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[Wilfred Donald Stein](#) *

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Article

An Orthologics Study of the Notch Signalling Pathway

Wilfred D. Stein

Silberman Institute of Life Sciences, Hebrew University, Jerusalem, Israel 91904; wdstein@mail.huji.ac.il

Abstract: The Notch signaling pathway plays a major role in embryological development and in the ongoing life processes of many animals. Its role is to provide cell-to-cell communication in which a Sender cell, bearing membrane -embedded ligands, instructs a Receiver cell, bearing membrane-embedded receptors, to adopt one of two available fates. Elucidating the evolution of this pathway is the topic of this paper, which uses an orthologs approach to build a firm base for such a study. Using BLAST searches, orthologs were identified for all of the 49 listed components of the Notch signaling pathway. The historical time course of the integration of these proteins as the evolution of the animals provided was elucidated. Insofar as cell -to-cell communication is only of relevance in multicellular animals, it is not surprising that the Notch system reached a fully-working configuration only with the evolutionary appearance of the Metazoa, the first multicellular animals. The roles of the Notch system's 49 components in modern descendants of the ortholog-contributing ancestors were ascertained from a survey of the literature. A single protein, the protein tyrosine kinase (PTK) of the protozoan *Ministeria vibrans* was identified as a possible pre-Metazoan ancestor of the DLL, JAG, and NOTCH proteins. A scenario for the evolution of the Notch signaling pathway is presented and described as the co-option of its components, clade by clade, in a repurposing of genes already present in the ancestral unicellular organisms.

Keywords: evolution; orthologs; notch signaling system; cell-to-cell communication; origin of multicellularity; co-option

Introduction

The Notch signaling pathway is one of the many signaling pathways in biological systems (the collection in <https://www.tocris.com/signaling-pathways> lists 14 of them).

The Notch pathway plays a major role in embryological development and the ongoing life processes of many animals. Among its many roles, one may list:

Neuronal function and development [1], fate determination in angiogenesis [2], and in cardiac development [3], control of the differentiation of both endocrine and exocrine pancreas [4], cell fate decisions between the secretory and absorptive lineages in the gut [5], in the development of mammary glands [6] and of alveoli in the lung [7].

In many of these cases, the Notch pathway acts as an arbiter between two alternative cell-fate decisions: "Notch acts in a permissive manner: It does not determine the fate of a cell but rather whether a given fate is adopted" (Ehebauer et al [8]).

Figure 1 Copy of WSP268, the version of the Notch signaling pathway depicted in Wikipedia <https://www.wikipathways.org/pathways/WP268.html>.

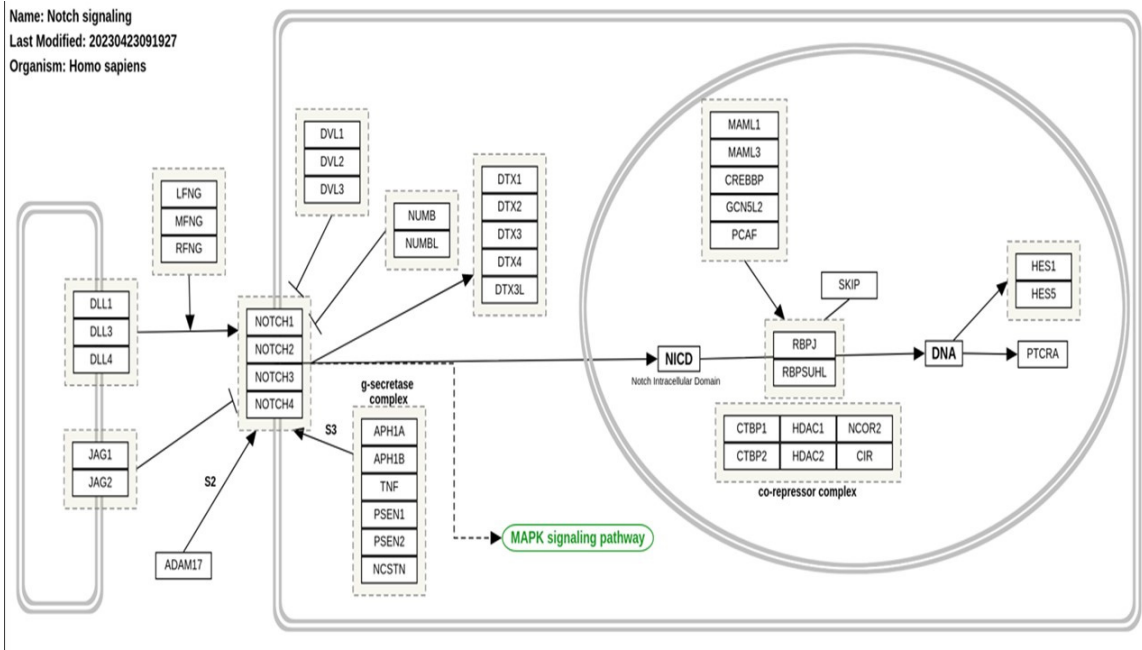


Figure 1. displays a version of the Notch Signaling Pathway listed by Wikipedia as WSP268:.

This scheme shows two cells; the one on the left is the cell that we will call the SENDER cell, that on the right is the RECEIVER cell. Embedded in the membrane of the sender cell are the DLL and JAG proteins, which are the ligands for the NOTCH proteins, also membrane- embedded, of the receiver cell. On binding of the ligands to the NOTCH proteins, these receptors are hydrolytically cleaved and the intracellular portion of the NOTCH proteins move to the cell nucleus (depicted as the circle with in the receiver cell) where they interact with the RBP proteins that regulate the HES transcription factors. This transcription determines the expression or repression of targeted proteins in the receiver cell and hence its fate. The central core of the system is thus the DLL/JAG – NOTCH – RBPJ – HES chain that enables the sender cell to control the expression of proteins in the adjacent receiver cell. Additional members of the pathway activate or repress other components of the chain. An example is the co-repressor complex depicted in Figure 1 as being within the nucleus of the receiver cell. Another is the cytoplasmic γ -secretase complex, also depicted in the figure. The DVL and NUMB proteins interact with, and repress the NOTCH proteins, as do the proteins of the DTX family (although these can also activate NOTCH proteins). The extent of these activating or pressing processes are, of course, determined by the epigenetic status of the receiver cell. Finally, the Fringe proteins (LFNG, MFNG, and RFNG) are glucosaminyltransferases that have been shown to modulate Notch activity.

The evolution of such an important system is an intriguing question and has been tackled before. Gazave etal [9] presented an impressive analysis of the question, but their study comprised less than half of the Notch system proteins depicted in Figure 1. The comprehensive work of Lv etal [10] is confined to Notch signaling in the invertebrates, while the study of Babonis and Martindale [11], that considers a broad range of signaling systems, deals with only twenty components of the Notch system. The present work considers all 49 of the Notch pathway components (46 that appear in Figures 1 and 3 that were identified after Figure 1 was built) and considers both vertebrate and invertebrate animals. It is based on the use of orthologs. This provides a firm foundation to elucidating the appearance of the proteins of the Notch signaling system, evolutionary level by evolutionary level, from the first protozoa to modern humans. Only with our own appearance does the recruiting to the system reach completion.

Methods

Searches for orthologs were performed using the protein BLAST (Basic Local Alignment Search Tool) program of the NCBI (National Center for Biotechnology Information of the National Library of Medicine): <https://blast.ncbi.nlm.nih.gov/Blast.cgi> with the following search parameters: Max target sequences 1000, Expect threshold 200000, Word size 2, Max matches in a query range 0, Matrix BLOSUM62, Gap Costs Existence: 11 Extension: 1, No compositional adjustments, No Low complexity regions filter.

To be recognised as an ortholog in the present study, a sequence found in the databases had to be annotated with the same name as the probe sequence from the human genome. A sequence annotated as “-like” was rejected. Of course, by the definition of an ortholog, the probe sequence had to be absent when earlier -appearing clades were searched.

Sequence comparisons between two proteins and dot plots of the comparisons were made using the BLAST 2-sequences tool of the BLAST program, using the same search parameters as above. All one-on-one Expect values recorded in this paper were results of BLAST 2-sequence comparisons between the two proteins.

Alignments between protein sequences were established and dot plots generated using the COBALT (Constraint-Based Alignment Tool) aligner at the NCBI <https://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?CMD=Web>.

Properties of the orthologs listed in Supplementary Table S1 were taken from the listings in GeneCards <https://genealacart.genecards.org/Result>. GeneCards proved useful also when the aliases of genes discussed in the literature had to be interpreted to provide HGNC symbols.

The clades leading up to the emergence of the primates are numbered as Phylostratum Levels in the following scheme, which follows the formulation by Domazet-Loso and Tautz [12].

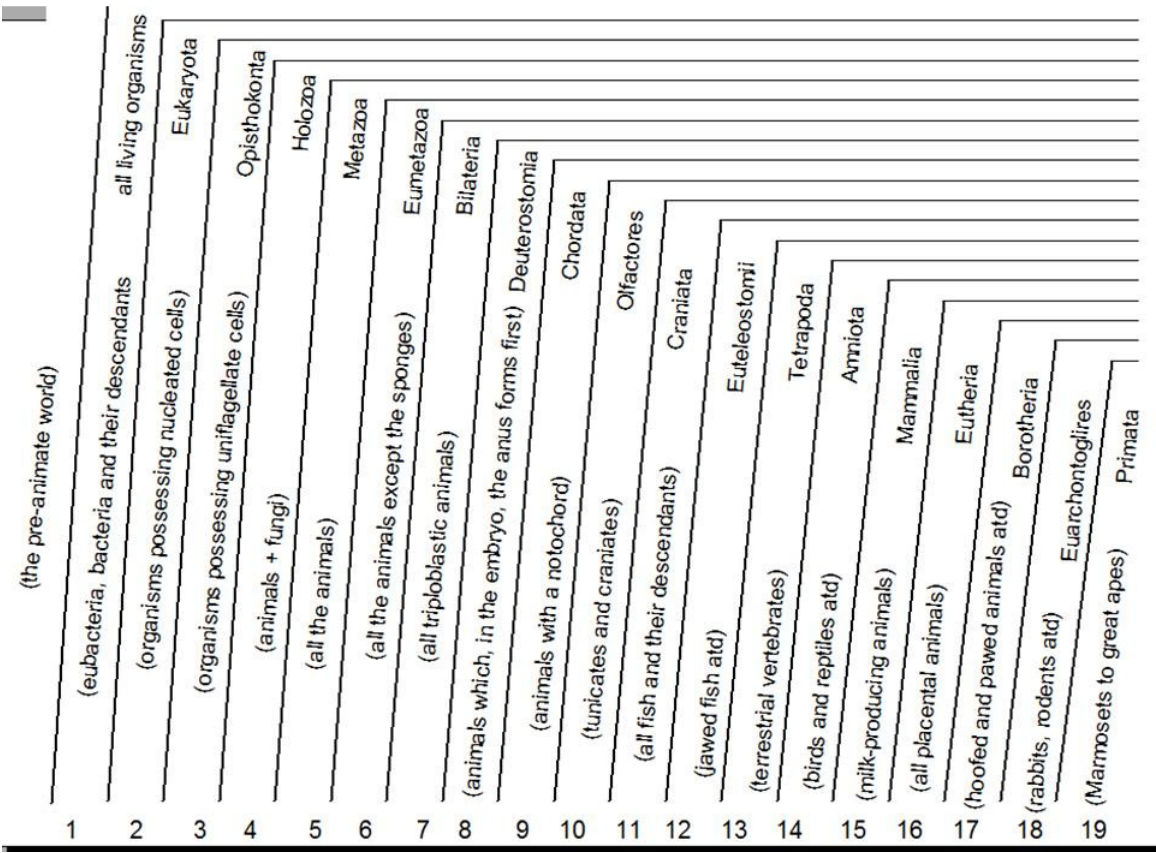


Figure 2. The clades leading to Homo sapiens. The numbering of the 19 Phylostratum Levels follows that of [12].

Results

Figure 1 above depicts 46 proteins of the NOTCH signaling pathway. These are listed in Table 1a,b together with the orthologs found by BLAST searches (see Methods). Included in the tables are the three recently found paralogs of the NOTCH2NL gene, that appeared in human evolution some three million years ago [13]. Also listed in the tables are the HGNC symbol for each protein (in cases where the name listed in Figure 1 is not an HGNC symbol), the Uniprot symbol for the protein, the ortholog level, the Phylostratum number, and the Expect value obtained by a BLAST 2-sequence comparison between this ortholog and the corresponding protein in *H.sapiens*.

Table 1.

TABLE 1a ORTHOLOGS OF THE NOTCH SIGNALLING GENES (A to H)				
HGNC (or name on WP268)	Uniprot	Ortholog found in:	PSn*	E value**
ADAM17	P78536	Branchiostomatidae	9	6.00E-178
APH1A	Q96BI3	Fungi	3	1.00E-34
APH1B	Q8WW43	Fungi	3	3.00E-40
CIR1 (CIR)	Q86X95	Porifera	5	1.00E-63
CREBBP	Q92793	Porifera	5	0.00E+00
CTBP1	Q13363	Bilateria	7	0.00E+00
CTBP2	P56545	Branchiostomatidae	9	0.00E+00
DLL1	O00548	Cnidaria	6	2.00E-110
		Elasmobranchi sharks and		
DLL3	Q9NYJ7	rays	12	1.00E-111
DLL4	Q9NR61	Branchiostomatidae	9	3.00E-180
DTX1	Q86Y01	Bilateria	7	5.00E-100
DTX2	Q86UW9	Porifera	5	6.00E-83
DTX3	Q8N9I9	Porifera	5	8.00E-63
DTX3L	Q8TDB6	Elasmobranchi sharks and rays	12	9.00E-59
DTX4	Q9Y2E6	Cnidaria	6	2.00E-87
DVL1	O14640	Elasmobranchi sharks and rays	12	0.00E+00
DVL2	O14641	Elasmobranchi sharks and rays	12	0.00E+00
DVL3	Q92997	Cyclostomata	11	0.00E+00
KAT2A (GCN5L2)	Q92830	Porifera	5	0.00E+00
HDAC1	Q13547	Fungi	3	0.00E+00
HDAC2	Q92769	Protista	2	0.00E+00
HES1	Q14469	Cnidaria	6	6.00E-50
HES5	Q5TA89	Elasmobranchi sharks and rays	12	5.00E-43
Footnotes: *Phylostratum number ** Expect value in a BLAST 2-sequence comparison				
TABLE 1b ORTHOLOGS OF THE NOTCH SIGNALLING GENES (J to T)				
HGNC (and name on WP268)	Uniprot	Ortholog found in:	PSn*	E value**
JAG1	P78504	Cnidaria	6	0.00E+00
JAG2	Q9Y219	Elasmobranchi sharks and rays	12	0.00E+00
LFNG	Q8NES3	Porifera	5	8.00E-51
MAML1	Q92585	Elasmobranchi sharks and rays	12	0.00E+00

MAML3	Q96JK9	Elasmobranchi	sharks and rays	12	0.00E+00
MFNG	O00587	Cnidaria		6	3.00E-91
					2.20E-
NCOR2	Q9Y618	Cnidaria		6	117
					1.00E-
NCSTN	Q92542	Porifera		5	106
NOTCH1	P46531	Cnidaria		6	0.00E+00
NOTCH2	Q04721	Porifera		5	0.00E+00
NOTCH2NLA	Q7Z3S9	Humans		19	Identical
NOTCH2NLB	P0DPK3	Humans		19	Identical
NOTCH2NLC	P0DPK4	Humans		19	Identical
	Q9UM4				
NOTCH3	7	Elasmobranchi	sharks and rays	12	0.00E+00
NOTCH4	Q99466	Amphibia		13	0.00E+00
NUMB	P49757	Porifera		5	2.00E-47
					4.00E-
NUMBL	Q9Y6R0	Bilateria		7	111
KAT2B (PCAF)	Q92831	Cnidaria		6	0
					2E-132
PSEN1	P49768	Porifera		5	as PSN
PSEN2	P49810	Porifera		5	3E-122
PTCRA	Q6ISU1	Amniota		14	1.00E-34
RBPJ	Q06330	Fungus		3	9.00E-53
	Q9UBG				2.00E-
RBPJL (RBPSUHL)	7	Porifera		5	150
RFNG	Q9Y644	Cnidaria		6	7.00E-88
SNW1 (SKIP)	Q13573	Fungus		3	2E-170
TNF	P01375	Elasmobranchi	sharks and rays	12	5.00E-34

Footnotes: *Phylostratum number ** Expect value in a BLAST 2-sequence comparison.

Supplementary Table S1 lists these 49 proteins and the annotations obtained by querying Genecards (<https://www.genecards.org/>).

Figure 3 depicts the contribution of the orthologs at each phylostratum level (see the definitions of the phylostratum levels in Methods), while Figure 4 shows the data as the accumulated total of orthologs as a function of phylostratum level:

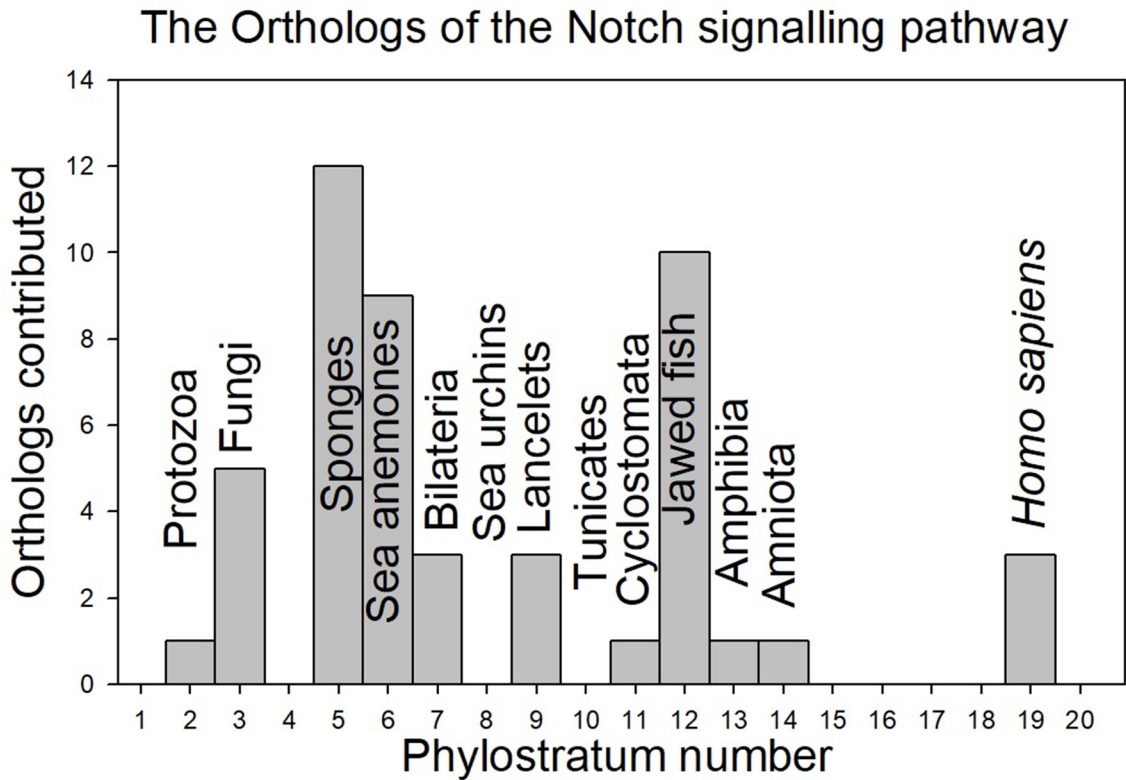


Figure 3. Contribution of orthologs of the proteins of the Notch signaling system at each phylostratum level. Data from Tables 1a and 1b.

Contributions begin already at the level of the protozoa with major additions occurring with the appearance of the sponges, the sea anemones and the jawed fish. Interestingly, it would appear that the sea urchins and tunicates did not contribute new proteins to the Notch signaling pathway.

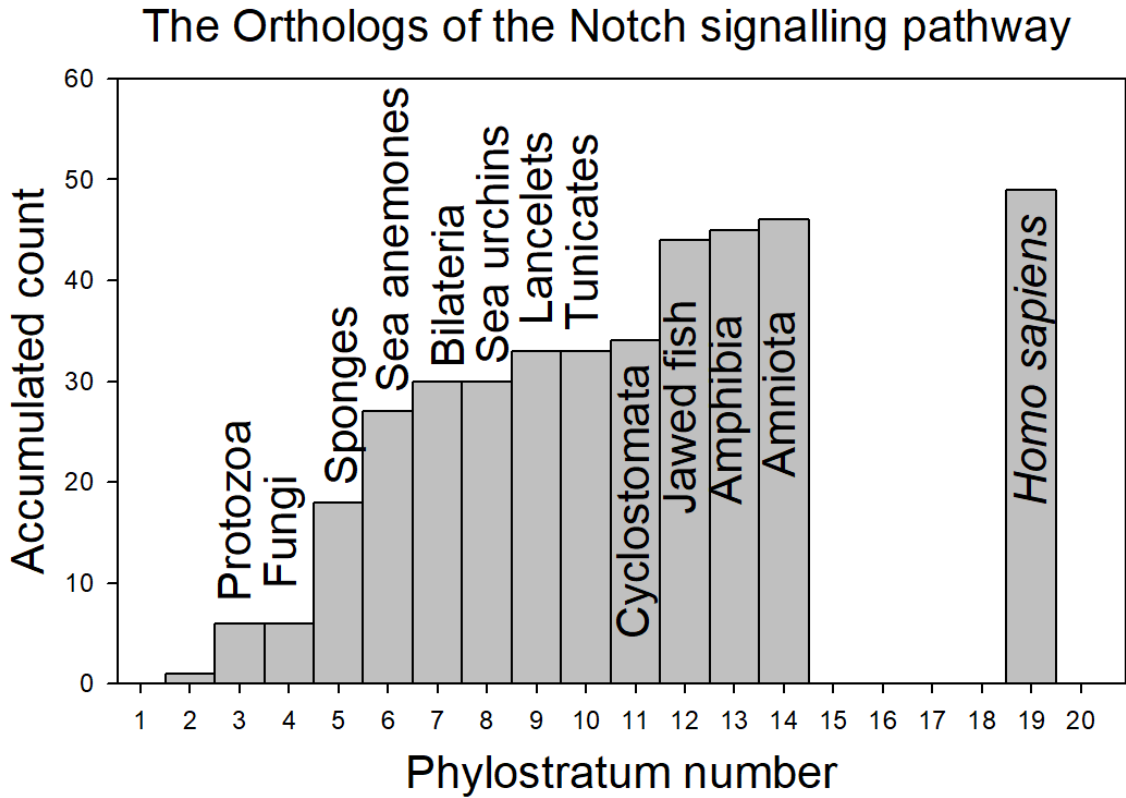


Figure 4. Accumulated contribution of orthologs of the proteins of the Notch signaling system per phylostratum level. Data from Table 1.

From Figure 4, it would appear that by the time of the arrival of the sea anemones, fully half of the Notch signaling proteins had accumulated to the evolutionary development of the pathway.

Figure 5 below shows the proteins that were to form the Notch signaling system as they appeared, clade by clade, with the evolution of each new clade in the early evolutionary history of the animals. It will be appreciated that these proteins accumulate- so that at each clade, those Notch signaling proteins that appeared in earlier clades will be, in general, still retained in the newly evolved organism.

Eumetazoa: (Cnidaria)	DLL1	RFNG	NOTCH1					
	JAG1	MFNG			DTX4		PCAF	HES1
							NCOR2	
Metazoa: (Porifera)	[DLL]	LFNG	NOTCH2	NUMB	DTX2		CREBBP	
				PSEN1	DTX3		GCN5L2	
				PSEN2			RBPSUHL	
				NCSTN			CIR	
Eukaryota: (Fungi)				APH1A			RBPI	
				APH1B			HDAC1	
							HDAC2	
							SNW1	

Figure 5. Contribution of orthologs of the proteins of the Notch signaling system for the lower half of the evolutionary trajectory of the animals. Data from Table 1.

(The genome of the primitive Metazoan, the Placazoa *Trichoplax adherens* possesses no Notch protein designated as such, but a sequence designated as “uncharacterized protein TRIADDRAFT_57304 “, when BLASTED against *H. sapiens* yields as the two top hits NOTCH1 and NOTCH2 - that show Expect values of 0.0 and 0.0, respectively, in BLAST 2-sequence analyses against this *Trichoplax* sequence).

The protein that appears as [DLL] among the Porifera components in Figure 5 above is designated in this fashion because the five DLL proteins of the sponges do not fit the definition of an ortholog as given in Methods: “To be recognised as an ortholog in the present study, a sequence found when searching the databases had to be annotated with the same name as the probe sequence from the human genome”. However, Figure 1 depicts only three DLL proteins, not five. To accommodate this complexity, the “ortholog” in the sponges is designated here as a single protein [DLL].

The sequences of two of the five DLL proteins of the sponge *Amphimedon Queenslandica* are compared with the sequence of DLL1 of *H. sapiens* in Figure 6 below.

✓ ADU54210.1 150 QISHTAIGVF NRVSLLSSVRLLCNVYGFDCSVLCIPYNDNTNGHYTCNSTTGAKKCREGWQNVTTNCTNVACNLV 226
 ✓ ADU54207.1 154 ERQSTVVGED GKISLELSYSLSCSQNYVYGSDCSQQCIPRNDNTNGHYTCNTTTGGIICREGWQNTTCTDVACHLV 230
 ✓ 000548.2 150 QR-HLTVGEE[8]GRDCLKYSYRFVCDHYVYGGCSVFCRPR-DDAFGHFTCG-ERGEKVCNPGWKG--PYCTEPICLPG 229

 ✓ ADU54210.1 227 CRSPGGTCTHNGTCQCTIGWSGDGCDIPQCIEGCHPQGGYCTEPYQCLCYNNWNGSLCNESF--CTVN--CSSIGGQCLV 302
 ✓ ADU54207.1 231 CQSPGGNCSSDGTCECNSNWFQDQCQNPNCIEGCHPEGGFCNMPNECLCHSNWNGTLCDELL--CNVN--CSSRGGECLV 306
 ✓ 000548.2 230 CDEQHGFCDKPGECKCRVGNQGRYCDCEIRYPGC--LHGTCCQPWQCNCQEGWGLFCNQDLnyCTHHkpCKN-GATCTN 306

Figure 6. Comparison of a portion of the protein sequences of three DLL proteins. The top two sequences are for Delta5 and Delta2 of *Amphimedon Queenlandica* while the lowest is that of DLL1 of *H. sapiens*.

Exploring the Deep Evolutionary Origins of the Notch, DLL and JAG Proteins

The Porifera are classified in the Metazoa, the earliest multicellular animals. Figure 5 shows that both a DLL protein and NOTCH2 had already appeared in these animals, giving them the possibility of cell-cell interactions, the DLL in the Sender cell and the NOTCH in the Receiver cell both being present. It is of interest to ask what might have been the ancestors of these fundamental proteins of the Notch signalling system. To answer this question, we performed a BLAST search, using the NOTCH protein of the glass sponge *Oopsacas minuta* as bait and querying all the animals below the Metazoa. The top hit was BAM76481.1, the annotation of a sequence in the protein tyrosine kinase (PTK) of the bacterivorous amoeba *Ministeria vibrans*. A BLAST 2- sequence comparison between this sequence and that of the sponge's NOTCH returned a convincing Expect value of $2E^{-103}$.

A comparison between the protein sequences of the NOTCH protein of the sponge *O. minuta*, the four NOTCH proteins of *H. sapiens* proteins and the protein tyrosine kinase of *M. vibrans* is shown in Figure 7 that follows:

BAM76481.1	53	TNINECEGV--TCMNGNCIDQVN-HFKCACVTGFTGVYCETNIDDCASSCSAYGTCQDGVASFFCACAP-GYTG---	124
APZ81095.1	355	EVIDNCPTS--SCEYG-ICQNSVG-SYTCDCSPGFMGTLCDAEINECHPDPCVTGD-CIDFVNGVYCNCTDaGYVGRNC	428
P46531.4	294	EDVDECQLM-PNACQNGGTCHNTHG-GYNCVCVNGWGTGDCSENIDDCASAACFHGATCHDRVASFYCECPH-GRTGLLC	370
Q04721.3	297	EDVDECLLQ-PNACQNGGTCANRNG-GYGCVCVNGWGTGDCSENIDDCAFASCTPGSTCIDRVASFSCMCPE-GKAGLLC	373
Q9UM47.2	196	NPAVPCA--PSPCRNGGTCRQSGDITYDCACLPGFEGQNCENVNDDCPGHRCLNGGTCVDGVNTYNCQCPP-EWTGQFC	271
Q99466.2	313	EDVDECETQgPPHCRNGGTCQNSAG-SFHCVCVSGWGGTSCENLDDCIAATCAPGSTCIDRVGSFSCLCPP-GRTGLLC	390

BAM76481.1	157	SAGYTGHCETDINECASNPCENGGTCDNHV--GSFSCNCAgTGFVGSTCNAQFDECv--SSPCQNSGVCFSGINYYSCN	232
APZ81095.1	511	QDGYEGSLCADDINECDLSPCNDGVCNSFH--GGFNCTCT-VGFEGDTCIDRTDYCAm-GNECANEATCVNNRFNYTCQ	586
P46531.4	441	LQGYTGPRCEIDVNECVSNPCQNDATCLDQI--GEFQCICM-PGYEGVHCEVNTDECA--SSPCLHNGRCLDKINEFQCE	515
Q04721.3	445	LKGYAGPRCEMDINECHSDPCQNDATCLDKI--GGFTCLCM-PGFKGVHCELEINECQ--SNPCVNNQCVDKVNRFQCL	519
Q9UM47.2	341	PMGKTGLLCHLD-DACVSNPCHEDAICDTNPvNGRAICTCP-PGFTGGACDQDVDECSigANPCEHLGRCVNTQGSFLCQ	418
Q99466.2	464	PPGYTGSRCEADHNECLSQPCHPGSTCLDLL--ATFHCLCP-PGLEGQLCEVETNECA--SAPCLNHADCHDLLNGFQCI	538

Figure 7. Comparison of a portion of the protein sequences of the protein tyrosine kinase of *M. vibrans*, the NOTCH protein of *Oopsacas minuta*, and the four NOTCH proteins of *H. sapiens* proteins. The top sequence in each group is that of the protein tyrosine kinase, the next is that of the sponge, while the next four sequences are for the NOTCH proteins of *H. sapiens*.

Using now as bait the Delta protein of, again, the glass sponge *O. minuta* (The glass sponge possesses a single DLL protein named Delta), and querying all the animals below the Metazoa, the same sequence BAM76481.1 of *M.vibrans* was returned. A BLAST 2-sequence comparison between this sequence and that of the sponge's DLL returned an Expect value of $4E^{-80}$.

A comparison between the protein sequences of the single Delta protein of the sponge *O. minuta*, three DLL proteins of *H. sapiens*, and the protein tyrosine kinase of *M. vibrans* is shown in Figure 8 that follows:

BAM76481.1	197	tGFVGSTCNAQFDECvSSPCQNSGVCf----SGINYYSCNCDgtGYTGTMCdVEVDECESSPCANGGVCsNLINAFsCSc	272
APZ81095.1	347	-GYEDKNCTEVIDNCPTSSC-EYGICQ---NSVGSYTCDCSP-GFMGTLCDAEINECHPDPCVTGD-CIDFVNGVYCNc	418
Q00548.2	357	-GFYGIKCELSAMTCADGPCFNGGRC--SDSPDGG-YSCRCVP-GYSGFNCEKKIDYCSSSPCSNGAKCVDLGDAYLCRC	431
Q9NYJ7.1	303	-GFYGLRCEVSGVTCADGPCFNGGLCVgGADPDSA-YICHCPP-GFQGSNCEKRVDRCSLQPCRNGGLCLDLGHALRCRC	379
Q9NR61.1	353	-GYYGLHCEHSTLSCADSPCFNGGSC--RERNQGANYACECPP-NFTGSNCEKKVDRCTSNPCANGGQCLNRGSPSRMRC	428

BAM76481.1	273	A-AGFVGTCQQTNTNECAS[37]SPCQNGGVCTDLVNS--FSCGCPNGFAGDVCEtNID--DC	AVAPCKNGATCVD-	376	
APZ81095.1	419	TdAGYVGRNCsLAAIDHPN[8]NSCLNGATCDTDGAEPfFNCSCLDGYSYGAYCGDNTD--NC[4]SSNPcDNDAVCID-		500	
Q00548.2	432	Q-AGFSGRHCDDNVDDCAS	SPCANGGTCDRGVND--FSCTCPPGYTGRNCsAPVS--RC	EHAPCHNGATCHE-	498
Q9NYJ7.1	380	R-AGFAGPRCEHLDLDCAG	RACANGGTCEVGGGA--HRCsCALGFGRDCRERAD--PC	AARPCAHGGRCYAh	447
Q9NR61.1	429	R-PGFTGTYCELVSDCAR	NPCAHHGGTCHDLENG--LMCTCPAGFSGRRCeVRTSIdAC	ASSPCFNrATCYTd	498

Figure 8. Comparison of a portion of the protein tyrosine kinase of *M. vibrans*, the Delta protein of the sponge *O. minuta* and the protein sequences of, three DLL proteins of *H. sapiens*. The top sequence in each group is that of the protein tyrosine kinase, the next is that of the sponge, while the next three sequences are for the DLL proteins of *H. sapiens*.

Finally, the two JAG proteins of the Notch signalling pathway, present also with the DLL proteins on the membrane of the Sender cell (Figure 1), again have strong sequence similarity with the protein tyrosine kinase of the amoeba *M. vibrans*, the BLAST 2 sequence Expect vales being $2E^{-120}$ for JAG1 and $3E^{-122}$ for JAG2, respectively. A comparison between the protein sequences of the JAG proteins of *H. sapiens* and the protein tyrosine kinase of *M. vibrans* is shown in Figure 9:



Figure 9. Comparison of a portion of the protein sequences of the protein tyrosine kinase of *M. vibrans*, and the JAG proteins of *H. sapiens*. The top sequence in each group is that of the protein tyrosine kinase, while the next two sequences are for JAG1 and JAG2 of *H. sapiens*.

A phylogenetic tree of the relationship between the protein tyrosine kinase (PTK) of *M. vibrans* and the JAG and DLL proteins of *H.sapiens* is shown in Figure 10:

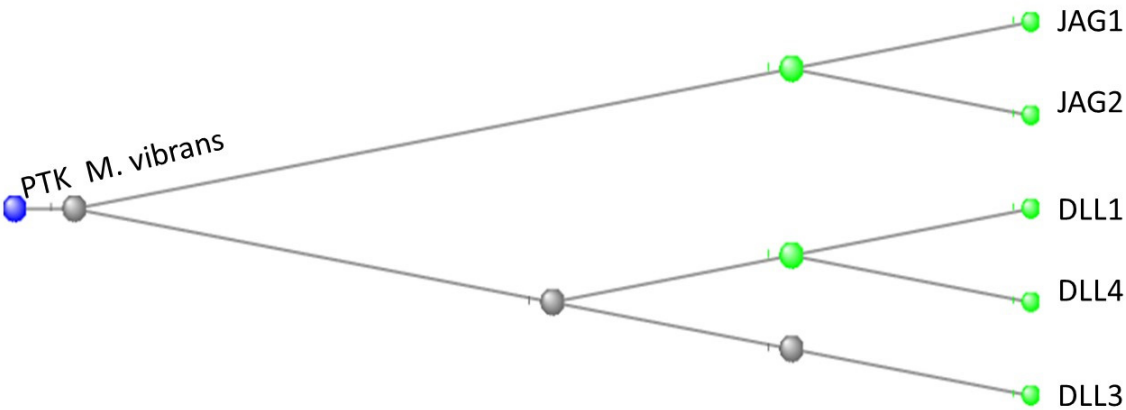


Figure 10. Phylogenetic tree depicting the relationship between the PTK of *M. vibrans* and the JAG and DLL proteins of *H.sapiens*. The phylogenetic tree is rooted at the PTK of *M. vibrans*.

Figure 11 that follows depicts the phylogenetic tree of the DLL and JAG proteins of *H. Sapiens*, the Delta protein of *O. Minuta* and the protein tyrosine kinase of *M. vibrans* (the same input as was

used in Figure 10), after dialling down the maximum sequence difference to 0.7. At a maximum sequence difference of 0.8, all 7 proteins were still present (data not shown).

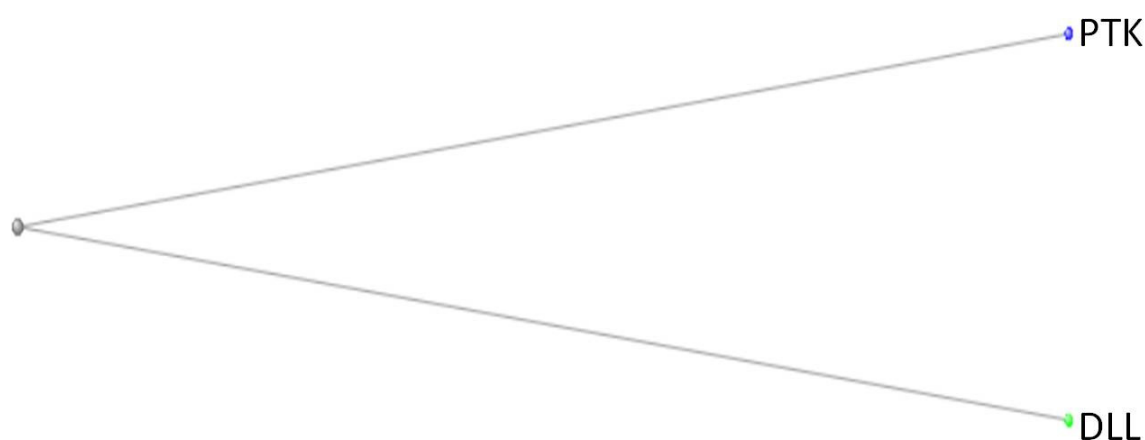


Figure 11. Phylogenetic tree depicting the relationship between the PTK of *M. vibrans* and the JAG and DLL proteins of *H. sapiens* and the DLL protein of *O. minuta*, after dialling down the maximum sequence difference routine of the COBALT program to 0.7. (At a maximum sequence difference of 0.8, all 7 proteins still appeared – data not shown).

This analysis of these sequences by dialling down in the maximum sequence difference routine of the COBALT program, where only the sponge's DLL and the PTL now remained, showed that the amoeba and sponge sequences were more closely related to each other than either was to the sequences of the *H. sapiens* proteins.

All of the sequences depicted in Figures 6 through 9 show a series of six or more repeating cysteine residues, characteristic of proteins of the EGF family. This will be considered further in the DISCUSSION section.

From Figure 5 it can be seen that by the time the Cnidaria appeared, the entire central core of the pathway was in place, with two of the membrane-bound ligands of the Sender cell (DLL1 and JAG1), and the nuclear-active terminal protein of the Receiver cell being present, as well as two NOTCH proteins – the receptors on the Receiver cell. In addition, some proteins serving as activators and repressors of the central core proteins had appeared, members of the co-repressor complex (Figure 5) and the γ -secretase complex, as well as the Fringe proteins (LFNG, MFNG, and RFNG). The further evolution of the Notch signaling pathway from the cephalochordate [14] through to the sharks and rays (the Elasmobranchi) and bony fish [15] was of proteins that contribute further to the control of the central core (listed in Table 1). Notch4, with a role in angiogenesis [2], and PTCRA (Pre T-Cell Antigen Receptor Alpha), which contributes to the regulation of early T-cell development [16] appeared with the amphibia and amniota, respectively. The three NOTCH2NL paralogs appeared during the recent evolution of the human species.

Discussion

In the evolutionary trajectory from the first living organisms to the emergence of the humans, genes were added to the accumulating genome at each Phylostratum Level. At many levels, these included genes that were later to form the Notch signaling system. It is of much interest to ask what was the function of such a “Notch signaling” gene in an animal that long preceded the appearance of the complete Notch system.

Figure 5 above shows the contribution of orthologs of the proteins of the Notch signaling system at each Phylostratum Level for the lower half of the evolutionary trajectory leading to the humans. At the level of the Eukaryota, we see four genes appearing: RBPJ, SNW1, HDAC1 and HDAC2. These four are found in the nucleus of the Receiver cell as can be seen in the scheme of Figure 1. The two HDAC genes are shown in the figure as being in the co-repressor complex while RBPJ is depicted as directly interacting with DNA.

Of these four genes, HDAC genes are expressed in the cell nucleus of the protozoan *Trypanosoma brucei*, where they participate in the expression of the VSG (Variant Surface Glycoprotein) genes that enable the parasite to evade the host's immune response [17]. Indeed, the paper [17] reported on attempts to use inhibitors of HDACs to combat infection by the parasite. For RBPJ, Prevorsev et al [18] found orthologs of RBPJ in several fungal species while Oravcova et al [19] showed that one such expressed protein (named in *Schizosaccharomyces pombe* as Cbf11) could act as a sequence-specific DNA binding activator of transcription. Finally, for SNW1, Halova et al [20] working with the fungus *Saccharomyces cerevisiae* showed that the splicing factor Prp45 (a homolog of SNW1) was expressed in the yeast. It is present in the spliceosome and takes part in co-transcriptional splicing.

Thus, all four genes have been shown to be expressed in fungi or protozoa and suggestions made as to their roles in these organisms. Yet hundreds of millions of years would pass before a complete sender-to-receiver Notch signaling system would appear in the Cnidaria (the sea anemones, corals, and jellyfish).

Figure 5 shows that, meanwhile, thirteen Notch signaling system orthologs were recruited with the appearance of the Porifera, the sponges. Schenkelaars et al [21], noting that the sponges were the first animals to be truly multicellular (the fungi forming mere syncytia), investigated whether the cell-to-cell Notch signaling system might already be fully present in a sponge. Investigating the Notch and Wnt signaling pathways in the glass sponge *O. minuta*, they showed that, while many components of the Notch pathway were already present in the sponge including the eponymous NOTCH, key components were not. The nuclear-active HES1 and HES5 of the Receiver cell were not identified nor were JAG1 or JAG2 of the Sender cell. Schenkelaars et al [21] did find a DLL of the Sender cell, shown as [DLL] in Figure 5 above. The DLL in the sponges are not named as either of DLL1, DLL3, or DLL4 – see above –, although they may play the role in the sponge as a Notch ligand [21]. The extracellular portion of the protein has a strong sequence similarity to that of the human DLL proteins – Figure 6 – and it is this portion of these molecules that bind to the NOTCH proteins. Richards and Degnan [22,23], studying the sponge *Amphimedon queenslandica*, followed the expression of Notch pathway components during the embryological development of this organism with results suggesting that classic roles for the pathway – like mediating the choice between cell fates (here between the path to an anterior pole cell or alternatively, to a flask cell) – are already operative at this stage of evolution.

The Cnidaria have a full Notch pathway, extending from DLL1 and JAG1 that are ligands in the Sender cell (Figure 1) to the nuclear-active HES1 in the Receiver. Pharmacological inhibition of Notch pathway components (inhibiting presenilin by treatment with the PSEN inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) or blocking the intracellular domain of NOTCH itself by treatment with the Notch inhibitor SAHM1 (stapled α -helical peptide derived from MAML) showed in *Hydra* that such treatment disturbed head regeneration and tentacle patterning. These effects demonstrate a functional role for the pathway in the animal's development [24]. Pan et al [25] extended these findings, showing that genetic modification of Notch pathway components (over-expressing the intracellular domain components of NOTCH, or knockdown of NOTCH) recapitulated the pharmacological effects. Note that PSEN1 and PSEN2, inhibited in the experiments of Munder et al [24], were added to the evolving genome already in the Porifera – thus showing that earlier -appearing components of the Notch pathway were integrated into the signalling system by the time that the Cnidaria appeared.

Our exploration of a possible pre-Metazoan origin of the Notch proteins suggested (see Results) a protein tyrosine kinase (PTK) of the amoeboid *Ministeria vibrans* as a possible candidate. To our

surprise, our subsequent explorations of such an origin for the DLL and JAG proteins came up with the same protein tyrosine kinase as possible candidate also for these proteins.

The PTK, DLL, JAG, and Notch proteins show extensive sequence similarity (Figures 6 through 9) and, in particular, a forty-membered sequence containing sets of six-fold repeats of Cysteine residues. This is a feature characteristic of the large family of EGF proteins [26] where these sequences form part of the extracellular ligand-binding domain of these proteins. The 42-long sequence GDKCQTDMECLSEPCCKNGGTCSYVNSYTCKCQAGFDGVHC in NOTCH2 includes the calcium-binding domain of this protein (GeneCards – see Methods).

Suga and his colleagues [27] performed an extensive survey of the PTK proteins of all pre-Metazoan animals. In particular, they analysed the Filasterea clade to which *Ministeria vibrans* belongs. (“Filasterea is a proposed basal Filozoan clade of single-celled amoeboid eukaryotes that includes Ministeria and Capsaspora.” – Wikipedia). The Filasterea are a sister group to the Metazoa and Choanoflagellata and are thus central to a discussion of the evolution of the Metazoa. Suga et al [27] concluded that the “divergence pattern of [PTKs] is consistent with the hypothesis that they act to detect changes in the extracellular environment or to recognize and catch prey, because each organism has to be adapted to its own environment and nutrient conditions”, a statement that provides a plausible answer to the question of what was the role of the PTKs in these pre-Metazoan animals.

The Filasterean PTKs have transmembrane sequences as do other members of the EGF protein family. It has not been shown that in these pre-Metazoan PTKs, dimerization of the transmembrane portions of the molecule plays an important role in their function as tyrosine kinases. This has been found, however, in Metazoan PTKs [28].

It is thus a possible scenario that, as the Metazoans diverged from the common Filasterean/Metazoan precursor, their protein tyrosine kinases evolved into forms that would recognize and bind to one another in the typical ligand/receptor mode. Thus, evolving from a pre-Metazoan protein tyrosine kinase, the DLL and JAG proteins became ligands on the extracellular surface of a Sender cell while the Notch proteins became the extracellular receptors on the Receiver cell.

Conclusions

Cell-to-cell communication is the ineluctable modality of multicellular animals. The Notch signaling system is a prime example of such an actor in cell-to-cell communication, with, as we have described, its Sender cell communicating instructions to the Receiver cell that determine the Receiver's fate. It is not surprising, therefore, that the Notch system reached a fully working configuration with the evolutionary appearance of the Metazoa, the first multicellular animals. Yet this configuration, as the present paper documents, includes many proteins whose appearance in evolution long preceded the appearance of the Metazoa. At every evolutionary level at which these components first appeared in an animal, they play a discernible role in the life processes of the contemporary descendants of that ancestral animal. But these roles are not concerned with cell-to-cell communication, and could not have been so until multicellular animals themselves appeared. Such a linking together of pre-existing components into a workable system is a typical case of the co-option that is so often found in evolution, “the repurposing of genes [that are] present in ancestral unicellular organisms owing to the changed scale of the multicellular state” as Newman describes it in his cogent discussion of the major transitions in animal evolution [29].

References

1. Jin, M.; Zhang, H.; Xu, B.; Li, Y.; Qin, H.; Yu, S.; He, J. Jag2b-Notch3/1b-mediated Neuron-to-glia Crosstalk Controls Retinal Gliogenesis. *EMBO Rep.* **2022**, *23*, e54922, doi:10.15252/embr.202254922.
2. Eng, J.W.-L.; Kato, Y.; Adachi, Y.; Swaminathan, B.; Naiche, L.A.; Vadakath, R.; Sakamoto, Y.; Nakazawa, Y.; Tachino, S.; Ito, K.; et al. Inhibition of Notch4 Using Novel Neutralizing Antibodies Reduces Tumor Growth in Murine Cancer Models by Targeting the Tumor Endothelium. *Cancer Res. Commun.* **2024**, *4*, 1881–1893, doi:10.1158/2767-9764.crc-24-0081.

3. MacGrogan, D.; Münch, J.; Pompa, J.L. de la Notch and Interacting Signalling Pathways in Cardiac Development, Disease, and Regeneration. *Nat. Rev. Cardiol.* **2018**, *15*, 685–704, doi:10.1038/s41569-018-0100-2.
4. Kuriyama, K.; Kodama, Y.; Shiokawa, M.; Nishikawa, Y.; Marui, S.; Kuwada, T.; Sogabe, Y.; Kakiuchi, N.; Tomono, T.; Matsumori, T.; et al. Essential Role of Notch/Hes1 Signaling in Postnatal Pancreatic Exocrine Development. *J. Gastroenterol.* **2021**, *56*, 673–687, doi:10.1007/s00535-021-01779-y.
5. Demitrack, E.S.; Samuelson, L.C. Notch Regulation of Gastrointestinal Stem Cells. *J. Physiol.* **2016**, *594*, 4791–4803, doi:10.1113/jp271667.
6. Chen, W.; Wei, W.; Yu, L.; Ye, Z.; Huang, F.; Zhang, L.; Hu, S.; Cai, C. Mammary Development and Breast Cancer: A Notch Perspective. *J. Mammary Gland Biol. Neoplasia* **2021**, *26*, 309–320, doi:10.1007/s10911-021-09496-1.
7. Kiyokawa, H.; Morimoto, M. Notch Signaling in the Mammalian Respiratory System, Specifically the Trachea and Lungs, in Development, Homeostasis, Regeneration, and Disease. *Dev., Growth Differ.* **2020**, *62*, 67–79, doi:10.1111/dgd.12628.
8. Ehebauer, M.; Hayward, P.; Arias, A.M. Notch, a Universal Arbiter of Cell Fate Decisions. *Science* **2006**, *314*, 1414–1415, doi:10.1126/science.1134042.
9. Gazave, E.; Lapébie, P.; Richards, G.S.; Brunet, F.; Ereskovsky, A.V.; Degnan, B.M.; Borchellini, C.; Vervoort, M.; Renard, E. Origin and Evolution of the Notch Signalling Pathway: An Overview from Eukaryotic Genomes. *BMC Evol. Biol.* **2009**, *9*, 249, doi:10.1186/1471-2148-9-249.
10. Lv, Y.; Pang, X.; Cao, Z.; Song, C.; Liu, B.; Wu, W.; Pang, Q. Evolution and Function of the Notch Signaling Pathway: An Invertebrate Perspective. *Int. J. Mol. Sci.* **2024**, *25*, 3322, doi:10.3390/ijms25063322.
11. Babonis, L.S.; Martindale, M.Q. Phylogenetic Evidence for the Modular Evolution of Metazoan Signalling Pathways. *Philos. Trans. R. Soc. B: Biol. Sci.* **2017**, *372*, 20150477, doi:10.1098/rstb.2015.0477.
12. Domazet-Lošo, T.; Tautz, D. Phylostratigraphic Tracking of Cancer Genes Suggests a Link to the Emergence of Multicellularity in Metazoa. *BMC Biol.* **2010**, *8*, 66, doi:10.1186/1741-7007-8-66.
13. Fiddes, I.T.; Lodewijk, G.A.; Mooring, M.; Bosworth, C.M.; Ewing, A.D.; Mantalas, G.L.; Novak, A.M.; Bout, A. van den; Bishara, A.; Rosenkrantz, J.L.; et al. Human-Specific NOTCH2NL Genes Affect Notch Signaling and Cortical Neurogenesis. *Cell* **2018**, *173*, 1356–1369.e22, doi:10.1016/j.cell.2018.03.051.
14. Bertrand, S.; Petillon, Y.L.; Somorjai, I.M.L.; Escrive, H. Developmental Cell-Cell Communication Pathways in the Cephalochordate Amphioxus: Actors and Functions. *Int. J. Dev. Biol.* **2017**, *61*, 697–722, doi:10.1387/ijdb.170202sb.
15. Sánchez-Iranzo, H.; Halavatyi, A.; Diz-Muñoz, A. Strength of Interactions in the Notch Gene Regulatory Network Determines Patterning and Fate in the Notochord. *eLife* **2022**, *11*, e75429, doi:10.7554/elife.75429.
16. Materna, M.; Delmonte, O.M.; Bosticardo, M.; Momenilandi, M.; Conrey, P.E.; Muylder, B.C.-D.; Bravetti, C.; Bellworthy, R.; Cederholm, A.; Staels, F.; et al. The Immunopathological Landscape of Human Pre-TCR α Deficiency: From Rare to Common Variants. *Science* **2024**, *383*, eadh4059–eadh4059, doi:10.1126/science.adh4059.
17. Zuma, A.A.; Souza, W. de Histone Deacetylases as Targets for Antitrypanosomal Drugs. *Futur. Sci. OA* **2018**, *4*, FSO325, doi:10.4155/fsoa-2018-0037.
18. Převorovský, M.; Půta, F.; Folk, P. Fungal CSL Transcription Factors. *BMC Genom.* **2007**, *8*, 233, doi:10.1186/1471-2164-8-233.
19. Oravcová, M.; Teska, M.; Půta, F.; Folk, P.; Převorovský, M. Fission Yeast CSL Proteins Function as Transcription Factors. *PLoS ONE* **2013**, *8*, e59435, doi:10.1371/journal.pone.0059435.
20. Hálová, M.; Gahura, O.; Převorovský, M.; Cit, Z.; Novotný, M.; Valentová, A.; Abrhánová, K.; Půta, F.; Folk, P. Nineteen Complex-Related Factor Prp45 Is Required for the Early Stages of Cotranscriptional Spliceosome Assembly. *RNA* **2017**, *23*, 1512–1524, doi:10.1261/rna.061986.117.
21. Schenkelaars, Q.; Pratlong, M.; Kodjabachian, L.; Fierro-Constain, L.; Vacelet, J.; Bivic, A.L.; Renard, E.; Borchellini, C. Animal Multicellularity and Polarity without Wnt Signaling. *Sci. Rep.* **2017**, *7*, 15383, doi:10.1038/s41598-017-15557-5.
22. Richards, G.S.; Degnan, B.M. The Dawn of Developmental Signaling in the Metazoa. *Cold Spring Harb. Symp. Quant. Biol.* **2009**, *74*, 81–90, doi:10.1101/sqb.2009.74.028.
23. Richards, G.S.; Degnan, B.M. The Expression of Delta Ligands in the Sponge Amphimedon queenslandica Suggests an Ancient Role for Notch Signaling in Metazoan Development. *EvoDevo* **2012**, *3*, 15, doi:10.1186/2041-9139-3-15.
24. Münder, S.; Tischer, S.; Grundhuber, M.; Büchels, N.; Bruckmeier, N.; Eckert, S.; Seefeldt, C.A.; Prexl, A.; Käsbaier, T.; Böttger, A. Notch-Signalling Is Required for Head Regeneration and Tentacle Patterning in Hydra. *Dev. Biol.* **2013**, *383*, 146–157, doi:10.1016/j.ydbio.2013.08.022.
25. Pan, Q.; Mercker, M.; Klimovich, A.; Wittlieb, J.; Marciniak-Czochra, A.; Böttger, A. Genetic Interference with HvNotch Provides New Insights into the Role of the Notch-Signalling Pathway for Developmental Pattern Formation in Hydra. *Sci. Rep.* **2024**, *14*, 8553, doi:10.1038/s41598-024-58837-7.

26. Appella, E.; Weber, I.T.; Blasi, F. Structure and Function of Epidermal Growth Factor-like Regions in Proteins. *FEBS Lett.* **1988**, *231*, 1–4, doi:10.1016/0014-5793(88)80690-2.
27. Suga, H.; Dacre, M.; Mendoza, A. de; Shalchian-Tabrizi, K.; Manning, G.; Ruiz-Trillo, I. Genomic Survey of Premetazoans Shows Deep Conservation of Cytoplasmic Tyrosine Kinases and Multiple Radiations of Receptor Tyrosine Kinases. *Sci. Signal.* **2012**, *5*, ra35, doi:10.1126/scisignal.2002733.
28. Li E, Hristova K. Receptor tyrosine kinase transmembrane domains: Function, dimer structure and dimerization energetics. *Cell Adh Migr.* 2010, 249-54. doi: 10.4161/cam.4.2.10725.
29. Newman, S.A. 'Biogeneric' Developmental Processes: Drivers of Major Transitions in Animal Evolution. *Philos. Trans. R. Soc. B: Biol. Sci.* **2016**, *371*, 20150443, doi:10.1098/rstb.2015.0443.

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