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Supplemental Data

Spontaneous Generation of Prion Infectivity in Fatal Familial Insomnia Knockin Mice

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of *Prnp* targeting constructs and ESCs.

Genomic plasmids VectorA, VectorC, and p129PrP were described in (Moore et al., 1995) and used as starting materials for our targeting constructs. We did not modify the knock-out clone (Vector C). To simplify the generation of several exchange constructs, we first added small linkers flanking the open reading frame (ORF) of Prnp. This was done by cloning a 2.2kb EcoRI fragment from the p129PrP into the EcoRI site of pBTIIKS(+), making pWJPrP31. An EagI restriction site was added downstream of the ORF by PCR site directed mutagenesis (PCRSDM) of pWJPrP31 following the kit manufacturer's protocol using primers (5'-GTGGGATGAGGGCGGCCGTCCTGCTTGTTC-UPPER) (5'-GAACAAGCAGGACGGCCGCCCTCATCCCAC-LOWER) and a ClaI site was created by using primers (5'-GCCAAGGTTCGCCATCGATGACTGATCTGC-UPPER) (5'-GCAGATCAGTCATCGATGGCGAACCTTGGC-LOWER), inserting a guanine immediately before the translation start site yielding plasmid pWJPrP34. Vector A is an intermediate construct that has the extreme ends of the targeting homology but lacks the EcoRI fragment mentioned above. ClaI and EagI restriction sites in the backbone of

Vector A were mutated by digestion with the corresponding restriction enzymes followed by blunting using mung bean nuclease for EagI or Klenow plus dNTP for ClaI, to make pWJPrP32. A 2.2kb EcoRI fragment was isolated from pWJPrP34 and ligated into the corresponding site of pWJPrP32, yielding pWJPrP38. A mouse PrP ORF containing methionine codons for positions 108 and 111 was cloned into pBTIIKS (+) in a manner where the ClaI site is next to the 5' end of the PrP ORF, and EagI site is next to the 3' end of the ORF, yielding pWJPrP1. pWJPrP1 was then modified by PCRSDM to make the D177N mutation by using the following primers; (5'-CAGAACAACTTCGTGCACAATTGCGTCAATATCACCATC-UPPER) and (5'-GATGGTGATATTGACGCAATTGTGCACGAAGTTGTTCTG-LOWER) to make pWJPrP5. 0.8 kb EagI-ClaI fragments were isolated from pWJPrP1 and pWJPrP5 and ligated into the corresponding sites of pWJPrP38 to generate exchange targeting constructs pWJPrP41 and pWJPrP43, respectively. The result is the modification to the PrP ORF as well as the inclusion of the following linker sequences; GATAAGCTT between base -1 and +1 with respect to translation start site, and ATCCACTAGTTCTAGAAG at the third base pair position downstream of the translation termination sequence. These constructs were linearized by XhoI prior to electroporation. All enzymes purchased from New England Biolabs, Ipswich, MA. DNAs subjected to PCR or restriction digestion and ligation were sequenced.

A *Prnp* knock-out ESC line was generated by electroporation (300V, 250 μF) of $1x10^7$ HM-1 ESCs (Magin et al., 1992; Selfridge et al., 1992) in 1 mL of ESC media, using 50 μg of ClaI linearized vector C (Moore et al., 1995) and plated onto tissue culture plates pre-coated with gelatin and feeder cells. The next day, fresh media supplemented

with HAT and Gancylovir selection agents was added. Selection was maintained for six days. Resistant colonies were tested by PCR and Southern analysis (described below). Properly targeted cells were then electroporated with exchange constructs in a two electroporation process. Cells were resuspended in cytomix (120 mM KCl; 0.15 mM $CaCl_2$; 10 mM K_2HPO_4/KH_2PO_4, pH 7.6; 25 mM Hepes, pH 7.6; 2 mM EGTA, pH 7.6; 5 mM $MgCl_2$; plus 2mM ATP, pH 7.6 and 5 mM glutathione added immediately prior to resuspension)(van den Hoff et al., 1992) at $1.25x10^7$ cells/mL. 0.8 mL of cells was added to a cuvette with 15 ug exchange construct. Cells were electroporated first with 400V at 950 μF, then at 400V at 500 μF, with 15 μg more exchange construct added between electroporations. Cells were plated as before, and split five days after electroporation into media containing 6-thioguanine. Resistant colonies were picked two weeks later and screened by PCR and Southern analysis for proper gene-targeting. The electroporator (GenePulser Xcell with the capacitance extender (CE) module) and 4 mm electroporation cuvettes were from Biorad, Hercules, CA.

Genotyping

PCR is performed using primers that flank the downstream insertion site with the following sequences (5'-GAGCAGATGTGCGTCACCCAG-upper) and (5'-GAGCTACAGGTGGATAACCCC-lower). PCR products from unmodified alleles are 207 bp and those from knock-in alleles are 225 bp, easily resolved with 3% agarose gels. The third band produced in PCR with heterozygous cells or animals is due to impaired migration of heteroduplex DNA. These heteroduplexes can also be formed by mixing PCR products from homozygous WT and knock-in mice, melting the DNA and

allowing it to renature, indicating there is not an unexpected chromosome rearrangement in heterozygous animals.

PCR positive ESC clones were further tested by Southern blotting using conventional methods (Sambrook et al., 1989). A 3' probe was isolated from p129PrP using EcorV and BamHI. This probe shares no homology with the exchange or knock-out vectors and therefore only detects chromosomal ESC DNA. Genomic DNA was digested with BamHI, to detect a new restriction site in the modified allele. These 129/Ola (Ola) derived ESCs were then injected into C57Bl/6Taconic (B6) 3.5 day old blastocysts to generate chimeric animals. Chimeras were bred to B6 mice to generate F1 animals. F1 animals were bred to B6 mice to generate F2 animals. F2 heterozygous animals were intercrossed to generate mice that were homozygous knock-in or homozygous WT. Homozygous mice were then bred to produce large enough litters for experiments. During the crossing with B6 mice, in addition to tracing PrP alleles, we followed the inheritance of the wild-type *Hprt* allele using a PCR assay (McEwan and Melton, 2003) in order to eliminate the *Hprt* deletion from the HM-1 cells from our colony.

Husbandry

The animal facility is designated as specific pathogen free, with a series of barriers, including restricted access, multiple doorways, isolation cubicles, and filter-topped mouse cages. Sentinel mice are tested regularly for pathogens. Technicians check cages daily with respect to water, food (provided *ad libitum*), and cleanliness as well as the general health of the animals. Cages are changed twice weekly. Technicians do not interact with animals during video recording. Three different rooms are used to separate

1) scrapie infected mice from 2) the general population of mice and mice in 3) behavioral experiments and mice involved in *de novo* FFI prion transmission experiments. Mice in room 3 are divided into separate cubicles.

Supplemental Tables

Supplementary table 1 Transmission of ki-3F4-FFI disease

innoculum	Ki-3F4-WTvs.:	P value[a]	Tga20 vs.:	P value[a]
Primary-#1	WT	.0065	WT	.014
	KO	.0027	KO	.0067
Primary-#2	WT	.0175	WT	.0027
	KO	.0015	KO	.0027
Primary-#3	WT	.0001	WT	.0002
	KO	.0029	KO	.0060
Secondary-#1	KO	.0001		
Secondary-#2	KO	.0132		

[a] P value calculated by Log-rank test

Supplementary table 2 Scrapie transmission

Corresponding figure	Comparison	P value [a]
5a	22L hi WT vs. 22L hi ki-3F4-WT	<.0001
5a	RML hi WT vs. RML hi ki-3F4-WT	<.0001
5a	22L low WT vs. 22L hi ki-3F4-WT	<.05
5a	RML low WT vs. RML hi ki-3F4-WT	<.005
5b	22L WT vs. 22L ki-3F4-WT	<.005
5c	22L hi ki-3F4-WT vs. 22L hi ki-3F4-FFI	<.0001
5c	RML low ki-3F4-WT vs. RML low ki-3F4-FFI	>.1
5d	1% 22L vs. 1% NBH	<.0005
5d	1% 22L vs. uninoculated	<.0001
5d	1% NBH vs. uninoculated	>.1
5f	263K WT vs. 263K ki-3F4-WT	>.5
5f	263K WT vs. 263K ki-3F4-FFI	>.5
5f	263K ki-3F4-WT vs. 263K ki-3F4-FFI	>.5

[a] P value calculated by Log-rank test

Supplemental Figure Legends

Figure S1. Two step gene-replacement approach to generate knock-in ES cells and mice. Horizontal lines with labeled boxes superimposed on them represent double stranded DNA. The boxes correspond with exon 3 of the mouse Prnp gene. The box labels indicate the protein coding sequence for that DNA sequence. Long horizontal lines with diagonal breaks near the ends represent genomic DNA. The diagonal breaks represent conceptual breaks in the continuity of the genomic DNA to indicate the genomic DNA represented in the schematic is only a tiny portion of the entire chromosome. Short horizontal lines represent recombinant DNA plasmids used to manipulate genomic DNA. The two step "tag and exchange" method of gene-targeting was used to replace the entire protein coding sequence of Prnp with DNA sequence encoding mutated PrP open reading frames in mouse embryonic stem cells (ES cells). For the first step, a "tag construct" undergoes homologous recombination (depicted by large "X" like crosses) with one endogenous *Prnp* allele (labeled "endo"), resulting in deletion of a section of *Prnp* and insertion of a hypoxanthine phosporibosyltransferase (*Hprt*) minigene, resulting in a knock-out allele (KO). The presence of the *Hprt* minigene confers resistance to HAT media which will kill ES cells lacking the *Hprt* minigene. For the second step, KO ESCs were then modified with an exchange construct which replaces the Hprt minigene with the mutated PrP open reading frame sequence via homologous recombination. The "knock-in PrP" allele differs from the wild-type Prnp allele by the inclusion of a BamHI restriction endonuclease site (B) adjacent to the open reading frame ("3F4-PrP" boxed), which can be detected by genomic Southern analysis using a probe (P) which is homologous to a region of Prnp downstream of the targeting constructs. Correctly

targeted ESCs were injected into C57Bl/6 Taconic embryos 3.5 days post fertilization. Injected embryos were then implanted into pseudopregnant females (CD1). Resulting chimeric mice derived from host embryo and donor ES cells were then bred to C57Bl/6 mice to establish the knock-in mouse lines.

Figure S2. Additional examples of MEMRI scans of knock-in mice.
Top, MEMRI scan of another ki-3F4-WT mouse. Lower, MEMRI scans of 3 more ki-3F4-FFI mice. Mice with prominent clinical signs (ataxia, reduced body condition, or kyphosis) showed the most prominent MEMRI abnormalities.

Figure S3. Heavily vacuolated deep cerebellar white matter in ki-3F4-FFI mice.
Aged but otherwise normal ki-3F4-WT cerebellum (left) has very few vacuoles. H&E stained paraffin sections show vacuolation in a ki-3F4-FFI mouse (right).

Figure S4. Severe loss of thalamic neurons.
Nissl stained stained 30 μm thick sagittal sections, from ki-3F4-WT (left), and from ki-3F4-FFI mice (right). Note the ki-3F4-FFI sections have fewer neurons (large white spots) yet more non-neuronal cells (small white spots), most of which are likely glial cells. Dark areas in top right of each panel are ventricles. Distance from midline is indicated to the left of each row.

Figure S5. Phenotypic arrays of AMBA data.

(A) Tile panels showing the fold difference (left) and P-values (right) from comparisons ki-3F4-WT and WT mice. This comparison was performed to gage the amount of noise intrinsic to this technique. The colored P-values tiles are scarce and randomly distributed.

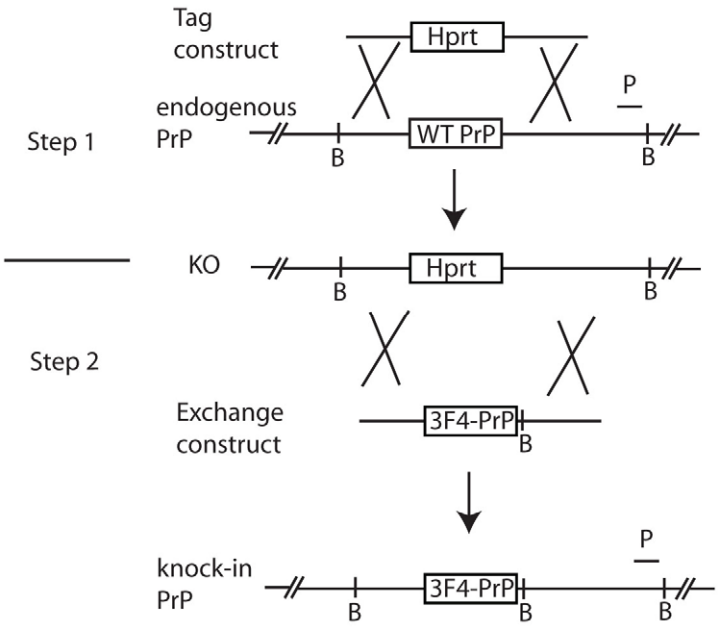
(B) A pair of tile panels showing the fold difference (left) and P-values (right) from comparisons of ki-3F4-WT and ki-3F4-FFI mice. For all panels, yellow tiles represent behaviors performed more by the indicator line (WT or ki-3F4-FFI mice) than the standard line (ki-3F4-WT mice), and blue tiles represent behaviors performed less by the indicator lines. The age in months is indicated immediately below each panel, the number of mice used in each comparison is immediately above each panel. Behaviors reported in this figure show all possible behaviors analyzed (although "urinate" was not analyzed and "circle" is prone to error). A similar panel is displayed in Fig. 3 of the main manuscript, differing in that behaviors there were combined to allow for a simpler array (details in methods section). P-values are calculated using a non-parametric (Wicoxon rank-sum) test to buffer against effects of highly uncharacteristic scores which are inevitable in this type of non-task dependent behavioral assay. The following behaviors were condensed to give the corresponding behavior names in Fig. 3b: pause and stationary condensed to still; rear up to partially reared, remain partially reared, and come down to partially reared were condensed to half reared; rear up from partially reared, rear up, and remain rear up were condensed into fully reared; come down from partially reared and come down were condensed to return to floor; hang vertically from rear up, remain hang vertically, and hang vertically from hang cuddled were condensed to vertical

hang; hang cuddled and remain hang cuddled were condensed to cuddled hang; walk slowly, walk left, and walk right were condensed to travel; unknown behavior and no data were condensed to unassigned. Hang vertically and urinate are not analyzed and circle scoring is unreliable.

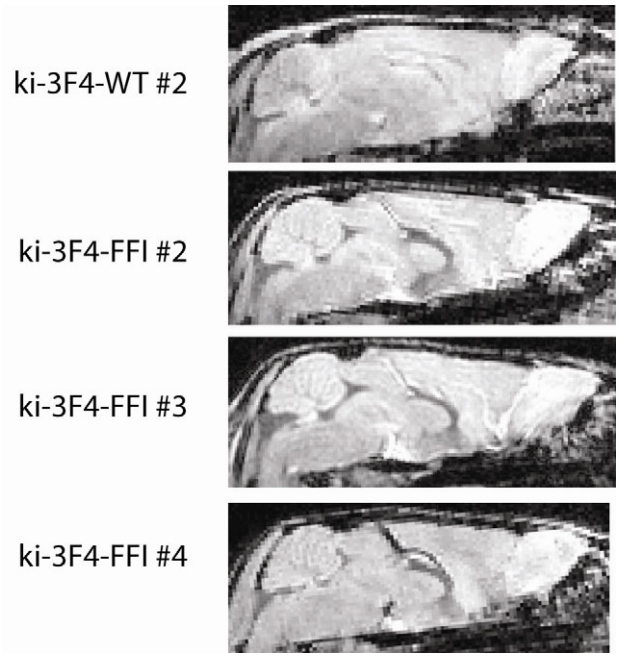
Figure S6. Fluctuation in Tb of ki-3F4-FFI mice.

(A) Tb of an individual ki-3F4-WT mouse (WT 2, red) overlayed on a line representing the average Tb of 5 ki-3F4-WT mice (WT avg, black), top left. Tb of 3 ki-3F4-FFI mice (FFI 3, 4 , 5, blue lines top right and both bottom panels) plotted over the same WT avg line (black) as the top left panel and the 3 "c" panels in Fig. 3 of the main manuscript. Temperatures are recorded every 5 minutes, 288 measurements per day.

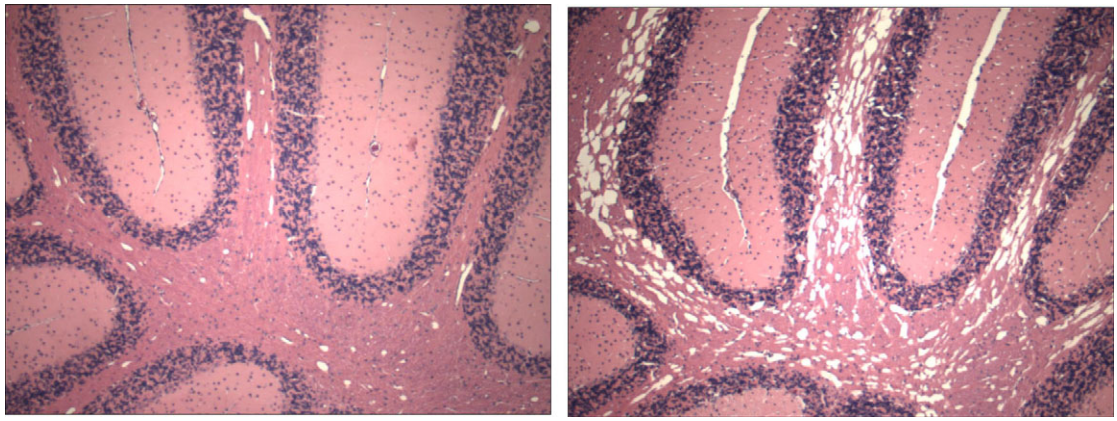
(B) Bar charts of the above mentioned data showing the average number of epochs during which the temperature change was less than 0.1°C (left), or greater than 1°C (right). Epoch lengths considered are labeled below the charts. Error bars indicate standard error of the mean. P-values calculated by Student's two-tailed T test. One star indicates $P< 0.05$, two stars indicate $P< 0.01$.



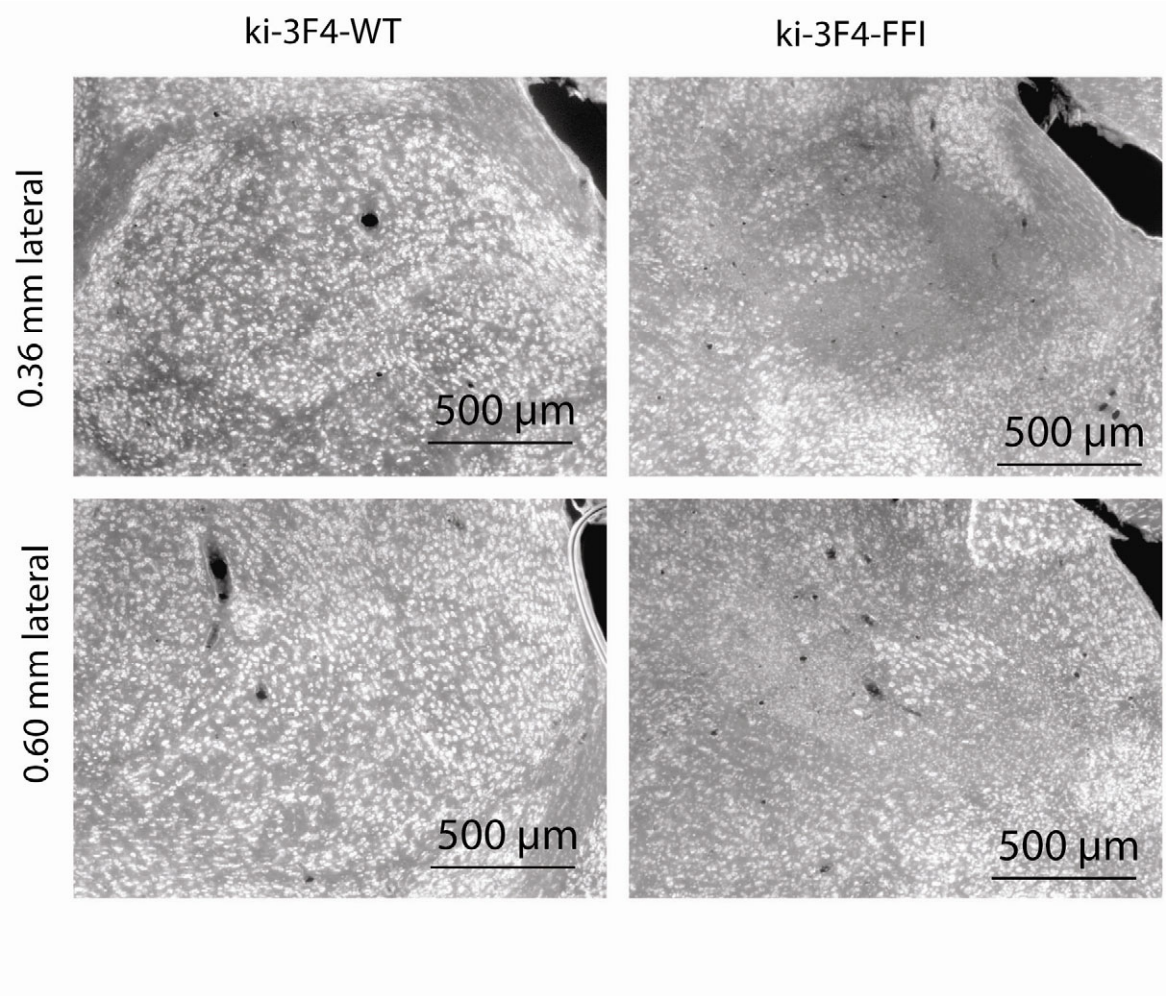
Supplemental Figure 1



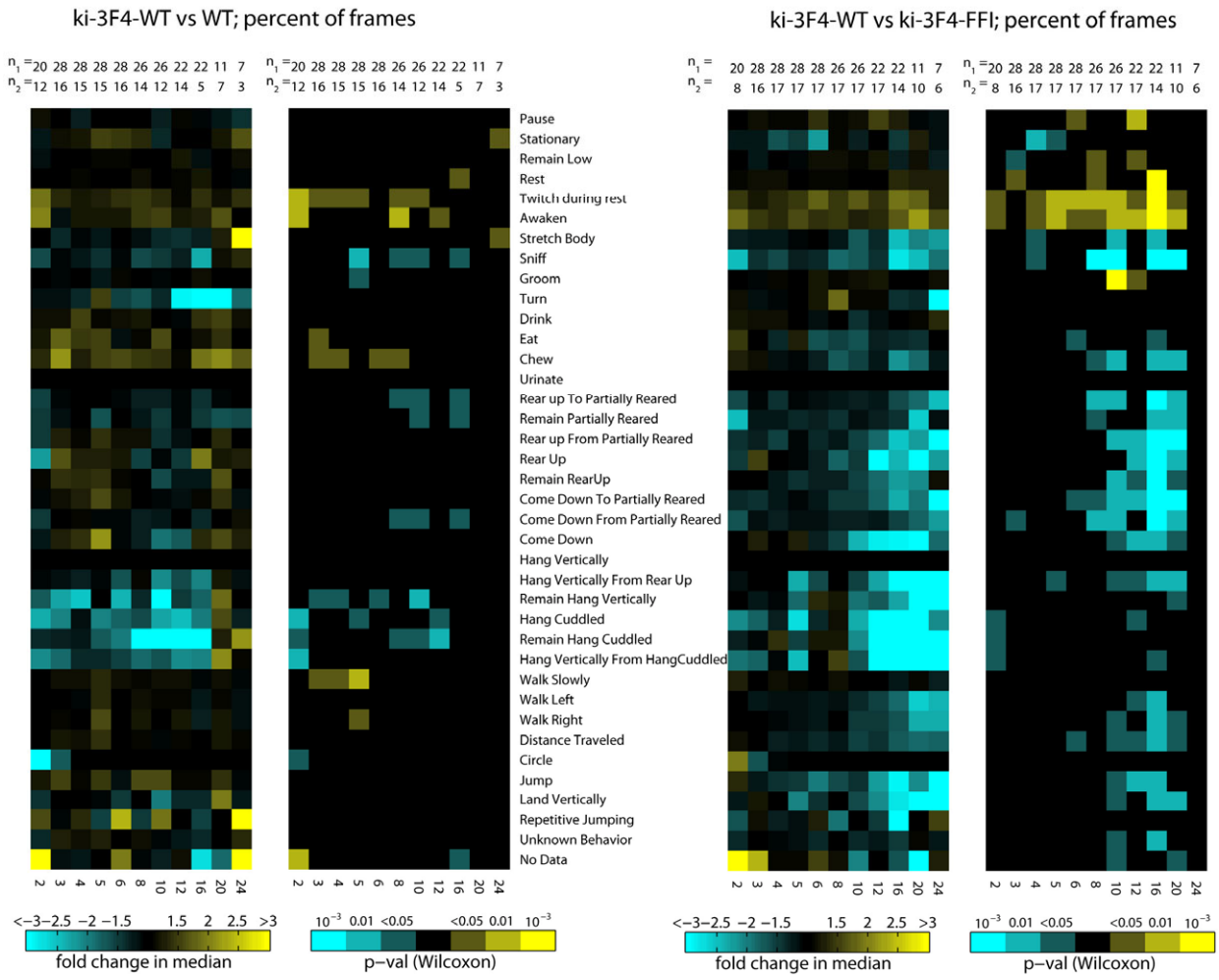
Supplemental Figure 2



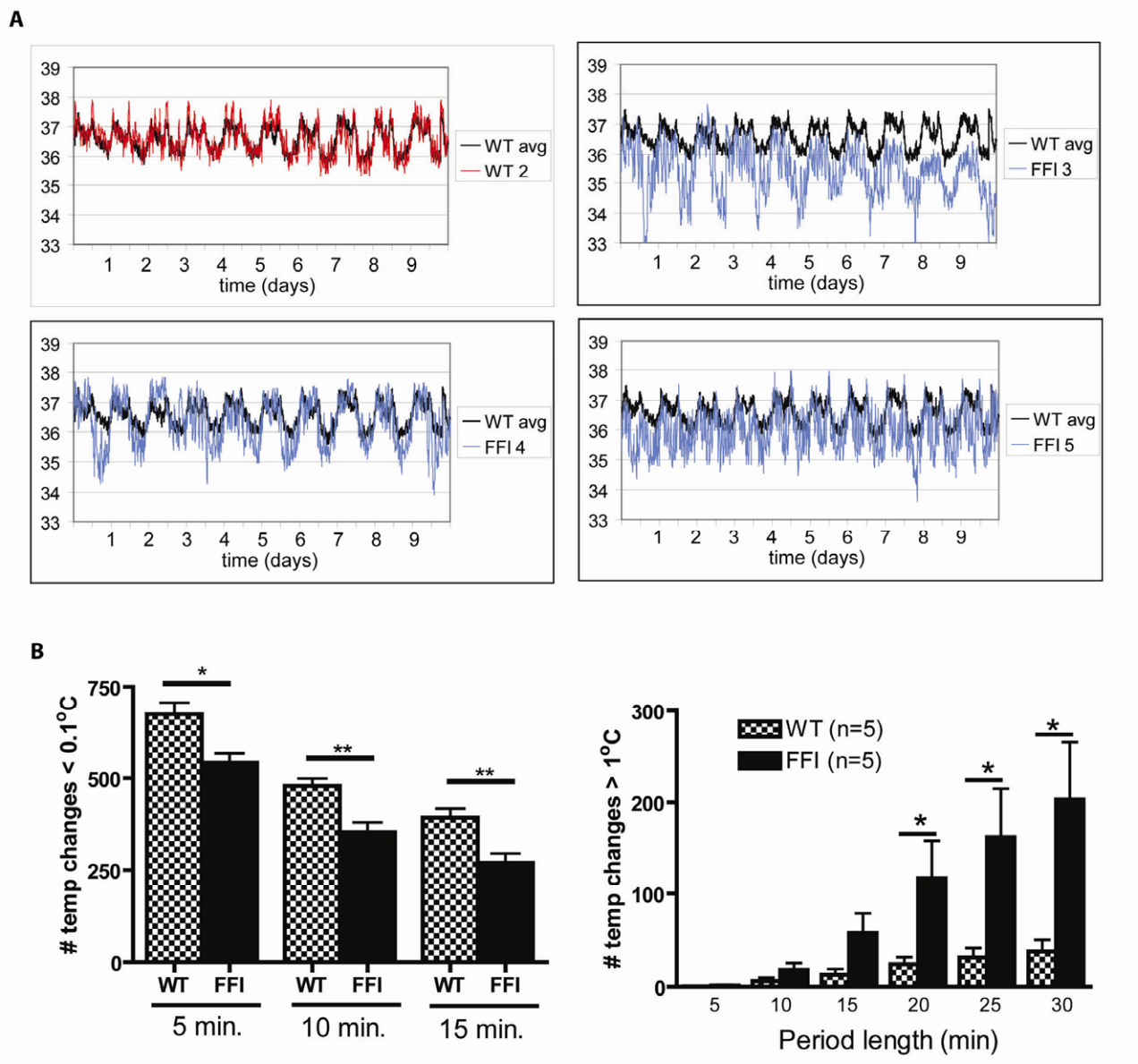
Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure 6

Supplemental References

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