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9. Preparation of ubiquitin-Sepharose and cytosolic fraction II, and performance of ubiquitin affinity chromatography, were carried out essentially as described [S. Jentsch, J. P. McGrath, A. Varshavsky, *ibid.* **329**, 131 (1987)]. Ubiquitin-Sepharose was loaded with fraction II in the presence of 2.0 mM ATP; washed repeatedly with 25 mM tris (pH 7.4), 5 mM magnesium chloride, 2.0 mM ATP, and 0.2 mM DTT; and split into two halves. In parallel, microsomes were solubilized on ice with 25 mM tris (pH 7.5), 250 mM potassium acetate, 5 mM magnenesium chloride, 0.2 mM DTT, 2.0 mM ATP, and 1.0% CHAPS. One-half of the ubiquitin-Sepharose was loaded with the solubilized membrane proteins, the other with the solubilization buffer. After being washed with 25 mM tris (pH 7.5), 1.0 M potassium chloride, and 1.0% CHAPS, the columns were washed repeatedly with solubilization buffer. Elution of bound material was performed with solubilization buffer without ATP but containing 20 mM DTT. These eluates were further fractionated by RP-HPLC on an Aquapore 300 C8 column (Applied Biosystems) with an acetonitrile gradient. Fractions were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE).
10. Microsome and cytosol preparations were performed essentially as described (*4*) [S. Panzner, L. Dreier, E. Hartmann, S. Kostka, T. A. Rapoport, *Cell* **81**, 561 (1995)].
11. *CUE1* was isolated from a genomic library (generated in the plasmid pSEY8) by hybridization with oligonucleotides. *CUE1* is located on chromosome XIII (YMR264W). A null allele of *CUE1* was constructed by amplification of 400 base pairs (bp) 5′ from the start codon and 650 bp 3′ from the stop codon by polymerase chain reaction (PCR) and insertion of the 2.2-kb fragment containing *LEU2*. A second disruption allele carries the *HIS3* gene inserted into the singular Eco RI site of *CUE1*. Null alleles were introduced into yeast cells by the one-step gene disruption method and monitored by Southern (DNA) hybridization [F. M. Ausubel *et al.*, *Short Protocols in Molecular Biology* (Greene Publishing Associates, New York, 1992)] Both disruptions showed no obvious growth defect (*23*).
12. Cue1p antibodies were raised against the peptide CKLQSDKDLQSLLT (*24*), corresponding to the COOH-terminus of the protein plus an additional NH_2-terminal cysteine. Affinity purification and immobilization of the antibodies were carried out as described (*15*). The antibodies detected a single protein of about 23 kD exclusively in crude microsomes of wild-type but not of Δ*cue1* cells (*23*). Protease protection assays of intact microsomes revealed that the COOH-terminus of Cue1p was oriented toward the cytosol (*23*).
13. Microsomes (*10*) were extracted as in (*3*).
14. GST-ubiquitin was expressed and immobilized as described [M. Scheffner, J. M. Huibregtse, R. D. Vierstra, P. M. Howley, *Cell* **75**, 495 (1993)]. Binding of fraction II and CHAPS-solubilized membrane proteins was done on a small scale but essentially as in (*9*). The first elution was performed with 3.0 M urea and 1.0% CHAPS at room temperature, the second with nonreducing sample buffer containing 4.0% SDS. Bound GST-ubiquitin was not eluted with 3.0 M urea but with SDS (*23*). Binding of Cuep1 to GST-ubiquitin was dependent on the preloading step with fraction II (*23*). Fractions were analyzed by immunoblotting.
15. Immunoaffinity chromatography was performed essentially as described [D. Görlich, S. Prehn, E. Hartmann, K. U. Kalies, T. A. Rapoport, *Cell* **71**, 489 (1992)]. Solubilization of microsomes and chromatography were performed in 25 mM tris (pH 7.5), 125 mM potassium acetate, 5 mM magnesium chloride, 1.0% Triton X-100 (Fluka, Buchs, Switzerland), 250 mM sucrose, 0.1 mM DTT, bovine serum albumin (1.0 mg/ml), 0.5 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), and chymostatin (5 μg/ml).
16. To tag the NH_2-terminus of Ubc7p with the myc epitope, a unique Sph I site was introduced into *UBC7* by mutagenesis [C. Papworth, J. C. Bauer, J. Braman, *Strategies* **9**, 3 (1996)]. A PCR fragment containing three successive myc epitopes [B. L. Schneider, W. Seufert, B. Steiner, Q. H. Yang, B. Futcher, *Yeast* **11**, 1265 (1996)] was introduced into this site. $Ubc7p^{myc}$ was expressed under control of its native promotor from pRS416 (ARS/CEN) or pRS426 (2 μm). This version of Ubc7p was fully functional in degradation of mutant Sec61p (*23*). Using $Ubc7p^{myc}$, we also performed immunfluorescent microspcopy. A perinuclear ring-shaped staining was visible with antibodies to myc, which was indistinguishable from that of Kar2p (the ER-lumenal homolog of mammalian BiP). Cells that do not express the myc epitope showed virtually no staining (*23*).
17. Pulse-chase experiments and immunoprecipitation were done as described (*4*, *19*). The wild-type strain used in this study was YWO2 (mat**a**, *trp1-1(am), his3-Δ200, ura3-52, lys2-801, leu2-3,-112*). RSY521 (matα, *leu2-3,-112, ura3-52, trp1-1, his4-401, HOL1-1*) and YFP338 (matα, *sec61-2, leu2-3,-112, ura3-52, ade2-3, pep4-3*) were kindly supplied by M. Rose and R. Schekman. YTX5 (matα, *Δubc6::HIS3, trp1-1(am), his3-Δ200, ura3-52, lys2-801, leu2-3,-112*) and YTX93 (matα, *sec61-2, Δubc7::LEU2, leu2-3,-112, ura3-52, ade2-3, pep4-3*) were as described (*4*). Mutants used in this study [YTX105 (matα, *Δcue1::HIS3, trp1-1(am), his3-Δ200, ura3-52, lys2-801, leu2-3,-112*), YTX106 (matα, *Δubc7::LEU2, trp1-1(am), his3-Δ200, ura3-52, lys2-801, leu2-3,-112*), and YTX121 (matα, *sec61-2, Δcue1::LEU2, leu2-3,-112, ura3-52, ade2-3, pep4-3*)] were generated by direct transformation of null alleles (*11*). Null alleles of *UBC6* and *UBC7* have been described previously (*3*, *20*). The *prc1-1* allele was introduced into haploid wild-type cells as described (*7*) to generate YTX140 (mat**a**, *prc1-1, trp1-1(am), his3-Δ200, ura3-52, lys2-801, leu2-3,-112*). Multiple mutants were generated by a second round of transformation [YTX141 (matα, *Δcue1::HIS3, prc1-1, trp1-1(am), his3-Δ200, ura3-52, lys2-801, leu2-3,-112*), YTX142 (mat**a**, *Δcue1::HIS3, Δubc7::LEU2, Δubc6::TRP1, prc1-1, trp1-1(am), his3-Δ200, ura3-52, lys2-801, leu2-3,-112*)] or by crossing of single mutants and subsequent tetrad dissection [YTX133 (mat**a**, *Δcue1::HIS3, Δubc7::LEU2, trp1-1(am), his3-Δ200, ura3-52, lys2-801, leu2-3,-112*)].
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20. Growth of Δ*cue1* and Δ*cue1* cells overexpressing Ubc7p from a multicopy vector on minimal media containing cadmium (*23*) was tested as decribed in J. Jungmann, H. A. Reins, C. Schobert, S. Jentsch, *Nature* **361**, 369 (1993).
21. Pulse-chase (*4*) and protease protection assays (*3*) were done essentially as described. The cells were lysed in 50 mM tris (pH 7.5), 250 mM sucrose, and 10 mM EDTA with one volume of glass beads by four repeated cycles of mixing with a Vortex for 30 s at maximum speed, interrupted by 30-s incubations on ice. In every pulse-chase experiment, the extracts were untreated, treated with proteinase K (0.1 mg/ml), or treated with proteinase K (0.1 mg/ml) and 0.4% Triton X-100 on ice for 15 min. No immunoprecipitable CPY* was detected after treatment with proteinase K and detergent (*23*).
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24. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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Multistep Control of Pituitary Organogenesis

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Lhx3 and *Lhx4* (*Gsh4*), two closely related LIM homeobox genes, determine formation of the pituitary gland in mice. Rathke's pouch is formed in two steps—first as a rudiment and later as a definitive pouch. *Lhx3* and *Lhx4* have redundant control over formation of the definitive pouch. *Lhx3* controls a subsequent step of pituitary fate commitment. Thereafter, *Lhx3* and *Lhx4* together regulate proliferation and differentiation of pituitary-specific cell lineages. Thus, *Lhx3* and *Lhx4* dictate pituitary organ identity by controlling developmental decisions at multiple stages of organogenesis.

Pituitary organogenesis is driven by a series of developmental decisions controlled by transcription regulators. *Pit-1*/GHF-1 (*1–4*) and prophet-1 (*5*) direct establishment of certain pituitary cell lineages [for review, see (*6*)]. Targeted mutation of the *Lhx3* gene revealed its role in the specification of most pituitary lineages (*7*). This study focuses on earlier steps in pituitary organ formation. We analyze the effects of null mutations in *Lhx3* and *Lhx4* (*8*), a gene closely related to *Lhx3* (*8–11*), and show that both genes direct formation of the pituitary gland in mice.

The anterior and intermediate lobes of the pituitary are derived from the oral ectoderm that invaginates to form Rathke's pouch (*12*). Rathke's pouch gives rise to at least six pituitary-specific cell lineages (*6*, *12*).

Lhx3 and *Lhx4* are expressed throughout the invaginating pouch at day 9.5 of gesta-

tion (E9.5) (Fig. 1, a and e). However, at E12.5, *Lhx4* expression becomes restricted to the future anterior lobe of the pituitary gland, whereas *Lhx3* remains expressed in the whole pouch (Fig. 1, b and f). At E15.5, *Lhx4* expression diminishes; *Lhx3* expression is maintained (Fig. 1, c and g). In the adult pituitary, *Lhx3* is expressed at a higher level than *Lhx4* in the anterior and intermediate lobes (Fig. 1, d and h).

Null mutations of either *Lhx3* (*7*) or *Lhx4* (see below) do not prevent formation of Rathke's pouch. We generated $Lhx3^{-/-}/Lhx4^{-/-}$ mice carrying null mutations at both loci ($3^{-/-}4^{-/-}$ for short). In these mutants the oral ectoderm invaginates normally to form a pouch rudiment (Fig. 2d). The pouch rudiment grows no further after E12.5 (Fig. 2e), and by E15.5, as the cartilage of the sphenoid bone grows across the floor of the brain, the entire rudiment is observed pressed back toward the oral cavity (Fig. 2f).

After E9.5, normal pouch development proceeds from a rudiment to a definitive pouch that is characterized by an extension of the pouch into the brain cavity, where it abuts the infundibulum. In $Lhx3^{-/-}$ or $Lhx4^{-/-}$ mutants a definitive, albeit defective, pouch forms (*7*, *13*). Analysis of mutants for one gene that are heterozygous at the other locus revealed that one wild-type (WT) allele of either *Lhx3* or *Lhx4* is sufficient for formation of a definitive pouch (Fig. 2, g to l).

The next developmental step is commitment to the fate of the pituitary organ, which leads to formation of a proper pituitary structure and specification of pituitary lineages. This step is absolutely dependent on *Lhx3* and is not realized in mutants that lack *Lhx3* (Fig. 2, j to l) [see also (*7*)]. Histological analysis of pituitary development in mutants with intermediate genotypes showed that Rathke's pouch gave rise to a pituitary structure in the presence of at least one copy of *Lhx3* (Fig. 2, g to i) (*13*), but not in the absence of *Lhx3* (*7*). Commitment to an organ fate also implies that the primordium will eventually give rise to organ-specific cell lineages. Therefore, we assessed the ability of mutant primordia to differentiate into organ-specific cell lineages. Marker gene and protein expression was assessed by in situ hybridization and immunocytochemistry. Transcripts for the α-glycoprotein subunit (GSU), *Pit-1*, growth hormone (GH), and thyroid-stimulating hormone β subunit (TSHβ) are

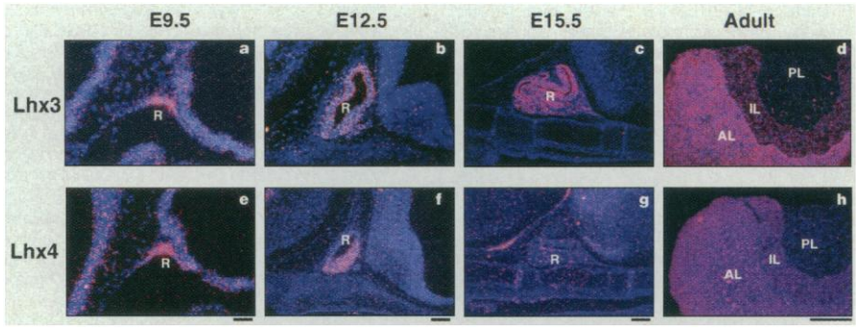

Fig. 1. Expression of *Lhx3* and *Lhx4* in developing and adult pituitary. Sagittal sections of Rathke's pouch or coronal sections of adult pituitary were hybridized to *Lhx3*-specific (*7*) or *Lhx4*-specific (*8*) riboprobes. Antisense riboprobes were transcribed from *Lhx3* or *Lhx4* cDNA with RNA polymerase (Ambion) in the presence of uridine 5′-[^{33}P]triphosphate (NEN). In situ hybridization was performed on tissue sections essentially as described (*16*). For photography, sections were stained with bisbenzimide (10 μg/ml) and simultaneously viewed in dark-field and ultraviolet illumination. R, Rathke's pouch; PL, posterior lobe; IL, intermediate lobe; AL, anterior lobe. Bars, 100 μm.

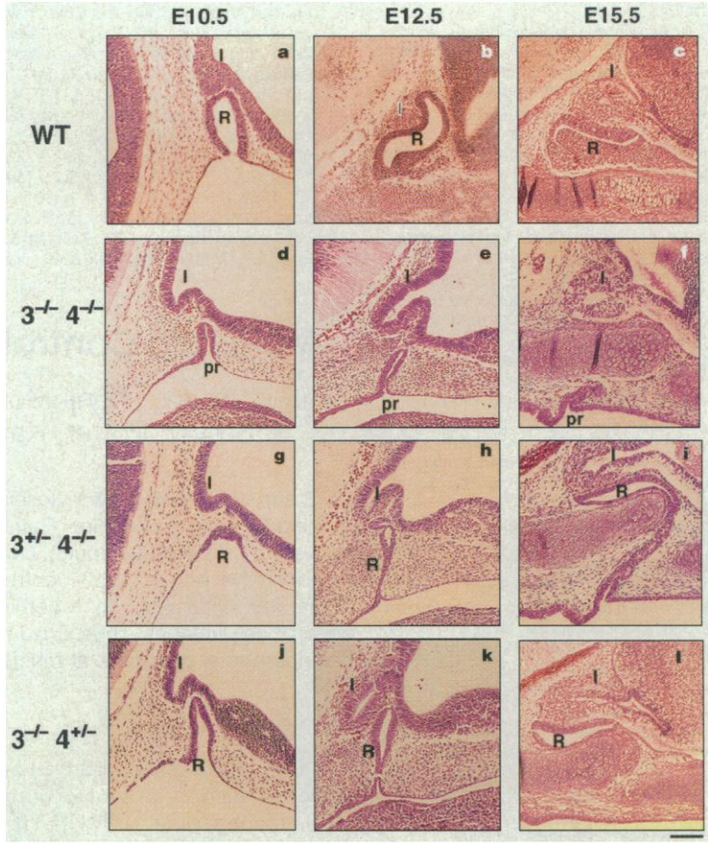

Fig. 2. Ontogeny of anterior and intermediate lobes of pituitary development in WT mice (**a** to **c**), in $3^{-/-}4^{-/-}$ double mutants (**d** to **f**), and in mutants for one gene that are heterozygous at the other locus (**g** to **l**). A pouch rudiment is formed in the absence of both *Lhx3* and *Lhx4* genes. However, this rudiment fails to grow into a definitive pouch in the double mutant. Downgrowth of the infundibulum occurs in the absence of a definitive pouch. I, infundibulum; R, Rathke's pouch; pr, pouch rudiment. Sagittal sections of embryos were stained with hematoxylin and eosin. *Lhx3* and *Lhx4* genotyping was done by Southern blotting and polymerase chain reaction, respectively, as described (*7*, *8*). Bar, 100 μm.

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present in the *Lhx4*$^{-/-}$ mutant but not in the *Lhx3*$^{-/-}$ mutant (*7*, *13*). In the 3$^{+/-}$4$^{-/-}$ pituitary, cells containing *Pit-1*, GSU, GH, and TSHβ transcripts are present, but there are fewer than in the 3$^{+/+}$4$^{-/-}$ pituitary (*13*). Therefore, the presence of one copy of the *Lhx3* gene is sufficient for specification of pituitary cell lineages.

In the *Lhx4*$^{-/-}$ mutant, immunohistochemistry revealed that only one of three mutant pouches contained a few luteinizing hormone–positive (LH^{+}) cells (Fig. 3A). In situ hybridization with probes specific for LH and for the receptor for gonadotropin-releasing hormone (GnRHR) showed similar results at both E15.5 and E18.5 (*13*). Thus, *Lhx4* may support, but is not required for, specification of gonadotroph cells.

We conclude that elaboration of a definitive pouch is directed by either *Lhx3* or *Lhx4*, and subsequent organ fate commitment is regulated solely by *Lhx3*. Formation of Rathke's pouch and commitment of cells to the fate of the pituitary organ are distinct developmental events. The function of *Lhx3* in the latter event is not replaceable by *Lhx4*.

All defective pouches display defects in cell proliferation (Figs. 2 and 3), with no detectable increase in programmed cell death (*13*). These defects in cell proliferation depend on *Lhx3* and *Lhx4* gene dosage. The severity of cell proliferation defects aligned as follows: 3$^{-/-}$4$^{-/-}$ > 3$^{-/-}$4$^{+/-}$ > 3$^{-/-}$4$^{+/+}$ > 3$^{+/-}$4$^{-/-}$ > 3$^{+/+}$4$^{-/-}$ > 3$^{+/-}$4$^{+/-}$ (Figs. 2 and 3) (*13*). In *Lhx3*$^{-/-}$ mutants, pituitary precursor cells cease proliferation before most lineage markers are expressed (*7*). In *Lhx4*$^{-/-}$ mutants, cell proliferation in the intermediate lobe is less affected, but the anterior lobe is distinctly hypoplastic (Fig. 3, A and B) and all five cell lineages in the anterior lobe show reduced numbers (Fig. 3A) (*13*). The GnRHR^{+} gonadotroph precursors and the Pit-1^{+} somatotroph, lactotroph, and thyrotroph precursors are present at E15.5 (Fig. 3C, b and d). This suggests that the reduction in terminally differentiated pituitary cell lineages in the *Lhx4*$^{-/-}$ mouse is caused by a cell proliferation defect at the precursor level and that proliferation of these precursors requires the function of the *Lhx4* gene.

Thus, pituitary organogenesis is a multistep process, and each step is controlled by a distinct genetic program (Fig. 4). Formation of Rathke's pouch involves at least two independent developmental decisions. At the beginning, a portion of the oral ectoderm apposing the neural ectoderm of the diencephalon diverges from its original ectodermal fate to become the pituitary an-

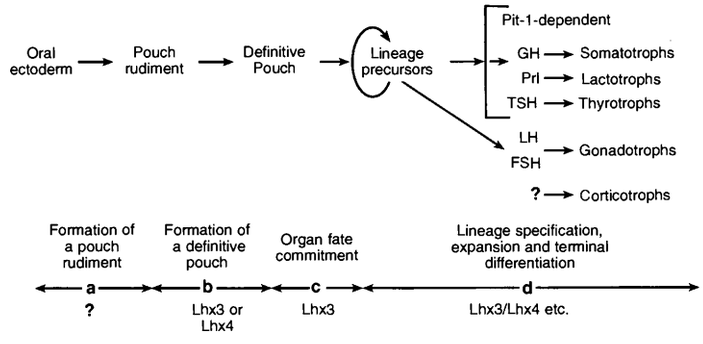

Fig. 4. Schematic illustration of ontogenetic events leading to pituitary formation.

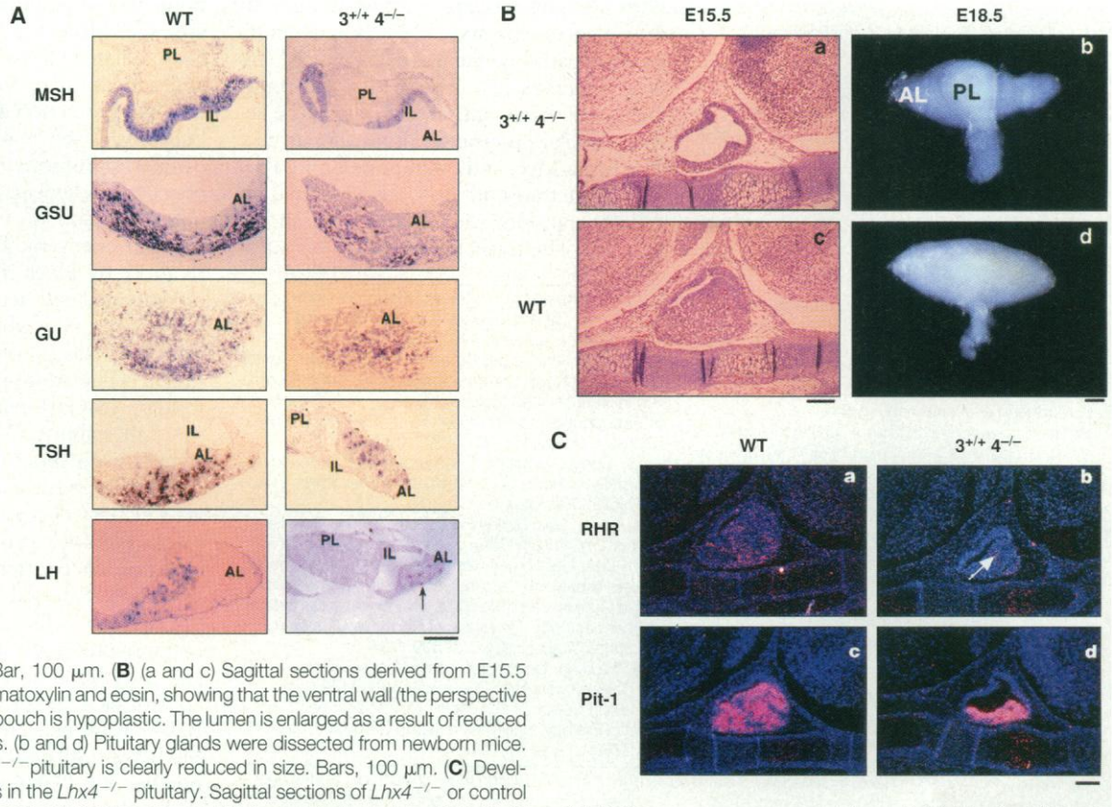

Fig. 3. Immunohistological (A), morphological (B), and in situ hybridization (C) analysis of pituitary development in the *Lhx4*$^{-/-}$ mutant. (**A**) Immunohistological analysis (*17*) of pituitary-specific lineage development in the *Lhx4*$^{-/-}$ mutant. Paraffin sections derived from E18.5 normal and mutant mice were stained with antibodies specific to adrenocorticotropic hormone (ACTH) (*13*), PRL (*13*), melanocyte-stimulating hormone (MSH), GSU, GH, TSH, and LH. All five anterior pituitary–specific cell lineages are present in the *Lhx4*$^{-/-}$ pituitary but in dramatically reduced numbers. There are about the same number of melanotrophs in the intermediate lobe of the *Lhx4*$^{-/-}$ pituitary and in the control. Bar, 100 μm. (**B**) (a and c) Sagittal sections derived from E15.5 embryos and stained with hematoxylin and eosin, showing that the ventral wall (the perspective anterior lobe) of the *Lhx4*$^{-/-}$ pouch is hypoplastic. The lumen is enlarged as a result of reduced proliferation of the precursors. (b and d) Pituitary glands were dissected from newborn mice. The anterior lobe of the *Lhx4*$^{-/-}$pituitary is clearly reduced in size. Bars, 100 μm. (**C**) Development of lineage precursors in the *Lhx4*$^{-/-}$ pituitary. Sagittal sections of *Lhx4*$^{-/-}$ or control mouse at E15.5 were hybridized to GnRHR-specific and *Pit-1*–specific riboprobes. Both *Pit-1*$^{+}$ and GnRHR^{+} precursors are present. Arrow highlights a few LH^{+} cells in the anterior lobe. Bar, 100 μm.

lage. Tissue recombination experiments have provided evidence that surrounding neural tissues are a source of inductive signals for determination of the primordium (*14*). Formation of the pouch rudiment does not require the function of either *Lhx3* or *Lhx4* (Fig. 4, arrow a). *Lhx3* or *Lhx4* controls development of the pouch rudiment into a definitive pouch (Fig. 4, arrow b). Commitment of precursor cells in Rathke's pouch to a pituitary organ fate is controlled by *Lhx3* (Fig. 4, arrow c). From E12.5 onward, cells begin to express lineage-specific molecules. *Lhx4* is required for proliferation of lineage precursors. In the $Lhx3^{-/-}$ mutant, pouch development is arrested before the appearance of most pituitary cell lineages (*7*), precluding an exhaustive evaluation of *Lhx3* function in lineage development. However, *Lhx3* regulates the expression of *Pit-1*, GSU, and, in synergy with *Pit-1*, GH and prolactin (PRL) in vitro (*7*, *15*), suggesting that *Lhx3* also regulates cellular differentiation (Fig. 4, arrow d). Because *Lhx3* is expressed in almost all pituitary precursor cells, it is unlikely that this gene determines cell type identity. Rather, it may act in concert with genes that are lineage restricted, such as *Pit-1*/GHF-1 (*1*–*4*) and prophet-1 (*5*).

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17. Antibodies to ACTH and MSH were obtained from Incstar (Minnesota). An antibody to human PRL was from Dako (Carpinteria, CA). The rest of the hormone antibodies were obtained from the National Hormone and Pituitary Program, NIDDK. Paraffin sections were incubated with hormone antibodies overnight at 4°C. Specific staining was visualized with alkaline-phosphatase–conjugated secondary antibodies (Boehringer Mannheim, Indianapolis, IN) and an alkaline-phosphatase substrate kit (Vector Laboratories, Burlingame, CA). After immunostaining, the slides were dehydrated in graded ethanol and mounted in Permount (Fisher Scientific).
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Requirement of NF-κB Activation to Suppress p53-Independent Apoptosis Induced by Oncogenic Ras

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The *ras* proto-oncogene is frequently mutated in human tumors and functions to chronically stimulate signal transduction cascades resulting in the synthesis or activation of specific transcription factors, including Ets, c-Myc, c-Jun, and nuclear factor kappa B (NF-κB). These Ras-responsive transcription factors are required for transformation, but the mechanisms by which these proteins facilitate oncogenesis have not been fully established. Oncogenic Ras was shown to initiate a p53-independent apoptotic response that was suppressed through the activation of NF-κB. These results provide an explanation for the requirement of NF-κB for Ras-mediated oncogenesis and provide evidence that Ras-transformed cells are susceptible to apoptosis even if they do not express the p53 tumor-suppressor gene product.

Mutations in a *ras* allele occur in 30% of all human tumors (*1*), making *ras* the most widely mutated human proto-oncogene. Both mitogen-activated protein (MAP) kinase–dependent and MAP kinase–independent pathways mediate Ras-induced cellular responses (*2*), and these signal transduction pathways ultimately control the activity of various transcription factors (*3*). The Ets, c-Myc, and c-Jun proteins are Ras-responsive transcription factors required for cellular transformation in vitro (*4*) and in vivo (*5*). The transcription factor NF-κB is also activated in response to oncogenic Ras (*6*), and this regulation occurs largely through the stimulation of the transcriptional function of the NF-κB RelA/p65 subunit (*7*). Moreover, NF-κB is required for Ras-mediated focus-forming activity (*7*), and activation of this transcription factor provides protection against apoptosis (*8*, *9*). Because NF-κB may play a direct role in cellular transformation (*10*) and because oncogenesis appears to require an anti-apoptotic function (*11*), we investigated whether oncogenic Ras requires NF-κB activation to block transformation-induced programmed cell death.

To determine whether the inhibition of NF-κB in Ras-transformed cells would initiate a cell death response, we used β-galactosidase (β-Gal) expression assays to measure cell viability. We inhibited NF-κB activity with a super-repressor form of IκBα (SR-IκBα), which cannot be phosphorylated (*12*) or degraded (*13*) and, therefore, blocks the nuclear translocation and subsequent transactivation of NF-κB–responsive genes (*8*). Parental NIH 3T3 and H-Ras–transformed cells (3T3 H-Ras[V12]) were cotransfected with a pCMV-LacZ reporter and with either an empty expression vector control or a vector encoding SR-IκBα. H-Ras–transformed NIH 3T3 cells expressing SR-IκBα displayed a decrease in the total number of β-Gal–positive cells as compared with cells transfected with the vector control (Fig. 1). In contrast, parental NIH 3T3

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