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# Nuclear Accumulation of NFAT4 Opposed by the JNK Signal Transduction Pathway

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**The nuclear factor of activated T cells (NFAT) group of transcription factors is retained in the cytoplasm of quiescent cells. NFAT activation is mediated in part by induced nuclear import. This process requires calcium-dependent dephosphorylation of NFAT caused by the phosphatase calcineurin. The c-Jun amino-terminal kinase (JNK) phosphorylates NFAT4 on two sites. Mutational removal of the JNK phosphorylation sites caused constitutive nuclear localization of NFAT4. In contrast, JNK activation in calcineurin-stimulated cells caused nuclear exclusion of NFAT4. These findings show that the nuclear accumulation of NFAT4 promoted by calcineurin is opposed by the JNK signal transduction pathway.**

JNK is a member of the mitogen-activated protein (MAP) kinase group that is activated in response to cytokines and exposure to environmental stress (*1*). Substrates of the JNK protein kinase include the transcription factors ATF2, Elk-1, and c-Jun (*2*). JNK phosphorylates each of these transcription factors within the activation domain and increases transcriptional activity (*2*). Genetic studies of JNK indicate that this signaling pathway involves multiple cellular processes (*3*). Here, we show that one of these processes is the regulated nuclear accumulation of NFAT4.

Substrate recognition by JNK requires the interaction of the kinase with a region of the substrate that is distinct from the sites that are phosphorylated (*2*). We used this specific binding interaction to identify JNK substrates with the two-hybrid method (*4*). Fourteen independent cDNA clones that encode the transcription factor NFAT4 were obtained from an embryonic cDNA library. NFAT4 (also known as NFATx and NFATC3) is a member of the REL domain family of transcription factors that are important mediators of immune responses (*5*, *6*). In contrast to the other members of the NFAT group, which are primarily expressed in peripheral T cells (NFATc and NFATp), the NFAT4 isoform is expressed in immature thymocytes and nonlymphoid tissues (*5*–*7*). Because JNK is implicated in both innate (*3*, *8*) and acquired (*9*) immune responses, the interaction between JNK and NFAT4 may be biologically important.

To test whether JNK and NFAT4 interact in mammalian cells, we performed coimmunoprecipitation assays using extracts prepared from transfected cells. Immunoblot analysis demonstrated the presence of JNK in NFAT4 immunoprecipitates (Fig. 1A). Control experiments showed that JNK was not detected in NFAT3 immunoprecipitates (Fig. 1A). These data indicate that JNK interacts with NFAT4 in vivo. To confirm that JNK binds directly to NFAT4, we performed in vitro binding assays with recombinant proteins. JNK2 bound strongly to NFAT4 but not to NFAT3 (Fig. 1B). Similar data were obtained in experiments using JNK1 (*10*). The amount of JNK binding to NFAT4 was approximately five times the amount of binding to c-Jun (*10*). Deletion analysis demonstrated that the mini-

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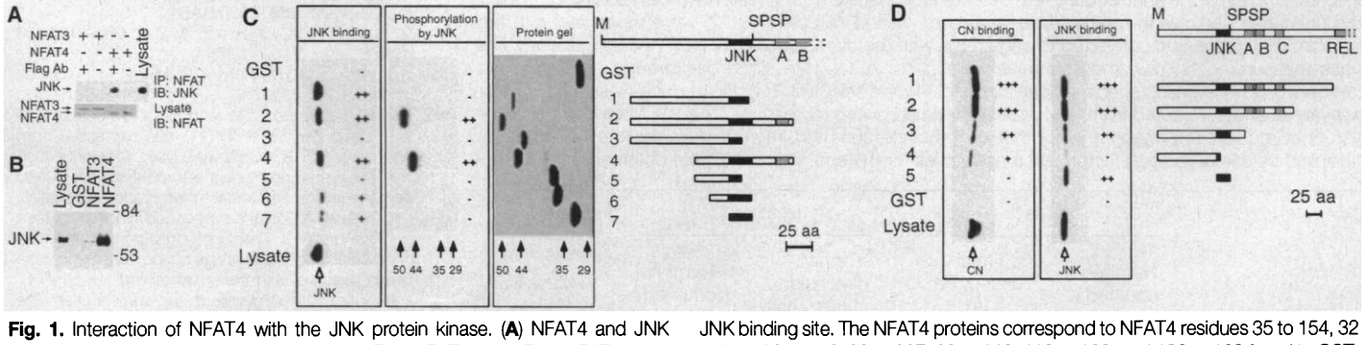


**Fig. 1.** Interaction of NFAT4 with the JNK protein kinase. (**A**) NFAT4 and JNK interact in vivo. Epitope-tagged HA-JNK2, Flag-NFAT3, and Flag-NFAT4 were expressed in COS cells. NFAT3 and NFAT4 were immunoprecipitated with mAb M2. HA-JNK2 in the immunoprecipitates (IP) was detected by protein immunoblot (IB) analysis with mAb 12CA5 (Boehringer-Mannheim). The NFAT3 and NFAT4 proteins in the cell lysates were detected by protein immunoblot analysis. (**B**) Binding of JNK to the $NH_2$-terminal region of NFAT4. NFAT4(1–207) and NFAT3(1–218) were fused to GST. The purified recombinant NFAT proteins were used to bind epitope-tagged HA-JNK2 (*17*). Bound JNK2 was detected by protein immunoblot analysis. Molecular size markers are indicated (in kilodaltons). (**C**) Identification of the JNK binding site. The NFAT4 proteins correspond to NFAT4 residues 35 to 154, 32 to 207, 32 to 146, 93 to 207, 93 to 146, 113 to 162, and 136 to 162 fused to GST. The purified recombinant NFAT4 proteins were examined by SDS-PAGE (Coomassie staining), as substrates for JNK2 (*17*), and in binding assays with JNK2 (*17*). The phosphorylation of NFAT4 was detected after SDS-PAGE by autoradiography. Bound JNK2 was detected by protein immunoblot analysis. (**D**) Binding of JNK and calcineurin to different sites on NFAT4. The NFAT4 proteins correspond to NFAT4 residues 1 to 413, 1 to 333, 1 to 207, 1 to 146, and 136 to 162 fused to GST. The immobilized NFAT proteins were incubated with cell extracts. Bound JNK2 and calcineurin were detected by protein immunoblot analysis.

mal region of NFAT4 required for interaction with JNK corresponds to residues 136 to 162 of human NFAT4 (Fig. 1C). This region of NFAT4 is not conserved in other members of the NFAT group.

The phosphatase calcineurin activates NFAT transcription factors by dephosphorylation (*7*). We therefore compared NFAT4-JNK binding with NFAT4-calcineurin binding. Deletion analysis demonstrated that the regions of NFAT4 required for interaction with JNK and calcineurin were distinct (Fig. 1D). The calcineurin binding site maps to a domain that includes three regions (A, B, and C boxes) conserved in the NFAT group of transcription factors (Fig. 1D). The calcineurin binding domain includes the glycogen synthase kinase–3 sites of NFAT phosphorylation that are dephosphorylated by calcineurin and have been implicated in the regulation of nuclear translocation (*11*).

Immune-complex kinase assays showed that NFAT4 is a substrate for JNK. Deletion analysis indicated that the sites of phosphorylation by JNK are located between NFAT4 residues 162 and 207 (Fig. 1C). Phosphoamino acid analysis demonstrated the presence of phosphoserine (Fig. 2A). Inspection of the NFAT4 sequence revealed two potential serine-proline motifs that might correspond to the sites of phosphorylation by JNK. These sites are located in a region of NFAT4 that is similar to the JNK phosphorylation sites located in the transcription factor ATF2 (*12*). Replacement of either of these sites ($Ser^{163}$ and $Ser^{165}$) with alanine decreased the shift in mobility of NFAT4 caused by phosphorylation by JNK during polyacrylamide gel electrophoresis (PAGE) (Fig. 2B). Moreover, replacement of both sites with alanine eliminated most of the phosphorylation of NFAT4 by JNK (Fig. 2B). These data indicate that $Ser^{163}$ and $Ser^{165}$ represent the major sites of in vitro phosphorylation of NFAT4 by JNK.

Phosphorylation of NFAT in vivo is associated with decreased mobility during PAGE (*7*). We therefore examined the mobility of wild-type and mutant ($Ala^{163,165}$) NFAT4 by immunoblot analysis. The mutant NFAT4 exhibited increased PAGE mobility relative to that of wild-type NFAT4 (Fig. 2C). In contrast, the mobility of these NFAT4 proteins was similar after dephosphorylation by calcineurin (Fig. 2C). Tryptic phosphopeptide mapping of wild-type and mutant NFAT4 confirmed that $Ser^{163}$ and $Ser^{165}$ are phosphorylated in vivo (Fig. 2D).

To examine the possible role of JNK in the regulation of NFAT4 function, we examined the effect of mutation of the JNK phosphorylation sites on the transcriptional activity of NFAT4. We used a luciferase reporter plasmid containing an NFAT site derived from the interleukin-2 (IL-2) promoter. The mutant NFAT4 had more transcriptional activity than did wild-type NFAT4 (Fig. 2E). We also fused the $NH_2$-terminal region of NFAT4 (residues 1 to 207) to the DNA binding domain of GAL4 and examined the transcriptional activity of the GAL4-NFAT4 fusion protein with a luciferase reporter plasmid containing GAL4 sites. Mutation of the JNK phosphorylation sites in the GAL4-NFAT4 fusion protein did not change the expression of the luciferase reporter gene (Fig. 2F). These data indicate that the effect of mutation of the JNK phosphorylation sites (that is, increased NFAT4 transcriptional activity) is not accounted for by changes in the function of the $NH_2$-terminal activation domain of NFAT4.

The NFAT transcription factors are sequestered in the cytoplasm until a sustained increase in intracellular calcium concentration leads to calcineurin-induced nuclear translocation (*7*). Changes in nuclear compartmentation could therefore contribute to the altered function of the mutant NFAT4. We tested whether the phosphorylation of NFAT4 might regulate nuclear accumulation. Immunoblot analysis of subcellular fractions demonstrated that the wild-type NFAT4 protein was cytoplasmic, whereas the NFAT4 protein lacking the JNK phosphorylation sites was located in the nucleus (*10*). These results were confirmed by immunofluorescence analysis (Fig. 3). NFAT4 was detected in the cytoplasm of control cells. Activated calcineurin caused nuclear accumulation of NFAT4. Control experiments showed that nuclear accumulation of NFAT3, NFATc, and NFATp occurred in response to activated calcineurin (Fig. 3). In contrast, the mutant NFAT4 protein was nuclear in both the presence and absence of activated calcineurin (Fig. 3). Thus, nuclear accumulation of NFAT4 can be mediated either by calcineurin or by the mutational

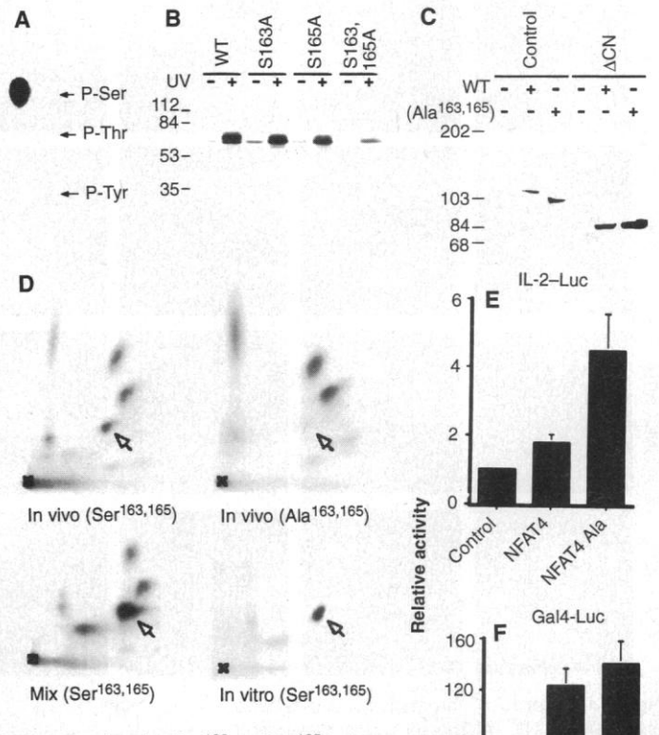


**Fig. 2.** Phosphorylation of NFAT4 by JNK. (**A**) JNK2 phosphorylates NFAT4 on serine residues. Recombinant NFAT4 was phosphorylated by JNK2 in vitro and examined by phosphoamino acid analysis, and phosphoserine was shown to be present. (**B**) Wild-type (WT) and mutated GST-NFAT4 (residues 1 to 207) were examined in protein kinase assays with JNK2 (*17*). The phosphorylated NFAT4 proteins were detected after SDS-PAGE by autoradiography (UV, ultraviolet light). (**C**) The effect of activated calcineurin (ΔCN) on wild-type NFAT4 and mutated ($Ala^{163,165}$) NFAT4 was examined in transfected COS-1 cells (*18*). The epitope-tagged NFAT proteins were detected by protein immunoblot analysis with mAb M2. (**D**) NFAT4 is phosphorylated on $Ser^{163}$ and $Ser^{165}$ in vivo. NFAT4 was isolated from MKK7-transfected COS cells labeled with [$^{32}$P]phosphate by immunoprecipitation. The NFAT4 proteins were digested with trypsin, and the peptides obtained were examined by phosphopeptide mapping (*12*). Maps of NFAT4 phosphorylated in vivo, NFAT4 phosphorylated by JNK2 in vitro, and a mixture of in vivo and in vitro phosphorylated NFAT4 are shown (horizontal dimension, electrophoresis; vertical dimension, chromatography; ×, origin). (**E**) The transcriptional activity of NFAT4 was examined in transfection assays in BHK cells by means of a luciferase reporter plasmid containing an NFAT site derived from the IL-2 promoter (*19*). The effect of replacement of $Ser^{163}$ and $Ser^{165}$ with Ala residues is shown. (**F**) The transcriptional activity of the GAL4 DNA binding domain (control) and GAL4-NFAT4 fusion proteins was examined with a GAL4-luciferase reporter plasmid in BHK cells (*19*).

removal of the JNK phosphorylation sites.

We also examined the effect of the calcineurin inhibitor cyclosporin A (*7*) on the nuclear accumulation of wild-type and mutant NFAT4. Cyclosporin A blocked the calcineurin-stimulated nuclear accumulation of NFAT4, NFAT3, NFATc, and NFATp (Fig. 3). Cyclosporin A also blocked the constitutive nuclear accumulation of the phosphorylation-defective ($Ala^{163,165}$) NFAT4 (Fig. 3). This nuclear accumulation is apparently mediated by a complex of NFAT4 with calcineurin (*13*).

Because mutational removal of the JNK phosphorylation sites leads to nuclear accumulation of NFAT4, the phosphorylation of NFAT4 on these sites may cause either nuclear export or cytoplasmic retention. To test this hypothesis, we examined the effect of JNK activation on the calcineurin-stimulated accumulation of NFAT4 in the nucleus. The MAP kinase kinase MKK7 (*14*) was used as a specific activator of JNK in these assays. JNK activation antagonized the nuclear accumulation of NFAT4 caused by calcineurin (Fig. 3) but did not block the nuclear accumulation of the mutated NFAT4 that lacks the JNK phosphorylation sites (Fig. 3). Moreover, the calcineurin-stimulated nuclear accumulation of NFAT3, NFATc, and NFATp was not affected by MKK7 (Fig. 3). These observations indicate that the JNK signal transduction pathway opposes the effect of calcineurin on the nuclear accumulation of NFAT4.

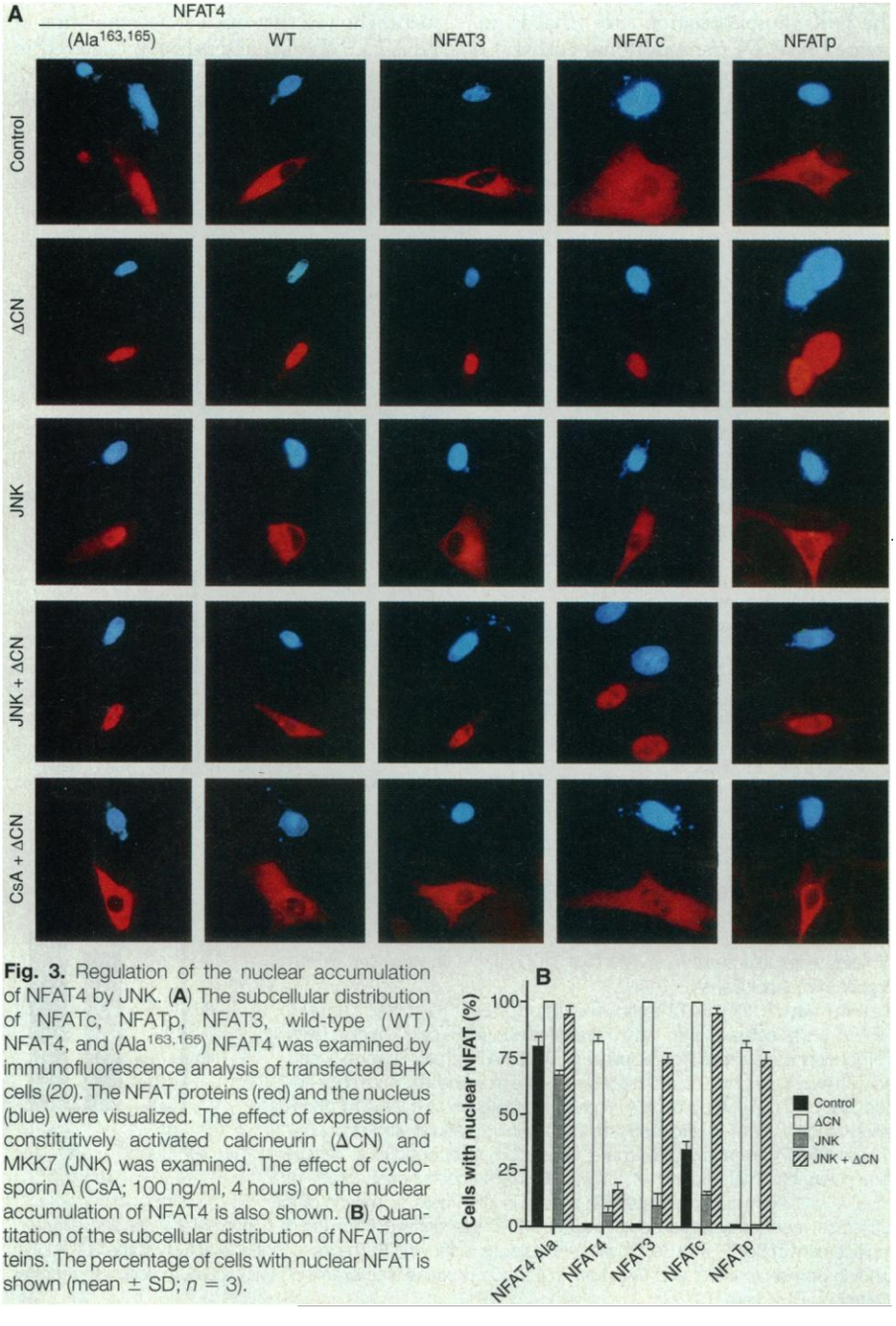


**Fig. 3.** Regulation of the nuclear accumulation of NFAT4 by JNK. (**A**) The subcellular distribution of NFATc, NFATp, NFAT3, wild-type (WT) NFAT4, and ($Ala^{163,165}$) NFAT4 was examined by immunofluorescence analysis of transfected BHK cells (*20*). The NFAT proteins (red) and the nucleus (blue) were visualized. The effect of expression of constitutively activated calcineurin (ΔCN) and MKK7 (JNK) was examined. The effect of cyclosporin A (CsA; 100 ng/ml, 4 hours) on the nuclear accumulation of NFAT4 is also shown. (**B**) Quantitation of the subcellular distribution of NFAT proteins. The percentage of cells with nuclear NFAT is shown (mean ± SD; $n = 3$).

Previous studies established that NFAT transcriptional activity requires both an AP-1 component and the nuclear translocation of cytoplasmic NFAT (*7*). The JNK signal transduction pathway has been shown to increase AP-1 transcriptional activity (*2*). It was therefore not expected that an activator of AP-1, like JNK, would block the nuclear accumulation of NFAT4. However, the JNK pathway represents only one mechanism of AP-1 activation (*15*). Other pathways—for example, the Ras-activated ERK pathway—increase AP-1 independently of the JNK pathway (*15*). These considerations indicate that the transcriptional activity of nuclear NFAT4 may be potentiated by the ERK signaling pathway rather than by JNK (*2*). To test this hypothesis, we examined NFAT4 transcriptional activity in the presence of activated Ras (*16*). We found that the transfection of cells with NFAT4 did not increase Ras-stimulated transcriptional activity in experiments using an NFAT4 reporter gene (Fig. 4). In contrast, transfection of cells with ($Ala^{163,165}$) NFAT4 increased Ras-stimulated NFAT transcriptional activity (Fig. 4). These data indicate that Ras activates the transcriptional activity of nuclear NFAT4 (Fig. 4).

The negative regulation of NFAT4 nuclear accumulation caused by JNK provides a mechanism for cell type–specific responses to extracellular stimulation. Thus, JNK activation causes increased AP-1 transcriptional activity (*2*) that cooperates with calcium-stimulated nuclear accumulation of NFAT3, NFATp, and NFATc (*7*). In contrast, JNK activation blocks NFAT nuclear

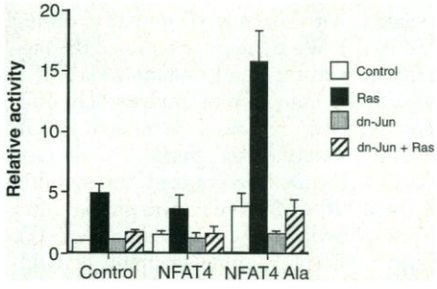


**Fig. 4.** Requirement for an additional signal to activate NFAT4. Jurkat cells were transfected without (control) and with NFAT4 or ($Ala^{163,165}$) NFAT4. Transcriptional activity was monitored with an IL-2 NFAT reporter plasmid (*19*). The effect of expression of activated Ras and dominant negative c-Jun (dn-Jun) was examined (*18*).

accumulation in cells that express NFAT4 (Fig. 3). The NFAT4 isoform therefore serves to integrate negative signals from JNK into the NFAT signaling pathway. Positive signals to calcium-stimulated NFAT4 derive, in part, from the Ras signaling pathway (Fig. 4). Together, these findings indicate that NFAT4 may mediate signal-specific responses in activated cells.

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18. Expression vectors for activated calcineurin [Y. Watanabe, B. A. Perrino, B. H. Chang, T. R. Soderling, *J. Biol. Chem.* **270**, 456 (1995)], JNK (*17*), MKK7 (*14*), Ras61L (*1*), and dominant negative c-Jun [TAM67(Δ3-122)] [U. R. Rapp, J. Troppmair, T. Beck, M. J. Birrer, *Oncogene* **9**, 3493 (1994)] have been described. The human NFAT3 and NFAT4 cDNAs were cloned in the polylinker of the expression vector pCDNA3 (Invitrogen). The human NFATc and NFATp cDNAs cloned in the vector pREP4 were obtained from T. Hoey (*5*). The polymerase chain reaction (PCR) was used to create mutations. GAL4 and glutathione-S-transferase (GST) fusion proteins were constructed by subcloning PCR fragments in the polylinker of pSG424 and pGEX-5X-1 (*17*).
19. Electroporation (Jurkat T cells) and lipofectamine (COS-1 and BHK cells) were used for transfection studies (Gibco-BRL). Luciferase reporter gene expression was examined in cotransfection assays. The IL-2 NFAT (*5*) and GAL4 (*17*) luciferase reporter plasmids have been described. Transfection efficiency was monitored with a β-galactosidase expression vector. Luciferase and β-galactosidase activities were measured in cell lysates 48 hours after transfection (*17*). The data are presented as the relative luciferase activity [mean ± SD ($n$ = 3) of the luciferase/β-galactosidase activity ratio].
20. BHK cells were grown on poly-D-lysine–coated cover slips, fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, and processed for immunofluorescence microscopy. The primary antibody used for epitope-tagged proteins was the mouse monoclonal antibody (mAb) M2 (IBI-Kodak). NFATc and NFATp were detected by means of a mAb (Affinity Bioreagents) and a rabbit polyclonal antibody (Upstate Biotechnology), respectively. The secondary antibodies were Texas Red–labeled goat antibody to mouse immunoglobulin (Ig) and rhodamine-labeled goat antibody to rabbit Ig (Jackson Immunoresearch).


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# Independent and Additive Effects of Central POMC and Leptin Pathways on Murine Obesity


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The *lethal yellow* ($A^Y/a$) mouse has a defect in proopiomelanocortin (POMC) signaling in the brain that leads to obesity, and is resistant to the anorexigenic effects of the hormone leptin. It has been proposed that the weight-reducing effects of leptin are thus transmitted primarily by way of POMC neurons. However, the central effects of defective POMC signaling, and the absence of leptin, on weight gain in double-mutant *lethal yellow* ($A^Y/a$) leptin-deficient ($lep^{ob}/lep^{ob}$) mice were shown to be independent and additive. Furthermore, deletion of the leptin gene restored leptin sensitivity to $A^Y/a$ mice. This result implies that in the $A^Y/a$ mouse, obesity is independent of leptin action, and resistance to leptin results from desensitization of leptin signaling.


Serum concentrations of the hormone leptin (*1*) correlate well with body mass index in both humans and rodents (*2*). The long form of the leptin receptor has been detected in multiple hypothalamic regions including the arcuate nucleus (*3*), where it completes a feedback loop that sends information on peripheral energy stores to the hypothalamus, altering both food intake and metabolic rate. Absence of leptin results in extreme obesity in the *obese* ($lep^{ob}/lep^{ob}$) mouse.

Obesity in another rodent model, the *lethal yellow* ($A^Y/a$) mouse (*4*), is caused by a dominantly inherited promotor rearrangement at the *agouti* locus that results in constitutive ectopic expression of the Agouti peptide (*5*). Agouti is a potent antagonist of the hypothalamic melanocortin-4 receptor (MC4-R) (*6*), and interruption of signaling at MC4-R increases feeding behavior in mice (*7–9*). Thus, desacetyl-α–melanocyte-stimulating hormone derived from arcuate nucleus POMC neurons, the primary source of ligand for MC4-R, appears to play a tonic inhibitory role in feeding and energy storage. Leptin levels in the $A^Y/a$ mouse, as well as other rodent obesity models, are elevated (*2*), reflecting the increase in adipose tissue. The $A^Y/a$ mouse is also resistant to leptin administered peripherally or intracerebroventricularly (*10*). Peripheral resistance to leptin occurs in other obese-rodent models (*2*, *11*), and it has been argued that both obesity in the $A^Y/a$ mouse as well as common forms of human obesity may result from genetically determined resistance to leptin feedback (*10*, *12*)

To study the relation between leptin and POMC signaling pathways on weight homeostasis and the potential dependence of leptin action on POMC signaling, we generated a *lethal yellow obese* ($A^Y/a$ $lep^{ob}/lep^{ob}$) double-mutant mouse. C57BL/6J mice with either the $A^Y/a$ or $lep^{ob}/+$ genotypes were crossed to create $A^Y/a$ $lep^{ob}/+$ breeders. These animals were bred to create the double-mutant $A^Y/a$ $lep^{ob}/lep^{ob}$ mice, which were identified by their yellow coat color and obese phenotype, and their genotype was confirmed by allele-specific oligonucleotide hybridization (*13*).

If defective POMC signaling in the $A^Y/a$ animal causes obesity solely by blocking the anorexigenic leptin signal, then introduction of the $A^Y$ allele into the leptin-deficient $lep^{ob}/lep^{ob}$ background should have no added effect on weight gain or metabolism in this model. However, because of the extreme rate of weight gain that results from leptin deficiency, the modest effects of the $A^Y$ allele on weight might be difficult to


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