suggest that there is space for up to three extended chains within the chamber. Second, little difference in the rates of endoproteolytic cleavage of these disordered substrates was detected between latent 20S and active 26S proteasomes (Figs. 1 to 4) in which the status of the gate that controls entry to the central axial channel of the proteasome is closed and open, respectively (Fig. 1C) (24, 30). Physiological regulators of the proteasome, such as PA700 (19S cap) and PA28, increase proteasome activity in part by opening this gate, thereby increasing access of substrates to the proteasome’s catalytic centers (24, 25). The ability of closed, latent 20S proteasome to catalyze cleavage of these natively disordered, physiological substrates suggests they possess certain features that also promote “gating” of the proteasome (Fig. 4E), features that folded proteins lack. This mechanism suggests a potential role for the free 20S proteasome found in the absence of bound regulatory proteins in many cells (33). It is possible that these inherent signals could target substrates directly for 20S proteasomal degradation without the need for polyubiquitin modification.

References and Notes
22. Materials and Methods are available as supporting material on Science Online.
28. The far-ultraviolet circular dichroism spectrum of the mixed population of circular and linear α-syns exhibits a predominant negative absorption at around 198 nm, characteristic of a random coil conformation.
29. GFP fluorescence was stable during the degradation of the linear and circular GFP-p21 substrates, as in Figs. 1 to 3.
34. We thank S. J. Elledge (Baylor College of Medicine) for his generous gift of p21cDNA; R. Nussbaum (National Human Genome Research Institute) for α-syn cDNA; C. Wigley, R. Stihlm, and S. Mualem for critical suggestions; and members of our laboratories for helpful comments. This work was supported by grants from the Welch Foundation [P.J.T.], Muscular Dystrophy Association [G.N.D.], and NIH [grants DK46818 (G.N.D.) and DK49835 (P.J.T.)].

Production of α,1,3-Galactosyltransferase–Deficient Pigs
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The enzyme α,1,3-galactosyltransferase (α,1,3GT or GGTA1) synthesizes α,1,3-galactose (α,1,3Gal) epitopes (Galα1,3Galβ1,4GlcNAc-R), which are the major xenogenantigen causing hyperacute rejection in pig-to-human xenotransplantation. Complete removal of α,1,3Gal from pig organs is the critical step toward the success of xenotransplantation. We reported earlier the targeted disruption of one allele of the α,1,3GT gene in cloned pigs. A selection procedure based on a bacterial toxin was used to select for cells in which the second allele of the gene was knocked out. Sequencing analysis demonstrated that knockout of the second allele of the α,1,3GT gene was caused by a T-to-G single point mutation at the second base of exon 9, which resulted in inactivation of the α,1,3GT protein. Four healthy α,1,3GT double-knockout female piglets were produced by three consecutive rounds of cloning. The piglets carrying a point mutation in the α,1,3GT gene hold significant value, as they would allow production of α,1,3Gal-deficient pigs free of antibiotic-resistance genes and thus have the potential to make a safer product for human use.

The enzyme α,1,3-galactosyltransferase (α,1,3GT or GGTA1) synthesizes α,1,3Gal epitopes (Galα1,3Galβ1,4GlcNAc-R) on the cell surface of almost all mammals with the exception of humans, apes, and Old World monkeys (1). α,1,3Gal epitopes are the major xenogenantigens causing hyperacute rejection (HAR) in pig-to-human xenotransplantation (2–4). Many reports have also indicated that α,1,3Gal epitopes are involved in acute vascular rejection (AVR) of xenografts (4–6). Piglets with α,1,3GT heterozygous knockout have been cloned by our group (7) and another team (8) in the last year. To produce homozygous α,1,3GT knockout piglets by natural breeding, assuming both male and female heterozygous knockout piglets are available at the same time and are fertile, is feasible but takes up to 12 months. However, by using a second-round knockout and clonning strategy, we could save up to 6 months and all cloned piglets would be α,1,3GT double knockout (DKO). We have selected and enriched for α,1,3GT DKO cells by using a bacterial toxin, toxin A from Clostridium difficile, which binds with high affinity to α,1,3Gal epitopes and produces a cytotoxic effect on cells that are α,1,3Gal-positive (9). Toxin A uses α,1,3Gal epitopes as a cell
surface receptor and causes “rounding” and lifting of the α1,3Gal-positive cells from the surface of the growth vessel (10, 11).

Heterozygous α1,3GT knockout fetal fibroblasts, 657A-I11 1-6 cells, were isolated from a day-32 pregnancy as described in (7). To avoid using a second antibiotic-resistance gene as a selection marker, we constructed an ATG (start codon)-targeting α1,3GT knock- out vector, pPL680 (12), which also contains a neo gene, to knock out the second allele of the α1,3GT gene. 657A-I11 1-6 cells were transfected by electroporation with pPL680 and selected for the α1,3Gal-negative phenotype with purified C. difficile toxin A (13). One colony (680B1) was isolated and expanded after toxin A selection. When the 680B1 cells were stained with a fluorescein-labeled α1,3Gal-specific lectin, GS-IB4, about 80% of the cells were found to be α1,3Gal-negative. The fact that fewer than 100% of the cells in the colony were negative with GS-IB4 staining indicated that this colony contained a mixture of α1,3Gal-negative and α1,3Gal-positive cells. We used 680B1 cells for somatic cell nuclear transfer (cloning) as described in (7). We transferred embryos to five recipient gilts, and three initial pregnancies were established, of which only one went beyond day 35 of gestation.

To determine whether all the fetuses cloned from 680B1 cells were α1,3GT DKO, we terminated the remaining pregnancy at day 39 and recovered four normal-sized fetuses. Fibroblast cell lines (680B1-1 to B1-4) were isolated from each of these four fetuses, and fluorescence-activated cell sorting (FACS) analysis with GS-IB4 staining was performed after toxin A selection. When the 680B1 cells were stained with a fluorescein-labeled α1,3Gal-specific lectin, GS-IB4, about 80% of the cells were found to be α1,3Gal-negative. The fact that fewer than 100% of the cells in the colony were negative with GS-IB4 staining indicated that this colony contained a mixture of α1,3Gal-negative and α1,3Gal-positive cells. We used 680B1 cells for somatic cell nuclear transfer (cloning) as described in (7). We transferred embryos to five recipient gilts, and three initial pregnancies were established, of which only one went beyond day 35 of gestation.

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To identify the nature of the inactivation event for the second allele, we subcloned and sequenced α1,3GT cDNAs from all four cell lines (B1-1, B1-2, B1-3, and B1-4). Sequencing results revealed that there was a T-to-G transversion at the second base pair of exon 9 in the non-targeted α1,3GT allele of B1-1.
B1-2, and B1-4 cells, but not in B1-3 cells or in the first knockout allele of all four cell lines. This T-to-G transversion in the α1,3GT coding region caused a single amino acid change from tyrosine to aspartic acid in the α1,3GT protein (Fig. 3). Although this mutation has not been observed in the inactivated α1,3GT gene of humans or higher primates (14), it is likely that the change of tyrosine, a hydrophilic amino acid, to aspartic acid, a hydrophobic amino acid, could disrupt α1,3GT function. Crystal structure analysis of bovine α1,3GT protein supports this speculation and shows that this tyrosine is at the center of the catalytic domain of bovine α1,3GT protein and is involved in uridine 5'-diphosphate–Gal binding (15, 16).

To further confirm that the mutated cDNA cannot make functional α1,3GT protein, we cloned α1,3GT CDNA s from the nontargeted allele of B1-1 to B1-4 cells and wild-type pig cells into an expression vector and transfected them into human HeLa cells, which normally do not express α1,3GT protein. HeLa cells transfected with cDNA expression vectors from B1-1, B1-2, and B1-4 cells were negative for GS-IB4 lectin staining, indicating that the transfected pig cDNA from these cells did not make functional α1,3GT protein. In contrast, HeLa cells transfected with the cDNA from B1-3 cells and wild-type pig cells were positive for GS-IB4 lectin staining, indicating that the transfected pig cDNA from these cells did make functional α1,3GT protein. Although toxin A selection was repeated several times on 657A-11-6 cells, with or without pPL680 vector transfection, no additional toxicon A-resistant colonies were detected.

We performed somatic cell nuclear trans-plantation (cloning) with all three DKO cell lines as respondents mutated and wild-type DNA sequences, respectively. Underlined capital letters denote intron and exon sequences, respectively. Small letters and capital letters denote intron and exon sequences, respectively. Underlined capital letters indicate α1,3GT nucleotide where the point mutation occurred. Amino acids deduced from the corresponding mutated and wild-type DNA sequence are underlined. No other mutations were found in the coding region of the α1,3GT gene from the second allele of the DKO pig fetuses in our genomic and reverse transcriptase–PCR libraries.

Thus, we have successfully produced four α1,3GT-deficient piglets by a toxin A–mediated selection method. Although our intent was to knock out the second allele of the α1,3GT gene by homologous recombination, this did not occur. Instead, because we used this powerful selection method, which allows us to isolate any event that results in loss of α1,3GT activity, we discovered a mutation in the second allele of the α1,3GT gene. Had we used standard selection methods with neomycin or hygromycin, we would not have found the mutation. Although the rate of spontaneous mutation in the pig genome is very low [about 4 × 10⁻⁸ for a spontaneous mutation per replication (18)] in a mammalian gene similar in size to the α1,3GT gene, toxin A selection still enabled us to detect this crucial mutation. Clearly inactivation of the α1,3GT protein by this point mutation is a better outcome than by gene targeting with the pPL680 vector. It provides the opportunity to produce α1,3GT-deficient pigs without any antibiotic-resistance genes or other foreign DNA sequences, which should facilitate regulatory approval and, potentially, make a safer product for human use. It is certain that this point mutation will be maintained in the genome of these DKO pigs and their offspring, just as the few critical point mutations in the α1,3GT gene of humans and higher primates have been maintained over 20 million years (14). This genomic stability is not only due to the rarity of a reverse mutation event [about 5 × 10⁻¹¹ per replication (18) for mammals] but, more importantly, the strong selection pressure against α1,3Gal-positive cells by the presence of antibodies to α1,3Gal in α1,3Gal-negative animals. Our results have demonstrated that removal of α1,3Gal epitopes on pig cells did not preclude development in utero, even though pig cells express up to 500 times the number of α1,3Gal epitopes as do mouse cells (4, 19). In addition, three consecutive rounds of cloning with rederived fetal cells did not appear to have a major detrimental effect on the overall development or health of the cloned pigs in this study. Analysis of tissues and organs from these α1,3GT DKO pigs in nonhuman primate models should provide clearer indications of the involvement of α1,3Gal in HAR, AVR, and chronic rejection.

Fig. 3. Sequencing analysis of the α1,3GT gene from wild-type pig cells and DKO porcine fetal fibroblasts. Upper and lower alignment show nucleotide sequence of the α1,3GT intron 8–exon 9 boundary from wild-type pig cells and the second allele of the DKO pig fetuses (B1-1, B1-2, and B1-4), respectively. Small letters and capital letters denote intron and exon sequences, respectively. Underlined capital letters indicate α1,3GT nucleotide where the point mutation occurred. Amino acids deduced from the corresponded mutated and wild-type DNA sequence are underlined. No other mutations were found in the coding region of the α1,3GT gene from the second allele of the DKO pig fetuses in our genomic and reverse transcriptase–PCR libraries.

References and Notes
11. pPL680 was made from three parts: a 1.8-kb PCR-
Recent Expansion of Toxoplasma Through Enhanced Oral Transmission

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The global predominance of three clonal Toxoplasma gondii lineages suggests that they are endowed with an exceptional trait responsible for their current parasitism of nearly all warm-blooded vertebrates. Genetic polymorphism analyses indicate that these clonal lineages emerged within the last 10,000 years after a single genetic cross. Comparison with ancient strains (~1 million years) suggests that the success of the clonal lineages resulted from the concurrent acquisition of direct oral infectivity. This key adaptation circumvented sexual recombination, simultaneously promoting transmission through successive hosts, hence leading to clonal expansion. Thus, changes in complex life cycles can occur rapidly and can profoundly influence pathogenicity.

Toxoplasma gondii is a member of the phylum Apicomplexa, an ancient group of ~5000 species of parasitic protozoa that infect a wide range of vertebrates (1–5). Most closely related members of this group have complex two-host life cycles that alternate between definitive (sexual propagation) and intermediate hosts (asexual replication) (4). T. gondii is remarkable among this group for the extremely wide range of birds and mammals that serve as intermediate hosts, although sexual propagation is limited to members of the cat family (Felidae) (4). Toxoplasmosis is a major cause of foodborne illness acquired through ingestion of contaminated water or infected meat (5). Human infections, although globally widespread, are primarily subclinical, except in immunocompromised individuals in whom they are often severe (6–8). T. gondii has a highly unusual, clonal population structure comprised of three widespread genotypes referred to as type I, type II, and type III (9–11). The genomewide rarity of polymorphisms within these lineages is suggestive of a recent and massive genetic “selective sweep,” in contrast to the otherwise ubiquitous and ancient nature of this group of parasites.

To estimate the age of T. gondii relative to other apicomplexans that form tissue cysts, we analyzed the small subunit (SSU) and internal transcribed spacer 1 (ITS1) regions of the ribosomal DNA cluster (12). Analysis of the SSU regions revealed that the genera Toxoplasma, Hammondia, and Neospora form a closely related triad, whereas other branches defined by Sarcocystis tenella, and the out-group Eimeria tenella, are quite distant (Fig. 1A)(13). Because there is no fossil record for the apicomplexans, we used the average SSU substitution rate calculated from a variety of taxa (14) to calculate the ages for lineages shown in Fig. 1. Notably, the node defined by the most recent common ancestor of T. gondii, N. caninum, and H. hammondii was estimated to be about 12 million years ago (Fig. 1A, table S1). To provide greater resolution between these closely related taxa, we analyzed the ITS1 region, which has fewer structural constraints and is therefore typically more variable than the SSU (Fig. 1B). Phylogenetic analysis of the ITS1 region clearly separated N. caninum from T. gondii and supported a paraphyletic origin for Hammondia, as described previously (15) (Fig. 1B). We sequenced the ITS1 region from a representative member of each of the three clonal lineages of T. gondii and found it to be identical at all 393 base pairs (bp), in agreement with a previous report (16) (Fig. 1B). By comparison, the amount of intraspecies sequence divergence in the ITS1 regions for several related Apicomplexa ranges from 1 to 6% (17–19). The lack of sequence divergence in the ITS1 region suggests that the major T. gondii lineages share a recent common ancestry.

Studies have shown that the three clonal lineages of T. gondii are highly similar, as estimated by restriction fragment length polymorphisms (10) and multilocus isoenzyme analysis (9). Furthermore, sequencing of individual genes indicates only 1 to 2% divergence (20–22). Sequencing of antigen-encoding genes established that the three clonal types are comprised of combinations of just two alleles at each locus, which indicates that they are the result of a recent cross between closely related parental strains (23). A small number (fewer than 5% of isolates) of recombinant strains, which have mixtures of the two-allele patterns, are also observed (10), and a few of these strains (~1%) contain unique polymorphisms (23–26). The latter strains are referred to here as “exotic.” Experimental crosses between different genotypes of T. gondii have demonstrated that genes are inherited in a Mendelian fashion and that many independent recombinants arise from a single cross (27, 28). Although recombination is apparently rare in nature, it might also be expected to give rise to a large number of distinct lineages. The predominance of just three clonal types in nature indicates that they have a trait or traits that allowed them to expand rapidly and dramatically after a recent origin.

To determine the relative divergence between T. gondii strains, we analyzed the frequency of single-nucleotide polymorphisms (SNPs) in noncoding regions consisting of 11 introns plus the ITS1 region that collectively constituted 4067 bp per strain. We compared the SNP frequencies among four type I, three type

References
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Supporting Online Material
www.sciencemag.org/cgi/content/full/1078942/DC1
Materials and Methods
Figs. S1 to S4