MAML1, a human homologue of Drosophila Mastermind, is a transcriptional co-activator for NOTCH receptors

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Notch receptors are involved in cell-fate determination in organisms as diverse as flies, frogs and humans. In Drosophila melanogaster, loss-of-function mutations of Notch produce a ‘neuromgenic’ phenotype in which cells destined to become epidermis switch fate and differentiate to neural cells. Upon ligand activation, the intracellular domain of Notch (ICN) translocates to the nucleus, and interacts directly with the DNA-binding protein Suppressor of hairloss (Su(H)) in flies, or recombination signal binding protein Jκ (RBP-Jκ) in mammals, to activate gene transcription. But the precise mechanisms of Notch-induced gene expression are not completely understood. The gene mastermind has been identified in multiple genetic screens for modifiers of Notch mutations in Drosophila. Here we clone MAML1, a human homologue of the Drosophila gene Mastermind, and show that it encodes a protein of 130 kD localizing to nuclear bodies. MAML1 binds to the ankyrin repeat domain of all four mammalian NOTCH receptors, forms a DNA-binding complex with ICN and RBP-Jκ, and amplifies NOTCH-induced transcription of HES1. These studies provide a molecular mechanism to explain the genetic links between mastermind and Notch in Drosophila and indicate that MAML1 functions as a transcriptional co-activator for NOTCH signalling.

We isolated MAM L1 (for mastermind-like-1), a human gene encoding a protein related to Drosophila Mastermind, particularly in the amino-terminal basic domain (35% amino acid identity and 51% similarity; Fig. 1a). Northern-blot analysis disclosed a single, 6-kb transcript in all tissues examined (Fig. 1b), and an additional 1-kb transcript in placenta.

We used a series of N-terminal truncations of MAML1 fused to green fluorescence protein (GFP) to examine subcellular localization. MAML1–GFP localized to nuclear dots (Fig. 2a). Deletion of the N-terminal 123 amino acids resulted in loss of punctate staining, but not nuclear localization, in COS7, HeLa and U2OS cells. A further truncation containing the putative nuclear localization signal resulted in both cytoplasmic and nuclear staining. An anti-MAML1 monoclonal antibody (8E6B10) confirmed punctate nuclear staining, indicating that localization to nuclear dots is not an artefact of fusion to GFP or overexpression (data not shown). In most cells, MAML1 colocalized with promyelocytic leukaemia (PML) protein (Fig. 2b).

We next looked for colocalization of MAML1 with ICN and RBP-Jκ. ICN–GFP localized to the nucleus in a diffuse pattern (Fig. 2c). Co-expression of MAML1 altered ICN–GFP distribution to the punctate pattern of MAML1. MAML1–GFP colocalized with ICN in these dots (Fig. 2d). RBP-Jκ also relocated into nuclear dots with MAML1 when ICN was present (Fig. 2e). Like MAML1, Mastermind also colocalized with ICN in nuclear dots in 293T cells (data not shown), suggesting conservation of function between Drosophila Mastermind and human MAML1, despite their limited sequence similarity.

These results indicate that MAML1, ICN and RBP-Jκ might form a complex. In support of this, MAML1 co-immunoprecipitated with ICN1, and this was enhanced by RBP-Jκ (Fig. 3a). MAML1 co-immunoprecipitated with RBP-Jκ, but only in the presence of ICN1. MAML1 similarly co-immunoprecipitated with ICN1. MAML1 co-immunoprecipitated with ICN1.

**Fig. 1** MAML1 structure and expression. a, Comparison of domain organization between MAML1 and Drosophila Mastermind, and schematic of full length (FL) and truncated mutant MAML1 proteins. Alignment of amino acid sequence of MAML1 and Drosophila Mastermind, generated by the Pileup program in GCG (Wisconsin Package Version 9.0) and refined by Boxshade 3.21 (http://www.ch.embnet.org/software/box_form.html; see Fig. A, http://genetics.nature.com/supplementary_info.html). The overall amino-acid identity and similarity was 24% and 27%, respectively. MAML1 was cloned from a HeLa cDNA library using a yeast two-hybrid system with the E6 oncoprotein of human papillomavirus as a bait. A possible nuclear localization signal (NLS), PGHKKTR, was identified at aa 135–141. MAML1 has a predicted molecular weight of 108 kD. The apparent molecular weight on reducing SDS–PAGE of endogenous and transfected MAML1 was ∼130 kD. b, Western blot analysis of the MAML1 expression in human tissues. Lane 1, spleen; lane 2, thymus; lane 3, prostate; lane 4, testis; lane 5, ovary; lane 6, small intestine; lane 7, colon; lane 8, peripheral blood leukocyte; lane 9, heart; lane 10, brain; lane 11, placenta; lane 12, lung; lane 13, liver; lane 14, skeletal muscle; lane 15, kidney; lane 16, pancreas. A single 6-kb transcript of MAML1 was present in these tissues with an additional 1-kb transcript in placenta (not shown). The hybridization with β-actin served as the RNA-loading controls.

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with ICN2, ICN3 and ICN4 (Fig. 3b). Co-immunoprecipitation required the N-terminal 123 amino acids of MAML1, and an N-terminal fragment of 302 amino acids from MAML1 was sufficient to form a complex with both ICN1 and RBP-Jκ (Fig. 3c).

We carried out in vitro binding studies to determine if MAML1 bound directly to ICN1-4. GST-MAML1 beads bound substantially more ICN1-4 than control GST beads (Fig. 4a). Binding of ICN1 to MAML1-GST was enhanced in the presence of RBP-Jκ, and required the presence of the ICN1 ankyrin repeats. The ability of MAML1 to associate with ICN1 and RBP-Jκ bound to DNA was evaluated with an electrophoretic mobility shift assay (Fig. 4b). A single complex was identified when partially purified RBP-Jκ was added to a labelled oligonucleotide containing RBP-Jκ binding sites. The further addition of both the RAM-ankyrin repeat region (RAM-ANK) of NOTCH1 (residues 1,760–2,126) and the ANK region without the RAM domain (residues 1,872–2,126) failed to shift the RBP-Jκ/DNA complex, even in the presence of MAML1. The higher molecular weight complex formed by RBP-Jκ, RAM-ANK and MAML1 was supershifted by an antibody specific for ICN1, confirming the presence of MAML1 in the complex. Overall, these studies are consistent with MAML1 acting to stabilize the association of ICN and RBP-Jκ through the formation of a ternary complex.

We investigated the functional importance of MAML1/ICN interaction by examining NOTCH-induced activation of a HES1 promoter construct. HES1 promoter activation induced by Jagged2 was increased 5–10-fold by co-transfection with MAML1 model in which the N-terminal basic domain of MAML1 forms a complex with ICN and RBP-Jκ, and activates transcription of NOTCH-dependent genes through its C-terminal TAD. We tested this model by determining if truncation mutants of MAML1 might function as dominant-negative inhibitors of NOTCH. Both MAML1(1–302) (defective in transactivation) and MAML1(124–1,016) (defective in Notch binding) reduced NOTCH-dependent reporter activity (Fig. 5a). MAML1 increased HES1 activity 2–3-fold in the absence of Jagged2. Similarly, co-expression of MAML1 with submaximal amounts of ICN1 led to a potentiation of reporter activity (Fig. 5b). A HES1 reporter lacking RBP-Jκ sites was not activated. MAML1 also increased HES1 reporter activity induced by ICN2, ICN3 and ICN4 by more than tenfold (Fig. 5c). These results suggested that MAML1 might function as a transcriptional co-activator for NOTCH receptors. A transcriptional activation domain (TAD) was identified in the carboxy terminus of MAML1 by fusing full-length or truncated MAML1 directly to the DNA-binding domain of GAL4 (Fig. 5d).

Previous studies of ICN1 have identified the ankyrin repeats and the C-terminal region of ICN1 as possible binding sites for co-activators. When over-expressed with MAML1, the ANK domain of ICN1 was sufficient to activate the HES1 reporter (data not shown). Thus, the binding of MAML1 may explain in part previous studies showing a critical role for this region.

Our experiments suggest a
GLP-1 and LIN-12, and the Caenorhabditis elegans Notch receptors, and to contain a transcriptional activation domain. Despite the functional similarities between MAML1 and LAG-3, however, there is minimal sequence similarity, and it is not clear if they are in the same gene family.

The role of nuclear bodies in NOTCH signalling warrants further investigation, as MAML1 causes the redistribution of ICN and RBP-Jκ to these nuclear structures. PML bodies have been linked to transcriptional regulation, and both transcription factors and transcriptional co-activators have been detected in these organelles; however, the functions of PML bodies and other nuclear dots remain largely unknown.

Overall, our results suggest a role for MAML1 in signalling of all four mammalian NOTCH receptors. Moreover, these studies provide a potential mechanism to explain some of the previously described genetic interactions of Drosophila Mastermind with the Notch pathway.

**Methods**

**Plasmids.** We obtained the bait construct used for yeast two-hybrid screening (pDBLeu-16E6) by PCR-amplifying the full-length E6 sequence derived from HPV type 16 E6 (cDNA was provided by P. Howley). We then cloned this cDNA into the SalI-NotI sites of pDBLeu (Gibco-BRL) in-frame with a sequence encoding the DNA-binding domain (DB) of GAL4. We cloned MAML1 full-length (FL), MAML1(124–1016), MAML1(719–1016), MAML1(842–1016) MAML1(124–841) and MAML1(1–302) sequences as SalI-NotI fragments into pFLAG-CMV-2 (Sigma), pEGFP-C3 (Clontech), pGEX-2TK (Stratagene) and pBIND (Promega) vectors. MSCV-GFP (ref. 24) is a derivative of MSCV (ref. 25). (Sigma), pEGFP-C3 (Clontech), pGEX-2TK (Stratagene) and pBIND (Promega) vectors. MSCV-GFP (ref. 24) is a derivative of MSCV (ref. 25). cDNAs encoding ICN1, ICN2, ICN3, ICN4 (Promega) vectors. MSCP-GFP (ref. 25) is a derivative of MSCP (ref. 24). cDNAs encoding ICN1, ICN2, ANK fragments of NOTCH1, and MAML1 (residues 1–300 with an HA tag) were cloned this cDNA into the SalI-NotI sites of pDBLeu (Gibco-BRL) in-frame with a sequence encoding the DNA-binding domain (DB) of GAL4. We cloned MAML1 full-length (FL), MAML1(124–1016), MAML1(719–1016), MAML1(842–1016) MAML1(124–841) and MAML1(1–302) sequences as SalI-NotI fragments into pFLAG-CMV-2 (Sigma), pEGFP-C3 (Clontech), pGEX-2TK (Stratagene) and pBIND (Promega) vectors. MSCP-GFP (ref. 25) is a derivative of MSCP (ref. 24). We also cloned MAML1 full-length (FL), MAML1(124–1016), MAML1(719–1016), MAML1(842–1016) MAML1(124–841) and MAML1(1–302) sequences as SalI-NotI fragments into pFLAG-CMV-2 (Sigma), pEGFP-C3 (Clontech), pGEX-2TK (Stratagene) and pBIND (Promega) vectors. MSCP-GFP (ref. 25) is a derivative of MSCP (ref. 24). cDNAs encoding ICN1, ICN2, ANK, MAML1 FL, MAML1(124–1,016), or MAML1(1–302), and analysed by immunoprecipitation and immunoblotting.

**Fig. 4** MAML1 binds directly to the anykyrin repeats of NOTCH. a, Detection of a MAML1, ICN and RBP-Jκ ternary complex in vivo. 35S-labelled polypeptides were generated by in vitro transcription/translation with the TNT reticulocyte lysate system, and binding of 35S-labelled proteins to GST or GST-MAML1 glutathione beads was measured. For comparison, 2.5% of the in vitro translation product is shown with precipitation on GST beads. The structures of the ICN1 polypeptides used here are shown in the diagram. b, Binding of MAML1 to ICN and RBP-Jκ in a DNA-binding complex by electrophoretic mobility shift assays. The indicated combinations of partially purified RBP-Jκ, purified ANK or RAM-ANK fragments of NOTCH1, and MAML1 (residues 1–300 with an HA tag) were added to 35S-labelled oligonucleotides containing a normal or mutated RBP-Jκ-binding site before gel electrophoresis (left). In a separate experiment, anti-HA antibody was added to supershift DNA binding complexes containing MAML1 (right). +, –, Presence or absence of the individual components listed on the left, respectively (+). The addition of RAM-ANK instead of ANK.
(Promega), which encode β-galactosidase and Renilla luciferase, respectively. pSG5-luc (Promega) is a firefly luciferase reporter plasmid that contains five copies of GAL4-binding site upstream of a minimal TATA box.

**Antibodies.** We purchased the following antibodies from commercial sources: mouse anti-Flag antibody (clone M2, Sigma); mouse anti-HA monoclonal antibody (clone HA.11, Babco); mouse anti-PM-1 monoclonal antibody (PG-M3), goat anti-NOTCH4, goat anti-mouse IGF and goat anti-rat IGF antibodies (Santa Cruz Biotechnology); horse-radish peroxidase (HRP)-coupled goat anti-mouse and goat anti-rabbit IGG antibodies (Amersham); and Rhodamine Red-X-conjugated F(ab’2) fragment goat anti-mouse antibody (Jackson ImmunoResearch Laboratories). Anti-Myc (clone 9E10) monoclonal antibody was obtained from J. Parvin.

**Cell culture and transient transfection.** We cultured human U2OS osteosarcoma cells in Dulbecco’s modified Eagle’s medium (DM EM) containing 10% FetalClone I serum (HyClone Laboratories), and COS7 cells in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). NIH 3T3 cells transfected with pBabe retrovirus were maintained in DM EM medium containing 10% FCS and puromycin (1 µg/ml). We carried out transfections using Superfect transfection reagent (Qiagen) according to the manufacturer’s instructions.

**Yeast two-hybrid screening.** Yeast two-hybrid screening (Proquest, Gibco-BRL) was performed on a HeLa cell expression library fused to the Ga4 activation domain (AD) in the Sall-NotI sites of the pPC86 vector according to the manufacturer’s instructions. We screened ~3×10⁶ transformants and obtained 20 positive clones. Plasmids containing the prey sequences were rescued and checked by retransformation with the bait into yeast. Finally, we sequenced the prey inserts obtained from the positive clones. In this screening, we obtained three novel proteins in addition to the known E6 binding proteins (E6AP and E6TP1). One of these, MAML1, was defined by four overlapping cDNAs. We sequenced the longest MAML1 cDNA (5.1 kb), and found that it was derived from a previously sequenced full-length cDNA (5.7 kb) generated randomly from a KG-1 library, KIAA0200, except MAML1 has Asn instead of Ser at position 1007. We obtained a full-length MAML1 cDNA by ligating a 709-bp Smal-BclI fragment of KIAA0200 (obtained from Kaza DNA Research Institute, Japan) to the appropriate site in the MAML1 E12 deletion cDNA.

**Northern-blot analysis.** Filter-immobilized polyadenylated RNAs from multiple human tissue blots (Clontech) were hybridized with 32p-labelled MAML1 (nt 369-2,160 of the MAML1 ORF) and β-actin cDNA probes according to the manufacturer’s instructions.

**Immunofluorescence staining.** We fixed cells grown on coverslips in 4% paraformaldehyde in PBS for 20 min. After permeabilization in 0.1% NP-40 in PBS for 15 min, non-specific binding sites were blocked with 5% non-immune goat serum in PBS for 15 min. We then incubated cells for 60 min with PBS/0.1% NP-40/5% goat serum containing primary antibody, washed cells extensively with PBS/0.1% N-P-40, and then incubated cells for 60 min with secondary antibody in PBS/0.1% NP-40/5% goat serum. After washing extensively, coverslips were mounted in Gel/medium (Biomedia) and photographed with an Olympus microscope and a SPOT camera (Diagnostic Instrument). Composite images were generated by SPOT software V2.2 (Diagnostic Instruments).

**Western-blot analysis and immunoprecipitation.** We seeded COS7 cells on 100-mm plates at ~10⁶ cells per plate one day before transfection, and transiently transfected cells with various combinations of expression plasmids. We kept the total amounts of plasmids constant by adding appropriate amounts of empty vectors without inserts. At 44-48 h post-transfection, we washed cells with ice-cold PBS and lysed cells in situ with a solution containing Tris (20 mM, pH 8.0), NaCl (150 mM), 1% NP-40 (w/v), 10% glycerol.

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*Fig. 5* MAML1 cooperates with NOTCH to activate the HES1 promoter. **a.** MAML1 augments ligand-induced NOTCH activation. U2OS cells were transfected with a β-galactosidase control plasmid (0.5 µg), HES1-luc (0.5 µg) and increasing amounts of pFLAG-CMV-2 plasmid encoding MAML1. Twenty hours post-transfection, 1×10⁶ NIH 3T3 cells expressing Jagged2 or NIH 3T3 cells infected with empty pBabe virus were added to each well. Expt as noted, in all HES1 transfection assays, cell extracts were prepared 44 h post-transfection. HES1 reporter luciferase activity, corrected for β-galactosidase activity, is expressed as fold activation relative to cells not expressing MAML1 that were co-cultured with control NIH 3T3 cells. Error bars indicate standard deviation of three independent experiments.

**b.** Activation of HES1 promoter by MAML1 requires ICN1 and RBP-J binding sites. U2OS cells were transfected with β-galactosidase control reporter construct, luciferase reporter construct, HES1-luc or HES1AAB-luc, and increasing amounts of pFLAG-CMV plasmid encoding MAML1, in the absence or presence of M5SV-ICN1 plasmid. Luciferase activity, corrected for β-galactosidase activity, was expressed as fold activation relative to cells with no expression of MAM and ICN1. When transfected with the ICN1 driven by strong promoter (a pDNA3 vector encoding ICN1), the enhancing effects of MAML1 on HES1 transcription were prominent at low levels of ICN1 expression, but decreased with higher, possibly non-physiologic, levels of ICN1 (levels where activation of the HES1 promoter by ICN1 alone was higher than what could be achieved with Jagged2 signalling; data not shown).
exchange chromatography on Mono-Q resin (Pharmacia) followed by gel filtration on a Superdex 200 column (Pharmacia). We purified RAM-ANK in an identical fashion, except that it was bound and eluted with an imidazole gradient from Ni-NTA agarose beads (Qiagen) before the Mono-Q chromatography step. A CDNA encoding the N-terminal portion of MAML1(1–300) was cloned into a plasmid derived from pRSET (Invitrogen) that permits expression of polypeptides with a hexahistidine N-terminal tag. MAML1(1–300) was purified from BL21(DE3) cell lysates in a single step by Ni-NTA agarose chromatography using an imidazole gradient. We immunoprecipitated Myc-epitope–tagged RBP-Jκ from 293T cells transfected with a pcDNA3 expression plasmid, using monoclonal anti-Myc antibody 9E10 on protein A-Sepharose beads (Sigma), and eluted the immunoprecipitated RBP-Jκ–Myc by incubation for 60 min at 4 °C in Tris (10 mM, pH 8.0) containing EDTA (1 mM), BSA (0.2 mg/ml), 10% glycerol and Myc epitope peptide (1 mg/ml; Research Genetics). All purified proteins were snap-frozen in liquid nitrogen and stored at −80 °C until use.

EMSA. We incubated 32P-labelled oligonucleotides (104 cpm) containing a normal or mutated RBP-Jκ-binding site29 (for 30 min at 30 °C) in a 15 μl volume containing Hepes (20 mM, pH 7.9), KCl (160 mM), M gCl2 (5 mM), DTT (10 mM), BSA (0.2 mg/ml), dGdC (250 ng), 10% glycerol in the presence or absence (+ or −) of the following components: 1 μl immunopurified RBP-Jκ, 50 ng RAM-ANK, 50 ng ANK and 50 ng MAML1(1–300). We electrophoresed samples at 140 V in 4% Tris-glycine-EDTA gels, and then dried and analysed the gels by autoradiography.

**Fig. 6** MAML1 mutants lacking the NOTCH-binding site or the transcriptional activation domain block ligand-induced signalling. U205 cells were transfected with HES1-luc (0.5 μg) and TK-Renilla luciferase control reporter construct (0.5 μg), together with increasing amounts of the construct encoding MAML1(1–302) (a) or MAML1(124–1016) (b) in the absence or the presence of 0.25 μg of the plasmid encoding MAML1. Twenty hours after transfection, 1×105 NIH3T3 cells expressing Jagged2 ligands or infected with empty pBABE virus were added to each well. HES1 reporter luciferase activity, corrected for TK-Renilla luciferase activity, is expressed as fold activation relative to cells not expressing MAML1 that were cocultured with control NIH 3T3 cells.

**Protein purification.** We cloned cDNAs encoding the ANK domain alone (aa 1,872–2,126), or both the RAM and ANK domains (RAM-ANK, aa 1,760–2,126, with an additional hexahistidine tag at the C terminus to facilitate purification) of human NOTCH1 into the prokaryotic expression plasmid pET41-1. To purify ANK, we incubated GST-ANK from BL21(DE3) cell lysates with glutathione-Sepharose beads (Pharmacia). Following release by thrombin cleavage, ANK was purified by anion exchange chromatography on Mono-Q resin (Pharmacia) followed by gel filtration on a Superdex 200 column (Pharmacia). We purified RAM-ANK in an identical fashion, except that it was bound and eluted with an imidazole gradient from Ni-NTA agarose beads (Qiagen) before the Mono-Q chromatography step. A CDNA encoding the N-terminal portion of MAML1(1–300) was cloned into a plasmid derived from pRSET (Invitrogen) that permits expression of polypeptides with a hexahistidine N-terminal tag. MAML1(1–300) was purified from BL21(DE3) cell lysates in a single step by Ni-NTA agarose chromatography using an imidazole gradient. We immunoprecipitated Myc-epitope–tagged RBP-Jκ from 293T cells transfected with a pcDNA3 expression plasmid, using monoclonal anti-Myc antibody 9E10 on protein A-Sepharose beads (Sigma), and eluted the immunoprecipitated RBP-Jκ–Myc by incubation for 60 min at 4 °C in Tris (10 mM, pH 8.0) containing EDTA (1 mM), BSA (0.2 mg/ml), 10% glycerol and Myc epitope peptide (1 mg/ml; Research Genetics). All purified proteins were snap-frozen in liquid nitrogen and stored at −80 °C until use.

**Reportor assay.** We seeded U205 cells on six-well plates at 1×105 cells per well 1 d before transfection, and transiently transfected cells with various combinations of expression plasmid DNA (indicated in each figure legend). The total amounts of plasmids were kept constant by adding appropriate amounts of empty vectors without inserts. We collected transfected cells 44 h post-transfection and measured luciferase activities in a Berthold luminometer (Lumat LB9507). Relative luciferase activities were normalized to β-galactosidase activity (in the case of pCMX-lacZ) or Renilla luciferase activity (in the case of pRL-TK). We measured β-galactosidase activity using a β-gal assay kit (Invitrogen), and luciferase activities using either single or dual luciferase reporter assay systems (Promega).

**GenBank accession number.** MAML1 cDNA sequence, AF221759; Drosophila mastermind cDNA sequence, AF221759; X54251; KIAA0200, D83785.

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