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27. REF-52 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were transfected by the standard calcium phosphate ($CaPO_4$) method with 8 μg of pEXV3-$p42^{mapk}$-Myc (*24*) or pCMV5-Flag-JNK1 (*14*) and 1, 2, or 4 μg of pDCR-V^{12}Ras, supplemented with vector pDCR to total 16 μg of DNA. Alternatively, cells were transfected using 8 μg of pCG-HA-$p42^{mapk}$ or pSG5-Flag-JNK1 and 1 or 4 μg of pCEP4-MKP-1-Myc, supplemented with vector pCEP4 to give a total of 16 μg of DNA. After an 12-hour incubation with DNA-$CaPO_4$ precipitates, cells were incubated in DMEM containing FBS (0.5%) for 24 hours. Where indicated, cells were treated with TPA or UV (wavelength, 254 nm) before harvesting. Cells were lysed in lysis buffer [50 mM tris (pH 7.5), 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, 1 mM DTT, 1 mM vanadate, 1 μM okadaic acid, 1 mM benzamidine, 1 mM phenylethylsulfonyl fluoride, leupeptin (10 μg/ml), and aprotinin (10 μg/ml)], and lysates were clarified by centrifugation. Myc epitope–tagged $p42^{mapk}$ was immunoprecipitated with monoclonal antibody 9E10 (American Type Culture Collection), HA eptiope–tagged $p42^{mapk}$ was immunoprecipitated with monoclonal antibody 12CA5 (Babco), and Flag epitope–tagged JNK1 was immunoprecipitated with monoclonal antibody M2 (IBI-Kodak). Immune complexes were collected by binding to protein A–Sepharose, washed extensively in lysis buffer, then assayed for 10 min at 30°C in kinase assay buffer with either myelin basic protein or GST–c-Jun(5-89) as described above (*16*).
28. HeLa cells were cultured in DMEM supplemented with FBS (5%) and transfected by means of the lipofection method with 1 μg of 5XSRE-CAT reporter plasmid or its pm18 mutant derivative; 3 μg of vector pCEP4, pCEP4–MKP-1–Myc, or pCEP4-PTP1B; and 1 μg of pDCR or pDCR-V^{12}Ras, together with 2 μg of SV40–β-Gal. After an 18-hour incubation with Lipofectin (BRL), cells were incubated in DMEM containing FBS (0.5%) for 24 hours. Lysates were then prepared, normalized for β-galactosidase activity, and assayed for CAT activity. The sequence of SRE is 5′-GGATGTCCATATTAGGACATCT-3′, and its pm18 derivative contains a single G to T base change at position 2 (*18*).
29. REF-52 cells were plated onto glass cover slips and cultured in DMEM supplemented with FBS (10%). The cells were grown to confluence, then placed in starvation medium (DMEM with 0.5% FBS) for 24 hours before microinjection. A plasmid mixture containing 5XSRE-CAT (62 μg/ml), pDCR-V^{12}Ras (5 μg/ml), and pCEP4-PTP1B or pCEP4–MKP-1–Myc (62 μg/ml) in microinjection buffer [50 mM Hepes (pH 7.2), 100 mM KCl, and 5 mM $NaHPO_4$] was microinjected into cell nuclei. Sixteen hours after injection, cells were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 1 hour, then permeabilized with 0.1% Triton X-100 in PBS for 3 min at room temperature. The cover slips were incubated with a mixture of rat antibody to Ras (Y13-259) (*25*) and rabbit antibody to CAT (5 prime, 3 prime) in PBS containing BSA (2 mg/ml) and then with a mixture of fluorescein-conjugated goat antibody to rat immunoglobin G (IgG) and rhodamine-conjugated goat antibody to rabbit IgG (Cappel). PTP1B was detected with monoclonal antibody FG6 (Oncogene Sciences), and Myc epitope–tagged MKP-1 was detected with monoclonal antibody 9E10 and the appropriate fluorescein-conjugated secondary antibodies. To monitor DNA synthesis, quiescent REF-52 cells were microinjected with a plasmid mixture containing pDCR-V^{12}Ras (5 μg/ml) together with pCEP-PTP1B or pCEP4–MKP-1–Myc (62 μg/ml). Alternatively, bacterially expressed V^{12}Ras protein (1.7 mg/ml) (*26*) was injected together with plasmid pCEP4-PTP1B or pCEP4–MKP-1–Myc (62 μg/ml). Although microinjections are done into cell nuclei, some spillover to the cytoplasm occurs, thus enabling the recombinant proteins to be delivered to the cytosol. After injection, BrdU (10 μM) was added to the medium, and cells were fixed 28 hours after injection in acid alcohol (ethanol:water:acetic acid, 90:5:5) at −20°C for 1 hour. The cover slips were first incubated with rat antibody to Ras (Y13-259), then with mouse antibody to BrdU (Cell Proliferation Kit, Amersham), and finally with a mixture of fluorescein-conjugated goat antibody to rat IgG and rhodamine-conjugated goat antibody to mouse IgG. Alternatively, after antibody to BrdU was applied, horseradish peroxidase–conjugated antibody to mouse IgG was used and detected by histocytochemical color development with Amersham's Cell Proliferation Kit.
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31. We thank R. Davis for providing pCMV5-Flag-JNK1 plasmid, C. Franklin and A. Kraft for partially purified Jun kinases and bacterially expressed GST–c-Jun(5-89) protein, M. Gilman for 5XSRE-CAT reporter plasmid and its pm18 derivative, S. Kaplan and R. Whitaker for help with tissue culture, P. Guida for technical assistance, P. Renna and J. Duffy for photography and illustrations, and A. Flint and A. Garton for critical reading of the manuscript. H.S. is a recipient of a Damon Runyan–Walter Winchell Cancer Research Fund fellowship. N.K.T. is a Pew Scholar in Biomedical Sciences. Supported by grants CA53840 (to N.K.T.) and CA55360 (to D.B.-S.) from NIH and the Joyce Green Family Foundation.

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Restoration of X-ray Resistance and V(D)J Recombination in Mutant Cells by Ku cDNA

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Three genetic complementation groups of rodent cells are defective for both repair of x-ray–induced double-strand breaks and V(D)J recombination. Cells from one group lack a DNA end–binding activity that is biochemically and antigenically similar to the Ku autoantigen. Transfection of complementary DNA (cDNA) that encoded the 86-kilodalton subunit of Ku rescued these mutant cells for DNA end-binding activity, x-ray resistance, and V(D)J recombination activity. These results establish a role for Ku in DNA repair and recombination. Furthermore, as a component of a DNA-dependent protein kinase, Ku may initiate a signaling pathway induced by DNA damage.

X-rays and oxidative metabolism induce DNA double-strand breaks, which must be repaired if cells are to survive. In yeast, double-strand break repair (DSBR) occurs mainly by homologous recombination (*1*). However, in mammalian cells, DSBR appears to occur by a different pathway: X-ray–sensitive mutant cells fall into nine genetic complementation groups; three of these (groups 4, 5, and 9) are severely defective for DSBR (*2*) but show at most a mild deficit in homologous recombination (*3*, *4*).

V(D)J recombination is another pathway that involves the resolution of DNA double-strand breaks. The pathway rearranges DNA by cleavage and rejoining of segments from the immunoglobulin or T cell receptor genes in a way that is independent of extensive DNA homology (*5*). Little is known about the proteins or genes involved, but early steps require the lymphocyte-specific recombination-activating genes *RAG-1* and *RAG-2*, which are sufficient for activating V(D)J recombination in nonlymphoid cells.

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All three of the mammalian x-ray–sensitive complementation groups that are defective in DSBR are also defective in V(D)J recombination (*6, 7*). Cotransfections of *RAG-1* and *RAG-2* with V(D)J recombination substrates reveal reduced frequencies of coding and signal joint formation in cells from groups 4 and 5 and reduced frequencies of coding but not signal joint formation in group 9 (which includes the mouse *scid* mutation). In each case, reduced joint formation is accompanied by large deletions in the few joints that are recovered, suggesting that the defects in one of the groups might include a protein that binds and protects DNA ends from degradation.

Three hamster cell lines (xrs-5, XR-V15B, and XR-V9B) from x-ray complementation group 5 lack DNA end-binding (DEB) activity (*8*). This DEB activity shares similar nuclear localization, abundance, and DNA substrate specificities with the Ku autoantigen. Ku was first identified in patients with several autoimmune diseases (9) and is a heterodimer with subunits of 70 and 86 kD that binds to nicked DNA, double-stranded DNA ends, or duplex DNA ending in stem-loop structures (*10–12*). DEB factor is recognized by Ku antisera (*13, 14*), and the 70-kD subunit of Ku is deficient in group 5 cells (*14*). The antisera do not recognize the hamster 86-kD subunit, so similar information is not available for that subunit. To determine if the Ku genes encode DEB activity, we coexpressed the human cDNAs (*15–17*) for both Ku subunits in vitro. The protein product was specific for DNA ends and formed a protein-DNA complex that comigrated with DEB activity from human extracts in an electrophoretic mobility-shift assay (EMSA) (Fig. 1).

These studies raised the possibility that the phenotype of the group 5 mutants might be due to a defect in Ku. However, the primary defect in group 5 could be in a protein that affects the expression of Ku as well as distinct proteins involved in DSBR and V(D)J recombination. Alternatively, the defect could consist of a chromosomal deletion that includes Ku and other genes. These other possibilities would leave the cellular role of Ku undefined.

To establish a functional role for Ku in intact cells, it was critical to transfect Ku cDNA into the group 5 mutants. The Ku cDNAs were cloned into the pBJ5 expression vector (*18*) and transfected into cells together with pRSVneo by coprecipitation with calcium phosphate (*19*). The *neo* gene confers resistance to the antibiotic G418 and therefore allowed for the selection of the subpopulation of stably transformed cells (*20*). The xrs-5 and XR-V15B cells will revert spontaneously at a low frequency (*8, 14*), so there would be no a priori way to rule out spontaneous reversion for any given G418-resistant clone. Therefore, the G418-resistant cells were analyzed as pooled, stably transformed colonies rather than as individual clones.

Cell extracts from the stably transformed cells were analyzed for the successful expression of Ku by both immunoblot and EMSA. Immunoblot of extracts resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) showed that cells stably transformed with cDNAs encoding Ku p70 or Ku p86 had increased expression of the polypeptides (Fig. 2A). Thus, the expression vectors were active for the expression of the Ku cDNAs. The transformation of XR-V15B cells with Ku p86 also led to a small but reproducible increase in a band of 70 kD that comigrated with the hamster Ku p70.

We used EMSA to determine DNA end-binding activity in the stably transformed cells (*8, 14*). In contrast to wild-type cells, XR-V15B cells contained no detectable DEB activity (Fig. 2B). When XR-V15B cells were cotransformed with both p70 and p86, or with p86 alone, DEB activity was restored. However, when the cells were transformed with p70 alone, DEB activity remained absent. When the wild-type V79 cells were transformed with cDNAs for p70, p86, or both, the expression of DEB activity was increased only slightly, so that expression of the transfected human Ku was relatively weak compared with the expression of endogenous hamster Ku.

Although group 5 cell extracts contain little or no Ku p70, the primary defect is not in the Ku p70 gene. Instead, group 5 cells do not express functional Ku p86, because transformation with functional human Ku p86 alone restored DEB activity to the hamster extracts. Ku has DNA binding activity in vitro only when both p70 and p86 subunits are coexpressed (*21*). Thus, the restoration of DEB activity in group 5 cells by transfection of cDNA for Ku p86 but not p70 suggests that the cells are capable of expressing normal hamster p70 protein to form an active heterodimer with the transfected human p86 protein.

X-ray sensitivity was determined by exposing the stably transformed cells to gam-

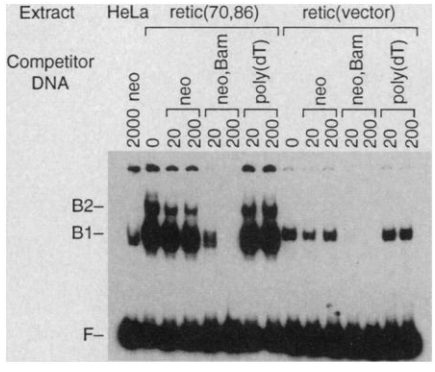

Fig. 1. The DNA end-binding activity of in vitro–synthesized human Ku protein. The Ku subunits p70 and p86 were transcribed and translated in vitro in a rabbit reticulocyte lysate [retic(70,86)] and analyzed by EMSA with different amounts of plasmid pRSVneo closed circular competitor (neo), plasmid competitor linearized with Bam HI (neo,Bam), or the single-stranded homopolymer poly(dT). HeLa extract (0.6 μg) was included as a control for wild-type human protein binding activity. Controls of reticulocyte lysate with the vector lacking an insert gene [retic(vector)] contained some background DNA end-binding activity. Numbers above the labels indicate the nanograms of competitor DNA added, and the lanes marked 0 indicate that no competitor DNA was added to the reaction. Each lane contained 1 μl of a reticulocyte lysate reaction (*28*). F indicates the position of the free DNA probe. B1 and B2 indicate binding activity specific for double-stranded DNA ends, because linearized plasmid DNA (neo,Bam) competed strongly for binding activity.

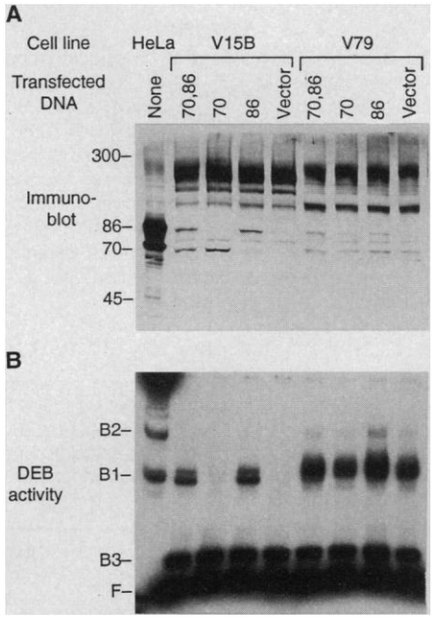

Fig. 2. Ku expression in transformed cells. (**A**) Immunoblot to measure the expression of Ku polypeptides in hamster cells transformed with Ku cDNAs. Shown are the expression of p70 and p86 together (70,86), p70 alone (70), p86 alone (86), and vector alone in pooled, G418-resistant XR-V15B (V15B) cells. HeLa shows the migration pattern of human p70 and p86. The hamster parental cell line V79 transformed with the Ku expression vectors is shown as a control (*29*). Molecular size standards are indicated at the left in kilodaltons. (**B**) Electrophoretic mobility-shift assay to measure DNA end-binding activity in hamster cells transformed with Ku cDNAs. HeLa and transformants of V79 are included for human and hamster reference controls (*30*). B3 indicates nonspecific binding activity and shows the presence of hamster extract in the lanes lacking DEB activity. F, B1, and B2 are as described in Fig. 1.

ma radiation from a ^{137}Cs source (Fig. 3). Transformation with the vector alone or with human Ku p70 did not rescue the x-ray–sensitive XR-V15B cells. However, transformation with human Ku p86 or both p86 and p70 together increased survival of the XR-V15B cells, although the restoration of x-ray resistance did not reach wild-type levels. Wild-type cells transformed with Ku cDNAs did not show increased survival after x-rays. Similar effects on x-ray survival were obtained in transformation experiments with xrs-5 cells, which also belong to group 5.

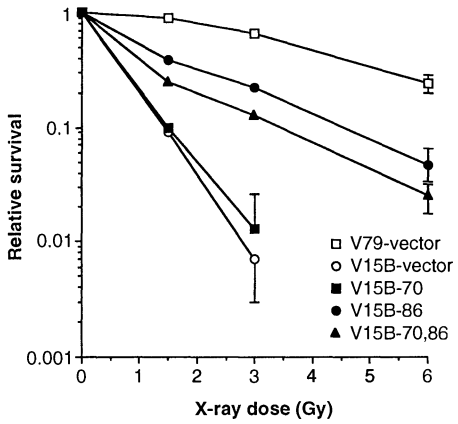

Fig. 3. X-ray survival of hamster cells transformed with Ku cDNAs. Mutant XR-V15B cells transformed with Ku p70, p86, or with p70 and p86 together (V15B-70, V15B-86, and V15B-70,86, respectively) were plated in triplicate and exposed to x-irradiation. After 14 days surviving colonies were stained with 10% Giemsa and counted. Transformations of wild-type V79 and XR-V15B cells with vector alone are shown as controls (V79-vector and V15B-vector). Gy, gray, the absorbed dose of ionizing radiation.

To determine competence for V(D)J recombination, we transiently transfected the pooled transformants with expression vectors for *RAG-1* and *RAG-2* together with V(D)J recombination substrates that were constructed to detect the formation of either signal joints or coding joints (*7*). The correct formation of signal and coding joints were verified by restriction analysis with Apa LI and Pvu II, respectively. As was observed for x-ray resistance, transformation of XR-V15B cells with Ku p86 or both p70 and p86 restored significant V(D)J recombination activity for both coding and signal joint formation (Table 1). Restoration of coding joints in the transformed cells was further confirmed by DNA sequencing. In each of eight cases, the DNA sequence revealed a bona fide coding joint with deletions ranging from 4 to 11 bases, similar to the joints analyzed previously from wild-type cells (*6*, *7*). Wild-type Ku transformants showed no differences from wild-type cells transformed with vector alone.

The rescue of x-ray resistance and V(D)J recombination activity by human p86 in hamster group 5 cells, though significant, was incomplete. There are at least three possible reasons for this. First, Ku expression may not have been sufficient in the transformants. Ku is a relatively abundant protein and wild-type amounts may be necessary for full restoration of both x-ray resistance and V(D)J recombination. The EMSA and protein immunoblot data show that Ku expression in the transformed cells remained significantly less than in the wild-type cells. Second, the differences between hamster and human Ku may lead to less than optimal protein-protein interactions in the DSBR and V(D)J recombination pathways when human Ku is transfected into hamster cells. Third, Ku may act downstream or even bypass the primary defect in group 5 cells.

In conclusion, Ku p86 cDNA restored DNA end-binding activity, x-ray resistance, and V(D)J recombination activity to the group 5 mutants. These results are consistent with previous mapping studies localizing a gene that rescues group 5 cells to human chromosome 2q35 (*22*, *23*) and the gene for Ku p86 to 2q33–35 (*24*), although such mapping studies are limited in resolution to several megabases. Most importantly, the rescue of group 5 mutant cells by Ku establishes a role for Ku in both DSBR and V(D)J recombination and identifies the group 5 mutants as key reagents for further studies of Ku function in intact cells.

The association of Ku with the DNA-dependent protein kinase, DNA-PK, suggests an additional role in signaling pathways that respond to DNA damage. DNA-PK is activated to phosphorylate multiple protein substrates when Ku binds to DNA ends, nicks, gaps, and stem-loop structures (*25*). In vitro substrates for DNA-PK include p53, cJun, cFos, cMyc, Sp1, RNA polymerase II, and Ku itself. DNA ends and nicks are induced by x-rays, and nicked intermediates are generated by the excision repair of damage induced by ultraviolet radiation and many other DNA damaging agents. The demonstration that Ku rescues x-ray–sensitive mutant cells suggests that the binding of Ku to its DNA substrates may activate signaling pathways that regulate the cellular response to DNA damage, perhaps initiating cell cycle arrest or apoptosis.

Note added in proof: Similar results were reported by Taccioli *et al.* (*26*).

Table 1. V(D)J recombination frequency in cells transfected with Ku cDNAs. V(D)J recombination activity was measured in stably transformed wild-type (V79) and x-ray–sensitive mutant (XR-V15B, listed as V15B) cells containing cDNAs for Ku p70, Ku p86, or both, or with vector lacking a cDNA insert. The V(D)J recombination frequency (RF) was calculated by dividing the number of Amp^RCam^R colonies by the number of Amp^R colonies and is shown for valid coding and signal joints, as determined by restriction enzyme analysis (*27*).

Cell line	Transfected cDNA	Number of Amp^RCam^R colonies	Number of Amp^R colonies	RF ($\times 10^{-3}$)
		Coding joints		
V15B	Vector	0	59,400	<0.02
	86	42	50,400	0.83
	70	0	80,500	<0.01
	70,86	37	66,400	0.55
V79	Vector	136	64,700	2.10
	86	41	16,700	2.46
	70	106	42,000	2.52
	70,86	59	42,100	1.40
		Signal joints		
V15B	Vector	0	55,000	<0.02
	86	41	54,100	0.76
	70	2	52,800	0.04
	70,86	20	68,800	0.30
V79	Vector	210	62,800	3.35
	86	145	50,600	2.86
	70	122	38,500	3.17
	70,86	130	36,700	3.55

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27. Pooled Ku transformants were transiently transfected with 8 μg each of expression vectors for *RAG-1* and *RAG-2* together with 4 μg of either pJH290 to test for coding joints or pJH200 to test for signal joints (*7*). DNA was transfected by coprecipitation with calcium phosphate (*19*). Plasmid DNA was harvested after 48 hours, digested with Dpn I to eliminate unreplicated plasmids, and electroporated into *Escherichia coli*. Unrearranged plasmids conferred resistance to ampicillin (Amp^R), whereas rearranged plasmids conferred resistance to both ampicillin and chloramphenicol (Cam^R). The column labeled Amp^RCam^R shows the number of doubly resistant colonies that also contained plasmids with correctly formed recombination joints, as determined by restriction analysis. (Pvu II creates a restriction fragment spanning the coding joint, and Apa LI cleaves at the site of a precisely formed signal joint.) The V(D)J recombination frequencies are expressed as the ratio of Amp^RCam^R colonies containing valid rearrangements to the total number of Amp^R colonies (*7*).
28. Ku p70 and p86 were cotranscribed from pcDNA3 vectors (Invitrogen) under control of the T7 promoter and cotranslated with the TnT rabbit reticulocyte lysate kit (Promega) according to the manufacturer's instructions. Extract was incubated with radiolabeled f148 probe and competitor DNA for 15 min, then resolved on a 5% acrylamide gel (*8, 14*).
29. Nuclear extracts prepared by the NP-40 lysis method (*8*) were resolved by SDS-PAGE, transferred to GSWP membrane (Millipore), and probed with human OM antiserum (1:5000 dilution) followed by horseradish peroxidase–conjugated goat antibody to human immunoglobulin G (TAGO). Antibody binding was detected by enhanced chemiluminescence (Amersham) (*8, 14*).
30. Nuclear extract (0.6 μg) was incubated for 15 min with radiolabeled f148 probe in the presence of 2 μg (HeLa) or 50 ng (V15B, V79) of closed circular plasmid competitor to mask the effect of nonspecific DNA binding proteins. Protein-DNA complexes were resolved on a 5% acrylamide gel (*8, 14*).
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Abnormal Fear Response and Aggressive Behavior in Mutant Mice Deficient for α-Calcium-Calmodulin Kinase II

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Mice deficient for the gene encoding α-calcium-calmodulin–dependent kinase II (α-CaMKII knockout mice) provide a promising tool to link behavioral and cellular abnormalities with a specific molecular lesion. The heterozygous mouse exhibited a well-circumscribed syndrome of behavioral abnormalities, consisting primarily of a decreased fear response and an increase in defensive aggression, in the absence of any measured cognitive deficits. Unlike the heterozygote, the homozygote displayed abnormal behavior in all paradigms tested. At the cellular level, both extracellular and whole-cell patch clamp recordings indicated that serotonin release in putative serotonergic neurons of the dorsal raphe was reduced. Thus, α-CaMKII knockout mice, in particular the heterozygote, may provide a model for studying the molecular and cellular basis underlying emotional disorders involving fear and aggression.

The recently developed mouse gene knockout technology has allowed us to use a multidisciplinary approach to analyze neurobiological abnormalities at the behavioral and cellular levels. Here we tested mutant mice, in which the α-CaMKII gene was disrupted (*1*), for abnormal behaviors associated with aggression and fear.

We first evaluated in CaMKII mutant and wild-type mice the fear response of freezing (*2*), as characterized by an immobile, crouching posture after footshock, which is an indicator of activation of the fear system (*3*). During training, wild-type animals exhibited a high frequency of freezing after footshock, whereas the heterozygous (*t* test, $P < 0.005$) and homozygous ($P < 0.007$) mutants froze significantly less often (Fig. 1A). On the following day, the animals were returned to the shock chamber (contextual conditioning). In the absence of footshock, the wild-type mice displayed freezing behavior, indicating retention of the fear response (Fig. 1B). In contrast, the heterozygotes showed a low percentage of freezing that rapidly decayed ($P < 0.0001$). The homozygotes showed no freezing at all, suggesting a deficiency in the fear response.

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To establish whether the lower rate of freezing in mutants is caused by a fear-specific abnormality or simply by modifications in freezing-related sensory or motor processing capacities, we examined (i) fear conditioning elicited by a different sensory cue, (ii) pain sensitivity to footshocks, (iii) an innate fear behavior, and (iv) a fear-associated autonomic response. The mice were subjected to another task in which simple tones were paired with footshocks (*2*). The heterozygotes ($P < 0.0001$) and homozygotes ($P < 0.0001$) displayed much less freezing than the wild-type mice during training (Fig. 1C), similar to the contextual conditioning (Fig. 1A). Both groups of mutant mice exhibited partial retention of the fear response to tones (compared with the wild-type mice, $P < 0.0001$; between two mutant groups, $P < 0.0002$) (Fig. 1D). Therefore, both groups of mutant mice display reduced freezing to contextual as well as tonal cues.

Because reduced pain would result in less freezing (*4*), we measured current thresholds for three reactions to nociceptive shock, namely, flinch, jump, and vocalization (*5*). For all responses, the heterozygotes exhibited similar pain thresholds to the wild-type (*t* test, $P < 0.18$), whereas the homozygotes had significantly lower pain thresholds and were thus more sensitive to all the nociceptive stimuli (flinch, $P < 0.005$; jump, $P < 0.001$; vocalization, $P < 0.002$) (Fig. 2A). Therefore, it is unlikely that the attenuated freezing response is caused by modified pain sensitivity.