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JB Review

Notch signalling in the nucleus: roles of Mastermind-like (MAML) transcriptional coactivators

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Notch signalling plays pivotal roles in development and homeostasis of all metazoan species. Notch is a receptor molecule that directly translates information of cell–cell contact to gene expression in the nucleus. Mastermind is a conserved and essential nuclear factor that supports the activity of Notch. Here, the past and current studies of the interplay between these factors are reviewed.

Keywords: MAML/Mastermind/Notch signalling/signal transduction/transcription.

Abbreviations: cHL, classical Hodgkin lymphoma; Dll1, Delta-like 1; DMam, *Drosophila* Mam; DNMAML1, dominant negative MAML1; EFs, embryonic fibroblasts; GSI, γ-secretase inhibitor; HPV, human papillomavirus; Mam, Mastermind; MAML1, human Mastermind-like 1; MAML2, human Mastermind-like 2; MAML3, human Mastermind-like 3; MZB, marginal zone B; NotchIC, intracellular domain of Notch; Su(H)), Suppressor of Hairless; T-ALL, T cell acute lymphoblastic leukaemia.

Notch signalling is one of a few highly conserved signalling systems that regulates the construction and maintenance of a variety of tissues and organs in multicellular animals. Signalling is initiated by direct physical contact between cells mediated by receptors (Notch) and ligands bound to the plasma membrane (Fig. 1). The signalling system appears mechanistically simple. However, the signalling outcomes are remarkably versatile and depend on the time and space in which the signalling system works. In some cases, the signalling causes inhibition of cellular differentiation, which preserves stem and/or progenitor cells in the tissue. However, in some cases, the signalling mediates choices between binary cell fates. Furthermore, in some cases, the signalling facilitates terminal differentiation of the cells. This variety in the signalling function is commonly attributed to its context dependency. The nature of the context is one of the current focuses in this research field (*2*, *3*).

In humans, abnormal gain or loss of components of Notch signalling causes a number of diseases including developmental (*e.g.* Alagille syndrome) and adult-onset (*e.g.* aortic valve disease) diseases and cancers (*4*, *5*). Likely a reflection of the pleiotropic effects of its normal functions mentioned earlier, Notch signalling is oncogenic in some tumours (*e.g.* T cell acute lymphoblastic leukaemia (T-ALL) and ovarian carcinoma) and suppressive in other tumours (*e.g.* squamous cell carcinoma, urothelial carcinoma and small cell lung cancer) (*6*–*8*).

The research field of Notch signalling is now wide and diverse. Nevertheless, there are excellent recent reviews comprehensively covering this subject (*2*, *3*). I will instead focus on one of its nuclear components, Mastermind (Mam), on which I have been working for over a decade, and describe its history and current studies in this review.

Discovery of Notch and Mastermind

The study of Notch signalling began when fruit flies with notched wings were found in Thomas Hunt Morgan's lab a century ago (*9*). The gene, whose heterozygous loss causes the phenotype, was named *Notch* and studied as a model for genetic control of animal development. The early studies of *Notch* later turned out to be pioneer works, as the Nobel Prize-awarded study done by Nusslein-Volhard and Wieschaus (*10*) definitively showed that genes control animal development.

Strains with mutations of the *mastermind* (*mam*) gene were identified by work from Nusslein-Volhard/Wieschaus, the first saturating mutagenesis of *Drosophila* (*10*, *11*). Homozygous mutants of *mam* and a couple of other genes (*almondex*, *big brain*, *neuralized*, *Delta*, *Enhancer of split*) exhibited an indistinguishable phenotype from *Notch* (hypertrophy of nervous system), and these genes were consequently called neurogenic genes (*11*). Since the neurogenic genes were later identified as components of Notch signalling, this work is considered the discovery of Notch signalling.

Cloning of the *Drosophila Notch* gene was reported in 1983 (*12*, *13*). In 1985–86, the sequence of the transcript was published, revealing that the product was a large single-pass Type I membrane protein (*14*, *15*). It was apparent in 1990–91 that vertebrates, including humans, possess *Notch* genes (*16*, *17*). From the late 1980s through the 1990s, various other signalling components were uncovered. *Delta*, along with *Serrate*/*Jagged*, was found to encode a ligand for Notch. *Enhancer of split* genes (*HES* genes in

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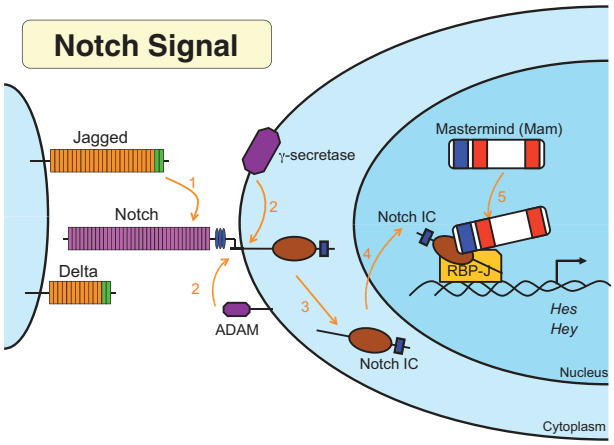

Fig. 1 Schematic diagram of Notch signalling. The Notch receptor is activated by binding of a ligand presented by a neighbouring cell. In response to binding, ADAM metalloproteases and the tetrameric γ-secretase complex, composed of presenilin, nicastrin, PEN2 and APH1, sequentially cleave Notch to release the NotchIC. In the absence of the NotchIC, a DNA-binding protein RBP-J associates with transcriptional corepressor proteins to repress transcription of target genes. When NotchIC enters the nucleus, it binds to RBP-J, which induces displacement of the co-repressors. The NotchIC/RBP-J interface is recognized by Mam, and this ternary complex recruits co-activators to assemble an active transcription complex on target promoters. This figure was modified from (*1*).

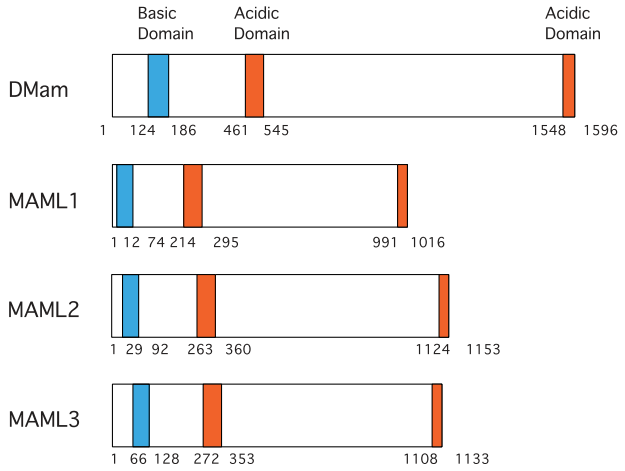

Fig. 2 Schematic diagram of Mastermind of *Drosophila* and humans. DMam, *Drosophila* Mam; MAML1, human Mastermind-like 1; MAML2, human Mastermind-like 2; MAML3, human Mastermind-like 3. All of the proteins contain an N-terminal basic domain and two acidic domains (N- and C-terminal). Numbers represent amino acid location in the primary structure. This figure was modified from (*1*, *21*).

mammals) were identified as signalling target genes. The product of *Suppressor of Hairless* (*Su(H)*) (*RBP-J* in mammals) was found to bind the regulatory sequences of *Enhancer of split* and also to the intracellular domain of Notch (NotchIC) (*18*). N-terminally truncated Notch, encompassing only its intracellular domain, accumulates in the nucleus and behaves as a constitutively active form of the receptor (*9*). Furthermore, the NotchIC associates with RBP-J and acts as a transcriptional activator of the *HES1* promoter (*19*). Later, in synchrony with ligand binding, Notch was shown to undergo sequential proteolysis that culminates in intra-membranous cleavage by γ-secretase complex, and its intracellular domain is released from the membrane (*2*, *9*) (Fig. 1).

Drosophila mam (DMam) cDNA was cloned in 1990 (*20*); however, no homologous sequence to the DMamprotein had been found in other species. The predicted primary structure of DMam contains no known sequence motif to suggest its function (*20*) besides clusters of basic or acidic amino acids (Fig. 2) (*22*). Using specific antibodies, it was determined that DMam is a nuclear protein and associates specifically with polytene chromosome regions, which often overlap with the region stained with anti-RNA Polymerase II (*20*, *23*). Thus, DMam was implicated in transcriptional regulation.

Discovery of Human Mastermind

Upon searching the database, we found a cDNA that encodes a protein now called Mastermind-like 1 (MAML1, also known as hMam-1) with a structural similarity to DMam (15% identity) (Fig. 2) (*24*). The arrangements of the basic and acidic amino acid clusters in the protein and DMam are conserved, implying that their higher-order structures are related. We first determined that MAML1 is a nuclear protein, like DMam, by immunocytochemistry. We thus used a luciferase reporter assay to see if its activity was related to the activation of the target promoters of Notch signalling, such as the *Hes* gene promoters. Although expression of the NOTCH1IC, the constitutively activated form, activates the *Hes* promoters, expression of MAML1 alone does not activate them. However, we found that co-expression of MAML1 potentiates the activity exerted by NOTCH1IC. In *Drosophila*, the expression of C-terminally truncated versions of DMam disrupts the Notch pathway by acting as a dominant negative form (*22*). Co-expression of a short truncated form of MAML1, which consists mostly of the N-terminal basic domain, reduced the NOTCH1IC-induced activation of the target promoters. These data suggested that MAML1, like DMam, may act as a positive regulator of Notch signalling. The data further suggested that MAML1 might act in concert with NotchIC.

Thus, we tested for the physical interaction of the MAML1 protein with the other nuclear components by using an electrophoretic mobility shift assay using RBP-J binding sites from the *Hes1* promoter as a probe (*19*, *24*). As shown in Fig. 3, cellular extract from cells transfected with Rbp-j exhibited two specific bands, which were identified as containing the Rbp-j protein based on their antibody reactivity (Lane 2). Co-transfection of NOTCH1IC with Rbp-j caused a slowly migrating smear in addition to the bands (Lane 3, asterisk). Interestingly, co-expression of MAML1 with these two proteins abolished all of these complexes and induced expression of two novel bands that migrate very slowly (Lane 4). These bands represent a ternary complex formed by MAML1, NOTCH1IC and Rbp-j based on their reactivity to antibodies and the mobility shift caused by expressing

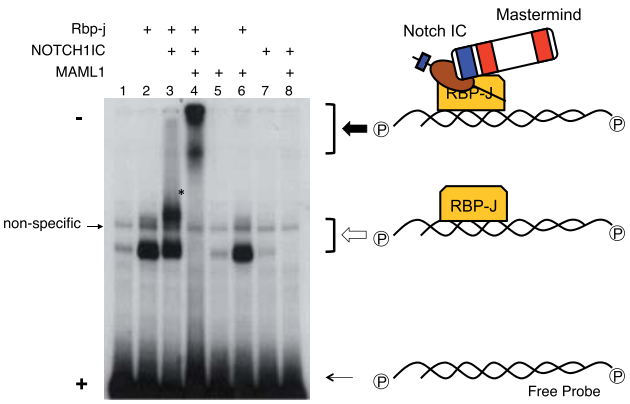

Fig. 3 Formation of the Mam-NotchIC-RBP-J complex (the Notch active transcriptional complex) on its target promoter elements. Extracts of transfected 293T cells exhibit activities that bind to the RBP-J element of the *Hes1* promoter. Cells were transfected with the expression vectors for the indicated proteins. The open arrow marks the Rbp-j-specific bands and the closed arrow marks the MAML1-NOTCH1IC-Rbp-j-specific bands. The asterisk indicates a slowly migrating smear in Lane 3. This figure was modified from (*1*, *24*).

deletion mutants of MAML1. Duality of the bands may reflect that the probe contains two binding sites and can be bound both by a single complex and by a dimeric complex (*25*). Furthermore, Lane 5 shows that MAML1 alone does not bind the probe, whereas Lane 6 shows that without the expression of NOTCH1IC, there is no evidence of interaction between Rbp-j and MAML1. Lanes 7 and 8 indicate that NOTCH1IC and MAML1 can recruit endogenously expressed RBP-J, although bands corresponding to the ternary complex are hardly visible in this exposure. Further, the MAML1-NOTCH1IC-Rbp-j ternary complex could be detected with an immunoprecipitation assay (*24*). In this assay, NOTCH1IC weakly bound to Rbp-j in the absence of MAML1. Thus, in the off state of the signal, MAML1 and RBP-J may not be associated. Upon activation, NotchIC, which was transported to the nucleus, would form weak contacts with RBP-J; subsequently, MAML1 participates and stabilizes the complex. The smear observed in Lane 3 of Fig. 3 may represent the unstable complex formed by NOTCH1IC and Rbp-j. Further, using the deletion mutants of MAML1, we found that the N-terminal basic domain is necessary and sufficient to form the ternary complex. However, the complex with the short fragment of MAML1 is less stable compared with that containing the full-length form. Moreover, we demonstrated that DMam forms a transcriptionally activating ternary complex with *Drosophila* NotchIC and *Drosophila* Su(H) (*24*). Thus, the mechanism of action of Mam is evolutionarily conserved. An essentially similar conclusion was reached in *Caenorhabditis elegans* and a mammalian system around the same time (*26*, *27*).

At that time, the 'nuclear Notch' model was controversial and not widely accepted (*28*). One reason it was not widely accepted was that Notch and RBP-J only weakly associated. The results from our lab and others provided the missing link and, I believe, contributed to the broad acceptance of the model.

Later, crystal structures of the core of the ternary complex bound to DNA were reported (*29–31*). In the structures, RBP-J and the ankyrin repeat domain of Notch combine to form an extended groove. The basic domain of Mam is settled as a long α-helix that makes widespread contacts with both proteins. The structure fit well with the model we constructed using biochemical analyses.

Mammalian Mastermind Family

The *Drosophila* genome encodes a single set of genes for most of the components of Notch signalling. However, in contrast, the mammalian genome encodes multiple paralogs for many of the components; Mam is not an exception. We identified two additional cDNA sequences (MAML2 and 3) that encode proteins with similarity to MAML1 (Fig. 2) (*21*). The arrangements of basic and acidic amino acid clusters in the Mam proteins are conserved. The overall identity between the MAML1 protein and the MAML2 and 3 proteins is 19 and 30%, respectively. In the murine sequences that were revealed later, identity between the Maml1 protein and Maml2 and 3 proteins is 21 and 29%, respectively. As paralog proteins in a mammalian species, the three Mam proteins, especially MAML2, seem to have significantly diverged compared with the other groups of paralogs. For instance, four murine Notch proteins (Notch1–4) show 30–51% identity in pairwise comparisons. Delta-like 1 (Dll1) and Dll4 ligand proteins exhibit 51% identity. Jagged1 and 2 ligands exhibit 54% identity. Thus, one may envisage that the three mammalian Mam proteins manifest distinct functional characteristics. However, both MAML2 and MAML3 form DNA-binding complexes with Rbp-j and NotchIC (*21*). In complex formation, the three Mam proteins exhibit little preference among all four kinds of mammalian Notch in the presence of Rbp-j, *i.e.* non-redundant in mammals (*21*). The expression of MAML2 and MAML3 augments the NotchIC-induced activation of the *Hes* target promoter with little appreciable difference (*21*). Moreover, the dependence on the N-terminal basic domain for the complex formation is a conserved feature of the proteins. Thus the three human Mam proteins show remarkable similarities in their functions while having unusual structural diversity. All three Mam mRNAs are expressed in many human tissues without striking specificity (*21*, *32*).

Function of Mammalian Mastermind *In Vivo*

To elucidate the *in vivo* function of Mam, we generated knockout alleles for *Maml1* (*33*) and *Maml3* (*34*) genes, the most structurally related pair in mouse. Mice deficient in Maml1 are growth retarded and die before weaning. The causes of the retardation and early death are unclear. We analysed haematopoiesis of the mice because roles of Notch signalling are well studied in mice. Among the Notch-dependent stages in haematopoiesis, Maml1 is cell-autonomously required for the development of marginal zone B (MZB) cells in the spleen and the efficient development of early T cells

in the thymus, steps that depend on Notch2 and Notch1, respectively (*33*). In contrast, Maml1 is dispensable for the generation of definitive haematopoiesis, the generation of the most primitive T cell progenitors in thymus, and a lineage choice between T/B lymphocytes in the thymus, steps that are all dependent on Notch1 (*33*). These results suggest that Maml1 is partially required for the canonical Notch signalling in mammals *in vivo* (*33*, *35*).

We also prepared a primary culture of embryonic fibroblasts (EFs) and evaluated the contribution of Maml1 in Notch signalling by a luciferase reporter assay (*33*). In the knockout cells, activation by Notch1IC was several-fold lower compared with in the wild-type and the heterozygous cells. By co-expressing MAML1 with Notch1IC, the promoter in the knockout cells was activated to a comparable level to those of wild-type and heterozygous cells. These results indicate that Maml1 is necessary to evoke the full magnitude of activity of Notch signalling in the fibroblasts and that a weak but certain degree of the signalling can be transmitted even in the absence of Maml1. These results harmonize with those of the phenotypic analyses of the knockout mice; both analyses concluded that the mice have a partial deficiency in Notch signalling (*33*). The reason why not all the 'Notch phenotype' was observed in the mice was later revealed to be due to the presence of other Mam family proteins, as described later.

Maml3-null mice showed no apparent abnormalities, including in the Notch signal-dependent steps in lymphocyte development (*34*). However, mice null for both Maml1 and Maml3 died during the early organogenic period with classic pan-Notch defects (*34*). They are namely imperfect cardiovascular development, defective somitogenesis and accelerated neurogenesis. Further, expression of the target genes for Notch signalling *Hes5*, *Hey1* and *Heyl* was reduced in the double knockout embryos of the period than in controls. Moreover, expression of the *Lfng* gene, which is strictly controlled by Notch signalling in the posterior presomitic mesoderm, was undetectable in this tissue of the double-null embryos, indicating that Notch signalling, in terms of target gene induction, was lost in the double-null mice at least in this tissue (*34*). Neither of the single-null embryos of Maml1 or Maml3 exhibited any of these phenotypes. Thus, disruption of just two of the three Mam genes, *Maml1* and *Maml3*, the most structurally related pair, disrupts signalling. Further, the results indicate that *Maml1* and *Maml3* are genetically equivalent and perform redundant roles in these developmental processes.

These results indicate that engagement of Mam is essential for Notch signalling and that the three Mam isoforms have distinct roles *in vivo*. These various roles of the three Mam proteins could be because of their differential physical characteristics and/or their spatiotemporal distributions.

Based on its expression pattern, Maml2 may have a role supporting Notch signalling later in life more than during the embryonic early organogenic period (*34*). There is no report of Maml2-deficient mice to date.

The pan-Notch phenotypes seen in the double-null mice were shown to be dependent mainly on Notch1. Thus, both Maml1 and Maml3 should be a part of the ternary complex containing Notch1 *in vivo*. However, as Maml1 is essential to the Notch2-dependent development of splenic MZB cells (*33*), Maml1 should also be a part of the ternary complex containing Notch2 *in vivo*. These data are consistent with the results of the luciferase assay using EFs, which showed that Maml1-deficiency affects signalling by all four Notch proteins (*34*), and with our previous results involving overexpression of these components to show that the three Mam proteins exhibit little preference among all four kinds of mammalian Notch (*21*).

Overall, each of the three mammalian Mam species seems to have a unique role *in vivo*, reminiscent of the case for the four mammalian Notch species. However, assignment of roles to the Notch species in various contexts seems to have occurred independently from that of the Mam species. Thus, different combinations of Mam-Notch might have distinct *in vivo* functions, depending on spatiotemporal criteria (*34*).

Roles of Mastermind in the Notch Active Transcriptional Complex and the Dominant Negative Form of Mastermind

As mentioned earlier, expression of the basic domain of MAML1 alone inhibits Notch-induced activation of the target promoters (*24*). A similar short form of MAML1 fused with GFP called dominant negative MAML1 (DNMAML1) was shown to efficiently inhibit Notch-dependent development of T lymphocytes in the thymus and MZB development in the spleen, when retro-virally introduced into the bone marrow progenitors of mice (*36*). The inhibition of the signalling by DNMAML1 was shown to non-specifically affect any kinds of Notch. Thus, it is a pan-Notch and a pan-Mam inhibitor and not only a specific inhibitor for MAML1 (*36*). Later, a transcription unit for DNMAML1 with a floxed cassette to allow conditional expression was knocked in to the *Rosa26* locus of the mouse genome (*37*). The mouse strain has been used to analyse the involvement of Notch signalling in various processes (*37*, *38*). Further, a synthetic 16-residue peptide of the sequence from the basic domain of MAML1 was modified to stabilize with the hydrocarbon staple and developed to suppress Notch signalling (*39*). This peptide efficiently inhibits growth of T-ALL cells both *in vitro* and *in vivo*.

In transcriptional transactivation assays using combinations of four kinds of Notch and three kinds of Mam, the strength of transcription largely depends on Notch but not on Mam (*21*, *32*). These results indicate that Notch but not Mam mainly co-activates transcription from the promoter in the ternary complex. A transcriptional activation domain in Notch1 has been identified which can interact with PCAF and GCN5 histone acetyltransferase (*40*).

Nevertheless, there is evidence that Mam also possesses transactivation domains. A deletion mutant of MAML1 devoid of the C-terminal acidic domain fails

to potentiate the activity of Notch1 in a cell-based assay (Kitagawa, M., unpublished data), while retaining the ability to participate in and stabilize the ternary complex (*24*). Another deletion mutant of MAML1 devoid of the N-side acidic domain also loses the ability to augment Notch activity (Kitagawa, M., unpublished data). Thus, Mam proteins also seem to participate in the activation. There is *in vitro* evidence that CBP/p300 histone acetyltransferase binds around the N-side acidic domain (*41*, *42*). The essential role of the C-terminal acidic domain remains unknown.

The dominant negative forms of MAML1 not only lose their co-activating activity but also shut down the activity of NotchIC almost to the basal level. Currently available data describe a model for the mechanism of action where the association of NotchIC with RBP-J is destabilized by sequestering endogenous Mam by the unaccompanied N-terminal basic domain that can only poorly stabilize NotchIC–RBP-J in comparison to its full-length counterpart (*24*, *33*).

In addition to the classic association factors mentioned earlier, more recent studies have expanded the number of potential interacting factors for NotchIC, and thus the NotchIC-Mam-RBP-J complex, to contribute to the transcriptional activation of target genes, including various histone-modifying enzymes (*e.g.* KDM5A, KDM1, and SIRT1) and chromatin remodelling factors (*e.g.* NURF, SWI/SNF, INO80 and CHD/NuRD). One notable study used the T-ALL cell line expressing a γ-secretase inhibitor (GSI)-sensitive mutant of *NOTCH1*. They identified GSI-sensitive (thus NOTCH1IC-depnedent) and -insensitive (thus constitutive) interacting factors of MAML1. The former class includes RBP-J, LSD1, PB1 and BRG1; the latter class includes PHF1 and Af4p12 (*43*). Factors of the former class may be associated either with NOTCH1IC or RBP-J, or recruited after formation of the ternary complex. Detailing these factors is beyond the scope of this review; for detailed descriptions, please refer to other recent reviews (*44*, *45*).

In the absence of NotchIC, RBP-J represses transcription. Classically, a general corepressor, the SMRT/HDAC1 complex and a specific corepressor, MINT (SHARP), constitutively associate with RBP-J and the target promoters. Upon Notch activation, NotchIC displaces the interaction between RBP-J and the corepressors (*46*, *47*). Recent studies have expanded the number of corepressors associating RBP-J (*e.g.* KDM5A and SIRT1-LSD1 complex) (*48*, *49*). However, recent studies have also shown that activation of Notch increases the occupancy of RBP-J on chromatin (*50*, *51*). These results are apparently contradictory, however, there may also be a possibility that both systems function concurrently. One possibility would be that the promoter repression by the RBP-J-corepressor complex might work in a portion of the binding sites.

Roles of Mastermind in Health and Disease

There is a growing list of studies describing involvement of Mam in health maintenance.

During the embryonic somitogenesis of mice, the rostro-caudal polarity within a somite is primarily determined by the on/off state of Notch signalling. A transcription factor, Mesp2, acts as an essential negative regulator of the Notch signalling pathway during this process by inducing degradation of Maml1 (*52*).

In a genome-wide association study for congenital heart malformations in humans, a significant association identified a SNP in the MAML3 gene (*53*), which is associated with heart development (*34*).

MAML2 is highly expressed in several types of B cell-derived lymphomas, including classical Hodgkin lymphoma (cHL) cells, relative to normal B cells. Knock-down of MAML2 in cHL cells resulted in downregulation of the NOTCH target genes, which are highly expressed in cHL cells, and in reduced proliferation. Thus, the deregulation of MAML2 in the lymphoma cells is important for NOTCH target gene regulation and growth of the cells (*54*).

Gene amplification and overexpression of *NOTCH3* had been found in a significant proportion of ovarian cancers (*55*). In cell lines with overexpressed *NOTCH3*, its inactivation by GSI or knock-down suppressed proliferation and induced apoptosis (*55*). In an extended study by a large consortium, mutation of *NOTCH3* was found in 11% of the cases, and mutations of either of the *MAML1-3* genes were found in 8% of the cases in total (*56*). Thus, Mam genes seem to behave as proto-oncogenes in the cancer.

In squamous cell carcinomas, Notch genes behave as tumour suppressors, especially for cutaneous squamous carcinoma, with ~75% of the cases having mutations in *NOTCH1* or *NOTCH2* genes (*57*). Notably, however, recurrent mutations are rarely identified in other components of the canonical Notch signalling pathway, including Mam genes for an unknown reason (*57*).

E6 oncoproteins of the α-genus human papillomavirus (HPV), such as HPV16 and HPV18 that cause cervical cancers, associate with and degrade the p53 tumour suppressor. In contrast, E6 proteins of the cutaneous β-HPVs, such as HPV8 and HPV17a that cause commensal infections or various types of warts, do not associate with p53. New studies identified MAML1 (*58–60*) and MAML3 (*59*) as associating partners of E6 from β-HPVs. They also found that the E6 proteins of β-HPVs but not α-HPVs hamper Notch signalling in cell-based assays. It should be noted that β-HPVs are associated with skin cancers of epidermodysplasia verruciformis or immunosuppressed patients.

A couple of recurrent chromosomal translocations in cancers target *MAML2* and *MAML3*. In mucoepidermoid carcinoma of salivary glands and other organs, *CRTC1-MAML2* and *CRTC3-MAML2* are recurrently found (*61*, *62*). In secondary acute myelogenous leukaemia and myelodysplastic syndrome, an *MLL-MAML2* fusion was found (*63*). A *PAX3-MAML3* fusion was found in biphenotypic sinonasal sarcoma (*64*). All the translocations cause the replacement of the first exon of Mam genes that encodes the N-terminal basic domain with a portion of other genes. Thus, the fusion proteins produced do not participate

Notch signalling but activate other genes. Mam genes may provide transcriptional activation domains.

Interaction of Mastermind with Other Transcription Factors

Besides Notch, there are indications that MAML1 coactivates other transcription factors, MEF2C (*65*), p53 (*66*), β-catenin (*67*) and NF-κB (*68*). This field may emerge as an important field in the future.

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Conflict of Interest

None declared.

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