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Structure of a pseudokinase-domain switch that controls oncogenic activation of Jak kinases

Angela V Toms[1,2], Anagha Deshpande[3,4], Randall McNally[1,2], Youngjee Jeong[1], Julia M Rogers[2,5], Chae Un Kim[6], Sol M Gruner[6,7], Scott B Ficarro[1,2,8], Jarrod A Marto[1,2,8], Martin Sattler[3,4], James D Griffin[3,4] & Michael J Eck[1,2]

The V617F mutation in the Jak2 pseudokinase domain causes myeloproliferative neoplasms, and the equivalent mutation in Jak1 (V658F) is found in T-cell leukemias. Crystal structures of wild-type and V658F-mutant human Jak1 pseudokinase reveal a conformational switch that remodels a linker segment encoded by exon 12, which is also a site of mutations in Jak2. This switch is required for V617F-mediated Jak2 activation and possibly for physiologic Jak activation.

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The receptors for diverse cytokines, including erythropoietin (EPO), growth hormone, interleukins and interferons, initiate intracellular signaling through one or more members of the Jak family of non-receptor tyrosine kinases[1,2]. Jak kinases contain a FERM domain, an SH2-like domain and a pseudokinase domain (also referred to as the Jak homology 2, or JH2, domain) adjacent to their C-terminal tyrosine-kinase domain (**Fig. 1a**). The pseudokinase domain has a protein-kinase fold, but key catalytic residues are not conserved, and the domain is thought to inhibit the adjacent tyrosine-kinase domain, perhaps through an intramolecular interaction[3]. Somatic gain-of-function mutations in Jaks underlie a number of hematologic malignancies[4], and the pseudokinase domain is the most frequent site of these activating mutations[5]. In particular, the V617F mutation in the Jak2 pseudokinase is found in ~95% of people with polycythemia vera (PV)[6–9], a myeloproliferative neoplasm characterized by EPO-independent overproduction of red blood cells due to constitutive signaling from Jak2 V617F in complex with the erythropoietin receptor (EPO-R)[10]. The corresponding mutation in Jak1 (V658F) has been identified in T-cell acute lymphoblastic leukemia[11] and also leads to constitutive catalytic activation[12]. A subset of PV-affected people who are V617F negative have mutations in exon 12 of Jak2 (refs. 13,14), which encodes a portion of the polypeptide chain that links the SH2-like and pseudokinase domains (SH2-PK linker).

To better understand the pseudokinase domain and the effects of pathogenic mutations, we determined crystal structures of a wild-type and V658F-mutant fragment of human Jak1 spanning the pseudokinase domain and a segment of the SH2-PK linker (residues 561–852). The wild-type and mutant structures were refined at resolutions of 1.8 Å and 1.9 Å, respectively (**Supplementary Table 1** and Online Methods). The overall architecture of the pseudokinase domain closely resembles that of typical tyrosine kinases, with an N-terminal lobe composed of five β-strands and the C helix, and a larger C-terminal lobe (**Fig. 1b**). Several features of the pseudokinase domain appear to be inconsistent with phosphotransfer activity. In particular, the region corresponding to the kinase activation loop adopts a well-defined conformation that is expected to preclude binding of polypeptide substrates (**Supplementary Fig. 1**). At the N terminus of the domain, the SH2-PK linker adopts a partially α-helical conformation and extends across the N lobe roughly perpendicular to the C helix. The Jak1 domain superimposes well with a recently reported structure of the Jak2 pseudokinase[15] (r.m.s. deviation 1.26 Å for 261 equivalent residues; **Fig. 1c**), but the Jak2 structure lacks the SH2-PK linker, which was truncated in the crystallized construct.

Val658 lies within the N lobe of the domain, at the end of strand β4. In the V658F mutant, the β4–β5 loop folds inward toward the C helix, and the mutant Phe658 packs in an edge-to-face manner with Phe636 in the C helix (**Fig. 2a**). Strikingly, the phenyl ring of Phe658 occupies precisely the same position occupied by Phe575 in the wild-type structure. Phe575 lies in the SH2-PK linker, and the V658F mutation induces a coordinated rearrangement of this segment. In the mutant, Phe575 is shifted by ~10 Å and becomes solvent exposed. Both the V658F and wild-type crystals contain two molecules in the asymmetric unit, and interestingly, we observe essentially the same rearrangement in molecule 'B' in the wild-type structure that we observe in the V658F mutant, results demonstrating that the wild-type pseudokinase domain can also adopt the Phe575-'flipped' conformation (**Fig. 2b**). In the V658F-mutant structure, both the 'A' and B molecules adopt the flipped conformation with Phe575 exposed. The electron density for the linker segments is clear and continuous in both the wild-type and V658F structures (**Supplementary Fig. 2a**,**b**).

Three residues appear to be centrally important for the observed rearrangement: Phe575 in the SH2-PK linker, Phe636 in the C helix and V658F, the site of the myeloproliferative-neoplasm mutation (V617F site). We refer to these three residues as the F-F-V triad. This triad is highly conserved in Jak-family kinases, and these residues appear to be evolutionarily 'coupled'; that is, substitutions in one position are almost invariably accompanied by changes in one of the

[1]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts, USA. [2]Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA. [3]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA. [4]Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA. [5]Committee on Higher Degrees in Biophysics, Harvard University, Cambridge, Massachusetts, USA. [6]Cornell High Energy Synchrotron Source, Cornell University, Ithaca, New York, USA. [7]Physics Department, Cornell University, Ithaca, New York, USA. [8]Blais Proteomics Center, Dana-Farber Cancer Institute, Boston, Massachusetts, USA. Correspondence should be addressed to M.J.E. (eck@red.dfci.harvard.edu).

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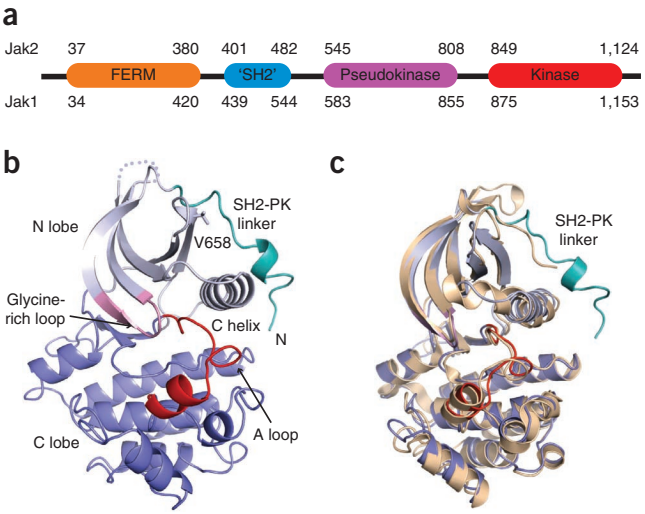

Figure 1 Structure of the Jak1 pseudokinase domain. (**a**) The domain structure of Jak-family kinases. 'SH2', SH2-like. (**b**) Structure of the Jak1 pseudokinase domain, with key structural motifs indicated. (**c**) The Jak1 pseudokinase domain superimposed on the structure of Jak2 pseudokinase domain (tan).

other residues in the triad (**Supplementary Data Set 1**). Interestingly, human Jak3 has a methionine at the V617F site, and it is the only human Jak kinase that is not activated by phenylalanine mutations at this site[12]. This pattern of conservation of residues composing the F-F-V triad, combined with our observation that the wild-type pseudokinase domain can also adopt the flipped conformation, leads us to speculate that the observed rearrangement is part of a structural switch controlling catalytic activation both in wild-type Jak kinases and in the context of the V617F-site mutation. Consistent with a model in which the mutation promotes a rearrangement related to that induced by cytokine, cells bearing Jak2 V617F are hyperresponsive to EPO, and even in the absence of EPO stimulation they proliferate at a rate equivalent to that of EPO-stimulated cells bearing wild-type Jak2[8]. In contrast, pseudokinase-domain deletions result in constitutive but diminished catalytic activation, and deletion mutants are not further stimulated by cytokine[3].

To examine the requirement for the observed phenylalanine-flipped rearrangement in Jak activation, we introduced point mutations in the SH2-PK linker and F-F-V triad and tested their effect on the ability of the V617F mutation in Jak2 to promote interleukin 3 (IL-3)-independent growth of Ba/F3 cells. We carried out this structure-function analysis in Jak2 because of the availability of the well-characterized Ba/F3 cell system (Ba/F3.EPO-R) in which the EPO-R is coexpressed with mutant Jak2 to allow its proper folding and maturation[16]. In the SH2-PK linker, mutation of the flipped phenylalanine (Phe537 in Jak2) to either valine or alanine abrogated V617F-driven proliferation (**Fig. 2c** and **Supplementary Fig. 3**). Residue Leu573 forms hydrophobic interactions that appear to stabilize the helical segment of the SH2-PK linker, and mutation of the corresponding residue in Jak2 from methionine to alanine (M535A) also reversed V617F-driven proliferation. In contrast, a conservative M535F substitution at this position was without effect (**Fig. 2c**). Phe636 in the C helix forms the platform for the observed rearrangement, and we found that mutation of the corresponding residue in Jak2 (Phe595) to alanine also abrogated the transforming effect of V617F (**Fig. 2c**), as previously observed[17,18]. The V615F mutation in Jak2, as compared with V617F alone, modestly enhanced proliferation, consistent with a role in promoting the SH2-PK linker rearrangement, but V615F was not activating on its own (**Fig. 2c** and **Supplementary Fig. 4f**). Collectively, the effects of these mutations support a role for the phenylalanine-flipped rearrangement in V617F-driven Jak2 activation. Analysis of these mutations in the context of wild-type Jak2 reconstituted in Jak2-deficient cells will be required to establish whether the rearrangement is also required for EPO-driven activation of Jak2.

We also probed a crystal-packing interaction between the two molecules in the asymmetric unit. This noncrystallographic dimer buries a large surface area (~2,080 Å^2) and involves both V658F and Phe575 in the SH2-PK linker, but we found no role for this interface in Jak regulation (**Supplementary Fig. 4**).

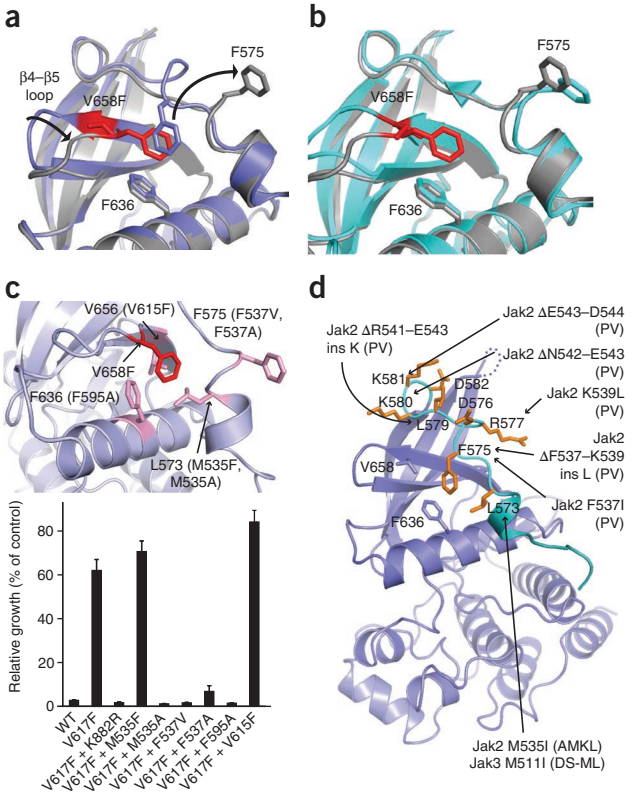

Figure 2 Crystal structure of the V658F-mutant Jak1 pseudokinase domain. (**a**) Superposition of wild-type Jak1 pseudokinase (blue) on V658F mutant (gray). Side chains of the F-F-V triad residues are shown. The mutant residue V658F displaces Phe575, rearranging the SH2-PK linker. (**b**) Superposition of crystallographic molecule B of the wild-type Jak1 pseudokinase (cyan) on the V658F mutant (gray), showing that the wild-type pseudokinase can adopt essentially the same conformation observed in the V658F mutant. (**c**) Mutagenesis of the linker-pseudokinase switch in Ba/F3 cells. Mutations were introduced in mouse V617F Jak2 in residues that appear to support the observed rearrangement of the F-F-V triad (top) and tested for their effect on IL-3–independent proliferation in Ba/F3 cells (bottom). Locations of mutations are illustrated on the Jak1 structure, with the mutations in the corresponding Jak2 residues in parentheses. K882R is a control mutation that renders the Jak2 kinase domain catalytically inactive. Error bars, s.d. of triplicate experiments. WT, wild type. Corresponding line graphs of daily proliferation measurements are presented in **Supplementary Figure 3**. (**d**) Patient-derived exon 12 mutations in Jak2 or Jak3 mapped on the Jak1 structure. For deletions, the first altered residue is indicated; ins denotes residues inserted at the site of the deletion. The clinical presentation associated with each alteration is abbreviated in parentheses (PV, polycythemia vera; AMKL, acute megakaryoblastic leukemia; DS-ML, Down Syndrome–associated myeloid leukemia).

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Somatic gain-of-function mutations in exon 12 of Jak2 map to the SH2-PK linker, and an activating mutation in Jak3 is also found in this region (**Fig. 2d**). These alterations are expected to disrupt the observed conformation of the linker and may promote repositioning of the β4–β5 loop, as we observe in the Jak1 V658F mutant (**Supplementary Fig. 2c**,**d**). Such a shift in the β4–β5 loop would in turn require rearrangement of the linker segment. Interestingly, an *in vitro* screen with Jak2 identified similar activating point mutations in the SH2-PK linker, prompting the conclusion that the SH2-PK linker is critical for mediating autoinhibition and cytokine-induced activation of Jak2 (ref. 19).

While this manuscript was under review, a structure of the wild-type Tyk2 pseudokinase domain was released in the Protein Data Bank (PDB 3ZON). The Tyk2 structure does include the SH2-PK linker and is very similar overall to the wild-type Jak1 structure described here (r.m.s. deviation 1.15 Å for 258 aligned residues); however, the linker conformation differs in detail (**Supplementary Fig. 5**). In Tyk2, the linker takes a similar path across the N lobe but does not adopt a helical conformation. Despite this difference, Phe581 (the equivalent of Phe575 in Jak1) is also in contact with the phenylalanine in the C helix and would also be expected to rearrange in the context of the V617F-site mutation. It is unclear whether the difference in linker conformation represents a genuine difference between Jak1 and Tyk2 or whether it may arise from crystal-packing interactions in this region in one or both structures.

In the Jak2 pseudokinase domain, stabilization of the C helix is proposed to mediate in part the activating effect of the V617F mutation[15]. The C helix is three residues longer at its N terminus in the V617F mutant, and a break in regular hydrogen-bonding near the center of the helix is also 'repaired' in the V617F structure. This break in the C helix appears to be unique to the Jak2 wild-type structure, as the C helix is continuous in all of our Jak1 structures and in Tyk2. However, we do observe a corresponding difference in C-helix length in molecule A compared to molecule B in the Jak1 pseudokinase structures, irrespectively of the presence of the V658F mutation. In the Tyk2 pseudokinase domain, the longer C helix is also observed in the wild-type context. Thus this is a conformationally labile region of the pseudokinase domain, but further work will be required to determine how alternate conformations of this segment may relate to Jak regulation.

Despite the lack of conserved active site residues, the Jak2 pseudokinase domain is reported to have catalytic activity and to autophosphorylate both Ser523 and Tyr570 (ref. 20). These are autoinhibitory phosphorylation sites, and the V617F mutation has been proposed to promote Jak2 activation in part by abrogating pseudokinase catalytic activity and therefore phosphorylation of these sites[15,20]. The Ser523 and Tyr570 phosphorylation sites are not conserved in Jak1 or in other family members, and we do not observe autophosphorylation of the Jak1 pseudokinase domain at alternate sites (**Supplementary Fig. 6**). Furthermore, the pseudokinase activation loop in Jak1 and Jak2, in both wild-type and V617F-site mutants, adopts a conformation that blocks the expected position of a phosphoacceptor (**Supplementary Fig. 1e**). Although we do not dismiss the possibility that catalytic activity of the pseudokinase domain has a role in regulation of Jak2 and perhaps other family members, we favor a model in which the V617F mutation promotes activation primarily through rearrangement of the pseudokinase domain and SH2-PK linker, as described here. The F-F-V triad, which is central to this rearrangement, is highly conserved among all Jak kinases. Furthermore, this model provides a unifying explanation for the activating effects of both the V617F and exon 12 mutations in myeloproliferative disorders.

An obvious outstanding question is how the rearrangement we observe here leads to activation of the adjacent tyrosine-kinase domain. The remodeled surface of the linker and pseudokinase unit could destabilize autoinhibitory interactions within the intact Jak kinase and also potentially promote activation by favoring dimerization or intramolecular interaction with the kinase domain proper. Structural studies of longer fragments of Jak including at a minimum both the pseudokinase and kinase domains will be required to definitively address this question at a structural level.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Coordinates and structure factors have been deposited in the Protein Data Bank, under accession codes 4L00 and 4L01.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.V.T., A.D., R.M. and J.M.R. designed and performed experiments and analyzed data. Y.J. carried out experiments. S.M.G. and C.U.K. contributed pressure cryocooling, and S.B.F. and J.A.M. contributed MS analysis. A.V.T., R.M., M.S., J.D.G. and M.J.E. designed experiments, analyzed data, and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Protein expression and purification. Constructs spanning residues 561–852 of human Jak1 bearing the wild-type sequence or V658F mutation were subcloned into a modified pTriEx vector (Novagen) for expression as N-terminal His_6 plus glutathione S-transferase (GST) fusion proteins with a tobacco etch virus (TEV) protease recognition sequence. The recombinant baculoviruses were generated with the BacVector3000 system (Invitrogen) and subsequently plaque purified. For induction of protein expression, the BIICs/TIPS method was used[21]. Hi5 cells were cultured in shaker flasks with Express Five serum-free medium (Invitrogen), and at a cell density of ~1.4 million/mL, cultures were infected with recombinant baculovirus. Infection was allowed to proceed for 60–66 h to achieve optimal expression of target protein before harvest by centrifugation. The cells were washed once in 20 mM Tris, pH 8.0, 150 mM NaCl and 5% glycerol and flash frozen in liquid nitrogen and stored at −80 °C.

For lysis, cells were thawed, resuspended in lysis buffer (100 mM Tris, pH 8, 300 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, 1% Nonidet P-40 and Roche Complete EDTA-free Protease Inhibitor Cocktail) and incubated on ice for 45 min. Cell debris was removed by centrifugation, and the supernatant was incubated for 45 min at 4 °C with Ni-NTA agarose (Qiagen). Beads were washed with a step gradient of Ni wash buffer (20 mM Tris, pH 8, 500 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol and 0.02% CHAPS) containing 0 mM, 10 mM, 20 mM or 40 mM imidazole and then eluted with elution buffer (Ni wash buffer with 250 mM imidazole). The eluate was supplemented with 5 mM DTT and then incubated with glutathione Sepharose (GE Healthcare) beads for 2 h at 4 °C. The beads were washed and protein eluted by GST elution buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 10% glycerol, 2.5 mM TCEP, 5 mM glutathione and 0.02% CHAPS). TEV protease was then added, and digestion was carried out at 4 °C overnight to remove the His_6-GST tag. The digested protein was supplemented with 15 mM imidazole and passed over a Ni-NTA agarose (Qiagen) column to remove His_6-GST tag and His_6-tagged TEV protease. The flow through was concentrated and run on a Superdex 200 gel-filtration column equilibrated in final buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 10% glycerol, 4 mM DTT and 0.02% CHAPS). The eluted peak was concentrated to 4 mg/ml, flash frozen in liquid nitrogen and stored at −80 °C.

Crystallization and structure determination. Crystals of both the wild-type and V658F Jak1 pseudokinase were grown by hanging-drop vapor diffusion at 20 °C by mixture of equal volumes of protein solution with a well solution consisting of 1.2–1.5 M sodium citrate (pH 6.3–6.6), 6 mM DTT and 0–30 mM NDSB 256. Before data collection, crystals were coated in NVH oil and then high-pressure cryocooled[22] or directly plunge frozen in liquid nitrogen. Data for Jak1 WT were collected at CHESS on beamline A1. Data for Jak1 V658F were collected at the Advanced Photon Source on beamline 24-C. Diffraction data were processed with the HKL suite[23]. The structure was determined by molecular replacement with the C lobe of focal adhesion kinase (PDB 1MP8 (ref. 24)) as a search model. The model was fit to the crystallographic data by manual model building with Coot[25] and refinement with CNS[26,27] and REFMAC[28,29], and that of the Jak1 WT structure was ultimately refined to a crystallographic R value of 18.2 (R_{free} = 20.9). Analogous procedures were used to obtain the structure of the V658F mutant.

Mass spectrometry. The Jak1 pseudokinase domain (residues 561–852) was incubated with 300 units/mL calf intestinal phosphatase (CIP) (New England Biosciences) at 37 °C for 30 min or 1 mM ATP and 10 mM $MgCl_2$ at 37 °C for 30 min followed by 300 units/mL CIP at 37 °C for 30 min. Treated and untreated Jak1 pseudokinase (5 μg) were injected onto a self-packed reversed-phase column (1/32-inch O.D. × 500 μm I.D. PEEK tubing with 5 cm of POROS 10R2 resin). After a desalting procedure, protein was eluted with an HPLC gradient (0–100% B in 1 min, A = 0.2 M acetic acid in water; B = 0.2 M acetic acid in acetonitrile; flow rate, 10 μL/min) into an LTQ ion-trap mass spectrometer (Thermo Fisher Scientific). Data were acquired in profile mode scanning *m/z* 300–2,000. Mass spectra were deconvoluted with MagTran1.03b2 (ref. 30).

Autoradiography. Jak1 pseudokinase at a concentration of 1 mg/mL was incubated at room temperature with 1 μCi [γ-^{32}P]ATP in a reaction buffer containing 20 mM Tris, pH 8.0, 300 mM NaCl, 10% glycerol, 4 mM DTT, 1 mM ATP and 10 mM $MgCl_2$ or $MnCl_2$ and then quenched with the addition of 100 mM EDTA. The autophosphorylation of EGFR kinase domain was measured under identical conditions as a positive control for detection of phosphorylation. Phosphorylation was monitored by SDS-PAGE and subsequent autoradiography.

Analysis of Jak2 mutants in Ba/F3 cells. Structure-function studies of Jak2 mutants in Ba/F3 cells bearing the EPO receptor were carried out essentially as previously described[31]. Briefly, point mutations were introduced in the pMSCV. JAK2V617F expression vector by site-directed mutagenesis with the QuikChange II XL Mutagenesis Kit (Agilent) and specific primers constructed. The sequence of the entire mutagenized cDNA was verified by DNA sequencing. Mouse Ba/F3 cells expressing EPO-R (Ba/F3.EPO-R) were maintained in RPMI 1640 containing 10% FBS and WEHI-3B conditioned medium. Ba/F3.EPO-R cells were transduced with the mutagenized Jak2 V617F with retroviral supernatant (293T cell method) by spin infection. Cells were grown for 2 d and sorted for GFP-positive cells. Proliferation was measured by growth of cells in 1-mL cultures with or without IL-3 for 3 d. The number of viable cells was determined daily with trypan blue exclusion.

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