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Genetically engineered minipigs model the major clinical features of human neurofibromatosis type 1

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Neurofibromatosis Type 1 (NF1) is a genetic disease caused by mutations in Neurofibromin 1 (NF1). NF1 patients present with a variety of clinical manifestations and are predisposed to cancer development. Many NF1 animal models have been developed, yet none display the spectrum of disease seen in patients and the translational impact of these models has been limited. We describe a minipig model that exhibits clinical hallmarks of NF1, including café au lait macules, neurofibromas, and optic pathway glioma. Spontaneous loss of heterozygosity is observed in this model, a phenomenon also described in NF1 patients. Oral administration of a mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor suppresses Ras signaling. To our knowledge, this model provides an unprecedented opportunity to study the complex biology and natural history of NF1 and could prove indispensable for development of imaging methods, biomarkers, and evaluation of safety and efficacy of NF1-targeted therapies.
F1 is a prevalent genetic disorder, occurring in one in every 3000 children born, with over two million cases worldwide. NF1 patients are born with mutations in one copy of the NF1 tumor suppressor gene, a negative regulator of the Ras signaling pathway. Over time, the remaining wild-type allele of NF1 may be lost in a rare cell, leading to abnormal cell growth and division in multiple body systems. For instance, the loss of heterozygosity (LOH) in the melanocyte lineage results in cafe au lait macules (CALMs), hyperpigmented patches of skin present in nearly all patients, and LOH in the Schwann cell lineage leads to the development of neurofibromas. NF1 patients are predisposed to other tumors, including malignant peripheral nerve sheath tumors, optic pathway gliomas (OPGs), astrocytomas, and juvenile myelomonocytic leukemia. While there has been considerable effort to develop targeted therapies for these tumors, few treatments show clinical efficacy.

In addition to tumor predisposition, NF1 patients also commonly suffer from skeletal abnormalities, scoliosis, short stature, learning disabilities, hypertension, and epilepsy.

Many murine models of NF1 have been developed, yet none fully recapitulates the disease spectrum seen in NF1 patients. The first mouse model of NF1 was a traditional germ line heterozygous knockout mouse, which developed some of the less common NF1-associated tumors, however, did not develop neurofibromas or other characteristic symptoms of NF1.

More complex mouse models have since been developed to replicate the more common features of NF1. For example, the Cre-lox system was employed to generate mice with bi-allelic loss of the tumor suppressor gene, a negative regulator of NF1, which leads to a targeted reduction in Ras signaling. This model provides a unique opportunity to study the complex biology and natural history of NF1 and could prove indispensable for preclinical evaluation of NF1-targeted therapies as well as development of imaging methods and diagnostic biomarkers.

Results

Generation of an NF1 minipig by gene editing. To generate a minipig model of NF1, we mimicked a recurrent nonsense mutation p.Arg1947*(R1947*) identified in 62 of 8100 (±8) unrelated and symptomatic NF1 patients. This mutation has also been described in several other studies. Generation of an NF1 minipig by gene editing

Recent technological advances in precision gene targeting and somatic cell nuclear transfer have given researchers the ability to produce genetically modified swine carrying exact disease alleles found in human patients. Genetically engineered swine as large animal biomedical models open a vast array of new opportunities for these tumors, few treatments show clinical efficacy.

We have developed an NF1 minipig that recapitulates the diverse phenotypes seen in NF1 patients, including the development of CALMs, neurofibromas, and OPG. NF1 minipigs exhibit spontaneous and cell-type-specific LOH, a critical step for both CALM and neurofibroma development in NF1 patients and a hallmark of NF1 that has not been observed in rodent models. We demonstrate that NF1 minipigs can be dosed orally with a small-molecule inhibitor, currently in clinical development for NF1, which leads to a targeted reduction in Ras signaling. This NF1 minipig model provides a unique opportunity to study the complex biology and natural history of NF1 and could prove indispensable for preclinical evaluation of NF1-targeted therapies.

NF1 minipigs display CALMs and other skin abnormalities. From birth, all NF1 F0 and F1 minipigs display CALMs and other skin abnormalities. Germ line transmission of the mutant NF1R1947* allele with Mendelian frequency. A minipig model of NF1, we mimicked a recurrent nonsense mutation p.Arg1947*(R1947*) identified in 62 of 8100 (±8) unrelated and symptomatic NF1 patients (Supplementary Table 1). This mutation has also been described in several other studies. From birth, all NF1 F0 and F1 minipigs exhibited spontaneous and cell-type-specific LOH, a critical step for both CALM and neurofibroma development in NF1 patients and a hallmark of NF1 that has not been observed in rodent models. We demonstrate that NF1 minipigs can be dosed orally with a small-molecule inhibitor, currently in clinical development for NF1, which leads to a targeted reduction in Ras signaling. This NF1 minipig model provides a unique opportunity to study the complex biology and natural history of NF1 and could prove indispensable for preclinical evaluation of NF1-targeted therapies as well as development of imaging methods and diagnostic biomarkers.
criterion for NF1. CALMs observed in NF1 minipigs increased in size and number with age. A subset of NF1 minipigs exhibited other abnormalities described in NF1 patients, including freckling (30.8%, 4/13 animals) and congenital hypopigmentation of the hair, with no underlying melanin changes in the skin (23.1%, 3/13 animals) (Supplementary Figure 1, Table 1). In contrast, no wild-type minipigs showed CALMs or evidence of pigmentation defects (N > 50).

**NF1 minipigs develop neurofibromas.** Neurofibromas are benign peripheral nerve sheath tumors composed of multiple cell types including Schwann cells, fibroblasts, and mast cells. These tumors are variable in size and number and represent a major source of pain and disfigurement in NF1 patients. Superficial tumors resembling neurofibromas were noted in 40% (2/5) of the NF1 F0 animals and 37.5% (3/8) of the NF1 F1 minipigs by 4 months of age (Table 1). Tumors were discrete and ranged in number from one to six per animal and in size from 1.8 to 6.0 centimeters in diameter (Fig. 2a–c). Hematoxylin and eosin (H&E) staining revealed areas of hypercellularity, confirmed to be clusters of Schwann cells by S100β and glial fibrillary acidic protein (GFAP) staining, surrounded by dense collagen and sparse fibroblasts (Fig. 2d, e, g). These tumors had a low Ki67 proliferative index and showed mast cell infiltration (Fig. 2f, h, i). Control immunohistochemistry was performed on sciatic nerve and intestinal tissue (Supplementary Figure 2). These data show that the tumors seen in NF1 minipigs share classic features of human neurofibromas. The skin-related abnormalities seen in NF1 minipigs closely resemble those seen in NF1 patients, and this model meets the NIH criteria for both CALMs and neurofibromas used to diagnose NF1 patients.

**NF1 minipigs develop OPG.** OPGs occur in 15–20% of children with NF1, where >80% are located within the optic nerves and chiasm. These slow-growing benign tumors are typically identified by magnetic resonance imaging (MRI), often revealing focal enlargement of the optic nerve and/or chiasm. One of seven NF1 minipigs imaged by MRI for OPG development exhibited a mass involving the optic chiasm that extended ventrally into the surrounding tissue (Fig. 3a, b, Table 1). Histopathology of this lesion revealed hypercellularity, microglial infiltration, and a low Ki67 proliferative index, similar to OPGs reported in NF1 patients and genetically engineered mouse models (Fig. 3c–k). Cells from NF1 minipigs undergo loss of heterozygosity. Bi-allelic inactivation of the NF1 gene through a "second-hit" mutation in a subset of Schwann cells has been demonstrated in neurofibromas from human patients. This phenomenon has also been described in melanocytes isolated from CALMs. To evaluate this in our minipig model, CALM-derived melanocytes and neurofibroma-derived Schwann cells were subjected to DNA sequencing. LOH at the NF1 locus was detected in a subset of CALMs and neurofibromas, by either a gene conversion event or large deletion (Fig. 4a). Interestingly, one CALM showed conversion to R1947* without RFLP incorporation, suggesting...
tumors with LOH compared to wild-type or levels of Ras activation were seen in Schwann cells isolated from analyzed by western blot for active Ras (Ras-GTP). Variable or sciatic nerves were starved overnight, serum stimulated, and NF1 associated with (Fig. 4c, d). To validate the biochemical defect in Ras signaling DNA sequencing results in Schwann cells isolated from tumors for neurotron microscopy (EM) of NF1 minipig optic nerves showed NF1 minipigs display other NF1-associated phenotypes (Figure 3). Despite >90% purity in Schwann cell cultures (Supplementary 4 COMMUNICATIONS BIOLOGY | DOI: 10.1038/s42003-018-0163-y | www.nature.com/commsbio were observed (Table 1). N/A not assessed

Table 1 Summary of NF1-related phenotypes seen in NF1 minipigs that underwent imaging

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Generation</th>
<th>Sex</th>
<th>Age</th>
<th>Imaging</th>
<th>CALM</th>
<th>Freckling</th>
<th>Hypopigmentation</th>
<th>Neurofibroma</th>
<th>OPG</th>
<th>Tibial diaphyseal narrowing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1728</td>
<td>F0</td>
<td>M</td>
<td>5.3 months</td>
<td>MRI, CT, X-ray</td>
<td>Present</td>
<td>Absent</td>
<td>N/A</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>1729</td>
<td>F0</td>
<td>M</td>
<td>19.8 months</td>
<td>MRI, CT, X-ray</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>1730</td>
<td>F0</td>
<td>M</td>
<td>16.7 months</td>
<td>MRI, CT, X-ray</td>
<td>Present</td>
<td>Absent</td>
<td>Present</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>1734</td>
<td>F0</td>
<td>M</td>
<td>3.7 months</td>
<td>MRI, CT, X-ray</td>
<td>Present</td>
<td>Absent</td>
<td>N/A</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>1735</td>
<td>F0</td>
<td>M</td>
<td>19.8 months</td>
<td>MRI, CT, X-ray</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>1228</td>
<td>F1</td>
<td>F</td>
<td>9.0 months</td>
<td>MRI, CT, X-ray</td>
<td>Present</td>
<td>Absent</td>
<td>N/A</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>1229</td>
<td>F1</td>
<td>M</td>
<td>16.1 months</td>
<td>MRI, CT, X-ray</td>
<td>Present</td>
<td>Absent</td>
<td>N/A</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>1232</td>
<td>F1</td>
<td>M</td>
<td>15.7 months</td>
<td>MRI, CT, X-ray</td>
<td>Present</td>
<td>Present</td>
<td>N/A</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>1233</td>
<td>F1</td>
<td>M</td>
<td>8.9 months</td>
<td>MRI, CT, X-ray</td>
<td>Present</td>
<td>Present</td>
<td>N/A</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>1236</td>
<td>F1</td>
<td>F</td>
<td>9.0 months</td>
<td>MRI, CT, X-ray</td>
<td>Present</td>
<td>Present</td>
<td>N/A</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>1342</td>
<td>F1</td>
<td>F</td>
<td>8.8 months</td>
<td>MRI, CT, X-ray</td>
<td>Present</td>
<td>Absent</td>
<td>N/A</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>1346</td>
<td>F1</td>
<td>M</td>
<td>9.9 months</td>
<td>MRI, CT, X-ray</td>
<td>Present</td>
<td>Absent</td>
<td>N/A</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>1359</td>
<td>F1</td>
<td>F</td>
<td>9.9 months</td>
<td>MRI, CT, X-ray</td>
<td>Present</td>
<td>Absent</td>
<td>N/A</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Prevalence of phenotype</td>
<td>13/13</td>
<td>4/13</td>
<td>2/3</td>
<td>3/13</td>
<td>5/13</td>
<td>1/7</td>
<td>2/13</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

NF1 minipigs as a preclinical model for pharmacology studies. It is useful to have a large animal disease model for testing safety and efficacy of novel pharmaceutical compounds prior to human studies. To demonstrate that NF1 minipigs represent a relevant preclinical model, we performed pharmacokinetic and pharmacodynamic analysis of PD0325901, a MEK inhibitor currently in clinical trials for NF1-related tumors (clinicaltrials.gov). A single dose of 0.79 mg kg⁻¹ PD0325901 was administered orally to four wild-type and four NF1 littermates and plasma was collected over time for pharmacokinetic analysis. PD0325901 was detectable in the plasma within 1 hour of administration, and the mean maximum plasma concentration of PD0325901 was 125 ± 47 ng mL⁻¹, a value higher than the reported PD0325901 plasma concentration (99 ng mL⁻¹) required for a pharmacodynamic effect in other preclinical models (Fig. 5a).\(^ {55}\) Notably, NF1 minipigs showed significantly higher plasma levels of PD0325901 than their wild-type littermate controls at 8 h (wild-type 59 ± 30 ng mL⁻¹ vs. NF1 104 ± 15 ng mL⁻¹) and 10 h (wild-type 48 ± 20 ng mL⁻¹ vs. NF1 87 ± 15 ng mL⁻¹) after drug administration, though estimated pharmacokinetic parameters showed no statistically significant differences between wild-type and NF1 minipigs (Fig. 5a, Supplementary Table 3). To measure the pharmacodynamic effect of PD0325901 in minipigs, peripheral blood mononuclear cells (PBMCs) were evaluated for suppression of phorbol-myristate acid (PMA)-stimulated ERK phosphorylation, a pharmacodynamic biomarker of MEK inhibitors\(^ {65,67}\). A single dose of PD0325901 was sufficient to suppress ERK phosphorylation in PBMCs by 98.1% (±0.7) in NF1 minipigs and 95.8% (±1.2) in wild-type minipigs (Fig. 5b). These results suggest that NF1 minipigs could serve as a valuable preclinical model for pharmacokinetic and pharmacodynamic analysis of targeted therapies.

NF1 minipigs display other NF1-associated phenotypes. Electron microscopy (EM) of NF1 minipig optic nerves showed myelin decompaction, a phenotype observed in mouse models and correlated with white matter enlargement and behavioral deficits in NF1 patients\(^ {23,34}\) (Supplementary Figure 4). Lesions resembling iris hamartomas, or Lisch nodules, were noted in two of the three NF1 minipigs examined by slit-lamp (Table 1, Supplementary Figure 5). Tibial diaphyseal narrowing was observed in 15.4% (2/13) of NF1 minipigs that underwent X-ray analysis (Table 1, Supplementary Figure 5). Blood pressure, weight, height, length, and head circumference were measured periodically in a cohort of NF1 minipigs and their wild-type littermate controls (Supplementary Figure 6). At 8 months of age, NF1 minipigs weighed significantly less than their wild-type siblings. All 12 NF1 minipigs (five F0 animals and seven F1 animals) that underwent MRI analysis also underwent full-body computerized tomography (CT) scanning and no other tumors were observed (Table 1).

Partial gene conversion or a point mutation in the wild-type allele (Fig. 4b). Schwann cells were subjected to western blot analysis for neurofibromin protein expression, which confirmed the DNA sequencing results in Schwann cells isolated from tumors (Fig. 4c, d). To validate the biochemical defect in Ras signaling associated with NF1 loss, Schwann cells from neurofibromas or sciatic nerves were starved overnight, serum stimulated, and analyzed by western blot for active Ras (Ras-GTP). Variable levels of Ras activation were seen in Schwann cells isolated from tumors with LOH compared to wild-type or NF1⁺/− Schwann cells, despite >90% purity in Schwann cell cultures (Supplementary Figure 3).

**Table 1** Summary of NF1-related phenotypes seen in NF1 minipigs that underwent imaging.
**Fig. 2** NF1 minipigs develop neurofibromas. a An example of an NF1 minipig harboring two dermal masses on its left side. The mass denoted by the white dotted box is enlarged in b and the mass denoted by the solid white box is enlarged in c. b The mass on the shoulder measured 3.5 cm in diameter. c The mass on the flank measured 4.2 cm in diameter. d H&E staining of a representative mass showing regions of hypercellularity. e Hypercellular regions stain positive for GFAP, a marker of Schwann cells. f Mast cell infiltration is shown by Toluidine blue metachromasia (purple). g Hypercellular regions stain positive for S100β, a marker of Schwann cells. h Hypercellular region showing minimal proliferation by Ki67 staining. i Mast cell infiltration is shown by c-Kit staining. Scale bars, 100 μM.

**Fig. 3** NF1 minipigs develop optic pathway glioma-like lesions. a Axial T1-weighted MRI demonstrates a lesion at the level of the optic chiasm (white arrow) in a 9-month-old NF1 minipig. b Axial T1-weighted MRI of a normal optic chiasm from a 16-month-old NF1 minipig. c H&E staining of the optic pathway lesion from an NF1 minipig shows hypercellularity. d H&E staining of an optic nerve from an NF1 minipig. e H&E staining of an optic nerve from a wild-type minipig. f Iba1 immunohistochemistry of the optic pathway lesion from an NF1 minipig shows increased microglial infiltration. g Iba1 immunohistochemistry of an optic nerve from an NF1 minipig. h Iba1 immunohistochemistry of an optic nerve from a wild-type minipig. i Ki67 staining of an optic pathway lesion from an NF1 minipig shows a low proliferative index. j Ki67 staining of an optic nerve from an NF1 minipig. k Ki67 staining of an optic nerve from a wild-type minipig. Scale bars, 50 μM.
Discussion

We describe here, the first minipig model of NF1 that exhibits the spectrum of clinical features seen in patients. NF1 minipigs meet NIH diagnostic criteria for NF1, and a subset of minipigs develop neurofibromas and OPG. This is also the first animal model of NF1 to exhibit CALMs, one of the most common clinical manifestations in humans. Both melanocytes and Schwann cells from NF1 minipigs undergo spontaneous LOH, a
critical step in disease progression. To our knowledge, spontaneous LOH in the cells of origin of neurofibroma and CALM has not been demonstrated in any other animal model of NF1. Interestingly, porcine Schwann cells with LOH show variable evidence of hyperactive Ras signaling in vitro. This may be indicative of a more complex biochemical process associated with NF1 loss in neurofibromas, such as activation of dual specificity phosphatases.

Similar to the human condition, our model shows variability in expression of NF1 phenotypes. For instance, NF1 minipig CALMs vary greatly in size and number between animals. Additionally, only a subset of minipigs develop OPG and neurofibromas. A clear strength of our system is that the NF1 minipig closely mimics the age of onset for NF1-related phenotypes in humans. In humans, CALMs are often present at birth, and neurofibromas tend to develop around puberty. CALMS were observed perinatally in all pigs, and minipig neurofibromas develop at around 4 months of age (puberty ~5–6 months). While OPG is typically diagnosed at a young age in humans, this phenotype was not noted in the NF1 minipig until 9 months of age (adulthood). However, it is likely that OPG was present at a young age and could not be diagnosed until MRI was performed, as these tumors are often asymptomatic.

While various model systems have been implemented to induce tumor development in mice, these have not proven to be representative of the complex natural history of NF1-associated tumorigenesis. For example, NF1+/− mice do not develop peripheral nerve sheath tumors or other hallmarks of NF1 syndrome (Table 2). Interestingly, a subset will develop less common NF1−associated phenotypes later in life, including neurofibrosarcoma, pheochromocytoma, and myeloid leukemia. It is possible that we would see these manifestations in the NF1 minipig, but Ossabaw minipigs have a lifespan of up to 15 years in captivity and were only monitored for 16 months for the purposes of this study. In comparison to the analogous mouse model described by Jacks et al. and Brannan et al., our NF1 minipig model manifests the more common hallmarks of the disease (Table 2). In fact, the original NF1+/− mouse models share no phenotypes with our NF1+/− minipig model (Table 1).

The genetic, anatomic, and physiologic similarities between minipigs and humans make them an optimal model for studying the biology and natural history of NF1. The minipig provides a human-sized platform for imaging studies to better evaluate tumor natural history and therapeutic efficacy and to develop methods for early detection of NF1-associated tumors. Their large size also makes it feasible to perform longitudinal blood and tissue sampling for identification of diagnostic biomarkers and evaluation of NF1-targeted therapies. The NF1 minipig may prove particularly valuable for studying long-term toxicity of targeted therapies in both pediatric and adult populations, as many drug-gable targets show significant homology between pig and human.

Table 2 Comparison of mouse, minipig, and human NF1-associated phenotypes

<table>
<thead>
<tr>
<th>NFI animal model</th>
<th>Age at puberty</th>
<th>Lifespan</th>
<th>Average age of onset</th>
<th>OPG</th>
<th>Cutaneous neurofibroma</th>
<th>Lisch nodule</th>
<th>CALM</th>
<th>Pheochromocytoma</th>
<th>Myeloid leukemia</th>
<th>Tibial diaphyseal narrowing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse (Mus musculus)</td>
<td>6–8 weeks</td>
<td>1–3 years</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>15–28 months</td>
<td>17–27 months</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>Ossabaw minipig (Sus scrofa)</td>
<td>5–6 months</td>
<td>15 years</td>
<td>9 months</td>
<td>4 months</td>
<td>Pre-puberty</td>
<td>Birth</td>
<td>n/a</td>
<td>Pre-puberty</td>
<td>39 years</td>
<td>2 years</td>
</tr>
<tr>
<td>Human</td>
<td>10–15 years</td>
<td>70–80 years</td>
<td>3–5 years</td>
<td>10–15 years</td>
<td>Pre-puberty</td>
<td>Birth</td>
<td>n/a</td>
<td>Pre-puberty</td>
<td>39 years</td>
<td>2 years</td>
</tr>
</tbody>
</table>

Further, this model provides the opportunity to evaluate drug penetration through the blood–nervé and blood–brain barriers. These studies will be critical in evaluating efficacy of treating NF1-related tumors including neurofibromas and OPGs.

Thus far, other important NF1-associated tumors including plexiform neurofibromas, malignant peripheral nerve sheath tumors, and gastrointestinal stromal tumors have not been observed in our model. We will continue monitoring NF1 minipigs as they age for the development of these and other clinical manifestations. To model more aggressive phenotypes, the NF1 minipig could be augmented with additional germ line or somatic mutations. For example, Tumor Protein p53 (TP53), a tumor suppressor gene that is often found co-mutated with NF1 in malignant peripheral nerve sheath tumors, is in close proximity to the NF1 gene (~9 MB) in the swine genome. This allows for the development of a minipig in which both NF1 and TP53 are mutated in cis, similar to previously described mouse models of malignant peripheral nerve sheath tumors.

Swine also harbor the Polycomb Repressive Complex 2 Subunit (SUZ12) gene, which is recurrently inactivated in NF1-associated malignancies and could be mutated or deleted with NF1 to drive the formation of additional tumor types. Future studies will also evaluate NF1-associated cognitive deficits and neurodevelopmental disabilities in our minipigs.

Methods

TALEN design, assembly, and RNA synthesis. All TALENs were designed using the TALE-NT software and assembled using standard methods. Linearized TALEN DNA was transcribed in vitro using the mMessage Machine T3 kit (Ambion). Synthesis reactions were assembled in a 20 µL reaction with 1 µg linearized plasmid DNA, 1× NTU/CAPs (Ambion), 1× reaction buffer (Ambion), and 2 µL enzyme mix (Ambion). Reactions were incubated for 2 h at 37 °C, treated with Turbo DNase (Invitrogen), then cleaned up with the RNeasy Mini Kit (Qiagen).

Cell culture and transfection of swine embryonic fibroblasts. Ossabaw minipig fibroblasts isolated from day 30–36 embryos were cultured in 1× high-glucose DMEM (Invitrogen) with 10% FBS (Atlas Biologicals), 2 mM l-glutamine (Corning), 10 mM HEPES buffer (Lonza), 1× penicillin/streptomycin solution (Corning), 5 µg mL−1 Apo-Transferin (Sigma), 20 ng mL−1 recombinant human IGF-1 (R&D Systems), and 25 ng mL−1 recombinant human EGF (R&D Systems) and transfected using the Neon Transfection System (Thermo Fisher Scientific). Briefly, each transfection reaction included 600,000 fibroblasts, 0.5 µg of RNA from each transcribed TALEN, and 0.2 nmol HDR oligonucleotide, and the transfection reaction was pulsed once at 1800 V for 20 ms using the Neon® transfection system (Thermo Fisher Scientific). Transfected cells were cultured 3 days at 30 °C, before splitting for RFLP analysis and plating for colony isolation at 38.5 °C. Individual colonies were collected in 10-cm dishes, where 80–250 transfected cells were plated and allowed to grow for 10–14 days and individual colonies were aspirated under gentle trypsinization.

Detection and sequence validation of gene modification. Transfected cells harvested at day three were prepared for PCR by pelleting and resuspending in PCR-safe lysis buffer (10 mm Tris-Cl, pH 8.0, 2 mM EDTA; 2.5% (vol/vol) Tween-20; 2.5% (vol/vol) Triton X-100; 100 µg mL−1 proteinase K) at ~1000 cells per µL.
followed by incubation at 50 °C for 60 min and 95 °C for 15 min. Typically, 1 μL of prepared lysis was used in a 2× AccuStart II PCR SuperMix (Quantabio); all other applications were according to the manufacturer’s protocol. Gene expression analysis in individual colonies was detected by RFLP analysis and direct sequencing of PCR amplicons, characterized by TOPO cloning (Invitrogen) and sequencing.

Animal husbandry and cloning. NF1 minipigs were produced under license of chromatin transfer technology from Hematech to Cooperative Resources International Center for Biotechnology (GRI, ICB, Verona, WI)63. All animal work was performed in Recombinetics facilities under its Animal Welfare Assurance #A4729-01 and the University of Minnesota under its Animal Welfare Assurance #A3456-01. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

H&E, toluidine blue, and immunohistochemical staining. H&E staining was performed on 3.5-μm-thick sections prepared from paraffin blocks of formalin-fixed tissues. For mast cell staining, a toluidine blue stock solution (10x) was prepared by diluting 1 g of toluidine blue O (Fisher Chemical, T161) in 100 mL of ethanol. The solution was freshly diluted in sodium chloride pH adjusted to 2.0–2.5 with glacial acetic acid. Sections were immersed in toluidine blue solution for 3 min, rinsed three times in distilled water, then quickly dehydrated and cleared in Clear-Rite (Thermo Scientific) before mounting. For immunohistochemistry, heat-induced epitope retrieval (HIER) was performed with target retrieval solution pH9 (Dako, S2367) except for S100 staining where no HIER was used. A standard protocol was used with primary antibodies incubated 1 h at room temperature (RT): c-Kit (1:200, Cell Signaling Technology, #3074), GFAP (1:3000, Dako, Z0334), Ki-67 (1:1000, BD Pharmingen, 556003), S100 (1:10000, Dako, Z0311). Biotinylated secondary anti-rabbit (1:2000 or 1:50,000), goat anti-mouse (1:2000 or 1:50,000), or goat anti-rabbit (1:2000 or 1:50,000) secondary antibodies were incubated 1 h at RT. Pictures were taken with an Axio Imager M1 microscope and Axiosvision software (Zeiss).

Minipig sedation, radiographic imaging, and euthanasia. Pigs were transported to the University of Minnesota Veterinary Medical Center (VMC) for housing and radiographic imaging. Minipigs were housed in stalls or kennels sized per the Guide for the Care and Use of Laboratory Animals. Minipigs were housed individually, fed a standard pig diet, and allowed water. Minipigs were fasted for 12–18 h prior to anesthesia. Anesthesia was induced with telazol (2.2–4.4 mg kg$^{-1}$), xylazine (2 mg kg$^{-1}$), and ketamine (20 mg kg$^{-1}$) intramuscularly (IM), then the animals were intubated with balloon-cuffed 7.5–10 mm endotracheal tubes. Anesthesia was maintained with 1.5–2.0% isoflurane in 100% oxygen with a volume of 340–1000 mL (depending upon size) and pressure 15–22 cm water, to maintain an end tidal CO2 of 35–45 mmHg. An 18–22-gauge IV catheter was placed in a peripheral ear vein or cephalic vein for administration of IV fluids and euthanasia solution. IV fluids (Lactated Ringers Solution or 0.9% saline) were given to maintain an arterial blood pressure and to continuously monitor arterial pressure, respiratory rate, and arterial oxygen saturation. CO2 level, and blood pressure were monitored continuously and recorded every 5–15 min.

Once anesthetized, minipigs were transported to the desired imaging area: X-ray, CT, or MRI. Typically, minipigs receiving all three modalities would start in CT, move to MRI, then X-ray. The bore of the MRI limited the size of minipigs which could be scanned. Therefore, minipigs over 90 kg were imaged by CT, then X-ray, then MRI of the head only post-mortem.

CT acquisition: minipigs were placed in the supine position and a single, non-contrast enhanced dataset of the chest–abdomen–pelvis region was acquired using a Toshiba Aquilion LB 160 CT scanner (Toshiba Medical Systems, software version V3.35ER006). Images were acquired in a helical mode using the full 64-row detector. Tissues were scanned with a 4.4 mg kg$^{-1}$–ketamine (20 mg kg$^{-1}$ −ketocon (10 mg kg$^{-1}$) was given. Optic pathway immunohistochemistry and pathological analysis. Formalin-fixed paraffin-embedded sections were subjected to immunohistochemistry using cell-specific antibodies45,46. Sections were deparaffinized, treated for citrate antigen retrieval, and incubated in 5% serum blocking solution for 1 h at RT. Slides were then incubated with rabbit primary antibodies overnight at 4 °C, followed by a 1:50 exposure to biotinylated species-specific secondary antibodies (Vector Laboratories) and development using the Vectorstain Elite ABC kit (Vector Laboratories) according to the manufacturer’s instructions. H&E staining was accomplished using standard methods. Images were acquired on an Olympus BX51 microscope using cellSens Entry imaging software.

Isolation and culture of primary minipig cell lines. Minipig skin fibroblasts were isolated from ear clips transported in 1× HBSS (Corning) with 1× antibiotic/antimycotic (AA; Corning), dispase II (2.5 mg mL$^{-1}$; GIBCO), and 1× AA (Corning). Hair and subcutaneous fat were trimmed from ear clips obtained from euthanized animals and fixed in formalin prior to paraffin embedding and sectioning. Five-micrometer sections were deparaffinized, treated for citrate antigen retrieval, and incubated in 5% serum blocking solution for 1 h at RT. Slides were then incubated with Iba1 (1:1000 dilution; Wako; 019–19741) or K67 (1:1000 dilution; Abcam; ab15580) primary antibodies overnight at 4 °C, followed by a 1:50 exposure to biotinylated species-specific secondary antibodies (Vector Laboratories) and development using the Vectastain Elite ABC kit (Vector Laboratories) according to the manufacturer’s instructions. H&E staining was accomplished using standard methods. Images were acquired on an Olympus BX51 microscope using cellSens Entry imaging software.
gentamicin (30 mg mL\(^{-1}\)), fungizone (2.5 mg mL\(^{-1}\)), penicillin–streptomycin (GIBCO), at 37 °C. Nerves were then mechanically dissociated by trituration using a 5 mL pipet, then centrifuged for 10 min at 1000 r.p.m., resuspended in PM, and incubated at 37 °C, 5% CO\(_2\) on a 1% FBS overnight before stimulation with complete media. A single oral dose was administered to four wild-type and four NF1 minipigs at 0.79 mg kg\(^{-1}\). The required volume of drug for each animal was based on individual body weight and sonicated to form suspension. A 10% dose was administered to four wild-type and four NF1 minipigs at 0.79 mg kg\(^{-1}\). The required volume of drug for each animal was based on individual body weight and sonicated to form suspension.

PD0325901 was formulated for oral administration in aqueous 0.5% (w/v) methylecylloside solution with 0.2% (v/v) polyserate 80 (TWEEN 80) to a concentration of 4 mg mL\(^{-1}\) and further formulated to form suspension. A single oral dose was administered to four wild-type and four NF1 minipigs at 0.79 mg kg\(^{-1}\). The required volume of drug for each animal was based on individual body weight and sonicated to form suspension.

Pharmacokinetic analysis of PD0325901. Whole blood was collected from the jugular vein into sodium heparin vacutainer tubes prior to PD0325901 administration (pre), and 1, 2, 3, 4, 6, 10, 12, 24, 36, and 48 h after PD0325901 administration. Samples (1.5 mL) were immediately transferred to a 1.7 mL microtube and centrifuged at 2000xg for 10 min in a tabletop refrigerated centrifuge at 4 °C. Plasma was subsequently transferred to a cryovial and stored at –80 °C until analysis.

Following the addition of an internal standard (30 ng of PLX4720), plasma samples (0.15 mL) were extracted with 1.25 mL of ethyl acetate using a multi-tube vortex for 10 min. Following centrifugation at 15,000xg for 5 min, the supernatant was removed and evaporated to dryness using a nitrogen evaporator (Zymark Turbo Vap LV, Hopkinton, MA) at 37 °C. The residue was reconstituted with 125 μL of mobile phase.

Detection and quantification of PD0325901 was performed using an high-performance liquid chromatograph (Agilent 1100 Series, Santa Clara, CA) coupled with an API 4000 triple quadrupole mass spectrometer (MDS-SCIEX, Concord, Ontario, Canada). The chromatographic separation was performed with an ACQUITY UPLC BEH C18 column, 50 mm × 2.1 mm, 1.8 µm (Milford, MA) with a mobile phase containing (40:60) DI water with 0.1% formic acid: acetonitrile with 0.1% formic acid, at a flow rate of 250 μL min\(^{-1}\), with the column temperature set at 30 °C. Mass spectrometric detection was performed using MRM (multiple reaction monitoring) in negative ionization mode. Source conditions were as follows: the turbo-gas temperature was set at 400 °C, and the ion spray needle voltage was optimized at –4500 V. The mass spectrometer was operated at unit resolution for both Q1 and Q3 in the MRM mode, with a dwell time of 100 ms per MRM channel. The precursor/product ion pairs monitored were m/z 481→398 for PD0325901 and m/z 412→305 for the internal standard (IS) (PLX4720). Ion source gases 1 and 2 were set at 50 and 20 arbitrary units, respectively; the curtain gas was set at 50 (arbitrary units) and the collision gas at 4 (arbitrary units). The collision energy was set at –22 eV for PD0325901 and –38 eV for the IS. Data acquisition was performed with analyst 1.4.1 software (MDS-SCIEX, Concord, Ontario, Canada).

Blood samples were collected immediately before PD0325901 administration and 4 h after PD0325901 administration. Whole blood was treated ex vivo with 200 nM 12-tetradecanoylphorbol-13-acetate or PBS for 10 min at 37 °C within 1 h of being drawn. Peripheral blood mononuclear cells (PBMCs) were isolated, washed, and dry pellets were snap-frozen and stored at –80 °C. Cells were lysed as described above. Relative quantification of p-ERK to total ERK was completed using an automated Simple Western apparatus, Wes (Protein Simple), following the manufacturer’s protocol. Primary antibodies used for this analysis were rabbit anti-p-ERK (CST #4370S), and rabbit anti-ERK (CST #4695S) monoclonal antibodies, which were obtained from Cayman Chemicals (Ann Arbor, Michigan). Minipig plasma for calibrators and standards were obtained from Valley Biomedical (Winchester, VA). The assay was linear in the range of 1–3000 ng mL\(^{-1}\), using 1X weighting. Method validation accuracy was 100.5% and the total variability was 6.8% (6.4% within day and 21% between days).

PD0325901 plasma concentration-time data from oral administration were analyzed using noncompartmental methods as implemented in R (version 3.4.1) R Studio PKNCA package (version 0.8.1)\(^{37}\). The pharmacokinetic parameters included area under the concentration-time curve from time 0 to 48 h (Emax, log dose), maximum concentration (Cmax), time to Cmax (Tmax), and half-life (t\(_{1/2}\)).

Pharmacodynamic analysis of PD0325901. Blood samples were collected immediately before PD0325901 administration and 4 h after PD0325901 administration. Whole blood was treated ex vivo with 200 nM 12-O-tetradecanoylphorbol-13-acetate or PBS for 10 min at 37 °C within 1 h of being drawn. Peripheral blood mononuclear cells (PBMCs) were isolated, washed, and dry pellets were snap-frozen and stored at –80 °C. Cells were lysed as described above. Relative quantification of p-ERK to total ERK was completed using an automated Simple Western apparatus, Wes (Protein Simple), following the manufacturer’s protocol. Primary antibodies used for this analysis were rabbit anti-p-ERK (CST #4695S)\(^{39}\) and rabbit anti-ERK (CST #4370S)\(^{37}\).
EM fixation and imaging. Porcine saphenous nerve and optic nerve were isolated immediately upon euthanasia and incubated for 24–48 h at 4 °C in Karnovsky’s fixation solution (4% paraformaldehyde and 3.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4–7.6), then transferred to sodium cacodylate buffer (EM Sciences). Tissue was blocked, osmicated, dehydrated, and embedded in LX 112 (EMS) and processed for routine semi-thin and ultrathin sectioning. Sections were stained in uranyl acetate and lead citrate and viewed on a Hitachi Model H-7600 microscope.

Blood pressure measurements. Pigs were sedated with 5.5 mg·kg⁻¹ Telazol and blood pressure measurements were taken using automated cuff (BP Accu-Gard, Vmed Technology) and Doppler (ES-100VX MINIDOP, KOVEN Hadeco).

Statistics. All statistical analysis was done using a Student’s t-test to generate two-tailed p values using GraphPad software or in R (version 3.4.3) R Studio PKNA package (version 0.8.17).


