Fv2 encodes a truncated form of the Stk receptor tyrosine kinase

Derek A. Persons[1]*, Robert F. Paulson[4]*, Melanie R. Loyd[2], Mark T. Herley[2], Sara M. Bodner[3], Alan Bernstein[5], Pamela H. Correll[4] & Paul A. Ney[2]

*These authors contributed equally to this work.

The Friend virus susceptibility 2 (*Fv2*) locus encodes a dominant host factor that confers susceptibility to Friend virus-induced erythroleukaemia in mice. We mapped *Fv2* to a 1.0-Mb interval that also contained the gene (*Ron*) encoding the stem cell kinase receptor (Stk). A truncated form of Stk (Sf-stk), which was the most abundant form of Stk in *Fv2*-sensitive (*Fv2^{ss}*) erythroid cells, was not expressed in *Fv2* resistant (*Fv2^{rr}*) cells. Enforced expression of Sf-stk conferred susceptibility to Friend disease, whereas targeted disruption of *Ron* caused resistance. We conclude that the *Fv2* locus encodes *Ron*, and that a naturally expressed, truncated form of Stk confers susceptibility to Friend virus-induced erythroleukaemia.

Introduction

Mice infected with Friend virus complex develop acute erythroblastosis that rapidly progresses to erythroleukaemia[1]. In the early stages of the disease, proliferating erythroblasts form discrete foci in the spleen that become confluent, causing massive splenic enlargement[2]. Depending on the strain of virus (FVA or FVP), the proliferating erythroblasts undergo differentiation, and either anaemia or polycythaemia develops. In the later stages of the disease, clones of fully transformed erythroblasts emerge, leading to erythroleukaemia[3]. This progression from uncontrolled polyclonal proliferation to erythroleukaemia is accompanied by the acquisition of additional genetic mutations, including activation of Pu.1 (Spi-1), and inactivation of p53 (refs 4,5). The multistage nature of Friend disease has made it an important experimental model to identify the viral and host cellular events involved in leukaemic transformation and malignant progression[6].

Friend virus complex consists of a replication-competent helper virus, Friend murine leukaemia virus (F-MuLV) and a replication-defective virus, spleen focus-forming virus[7] (SFFV). Acute erythroblastosis in the early stages of Friend disease is caused by SFFV (refs 8,9). In contrast to other acutely oncogenic retroviruses, SFFV does not contain a mutated cellular proto-oncogene[10,11]. Rather, pathogenicity depends on a chimaeric retroviral envelope protein, gp55 (ref. 12). The mechanism of gp55-mediated erythroblastosis involves constitutive activation of the erythropoietin receptor (Epor). Membrane-bound gp55 dimers, representing less than 5% of gp55 protein produced, are required for pathogenicity[13]. gp55 from the polycythaemic strain of SFFV associates with and activates the mouse Epor in Ba/F3 cells[14]. Furthermore, gp55 can replace erythropoietin (Epo) in supporting the formation of erythroid colonies *in vitro*, but only in the presence of the Epor (ref. 15).

A number of host genes have been identified that affect susceptibility to Friend disease. These can be divided into several categories based on the mechanism of resistance. The first group consists of genes that interfere with the infection of target cells by Friend virus. *Fv4* encodes an endogenous retroviral envelope protein that blocks cell-surface receptors[16]. *Fv1* encodes a gag-related protein that interferes with the retroviral life cycle by an unknown mechanism[17]. The second group consists of genes that alter the immune response to Friend virus infection. *Fv3* affects susceptibility to immunosuppression by F-MuLV, and two *H2*-linked loci, *Rfv1* and *Rfv2*, affect the recovery from Friend virus infection[18,19]. The third group consists of genes that affect the progression of Friend disease. *Fv5* determines whether FVP causes anaemia or polycythaemia in certain strains of mice[20]. *Fv3* and *Fv5* have not been clearly established as independent loci governing the response to Friend virus infection. Mutations in the genes flexed-tail (*f*), *Mgf* and *Kit* impede the progression of Friend disease[21–23]. These genes are required for normal erythropoiesis, emphasizing the importance of the normal erythropoietic machinery for the development of Friend disease.

Fv2 is another host gene that affects the progression of Friend disease[24]. *Fv2* does not interfere with retroviral entry into cells or with the retroviral life cycle[25]. Rather, *Fv2* appears to determine whether SFFV-infected erythroblasts proliferate in response to gp55. Experiments in chimaeric mice with *Fv2^{rr}* and *Fv2^{ss}* cells demonstrated that Friend virus infection causes selective expansion of *Fv2^{ss}* erythroid cells[26]. Thus, the effects of *Fv2* are cell autonomous, not a property of the cellular environment. There is indirect evidence that Fv2 and gp55 interact, and that interaction of these proteins with Epor causes erythroid proliferation. *Fv2*-mediated resistance can be circumvented by deletions in the ecotropic domain of gp55, but the same mutants are less active than wild-type gp55 in *Fv2^{ss}* mice[27]. To understand the role of

Departments of [1]Experimental Hematology, [2]Biochemistry and [3]Pathology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105, USA. [4]Department of Veterinary Science, Pennsylvania State University, University Park, Pennsylvania 16802, USA. [5]Program in Molecular Biology and Cancer, Samuel Lunenfeld Research Institute and Department of Molecular and Medical Genetics, Mount Sinai Hospital, University of Toronto, Toronto, Ontario, Canada. Correspondence should be addressed to P.A.N. (e-mail: paul.ney@stjude.org).

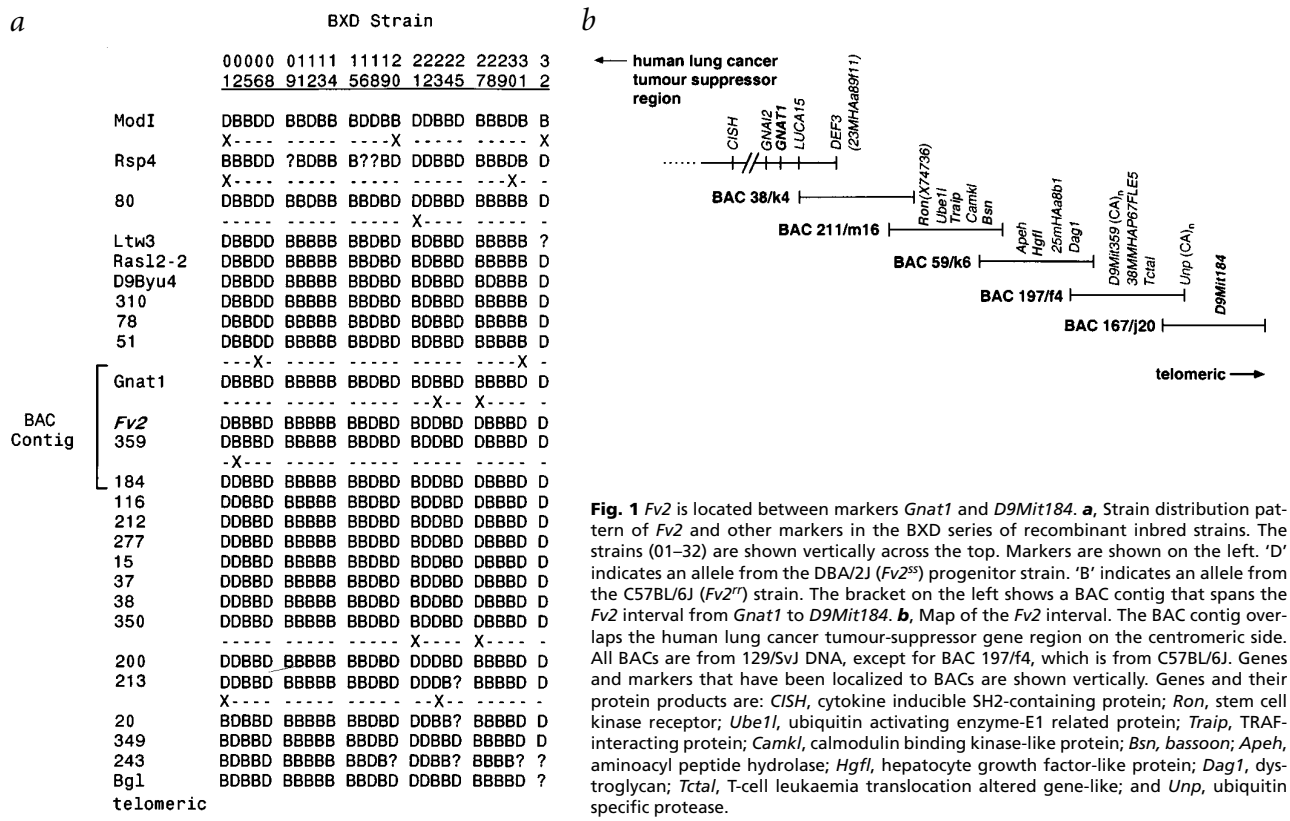

Fig. 1 *Fv2* is located between markers *Gnat1* and *D9Mit184*. ***a***, Strain distribution pattern of *Fv2* and other markers in the BXD series of recombinant inbred strains. The strains (01–32) are shown vertically across the top. Markers are shown on the left. 'D' indicates an allele from the DBA/2J ($Fv2^{ss}$) progenitor strain. 'B' indicates an allele from the C57BL/6J ($Fv2^{rr}$) strain. The bracket on the left shows a BAC contig that spans the *Fv2* interval from *Gnat1* to *D9Mit184*. ***b***, Map of the *Fv2* interval. The BAC contig overlaps the human lung cancer tumour-suppressor gene region on the centromeric side. All BACs are from 129/SvJ DNA, except for BAC 197/f4, which is from C57BL/6J. Genes and markers that have been localized to BACs are shown vertically. Genes and their protein products are: *CISH*, cytokine inducible SH2-containing protein; *Ron*, stem cell kinase receptor; *Ube1l*, ubiquitin activating enzyme-E1 related protein; *Traip*, TRAF-interacting protein; *Camkl*, calmodulin binding kinase-like protein; *Bsn, bassoon*; *Apeh*, aminoacyl peptide hydrolase; *Hgfl*, hepatocyte growth factor-like protein; *Dag1*, dystroglycan; *Tctal*, T-cell leukaemia translocation altered gene-like; and *Unp*, ubiquitin specific protease.

Fv2 in erythroid proliferation and leukaemic transformation, we undertook the positional cloning of *Fv2*. Here we report that *Fv2* encodes Stk, a member of the Met subfamily of receptor tyrosine kinases. Specifically, we show that susceptibility to Friend disease is conferred by expression of a truncated form of Stk that lacks an extracellular ligand-binding domain.

Results

Mapping and cloning the *Fv2* interval

Fv2 is located on the distal arm of mouse chromosome 9, approximately 61 cM from the centromere[28,29]. We mapped *Fv2* in the BXD series of recombinant inbred strains of mice[30]. The progenitor strains for this series are C57BL/6 ($Fv2^{rr}$) and DBA/2 ($Fv2^{ss}$). We confirmed the previously reported strain distribution pattern of *Fv2* and typed two strains not included in the original series[31] (Fig. 1*a*). To map *Fv2*, we selected 15 simple sequence length polymorphisms (SSLPs) from a group of markers (a bin) near *Fv2*, established in a generic cross of mice[32]. Most of these showed a single crossover with *Fv2* in the BXD2 strain and fell telomeric to *Fv2* (below *Fv2* in Fig. 1*a*). Some mapped still further telomeric of *Fv2* (*D9Mit200*, *D9Mit213*, *D9Mit20*, *D9Mit349* and *D9Mit243*). To obtain markers on both sides of *Fv2*, we studied SSLPs from an adjacent bin, namely *D9Mit51*, *D9Mit78* and *D9Mit80*. These SSLPs mapped centromeric to *Fv2* (above *Fv2* in Fig. 1*a*). Of all the markers tested, only *D9Mit359* matched the strain distribution pattern of *Fv2*. These results indicated that *Fv2* is in the interval between these two bins, which is 1.1 cM in the MIT backcross. Our results agree with those of a recent backcross that placed *Fv2* between *D9Mit78* and *D9Mit212* (ref. 29).

To clone the *Fv2* interval, we used bacterial artificial chromosomes (BACs) to walk from *D9Mit359* to the nearest recombinant markers on either side (Fig. 1*b*). For these experiments, we relied on a map provided by the MIT mouse genetic and physical mapping project consisting of YAC contigs anchored to SSLPs (ref. 32). *D9Mit359* was identified in a YAC contig (WC-1111) that included *D9Mit184* as well as several non-polymorphic markers (*23.MHAa89f11*, *X74736*, *25.mHAa8b1* and *38.MMHAP67FLE5*). These markers were used to probe 129Sv/J and C57BL/6 genomic DNA BAC libraries. We assembled four BACs into a contig that spanned the *Fv2* interval. On the telomeric end, BAC 167/j20 contained the nearest recombinant SSLP (*D9Mit184*). A CA-repeat in the ubiquitin-specific protease gene (*Unp* $(CA)_n$) also mapped telomeric of the *Fv2* interval, establishing this as the nearest recombinant marker on the telomeric side. On the centromeric end, we identified sequences that overlapped with the human lung cancer tumour-suppressor gene region[33]. The gene encoding the α-subunit of retinal transducin (*Gnat1*) is located 100 kb centromeric of this junction and is the nearest recombinant marker on the centromeric side.

Ron is a candidate gene for *Fv2*

Pulse-field gel electrophoresis showed that the BACs ranged 120–200 kb. Based on the number of BACs and their size, we estimated the *Fv2* interval to be less than 1.0 Mb. To identify candidate genes for *Fv2*, *Hin*dIII fragments of BACs in the *Fv2* region were randomly subcloned and sequenced. We identified 12 known genes by this approach; all of their human homologues were located in the syntenic region of human chromosome 3p21.3. One of the genes we identified was *Ron*. Mouse Stk is a member of the Met subfamily of receptor tyrosine kinases[34]. Stk is closely related to RON (its human homologue), avian v-sea and mouse Met (88%, 70% and 66% identical in the kinase domain, respectively). The relationship to v-*sea* increased our interest in Stk, because v-*sea* causes erythroblastosis and anaemia in chickens[35].

Stk was cloned from $Lin^{-}KIT^{+}Sca^{+}$ mouse bone marrow cells by PCR with degenerate primers to conserved sequences in the tyrosine kinase domain[34]. *Ron* has 19 exons[36] (Fig. 2*a*), but in

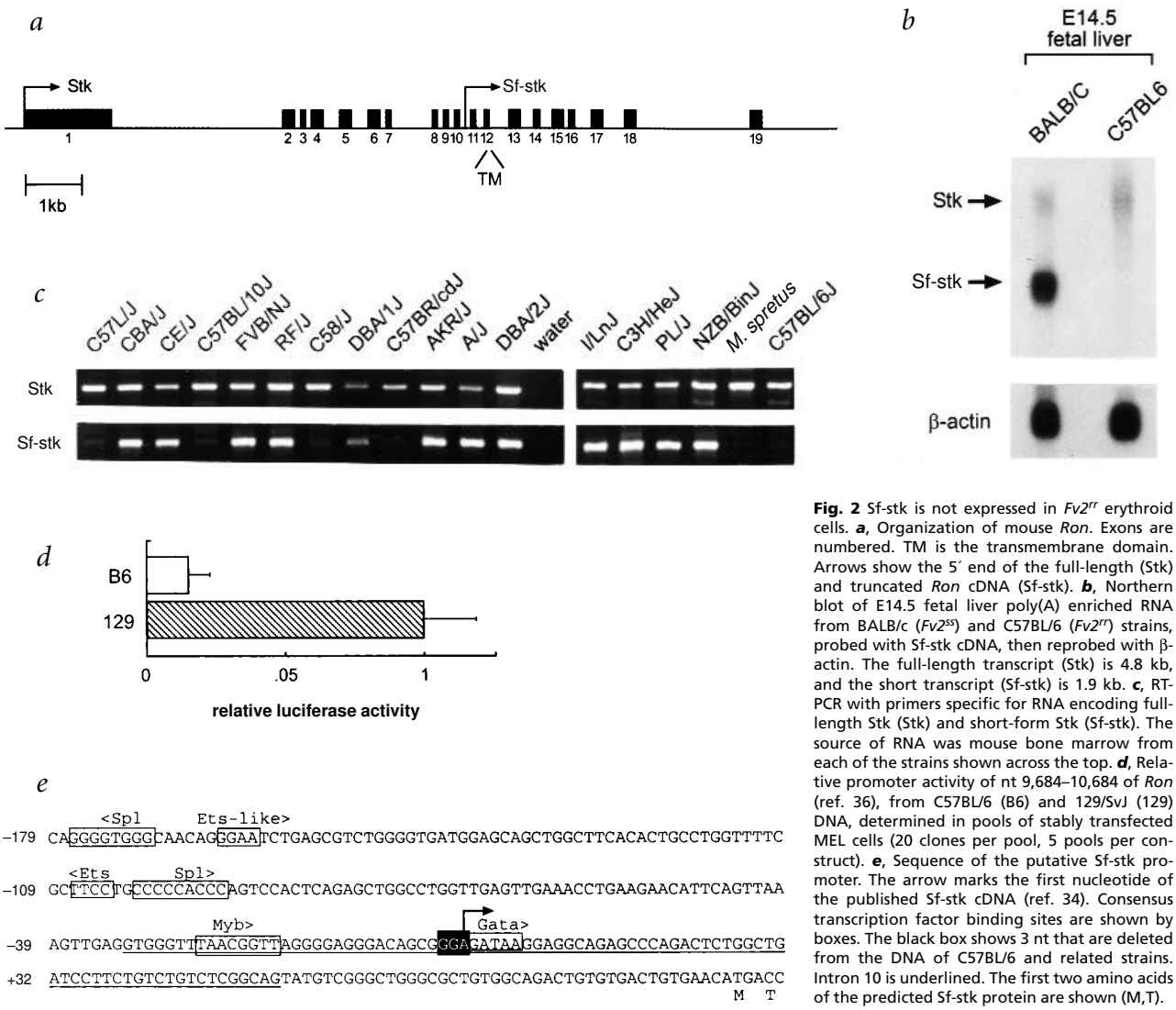

Fig. 2 Sf-stk is not expressed in *Fv2*^{rr} erythroid cells. ***a***, Organization of mouse *Ron*. Exons are numbered. TM is the transmembrane domain. Arrows show the 5′ end of the full-length (Stk) and truncated *Ron* cDNA (Sf-stk). ***b***, Northern blot of E14.5 fetal liver poly(A) enriched RNA from BALB/c (*Fv2*^{ss}) and C57BL/6 (*Fv2*^{rr}) strains, probed with Sf-stk cDNA, then reprobed with β-actin. The full-length transcript (Stk) is 4.8 kb, and the short transcript (Sf-stk) is 1.9 kb. ***c***, RT-PCR with primers specific for RNA encoding full-length Stk (Stk) and short-form Stk (Sf-stk). The source of RNA was mouse bone marrow from each of the strains shown across the top. ***d***, Relative promoter activity of nt 9,684–10,684 of *Ron* (ref. 36), from C57BL/6 (B6) and 129/SvJ (129) DNA, determined in pools of stably transfected MEL cells (20 clones per pool, 5 pools per construct). ***e***, Sequence of the putative Sf-stk promoter. The arrow marks the first nucleotide of the published Sf-stk cDNA (ref. 34). Consensus transcription factor binding sites are shown by boxes. The black box shows 3 nt that are deleted from the DNA of C57BL/6 and related strains. Intron 10 is underlined. The first two amino acids of the predicted Sf-stk protein are shown (M,T).

mouse erythroleukaemia (MEL) cells, the major expressed mRNA encodes a truncated form of the receptor, which starts in intron 10 (ref. 34). This short form of Stk (Sf-stk) lacks almost the entire extracellular domain, but retains the transmembrane and tyrosine kinase domains. In primary fetal liver cells, Sf-stk is the major expressed form of the gene (Fig. 2*b*). Notably, Sf-stk was expressed in the fetal liver cells of BALB/c (*Fv2*^{ss}), but not C57BL/6 (*Fv2*^{rr}), mice. To test the association between *Fv2* susceptibility and Sf-stk expression, we performed RT-PCR on bone marrow RNA from a panel of inbred strains of mice. Sf-stk was expressed in all *Fv2*^{ss} strains (Fig. 2*c*). In contrast, Sf-stk expression was decreased or absent in C57BL/6 (*Fv2*^{rr}) and related strains. Full-length Stk was expressed in all strains, regardless of *Fv2* status. Thus, *Fv2* susceptibility correlates with expression of Sf-stk in adult bone marrow cells, which are the target cells for Friend virus infection.

The strain-specific difference in Sf-stk expression we identified may be due to a difference in activity of an alternate Stk promoter. To examine this possibility, we linked 1 kb of DNA containing the putative Sf-stk promoter to the luciferase gene and stably transfected the construct into MEL cells. DNA from an *Fv2*^{ss} strain (129/SvJ) had promoter activity that was sixfold higher than that from an *Fv2*^{rr} strain (C57BL/6; Fig. 2*d*). This region contains consensus binding sites for Sp1, Ets, Myb and Gata proteins (Fig. 2*e*). Sequence analysis showed that three nucleotides were deleted from C57BL/6 DNA compared with 129/SvJ. This deletion included the 5′ end of the published Sf-stk cDNA, as well as part of a consensus WGATAR binding site[34]. To determine if this deletion was associated with resistance at the *Fv2* locus, we screened a panel of 48 different strains and substrains of mice. The deletion was present exclusively in C57BL/6 (*Fv2*^{rr}) and related strains. When the strain distribution pattern of this deletion was determined in the BXD series, it co-segregated with *Fv2*. Furthermore, when genomic DNA samples from a published backcross of 425 mice were screened for this deletion, it co-segregated with *Fv2* (ref. 29, and data not shown). These experiments confirm that *Fv2* and *Ron* are genetically tightly linked, and suggest that a 3-nt deletion in the Sf-stk promoter causes decreased Sf-stk expression and *Fv2* resistance in C57BL/6 and related strains.

Fv2 resistance was originally described in C57BL/6 mice but has also been reported in *Mus spretus*, wild-derived mice[37]. Because *M. spretus* are genetically distinct from C57BL/6 mice, this provided an opportunity to further test the correlation between Sf-stk expression and *Fv2* susceptibility. Sf-stk expression was decreased in *M. spretus* bone marrow cells (Fig. 2*c*). The three nucleotides that were deleted in C57BL/6 strain DNA were present in *M. spretus* DNA, but there was a point mutation in a

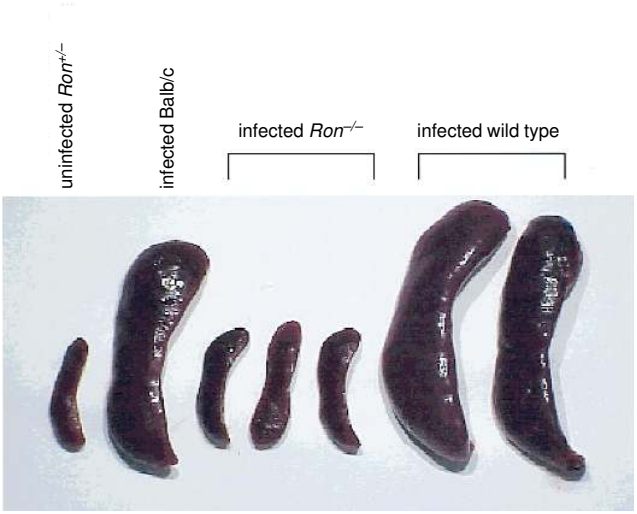

Fig. 3 Stk-deficient mice are resistant to Friend virus disease. Spleens are shown from control and Stk-deficient mice, 14 days after infection with FVP. The first spleen, on the left, is from an uninfected *Ron*$^{+/-}$ littermate control (0.11 g). The next is from an FVP-infected BALB/c mouse (1.31 g). The next three spleens are from FVP-infected *Ron*$^{-/-}$ mice (0.17, 0.17 and 0.18 g). The last two, on the right, are from FVP-infected wild-type littermate controls (2.51 and 1.81 g).

Myb consensus binding site (TAACGGTT→TAAAGGTT) that alters a contact for Myb binding[38]. Introduction of this mutation into 129/SvJ DNA reduced activity of the Sf-stk promoter to the level of C57BL/6 in MEL cells (data not shown). These results strengthen the correlation between Sf-stk expression and *Fv2* susceptibility and suggest that *M. spretus* have an allele of *Fv2*^{r} that is distinct from C57BL/6.

Stk is required for susceptibility to Friend disease

The absence of Sf-stk transcripts in C57BL/6 erythroid cells raised the possibility that Sf-stk expression is required for susceptibility to Friend virus infection, and hence that C57BL/6 mice are resistant because they fail to express the truncated Stk protein. To test this hypothesis, we examined the effect of Stk deficiency on susceptibility to Friend disease. We previously described mice with a targeted null mutation of the *Ron* locus[39]. Targeted mutation of exon 1 leads to an enhanced inflammatory response and deregulated activation of macrophages[39]. This mutation causes decreased Sf-stk expression, similar to the decrease in C57BL/6 mice (data not shown). We made the mutation on an *Fv2*^{ss} ([129/Sv×129/SvJ]F1) background and maintained the mice through backcrosses to an *Fv2*^{ss} strain (CD-1). Stk-deficient mice and littermate controls were injected with FVP. Stk-deficient mice were resistant to Friend virus-induced erythroblastosis, whereas wild-type littermate controls were fully sensitive (Fig. 3). Thus, Stk is required for susceptibility to Friend virus disease.

Sf-stk confers susceptibility to Friend disease

To determine if Sf-stk confers susceptibility to Friend disease, we introduced cDNA encoding Sf-stk into *Fv2*^{rr} bone marrow by retroviral-mediated gene transfer, and transplanted the transduced bone marrow cells into *Fv2*^{rr} mice. We subcloned Sf-stk cDNA into a bicistronic retroviral vector containing enhanced green fluorescent protein[40] (GFP). Retroviral producer cells expressed Sf-stk/GFP transcripts and Sf-stk protein (data not shown). When supernatant from these cells was used to transduce a polyclonal population of NIH3T3 cells, they became spindle-shaped and accumulated in a thick layer of cells but did not form foci. Similar changes have been described with expression of Tpr-Sea and Tpr-Ron in fibroblasts, indicating that our construct was expressing functional Sf-stk protein in target cells[41].

Bone marrow from C57BL/6 (*Fv2*^{rr}) mice was transduced with the Sf-stk/GFP retrovirus and transplanted into lethally irradiated C57BL/6 recipients[40]. We transduced a control group with a retrovirus that only expressed GFP. Experiments in chimaeric mice have shown that there is a threshold of 15–20% *Fv2*^{ss} cells that must be exceeded for Friend disease to develop[26]. T-cell depletion, however, can reveal the presence of a smaller *Fv2*^{ss}

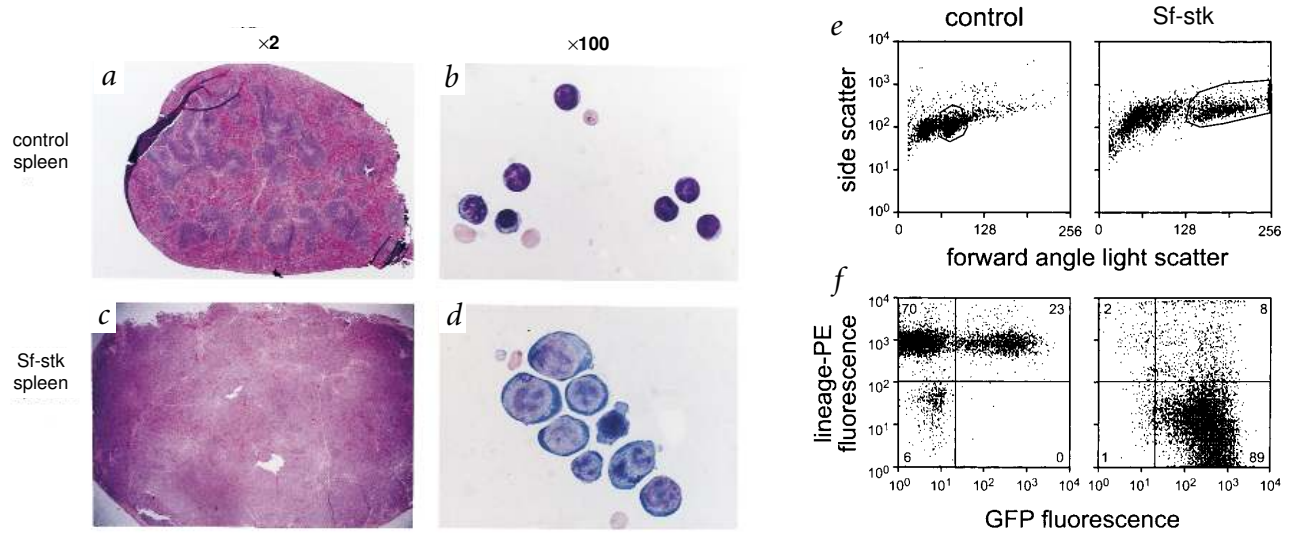

Fig. 4 Acute erythroblastosis in Sf-stk transplanted mice infected with FVP. ***a***,***c***, Cross-sections of spleens from control and Sf-stk transplanted mice, 17 d after infection with FVP (stained with haematoxylin and eosin, ×2 magnification). ***b***,***d***, Cytospin preparations of the same specimens (stained with Wright-Giemsa, ×100 magnification). *e*,*f*, Friend virus infection causes selective expansion of Sf-stk/GFP-expressing cells. FACS analysis was performed on splenic cells from control and Sf-stk mice 17 days after infection with FVP. ***e***, Graphs of forward versus side scatter. The gates were selected to include the predominant cell populations; events to the far left are caused by cellular debris. ***f***, Graphs of lineage expression versus GFP expression in the gated populations. The cells were stained with a combination of lineage markers that included Thy1.2, CD45R, CD11b and Gr-1.

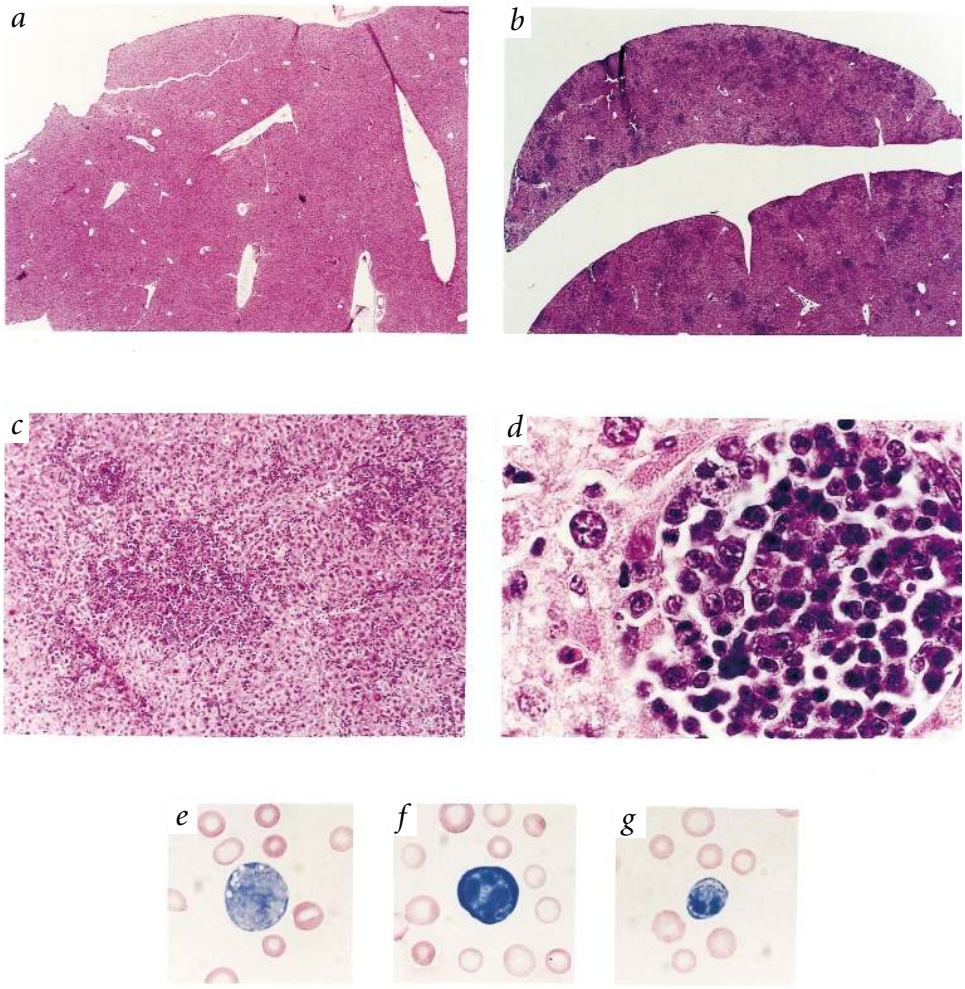

Fig. 5 Erythroleukaemia in Sf-stk transplanted mice infected with FVP. Representative specimens are shown from control and Sf-stk transplanted mice, two months after infection with FVP. ***a***, Cross-section of liver from a control transplanted mouse (stained with haematoxylin-eosin, ×2 magnification). ***b***,***c***,***d***, Cross-sections of liver from an Sf-stk transplanted mouse, showing intrasinusoidal spread of immature erythroblasts (×2, ×10 and ×100 magnification, respectively). ***e***,***f***, Poorly differentiated erythroblasts, and Friend cell (***g***) as described[44] (stained with Wright-Giemsa, ×100 magnification).

population[42]. Therefore, we T-cell depleted Sf-stk and control mice with anti-Cd4 and anti-Cd8 antibodies. We evaluated one-half of the mice from each group for the acute phase of disease 17 days after infection with FVP. Spleens from the Sf-stk transplanted mice were enlarged compared with controls (Sf-stk, 0.74±0.15 g, n=6; GFP control, 0.15±0.06 g, n=7; Sf-stk (no FVP), 0.06±0.01 g, n=3). Sections of spleens from control mice showed focal areas of erythroblast proliferation with preservation of the splenic architecture (Fig. 4*a*). In contrast, spleens from Sf-stk mice were effaced with rapidly proliferating, immature erythroblasts. By fluorescence-activated cell sorting (FACS) analysis, the cells in the spleens of infected control mice were small and positive for lineage markers (myeloid, lymphoid or macrophage; Fig. 4*e*,*f*); 23–44% were GFP positive. In contrast, the predominant cell type in the spleens of infected, Sf-stk transplanted mice were large and lineage negative. These cells were approximately 98% GFP positive. GFP and lineage marker expression in the residual population of small cells in these spleens was comparable to controls. Histochemical staining of spleen sections from Sf-stk transplanted mice for GFP showed diffuse staining of blastic cells (data not shown). These results indicate that Friend virus infection caused selective expansion of immature, Sf-stk/GFP-expressing erythroblasts.

In the second phase of Friend disease, fully transformed clones emerge[3], and by 2–3 months almost all infected mice have died of leukaemia[1]. At two months, the control mice in our series showed moderately elevated haematocrits (61±7%, n=8), suggestive of an indolent form of Friend disease. This was likely due to T-cell depletion, which permits continued replication of the virus in $Fv2^{rr}$ mice[43]. Otherwise, these mice were healthy. In contrast, 6 of 9 Sf-stk transplanted mice died or appeared ill. These mice had severe anaemia (15±4%) and leukaemic involvement of the liver (Fig. 5). Leukocyte counts were not elevated, but the blood contained cells that are typical of Friend erythroleukaemia, including large immature erythroblasts, Friend cells and basophilic fragments of cytoplasm[44]. Thus, Friend virus-infected, Sf-stk transplanted mice developed acute erythroblastosis that rapidly progressed to erythroleukaemia.

Discussion

In this study we demonstrated that a truncated form of the Stk receptor tyrosine kinase, encoded by the *Fv2* locus, is required for susceptibility to Friend virus-induced erythroleukaemia. Our evidence is as follows: (i) *Ron* and *Fv2* co-segregate in a backcross of 425 mice; (ii) expression of a truncated form of Stk in erythroid cells correlates with susceptibility to Friend disease; (iii) mice with targeted disruption of *Ron* are resistant to Friend disease; and (iv) retroviral-mediated introduction of Sf-stk into $Fv2^{rr}$ bone marrow cells confers susceptibility to erythroleukaemia induction by Friend virus. We conclude that the *Fv2* locus encodes *Ron*, and that a naturally expressed, truncated form of Stk confers susceptibility to Friend disease in most inbred strains of mice.

Two mechanisms have been proposed to explain *Fv2*-mediated susceptibility. The first, based on the effect of *Fv2* on killing of BFU-E by hydroxyurea or ^{3}H-thymidine, is that *Fv2*

regulates the cell cycle of target cells for Friend virus infection[45]. We have evidence that cell-cycle control of BFU-E and susceptibility to Friend virus are encoded by distinct but closely linked genes at the *Fv2* locus (R.F.P., manuscript in preparation). The second model, based on the isolation of deletion mutants of gp55 that cause Friend-like disease in $Fv2^{rr}$ mice, is that *Fv2* encodes a component of the Epor signalling complex[27]. It has been proposed that the $Fv2^{r}$ allele encodes a molecule that blocks the interaction of gp55 with the Epor, and that this can be circumvented by deletions in the ecotropic domain of gp55 (ref. 46). Our results agree with this model; however, they indicate that the $Fv2^{s}$ allele encodes a positive-acting molecule, Sf-stk, which is lacking in $Fv2^{rr}$ erythroid cells.

A comparison of *Ron* to the avian oncogene v-*sea*, which causes erythroblastosis and anaemia in chickens, may provide insight into the mechanism of action of Sf-stk. v-sea is a transmembrane protein with an extracellular domain that is related to the envelope protein of an avian retrovirus and a cytoplasmic tyrosine kinase domain that is related to Sf-stk. The structure of v-sea suggests that activation of the Epor by gp55 may depend on the formation of gp55/Sf-stk complexes that are functionally equivalent to v-sea. Sf-stk might exist in pre-assembled Epor/Sf-stk complexes, that undergo gp55-mediated oligomerization. Alternatively, Sf-stk may be recruited to the Epor by gp55. If Sf-stk, gp55 and the Epor are present in a trimeric complex, crosstalk could occur between Sf-stk and the Epor as well as other proteins associated with the complex (for example, Jak2). Recently, it has been shown that Stk co-localizes with and activates the common β-chain of the Il-3 receptor, demonstrating that there are productive interactions between Stk and members of the cytokine receptor family[47]. Additional experiments are planned to determine whether Sf-stk associates with the Epor, and whether this leads to receptor activation.

The requirement of Sf-stk for gp55-mediated activation of the Epor suggests that Stk might have a role in normal Epor signalling. Stk is a heterodimeric receptor for macrophage-stimulating protein/hepatocyte growth factor-like protein[48] (MSP/HGFL). Mice with targeted disruption of the first exon of *Ron* are viable and developmentally normal, but peritoneal macrophages from these mice produce elevated levels of nitric oxide in response to interferon-γ, and they are susceptible to endotoxic shock[39]. Under normal conditions, this mutation has no discernable effect on erythropoiesis. In response to phenylhydrazine-induced anaemia, however, expansion of BFU-E is impaired (P.H.C., unpublished data). This suggests that Stk has a role in the response to erythropoietic stress which is exploited by Friend virus in the early stages of infection.

Although the precise function of Stk in normal erythropoiesis remains to be determined, it is likely that Stk has a role in Epor signalling. In this regard, it is interesting that *Kit*, another host gene that regulates susceptibility to Friend virus, also encodes a receptor tyrosine kinase (Kit) essential for normal haematopoiesis[49]. Stem cell factor and Epo, the activating ligands for the Kit and Epo receptors, respectively, are potent co-mitogens for early erythroid progenitor cells, suggesting the possibility of crosstalk between the signalling pathways controlled by these receptors. The identification of additional genes that regulate the progression of Friend erythroleukaemia should continue to provide insights into the signalling pathways that regulate normal and leukaemic haematopoiesis.

Methods

Mapping and cloning the *Fv2* interval. To determine the strain distribution pattern of *Fv2*, we injected mice from the BXD series of recombinant inbred mice with 1.9×10^3 colony forming units of FVP into the tail vein. On day 9 post-injection, we killed the mice by cervical dislocation and determined the weight of their spleens. We obtained recombinant inbred mice from The Jackson Laboratory. We passaged the FVP (gift fromM. Bondurant) once in BALB/cByJ mice, and diluted the plasma (1:20) with Iscove's modified Dulbecco's medium for injection. To determine the strain distribution pattern of SSLPs, we performed PCR with primers obtained from Research Genetics and genomic DNA obtained from the Mouse DNA Resource Laboratory of The Jackson Laboratory. PCR conditions were 3 min at 94 °C for 1 cycle; 30 s at 94 °C, 1 min at 55 °C and 1 min at 72 °C for 35 cycles; and 7 min at 72 °C for 1 cycle. We resolved the PCR products by polyacrylamide gel electrophoresis. We mapped the 3-bp deletion of Stk on the *Fv2* backcross panel with primers 5′–GGTGGGTTTAACGGTTAGGG–3′ and 5′–TCTGGGCTCTGCCTCCTTAT–3′. PCR conditions were as described[29]. To clone the *Fv2* interval, we screened a 129/SvJ genomic DNA BAC library (Genome Systems) with primers to *D9Mit359*. We chromosome walked to the nearest recombinant markers by designing probes to the ends of BACs and rescreening the library. In addition, we screened the library with markers from the YAC contig WC-1111.

Analysis of *Ron* expression. To examine Stk expression, we obtained poly(A) enriched RNA from E14.5 fetal liver cells using oligo dT cellulose (Ambion). We analysed RNA (~10 μg) by northern blot, probing with SF-Stk cDNA, and re-probing with β-actin. We performed RT-PCR on total RNA (1 μg) from the bone marrow cells of different strains of mice. PCR primers to sequences encoding the extracellular domain of Stk were 5′–CAGCAGTGGACAGCCTGTTCA–3′ and 5′–ATGCCTTCCACTCGGAAGTGC–3′ (542-bp product). Sf-stk–specific PCR primers were 5′–TCTGGCTGATCCTTCTGTCTG–3′ and 5′–GCAGCAGTGGGACACTTGTCC–3′ (456-bp product). PCR conditions were 3 min at 94 °C for 1 cycle; 30 s at 94 °C, 1 min at 65 °C, and 1 min at 72 °C for 40 cycles; and 7 min at 72 °C for 1 cycle. We resolved the PCR products on a 2% agarose gel containing ethidium bromide (1 μg/ml).

Retroviral-mediated gene transfer. We subcloned SF-Stk cDNA into the first reading frame of the retroviral vector MSCVirGFP. We made SF-Stk/GFP producer cells as described[40]. The viral titer of conditioned media from SF-Stk/GFP producer cells was 1×10^6 particles/ml, assessed by transfer of GFP to NIH 3T3 cells. Southern-blot analysis demonstrated multiple copies of unrearranged provirus in the producer line, and unrearranged proviral transmission to NIH 3T3 target cells. We used the retroviral vector MSCVirGFP, which only expresses the GFP marker, as a control in the transplantation experiments. We harvested bone marrow from 8–12-week-old female C57BL/6 mice, 2 d after treatment with 5-fluorouracil (150 mg/kg). We stimulated bone marrow cells for 48 h with mouse Il-3 (20 ng/ml), human IL-6 (50 ng/ml) and mouse stem cell factor (50 ng/ml) in Dulbecco's modified Eagle's medium supplemented with 15% heat-inactivated FBS. Next, we co-cultured bone marrow cells for 48 h with irradiated (1,200 cGy) viral producer cells in the same culture medium supplemented with polybrene (6 μg/ml). We rinsed non-adherent bone marrow cells off the viral producer cell monolayers, and resuspended the cells in phosphate buffered saline containing 2% FBS. We injected 5×10^6 cells into the tail vein of lethally irradiated, recipient mice. Three to five months post-transplant, we T-cell depleted mice by intraperitoneal injection of anti-Cd4 and anti-Cd8 antibody cocktail, every other day, for a total of 11 injections[50]. On the day of the third anti-T-cell injection, we injected the mice with FVP.

Analysis of mice. To analyse spleen cells, we made a single cell supension by passing splenic tissue through a 70 μm nylon mesh and pipetting. We performed flow cytometry with a FACS Calibur, as previously described[40]. We made cytospins with a Cyto-Tek centrifuge (Miles Scientific). We obtained blood from anesthetized mice by retro-orbital puncture for morphology and haematocrit determination. We obtained sections of spleen, liver, kidney, heart, lung, thymus and bone marrow for routine and histochemical (GFP) staining.

Stk-deficient mice. Stk-deficient mice have been described[39]. Stk expression is disrupted by partial deletion of the first exon, and insertion of *lacZ* and a neomycin resistance cassette. We injected Stk-deficient mice and littermate controls with FVP at 5 months of age. We killed mice 14 d after injection with FVP to evaluate their spleens.

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