Perineuronal Nets Protect Fear Memories from Erasure

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Science **325**, 1258 (2009);
DOI: 10.1126/science.1174146

Perineuronal Nets Protect Fear Memories from Erasure

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In adult animals, fear conditioning induces a permanent memory that is resilient to erasure by extinction. In contrast, during early postnatal development, extinction of conditioned fear leads to memory erasure, suggesting that fear memories are actively protected in adults. We show here that this protection is conferred by extracellular matrix chondroitin sulfate proteoglycans (CSPGs) in the amygdala. The organization of CSPGs into perineuronal nets (PNNs) coincided with the developmental switch in fear memory resilience. In adults, degradation of PNNs by chondroitinase ABC specifically rendered subsequently acquired fear memories susceptible to erasure. This result indicates that intact PNNs mediate the formation of erasure-resistant fear memories and identifies a molecular mechanism closing a postnatal critical period during which traumatic memories can be erased by extinction.

Pairing an initially neutral stimulus (conditioned stimulus; CS) with an aversive stimulus (unconditioned stimulus; US) leads to the formation of a robust and long-lasting fear memory (*1*). In rats, such memories can last for the entire lifetime (*2*). Inhibition of conditioned fear responses can be achieved by repeated exposure to the CS in the absence of the US, a process called extinction (*3*). Unlike fear conditioning, in adult animals fear extinction is neither robust nor permanent. After extinction training, conditioned fear responses can recover spontaneously, after reexposure to the US (reinstatement), or in response to a context shift (renewal) (*3*–*5*). This strongly indicates that fear extinction does not erase previously acquired fear memories, but involves new learning eventually inhibiting conditioned fear behavior.

In stark contrast to adult animals, rats younger than 3 weeks do not exhibit reinstatement or context-dependent renewal of conditioned fear responses (*6*, *7*). During early postnatal development, extinction thus appears to be permanent and has been suggested to reflect an unlearning process that leads to the erasure of previously conditioned fear memories (*8*). Both in adults and in young animals, fear extinction depends on the amygdala (*3*, *8*). However, the neuronal mechanisms underlying the developmental regulation of fear extinction are not known.

Developmental regulation of brain plasticity is much better understood in sensory systems, such as the visual cortex. During the first few weeks of postnatal development, the so-called critical period, monocular sensory deprivation leads to long-lasting functional and structural changes (*9*). The absence of perineuronal nets (PNNs), a highly organized form of chondroitin sulfate proteoglycans (CSPG)–containing extracellular matrix (*10*), is considered to be a key permissive factor that allows the induction of ocular dominance plasticity during the critical period. The assembly of PNNs around parvalbumin (PV)-expressing inhibitory interneurons is thought to contribute to critical-period closure (*11*). Consistent with this notion, the degradation of PNNs in adults reenables the induction of ocular dominance plasticity (*11*). On the basis of these observations in sensory systems, we hypothesized that related developmental plasticity mechanisms may influence emotional learning processes in juveniles. Here, we examined whether in the amygdala, postnatal maturation of PNNs may underlie the developmental switch in fear extinction regulation.

We first quantified the time course of PNN formation in the basolateral amygdala (BLA) during the first 4 postnatal weeks (Fig. 1, A and B). The number of detectable *Wisteria floribunda* agglutinin (WFA)–stained PNNs increased markedly until postnatal day 28 (P28), when they were comparable to adult levels (Fig. 1B). Notably, the largest increase was observed between P16 and P21 (Fig. 1, A and B), the age around which the switch in the extinction phenotype occurs in rats (*8*).

We therefore compared spontaneous recovery and context-dependent renewal of extinguished

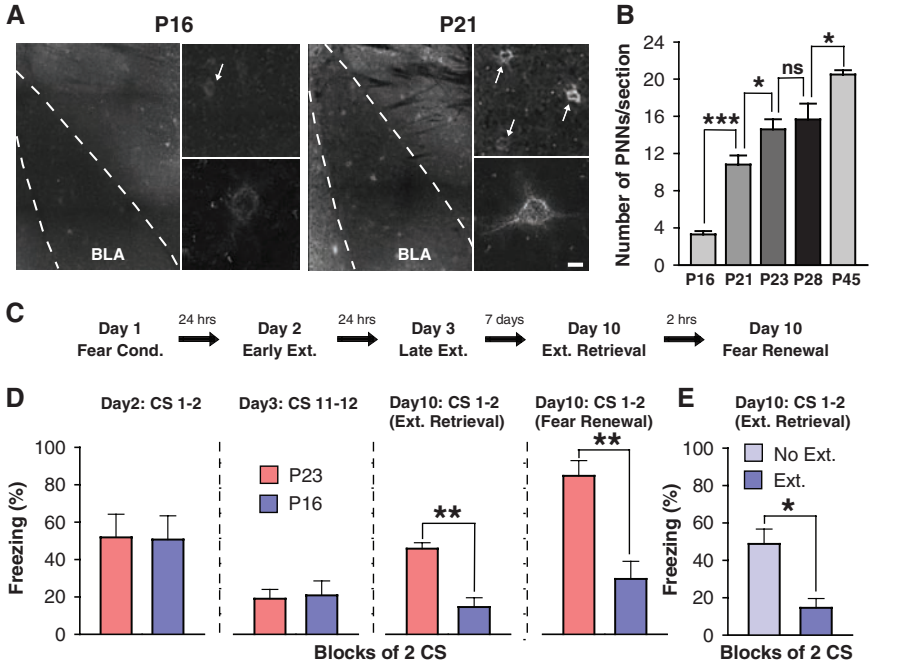

Fig. 1. Coincident developmental switch in PNN formation and in the susceptibility of fear memories to erasure. (**A**) Left panels: Overview of WFA staining in the BLA of P16 and P21 mice. Right panels: Higher-power images of PNNs in the BLA of the same age groups. Scale bar, 200 μm (left panels), 30 μm (top right panels), 10 μm (bottom right panels). (**B**) The number of WFA-positive PNNs in the BLA increases throughout postnatal development [one-way analysis of variance (ANOVA): $F_{(3,19)} = 23.4$, $P < 0.001$]. The largest increase is seen between P16 and P21 (Student-Newman-Keuls post-hoc tests). (**C**) Experimental protocol. Fear cond.: fear conditioning, Ext.: extinction. (**D**) One week after extinction training, comparison of freezing levels in mice conditioned at P16 ($n = 5$) or P23 ($n = 4$) revealed significant spontaneous recovery and context-dependent renewal in the P23, but not in the P16 group (percent of time spent freezing, extinction retrieval: P16: 14.6 ± 4.9, P23: 45.8 ± 3.2, $P < 0.01$; renewal: P16: 29.6 ± 9.4, P23: 84.8 ± 8.1; $P < 0.01$, two-tailed unpaired *t* test). In P23 mice, freezing levels during fear renewal were significantly greater than during extinction retrieval ($P < 0.05$, two-tailed paired *t* test).(**E**) In the absence of extinction training, P16 animals ($n = 7$) showed stable fear memory 10 days after conditioning (percent of time spent freezing: P16 No Ext: 48.7 ± 7.9, P16 Ext: 14.6 ± 4.9; $P < 0.05$, two-tailed unpaired *t* test). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ns, not significant.

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fear responses in young mice conditioned either before (P16) or after (P23) the age of 3 weeks (Fig. 1C). After fear conditioning, both groups exhibited equal levels of freezing behavior, which was strongly reduced by subsequent extinction training in a different context (Fig. 1D). Seven days after extinction training, mice were reexposed to the CS in both contexts. Mice conditioned at P23 exhibited significant spontaneous recovery and renewal when tested in the extinction context, or in the fear conditioning context, respectively (Fig. 1D). In contrast, in mice conditioned at P16, freezing levels did not increase compared with those measured at the end of extinction training, independent of the context in which they were exposed to the CS (Fig. 1D). The absence of spontaneous recovery and renewal did not reflect a passive loss of fear memory, because mice conditioned at P16 retained a stable fear memory for 10 days (Fig. 1E).

If PNNs in the BLA were causally related to the protection of fear memories from erasure by extinction, we hypothesized that it should be possible to convert the extinction phenotype of adult mice into a juvenile one by acutely destroying PNNs in adults. To test this, we locally injected the CSPG-degrading enzyme chondroitinase ABC (ChABC) (*11*, *12*) into the BLA of 3-month-old mice (fig. S1). Twenty-four hours after ChABC injection, no PNNs could be detected in the BLA (Fig. 2A). Mice fear conditioned 24 hours after ChABC injection (Fig. 2B) exhibited normal freezing levels compared with vehicle-injected controls when exposed to the CS 24 hours after conditioning (Fig. 2C). Moreover, both groups exhibited low freezing levels when exposed to an explicitly unpaired CS, indicating that conditioned freezing reflected CS-US associations rather than nonassociative sensitization processes (fig. S2).

After extinction training, freezing levels were equally reduced in ChABC- and vehicle-injected mice (Fig. 2C). However, when tested 7 or 28 days later, ChABC-injected mice, like juvenile mice conditioned at P16, exhibited a complete lack of spontaneous recovery and context-dependent renewal (Fig. 2C and figs. S3 and S4). Even though cued (CS-induced) fear behavior was strongly compromised, contextual fear memory was normal in ChABC-injected animals (Fig. 2C), which demonstrates that the absence of fear renewal cannot be explained by a deficit in context discrimination or by a lack of attention. Because contextual fear was not extinguished, this indicated that extinction training was necessary for the ChABC-induced permanent loss of conditioned fear behavior. To examine the time course of extinction-induced behavioral changes in ChABC-injected mice, we analyzed freezing levels during extinction training. Whereas a total of 24 CSs in two extinction training sessions distributed over 2 days was necessary to achieve full extinction of conditioned fear responses in control animals (Fig. 2D) (*13*), exposing ChABC-injected mice to just three CSs substantially decreased freezing levels, and after seven CS presentations, freezing behavior was already at baseline levels (Fig. 2D). We also examined the time course of extinction learning in juvenile mice conditioned at P16 or P23. Freezing behavior during extinction training was much more variable in juvenile animals compared with adults, but there was no significant group difference in the time course of extinction learning between juvenile mice conditioned at P16 or P23. The lack of accelerated within-session extinction in P16 mice could be related to the presence of diffuse WFA staining, which was not observed in adult animals treated with ChABC. Thus, in the absence of PNNs, extinction training triggers a rapid process that results in the acute and permanent loss of conditioned fear behavior, suggesting that PNNs in the amygdala prevent unlearning or erasure of fear memories in adults.

There are several alternative mechanisms that may explain the observed lack of conditioned fear responses in ChABC-injected mice. Because degraded PNNs take weeks to turn over after ChABC injection (*12*), one possibility is that degradation of PNNs could interfere with fear memory consolidation. Although the stability of contextual fear memory in ChABC-injected mice suggests otherwise (Fig. 2C), we specifically tested whether ChABC-injected mice could form a stable, long-term cued fear memory. Mice were injected with ChABC or vehicle (fig. S5) and fear conditioned 24 hours later. Long-term fear memory was ex-

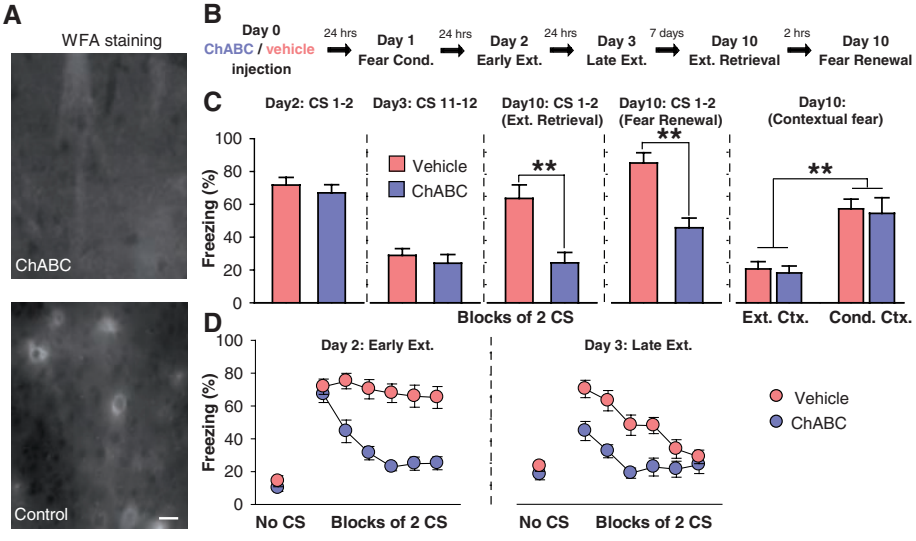

Fig. 2. Degradation of PNNs in adult mice abolishes spontaneous recovery and context-dependent renewal of conditioned fear. (**A**) ChABC injection eliminates WFA-positive PNNs in adult BLA. Control mice were injected with vehicle (phosphate-buffered saline). (**B**) Experimental protocol. (**C**) One week after extinction training, vehicle-injected mice (n = 10), but not ChABC-injected mice (n = 11), exhibited spontaneous recovery (measured in the extinction context) and renewal (measured in the fear conditioning context) of conditioned fear responses (percent of time spent freezing, extinction retrieval: vehicle: 63.5 ± 8.3, ChABC: 24.3 ± 6.3, $P < 0.01$; fear renewal: vehicle: 85.1 ± 6.3, ChABC: 45.7 ± 5.9; $P < 0.01$, two-tailed unpaired t test). ChABC-injected animals exhibited normal contextual fear responses (pre-CS freezing levels measured in the extinction context (Ext. Ctx.) versus conditioning context (Cond. Ctx.; $P < 0.01$ for both groups, two-tailed unpaired t tests). (**D**) ChABC-injected mice (n = 13), but not vehicle-injected mice (n = 13), exhibited a rapid reduction in freezing levels as early as the first day of extinction training (Day 2) [two-way ANOVA with repeated measures, (group × time); group: $F_{(1,24)} = 34.3$, $P < 0.001$; time: $F_{(1,5)} = 12.5$, $P < 0.001$; interaction between group and time: $F_{(5,120)} = 6.9$, $P < 0.001$]. Freezing levels between ChABC- and vehicle-injected mice were significantly different on the second block of extinction (percent of time spent freezing, second block of extinction: vehicle: 75.1 ± 4.7, ChABC: 44.5 ± 6.9; $P < 0.001$, two-tailed paired t test). At the end of the second day of extinction (Day 3), both groups reached similar levels of extinction (percent of time spent freezing, last block of extinction: vehicle: 28.8 ± 4.1, ChABC: 24.1 ± 5.3; $P = 0.51$, two-tailed paired t test). **$P < 0.01$.

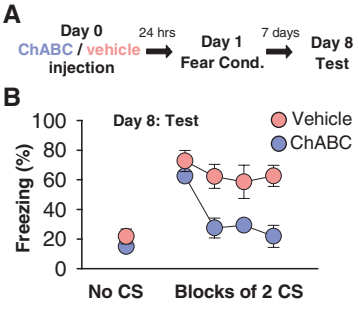

Fig. 3. Degradation of PNNs does not interfere with fear memory consolidation. (**A**) Experimental protocol for consolidation experiments. (**B**) Degradation of PNNs did not affect fear memory consolidation as measured 7 days after fear conditioning (percent of time spent freezing, first block: vehicle: 72.8 ± 7.1, n = 5; ChABC: 62.5 ± 3.8, n = 5; $P = 0.78$, two-tailed unpaired t test), yet rapid extinction was induced in ChABC-injected mice exposed to repetitive nonreinforced CSs (one-way ANOVA with repeated measures: vehicle: $F_{(4,3)} = 0.72$, $P = 0.56$; ChABC: $F_{(4,3)} = 18.5$, $P < 0.001$).

amined 7 days after conditioning (Fig. 3, A and B). Similar to the 24-hour time point, ChABC- and vehicle-injected mice did not differ in terms of their initial freezing levels even 7 days after fear conditioning (Fig. 3B). Still, repetitive CS exposure induced almost instantaneous extinction in ChABC-injected mice, whereas freezing levels remained constant in control animals (Fig. 3B).

A second possible explanation for the lack of spontaneous recovery and renewal could be that removal of PNNs enhanced new learning of inhibitory CS–no US associations during extinction training. That is, ChABC injections may have strengthened the extinction process per se rather than enabled fear memory erasure. Although difficult to test directly, we argued that if removal of PNNs acted by strengthening inhibitory learning during extinction training, the effect of ChABC injection should be independent of whether animals were injected before or after fear conditioning, as long as PNNs were destroyed before extinction training. We therefore compared extinction of conditioned fear memories acquired before or after ChABC injection in the same animals (Fig. 4, A and B; fig. S6). Degradation of PNNs after fear conditioning did not accelerate the time course of subsequent extinction learning (Fig. 4B). In the same animals, however, we observed rapid extinction of a second fear memory acquired in the absence of PNNs (Fig. 4B). Further, repeated retrieval of fear memories acquired before PNN degradation revealed stable freezing levels across several days, thus excluding a possible effect on memory reconsolidation (*14*, *15*) (Fig. 4C). Finally, we examined whether fear memories acquired before ChABC injection could be extinguished normally. Mice fear conditioned 24 hours before ChABC injection exhibited normal extinction and, when tested 1 week later, showed normal levels of spontaneous recovery and context-dependent renewal (Fig. 4, D and E).

Together, these findings demonstrate that loss of PNNs does not strengthen new inhibitory learning during extinction or impair memory reconsolidation, but renders fear memory traces susceptible to unlearning or erasure. Because ChABC injection had only anterograde, but no retrograde effects, we conclude that the state of fear memories acquired in the absence of PNNs fundamentally differs compared with the state of memories acquired in the presence of PNNs. Whereas in the presence of PNNs repeated nonreinforced CS presentations lead to the active inhibition of conditioned fear responses, in the absence of PNNs the same protocol leads to erasure of the fear memory.

What might be the mechanism by which degradation of PNNs enables fear memory erasure? Fear memories are acquired and stored, at least in part, by learning-induced strengthening of synaptic inputs to the lateral (LA) or basal (BA) nucleus of the amygdala through *N*-methyl-D-aspartate (NMDA) receptor–dependent long-term potentiation (LTP) (*1*, *16*, *17*). Thus, one possible mechanism could be that PNNs prevent fear memory erasure by rendering potentiated synapses resistant to LTP reversal, so-called depotentiation (*18*, *19*). However, because removal of PNNs after fear conditioning had no effect on extinction learning, this indicates that PNNs regulate network plasticity during fear conditioning in such a way that fear conditioning leads to the formation of an erasure-prone memory trace. To examine the effect of PNN removal on synaptic plasticity in the amygdala, we compared LTP at thalamo-LA inputs in slices obtained from ChABC- and vehicle-injected animals. LTP of monosynaptic excitatory inputs was completely abolished upon ChABC treatment, and LTP of disynaptic inhibition, which reflects synaptic plasticity at glutamatergic inputs onto local feedforward interneurons, was markedly reduced (fig. S7). Although these results may suggest less potent fear-acquisition mechanisms upon ChABC treatment, they are only correlative in nature. Different forms of thalamo-LA LTP, or plasticity at other intrinsic or extrinsic synaptic connections and pathways, could be more relevant to the observed behavioral phenotype. Consistent with our observations, however, in vitro removal of PNNs interferes with the induction of LTP and long-term depression in hippocampal slices (*20*). Underlying mechanisms might also involve changes in local GABA-mediated inhibition. PNNs primarily form around PV-positive GABA-containing interneurons (*10*), and GABA-mediated inhibition regulates various forms of synaptic plasticity in the BLA (*21*, *22*).

Our study indicates that qualitatively distinct neuronal mechanisms mediate acquisition and extinction of conditioned fear memories during early postnatal development and in adults. In adults, extinction memories coexist with previously acquired fear memories, both of which can be retrieved in a context-dependent manner. By contrast, in young postnatal animals, contextualization of fear memories does not occur—rather, fear memories are overwritten and deleted during extinction. Thus, contextualization of a memory by a second learning episode, a phenomenon that has been proposed to reflect a general principle governing the interaction between different memories (*23*, *24*), appears to be developmentally regulated. Consistent with this notion, the connectivity between brain areas implicated in contextualization of fear and extinction memories, including the amygdala, the prefrontal cortex, and the hippocampus, continues to develop during a protracted period of time, up to several months after birth (*25*, *26*). Functionally, our results may thus imply that during early postnatal development, chances of survival may be optimized by adhering to the most recently learned information. Such a strategy may also support learning from tutors.

Previous results implicate the formation of PNNs around PV-expressing interneurons in critical-period closure in the visual cortex (*9*, *27*).

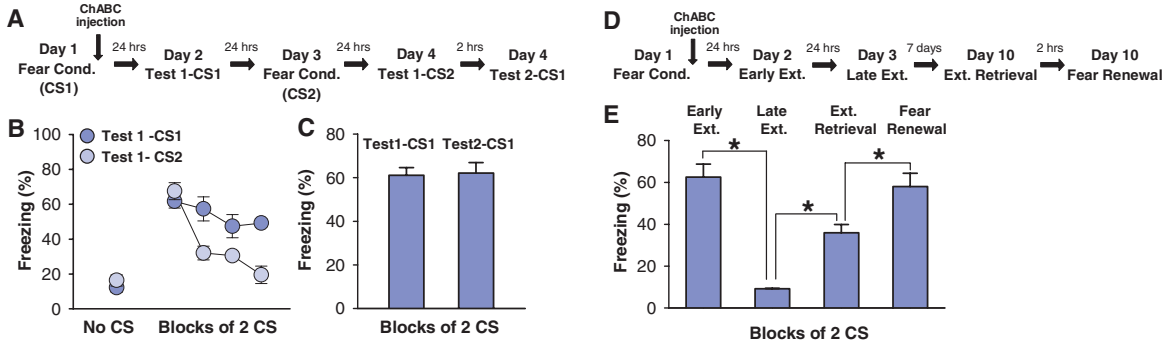

Fig. 4. Degradation of PNNs does not affect previously acquired fear memories or memory reconsolidation. (**A**) Experimental protocol. (**B**) Rapid extinction only occurred for conditioned fear responses that had been acquired after ChABC injection [Fear Cond. (CS2), $n = 6$], but not for those acquired before ChABC injection [Fear Cond. (CS1), $n = 9$, two-way ANOVA, (group × time); group: $F_{(1,13)} = 9.15$, $P < 0.01$; time: $F_{(1,3)} = 16.87$, $P < 0.001$; interaction between group and time: $F_{(3,39)} = 5.94$, $P < 0.05$). (**C**) Degradation of PNNs did not affect fear memory reconsolidation as measured by two consecutive tests separated by 48 hours. (**D**) Experimental protocol used to examine extinction in animals injected with ChABC after fear conditioning. (**E**) Normal extinction, spontaneous recovery and renewal in mice injected with ChABC after the acquisition of conditioned fear. Mice were injected with ChABC immediately after conditioning and submitted to fear extinction. At the end of extinction (Day 3), fear levels were significantly reduced (percent of time spent freezing, early extinction: 62.5 ± 6.3, late extinction: 9.2 ± 0.3; $P < 0.05$, two-tailed paired *t* test). One week later, ChABC-injected mice ($n = 3$) exhibited spontaneous recovery and renewal of conditioned fear responses when tested in the extinction and fear-conditioning context, respectively (percent of time spent freezing, recall: 36 ± 3.9; renewal: 58 ± 6.4; $P < 0.05$, two-tailed paired *t* test). *$P < 0.05$.

In the BLA, formation of PNNs marked the end of a developmental period during which fear memories could be erased by extinction and coincided with ability to form contextualized fear and extinction memories. This may suggest a general role for PNNs in mediating developmental changes in information storage in neuronal circuits. However, because degradation of PNNs in adult animals reenabled erasure of fear memories by extinction, this demonstrates that the mechanisms underlying extinction-induced fear memory erasure in juveniles are not lost in the adult, but that fear memories are actively protected from erasure by PNNs. Because context-dependent renewal of conditioned fear responses is believed to be an important factor contributing to the relapse of pathological fear in patients undergoing therapy for anxiety disorders (*28*), our findings may point to novel strategies in preventing the development of extinction-resistant pathological fear and anxiety.

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29. This work was supported by the Austrian Science Fund (FWF), Swiss National Science Foundation, and Novartis Research Foundation. We thank G. Cassasus for electrophysiological characterization of ChABC-injected animals and K. Scheffler for help with programming. We thank R. F. Westbrook and all members of the Lüthi lab for discussions and critical comments on the manuscript. The manuscript was prepared by N.G., P.C., A.L., and C.H. The authors declare that they have no competing financial interest.

Supporting Online Material

www.sciencemag.org/cgi/content/full/325/5945/1258/DC1
Materials and Methods
Figs. S1 to S7
References

27 March 2009; accepted 21 July 2009
10.1126/science.1174146

Activation of the PI3K Pathway in Cancer Through Inhibition of PTEN by Exchange Factor P-REX2a

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PTEN (phosphatase and tensin homolog on chromosome 10) is a tumor suppressor whose cellular regulation remains incompletely understood. We identified phosphatidylinositol 3,4,5-trisphosphate RAC exchanger 2a (P-REX2a) as a PTEN-interacting protein. P-REX2a mRNA was more abundant in human cancer cells and significantly increased in tumors with wild-type PTEN that expressed an activated mutant of *PIK3CA* encoding the p110 subunit of phosphoinositide 3-kinase subunit α (PI3Kα). P-REX2a inhibited PTEN lipid phosphatase activity and stimulated the PI3K pathway only in the presence of PTEN. P-REX2a stimulated cell growth and cooperated with a *PIK3CA* mutant to promote growth factor–independent proliferation and transformation. Depletion of P-REX2a reduced amounts of phosphorylated AKT and growth in human cell lines with intact PTEN. Thus, P-REX2a is a component of the PI3K pathway that can antagonize PTEN in cancer cells.

The *PTEN* (phosphatase and tensin homolog on chromosome 10) gene is frequently lost in cancers, and germline *PTEN* mutations are linked to inherited cancer predisposition syndromes (*1*). The PTEN protein dephosphorylates phosphatidylinositol 3,4,5-trisphosphate (PIP3), the critical lipid second messenger generated by phosphoinositide 3-kinase (PI3K) upon stimulation of cells by external mitogens (*2*, *3*). Inactivation of PTEN leads to accumulation of PIP3 and, as a consequence, increases activity of the kinase AKT, which promotes cellular survival, cell cycle progression, and growth, thereby contributing to oncogenesis. Studies of human tumors have revealed alterations in multiple components of the PTEN-PI3K-AKT axis, all of which result in increased signaling (*4*). There is mounting evidence that posttranslational modifications, including oxidation, phosphorylation, and ubiquitinylation, regulate PTEN (*5*, *6*). To further elucidate cellular regulatory mechanisms, we identified PTEN-interacting proteins by affinity purification. We postulated that a PTEN-mutant cell line would be a rich source for interacting proteins without competing endogenous PTEN and selected DBTRG-05MG, a human glioblastoma cell line, because it grows rapidly in culture and lacks detectable PTEN owing to an in-frame deletion of codons 274 to 342 (*7*). PTEN-binding proteins were purified from cytoplasmic extracts on an affinity column with PTEN as a glutathione *S*-transferase (GST) fusion protein and sequenced by mass spectrometry (Fig. 1A) (*8*, *9*). Associated proteins included PTEN-binding protein, major vault protein (MVP), and a second protein named P-REX2a (*10*).

P-REX2a is a guanine nucleotide exchange factor (GEF) for the RAC guanosine triphosphatase (GTPase), which was discovered in a search for proteins with sequence similarity to the leukocyte-specific RAC GEF, P-REX1 (*11*, *12*). P-REX1 GEF activity is critical for RAC-mediated formation of reactive oxygen species in response to PIP3 and signaling by the βγ heterodimer of heterotrimeric guanine nucleotide–binding protein (Gβγ signaling) in neutrophils (*13*). P-REX1 expression is increased in metastatic prostate cancers and has been shown to mediate a RAC-dependent metastatic and invasive phenotype in prostate cancer cell lines (*14*). P-REX2a is a widely expressed paralog of P-REX1 and contains an N-terminal Dbl homology and pleckstrin homology (DHPH) domain (which confers GEF activity), pairs of PDZ and DEP domains, and a C terminus with weak similarity to inositol 4-polyphosphate phosphatase.

To demonstrate an endogenous interaction, we coimmunoprecipitated P-REX2a and PTEN from HEK293 extracts using either a polyclonal

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