation and continuing for at least 16 weeks, blood was obtained from the tail veins of recipient mice, subjected to ammonium-chloride potassium red cell lysis, and stained with directly conjugated antibodies to CD45.2 (104), CD45.1 (A20), B220 (6B2), Mac-1 (M1/70), CD3 (KT31.1) and Gr-1 (8C5) to monitor engraftment.

BrdU incorporation by myeloid progenitors. Two and a half hours after BrdU administration, whole bone marrow cells were incubated first with anti-c-kit antibody conjugated to biotin (2B8) then with antibodies to lineage (Lin) markers including B220 (6B2), CD3 (KT31.1), CD5 (53-7-3), CD8 (53-6-7), Gr-1 (8C5), CD41 (MWR30) and Ter119 (Ter-119) that were conjugated to phycoerythrin (PE), anti-CD34 antibody (eBioscience, RAM34) conjugated to FITC, anti-CD16/ CD32 antibody (93) conjugated to PE-Cy7, streptavidin conjugated to Alexa700 (Invitrogen S21383), and anti-Scal antibody (D7) conjugated to PerCP/Cy5.5 (all antibodies were purchased from BioLegend unless otherwise stated). BrdU incorporation was measured by flow cytometry using the APC BrdU Flow Kit (BD Biosciences).

RESEARCH LETTER

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Extended Data Figure 1 | \( \text{Nras}^{G12D/+} \) increased HSC proliferation

a. The \( \text{Nras}^{G12D} \) allele was recombined in all HSCs after 3 doses (every other day) of piPc. Two weeks after the last dose of piPc was administered to \( \text{Mx1-cre; Nras}^{G12D/+} \) mice, the mice were killed and individual CD150+CD48-LSK HSCs were sorted into methylcellulose cultures in 96-well plates. The cells were cultured for 14 days then DNA was extracted from individual colonies and genotyped by PCR. The size of the recombined \( \text{Nras}^{G12D} \) allele (G12D) was 550 base pairs (bp) and the \( \text{Nras} \) allele (wild-type, WT) was 500 bp. \( \text{Nras} \) recombination was observed in 22 out of 22 HSC colonies examined. Blot is representative of three independent experiments.

b. Cell cycle analysis of HSCs by pyronin Y and DAPI staining. CD150+CD48-LSK HSCs were sorted from \( \text{Mx1-cre; Nras}^{G12D/+} \) mice and littermate controls into 100% ethanol and stained with pyronin Y and DAPI to identify cells in G0 (left lower quadrant), G1 (left upper quadrant) and S/G2/M (right upper and lower quadrants). Data represent mean ± s.d. Statistical analysis was performed with a two-way ANOVA (\( P < 0.01, n = 4 \)) followed by pairwise post hoc t-tests.
Extended Data Figure 2 | HSC competitiveness is increased in Vav1-cre; NrasG12D/+ mice. a, Frequencies of CD150⁺CD48⁻ LSK HSCs, CD150⁺CD48⁻ LSK MPPs, and LSK cells in the bone marrow (BM, top) and spleen (SP, bottom) of Vav1-cre; NrasG12D/+ (G12D/+) or littermate control mice (n = 4) at 6–10-weeks of age. b, Donor bone marrow cells (5 × 10⁵) from Vav1-cre; NrasG12D/+ (G12D/+) or littermate control mice at 6–10-weeks of age were transplanted into irradiated recipient mice along with 5 × 10⁵ recipient bone marrow cells (3 donors per genotype were each transplanted into 4 recipients per donor). c, Secondary transplantation of 3 × 10⁶ bone marrow cells from primary recipient mice in Extended Data Fig. 2b at 20 weeks after transplantation (2 primary recipients per genotype were each transplanted into 4 secondary recipients per primary recipient). Data represent mean ± s.d. Two-tailed Student’s t-tests were used to assess statistical significance. *P < 0.05, **P < 0.01, ***P < 0.001.
Extended Data Figure 3 | HSCs from Mx1-cre; Nras<sup>G12D/+</sup> mice were not immortalized. A fifth round of serial transplantation of 3 × 10<sup>6</sup> bone marrow cells from the quaternary recipients of Nras<sup>G12D/+</sup> (G12D/+) bone marrow cells shown in Fig. 2c showed that the Nras<sup>G12D/+</sup> HSCs eventually exhausted all of their HSCs and MPPs and were able to only give low levels of lymphoid reconstitution. Four donor mice from Fig. 2c were transplanted 20 weeks after the fourth round of transplantation into 4 recipients per quaternary donor. The data represent mean ± s.d. for donor blood cells in the myeloid (Gr-1<sup>+</sup> or Mac-1<sup>+</sup>) cells), B (B220<sup>+</sup>), and T (CD3<sup>+</sup>) cell lineages.
Extended Data Figure 4 | Nras\textsuperscript{G12D} (G12D/+) expression increased the reconstituting potential of CD150\textsuperscript{−}CD48\textsuperscript{−} LSK MPPs but did not affect the reconstituting potential of CD150\textsuperscript{+}CD48\textsuperscript{+} LSK, or CD150\textsuperscript{−}CD48\textsuperscript{−} LSK progenitors in irradiated mice. a–c, Ten donor MPPs (a), 25 CD150\textsuperscript{−}CD48\textsuperscript{−} LSK progenitors (b), or 100 CD150\textsuperscript{−}CD48\textsuperscript{−} LSK progenitors (c) from Mx1-cre; Nras\textsuperscript{G12D/+} (G12D/+) or littermate control mice at 2 weeks after pIpC treatment were transplanted into irradiated recipient mice along with \(3 \times 10^5\) recipient bone marrow cells. Data represent mean ± s.d. for donor blood cells in the myeloid (Gr-1\textsuperscript{+} or Mac-1\textsuperscript{+} cells), B (B220\textsuperscript{+}) and T (CD3\textsuperscript{+}) cell lineages. Two-tailed Student’s t-tests were used to assess statistical significance. None of the time points were significantly different between treatments. The data represent two independent experiments with 4 recipient mice per donor.
Extended Data Figure 5 | Nras<sup>G12D</sup>-induced changes in HSC function were not associated with the development of leukaemia. a–d, White blood counts (WBC), hemoglobin (Hb) levels, platelet counts and spleen masses for recipient mice from primary transplants (a, from Fig. 1d), secondary transplants (b, from Fig. 2a), tertiary transplants (c, from Fig. 2b) and quaternary transplants (d, from Fig. 2c). In all cases, these blood cell counts were collected from mice after the analysis of blood cell reconstitution was complete (at least 20 weeks after transplantation). The transplanted mice were observed for a median time of 260 (162–315) days for primary recipient mice, 194 (122–264) days for secondary recipient mice, 224 (176–336) days for tertiary recipient mice, and 280 (279–280) days for quaternary recipient mice. We never observed evidence of leukaemia or MPN by histology in these mice. Across all of the experiments, only two recipients of Nras<sup>G12D</sup> cells and two recipients of control cells died spontaneously. Data represent mean ± s.d. Two-tailed Student’s t-tests were used to assess statistical significance and none of the comparisons showed significant difference.
Extended Data Figure 6 | NrasG12D/+ had a bimodal effect on HSC cycling but increased the rate at which MPPs divide. a, Flow cytometric analysis of GFP expression in whole bone marrow cells from NrasG12D/+ or littermate control mice after 12 weeks of chase without doxycycline. b, Median GFP fluorescence intensity of H2B–GFP, H2B–GFPlo and H2B–GFPhi HSCs from wild type and NrasG12D/+ mice (n = 8 mice per genotype). GFP levels in control HSCs were set to one for comparison to relative levels in NrasG12D/+ HSCs. c, NrasG12D increased the rate of division by MPPs. Flow cytometric analysis of GFP expression in CD150−CD48−LSK MPPs from Mx1-cre; NrasG12D/+; Col1A1-H2B–GFP; Rosa26-M2-rtTA mice (G12D/+) and littermate controls (con) after 12 weeks of chase (n = 8 mice per genotype). Relative to control MPPs, NrasG12D/+ MPPs included significantly more H2B–GFP frequently cycling cells and significantly fewer H2B–GFPlo MPPs (P < 0.05 by two-way ANOVA and post hoc pairwise t-tests). d, We continuously administered BrdU to Mx1-cre; NrasG12D/+ versus control mice for 1 to 30 days and determined the frequency of BrdU+/HSCs (1 day BrdU data are from Fig. 1a). Data represent mean ± s.d. Two-tailed Student’s t-tests were used to assess statistical significance unless stated otherwise. *P < 0.05, **P < 0.01, ***P < 0.001.
Extended Data Figure 7 | Gene expression profiling demonstrates different transcriptional responses to Nras activation in quiescent as compared to frequently dividing HSCs. a, CD150^+CD48^-LSK HSCs and CD150^-CD48^-LSK MPPs were isolated from three pairs of Mx1-cre;NrasG12D/1 and littermate controls and gene expression profiling was performed with Affymetrix mouse genome 430 2.0 microarrays. The Venn diagram shows the number of genes that were differentially expressed between NrasG12D/1 and controls cells within each cell population (fold change $\geq 2$).

Extended Data Table 1 | Fold change of genes differentially expressed in NrasG12D/1 and control cells. The fold change value is calculated by dividing the gene expression level in NrasG12D/1 cells by that in control cells. Genes that are consistently increased or decreased in expression in response to Nras activation in HSCs, MPPs, GFP^-HSCs and GFP^hi HSCs (fold change $\geq 2$ and $P \leq 0.05$ in each cell population).

Extended Data Figure 7 | b, Venn diagram of genes that were differentially expressed between NrasG12D/1 and control GFP^-HSCs and GFP^hi HSCs isolated from 3 pairs of Mx1-cre;NrasG12D/1;Col1A1-H2B–GFP;Rosa26-M2-rtTA mice and littermate controls (fold change $\geq 2$ and $P \leq 0.05$). c. Genes that were consistently increased or decreased in expression in response to Nras activation in HSCs, MPPs, GFP^-HSCs and GFP^hi HSCs (fold change $\geq 2$ and $P \leq 0.05$ in each cell population). d-f, Gene set enrichment analysis (GSEA) of cell cycle genes (d), DNA replication genes (e) and RNA polymerase genes (f).
Extended Data Figure 8 | Nras activation increases STAT5 phosphorylation. a, Western blot for phosphorylated ERK (pERK) in LSK stem/progenitor cells, Lin− c-kit+ Sca1− progenitor cells, or whole bone marrow (WBM) cells from Mx1-cre; NrasG12D+/− (G12D+/−) mice, Mx1-cre; NrasG12D/G12D (G12D/G12D) mice, or littermate controls 2 weeks after plpC treatment. b, Western blot of pERK and total ERK in 10^6 uncultured splenocytes from Mx1-cre; NrasG12D+/− (G12D+/−) or control mice after 8 days of treatment with PD0325901 MEK inhibitor or vehicle (blot is representative of four independent experiments). c, The frequency of BrdU1 CD1501 CD482 LSK HSCs after a 24-h pulse of BrdU to Mx1-cre; NrasG12D+/− (G12D+/−) or control mice after 7 days of PD0325901 MEK inhibitor or vehicle (mean ± s.d. from four experiments). d, Western blot of pERK and total ERK in 10^6 uncultured bone marrow cells from Mx1-cre; NrasG12D+/− (G12D+/−) or control mice after 8 days of AZD6244 MEK inhibitor or vehicle (blot is representative of four independent experiments). e, The frequency of BrdU1 CD1501 CD482 LSK HSCs after a 24-h pulse of BrdU to Mx1-cre; NrasG12D+/− (G12D+/−) or control mice after 7 days of AZD6244 MEK inhibitor or vehicle (mean ± s.d. from four experiments). f, Western blot for phosphorylated Akt (pAkt) in CD481 LSK HSCs and MPPs, CD481 LSK progenitors, or WBM cells from Mx1-cre; NrasG12D+/− (G12D+/−) mice, Mx1-cre; Pten−/− (Pten−/−) mice, or littermate controls 2 weeks after plpC treatment. g, Soc2 transcript levels in HSCs and MPPs from Mx1-cre; NrasG12D+/− (G12D+/−) or control mice by microarray analysis (top, n = 3) and qRT–PCR (bottom, n = 7). h, i, Socs2 transcript levels in GFP2 and GFP3 HSCs from Mx1-cre; NrasG12D+/−; Col1A1-H2B-GFP, Rosa26-M2-rtTA mice and littermate controls by microarray (b, n = 3) and qRT–PCR (i, n = 3). j, Western blotting showed that pSTAT5 levels were significantly increased in CD481 LSK HSCs and MPPs from Mx1-cre; NrasG12D+/− mice as compared to control mice. Left panel shows western blots of pSTAT5 and total STAT5 from three independent experiments (signals were quantitated using NIH ImageJ software). Blot 1 was shown in Fig. 4e. k, Western blot showing that STAT5 levels were reduced in CD481 LSK HSCs/MPPs from Mx1-cre; Stat5a−/− or Mx1-cre; NrasG12D+/−; Stat5a−/− mice as compared to control and Mx1-cre; NrasG12D+/− mice (blot is representative of four independent experiments). l, BrdU incorporation into common myeloid progenitors (CMPs; Lin− Sca1− c-kit− CD48− CD16/32+CD150−CD48+), granulocyte macrophage progenitors (GMPs; Lin− ‘Sca1− c-kit+ CD48− CD150−CD48-‘), and megakaryocyte erythroid progenitors (MEPs; Lin− Sca1− c-kit+ CD48− CD150−CD48-‘) from control, Mx1-cre; Stat5a−/−, Mx1-cre; NrasG12D+/−, Mx1-cre; NrasG12D+; Stat5ab−/−; Stat5a−/− mice after a 2.5-h pulse of BrdU (n = 4 mice per treatment). Data represent mean ± s.d. Two-tailed Student’s t-tests were used to assess statistical significance.