Review

Decoding the PTM-switchboard of Notch

Daniel Antfolk\textsuperscript{a}, Christian Antil\textsuperscript{a}, Kati Kemppainen\textsuperscript{a}, Sebastian K.-J. Landor\textsuperscript{a,⁎}, Cecilia Sahlgren\textsuperscript{a,b,⁎}

\textsuperscript{a} Faculty of Science and Engineering, Cell Biology, Åbo Akademi University, Turku, Finland
\textsuperscript{b} Department of Biomedical Engineering, Institute for Complex Molecular Systems, Eindhoven University of Technology, Eindhoven, the Netherlands

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\section*{ABSTRACT}

The developmentally indispensable Notch pathway exhibits a high grade of pleiotropism in its biological output. Emerging evidence supports the notion of post-translational modifications (PTMs) as a modus operandi controlling dynamic fine-tuning of Notch activity. Although, the intricacy of Notch post-translational regulation, as well as how these modifications lead to multiples of divergent Notch phenotypes is still largely unknown, numerous studies show a correlation between the site of modification and the output. These include glycosylation of the extracellular domain of Notch modulating ligand binding, and phosphorylation of the PEST domain controlling half-life of the intracellular domain of Notch. Furthermore, several reports show that multiple PTMs can act in concert, or compete for the same sites to drive opposite outputs. However, further investigation of the complex PTM crosstalk is required for a complete understanding of the PTM-mediated Notch switchboard. In this review, we aim to provide a consistent and up-to-date summary of the currently known PTMs acting on the Notch signaling pathway, their functions in different contexts, as well as explore their implications in physiology and disease. Furthermore, we give an overview of the present state of PTM research methodology, and allude to a future with PTM-targeted Notch therapeutics.

\section*{1. Introduction}

The Notch signaling pathway is an evolutionarily conserved cell-cell contact dependent signaling mediator with pivotal roles in development, including orchestration of complex tissue patterning, and determination of cell fate. A Notch signal is initiated when transmembrane ligands and receptors on signal-sending and signal-receiving cells interact, leading to a chain of sequential cleavages of the receptors (reviewed in [1]). Initial pull of the ligand-receptor pair opens up the S2 cleavage site for ADAM metalloproteases (ADAM-10 and ADAM-17), yielding the truncated membrane-bound form of Notch called Notch extracellular truncation (NEXT). This in turn reveals the S3 site to be cleaved by γ-secretase, which ultimately leads to the release of the Notch intracellular domain (NICD) fragment. NICD can then translocate to the nucleus where it binds the Notch transcriptional regulator CSL (CBF1, Suppressor of Hairless, Lag-1), also known as RBPJ (Recombination signal binding protein for immunoglobulin kappa J region), which together with cofactor MAML forms a trimeric activation complex to initiate Notch downstream target gene expression.

Despite its deceivingly simple linear stoichiometry with one transcriptional regulator (CSL), the Notch pathway exhibits an immense pleiotropism, demonstrated by its vast involvement in different developmental settings as well as its involvement in all of the hallmarks of cancer (reviewed in [2]). The multifaceted character of Notch is partially explained by the many paralogs of Notch receptors and ligands that are expressed in the metazoan organisms as well as by differential binding of the receptors intracellular domains to the transcriptional regulator CSL. In metazoans, the Notch pathway consists of four receptors (Notch1–4) and five ligands (Jagged1, 2 and Dll1, 3, and 4) with paralog-specific outputs, and due to the context-dependent character in the developmental cell, Notch mutations and deregulations are often implicated in different cancers (reviewed in [295]).

An additional layer of complexity comes from numerous reports of post-translational modifications (PTMs) of Notch further broadening the seemingly simple core signaling pathway [3]. PTMs are a set of reversible modifications of proteins that modulate their activity, localization and stability in a cell. The PTMs include both enzymatic and non-enzymatic additions of different groups to target protein amino acid side chains. These PTMs are intrinsic modifiers of protein function and signaling output, with current predictions indicating that as much as 5% of our proteome are enzymes that perform these modifications [287].
Table 1

| PTM | Protein Function | Discovered sites | Corresponding sites in human | Effect on Notch signaling | Implicated in cancer/disease |
|-----|------------------|------------------|-----------------------------|--------------------------|----------------------------|
| Acetylation | PCAF/KAT2B (Histone) acetyltransferase | Mouse: K1764, K1770, K1771, K1785, K1935, K2050, K2068, K2146, K2147, K2150, K2154, K2161, K2164 | Human: K1774, K1780, K1781, K1782, K1795, R2060, K2078, K2156, K2157, K2160, K2164, K2171, K2174 | Stabilizes the N1-ICD by inhibiting its ubiquitination | NOTCH1 [88], [90] |
| | p300 (Histone) acetyltransferase | Mouse: K1692, K1731 | Human: K1691, K1730 | De-stabilizes the N3-ICD by promoting its ubiquitination | T-cell acute lymphoblastic leukemia (T-ALL), urothelial cancer |
| | | | | | NOTCH3 [89] |
| | | | | | Promotes Notch transcription complex formation and target gene induction by recruiting co-activator NACK |
| | GCN5/KAT2A (Histone) acetyltransferase | Mouse: K1764, K1770, K1771, K1785 | Human: K188, K189 | Promotes Notch signaling |
| | SIRT1 (Histone) deacetylase | Mouse: K1764, K1770 | Human: K188, K189 | De-stabilizes the N1-ICD by promoting its ubiquitination | NOTCH1 [88], [90] |
| | HDAC1 (Histone) deacetylase | Mouse: K1692, K1731 | Human: K1691, K1730 | Stabilizes the N3-ICD by inhibiting its ubiquitination | NOTCH3 [89] |
| | | | | | Brain tumor, breast cancer, liver cancer, oral squamous cell carcinoma, colorectal cancer, Dowling-Degos disease |
| | | | | | NOTCH1 [9,11,24,38,5–7,64,72,159,2–220,288] |
| | | | | | NOTCH2 (continued on next page) |
| PTM          | Protein Function | Discovered sites | Corresponding sites in human | Effect on Notch signaling | Implicated in cancer/ disease | Notch pathway component | Reference(s) |
|--------------|------------------|------------------|------------------------------|---------------------------|-------------------------------|------------------------|--------------|
| **POFUT1**   | O- fucosyltransferase | Mouse (consensus seq.): EGF1-6, 8, 9, 12, 18, 20, 21, 24-28, 30, 31, 34, 36 | S458, T466, T617, T692, S759, T767, S784, S797, T805, T900, S921, T997, T1035, T1159, T1197, T1362, T1379, T1402 | NOTCH3 | | |
| **POFUT1**   | O- fucosyltransferase | Mouse (consensus seq.): EGF4-8, 11, 17, 19, 21, 22, 24, 28-30, 34 | | | | | |
| **POGLUT1**  | O- glucosyltransferase | Mouse (consensus seq.): EGF2, 4, 10, 12-14, 16, 17, 19-21, 25, 27, 28, 31, 33 | | | Myeloid leukemia, T-ALL, Dowling-Degos disease | NOTCH1 | [9,10,15,22-24,63,68,72,28-9] |
| **POGLUT1**  | O- glucosyltransferase | Mouse (consensus seq.): EGF4, 10, 12-14, 16-21, 23, 25, 27, 30, 31, 36 | | | | | |
| **POGLUT1**  | O- glucosyltransferase | Mouse (consensus seq.): EGF1, 2, 5-9, 11, 12, 15, 18-21, 24, 25, 27, 29 | | | | | |
| **POGLUT1**  | O- glucosyltransferase | Mouse (consensus seq.): EGF10, 12-15, 18, 19, 20, 25, 27 | | | | | |
| **POGLUT2**  | O- glucosyltransferase | Mouse: Notch1: S435 on EGF1 Mouse: Notch3: S414 on EGF10 | Same in humans | O-glucose at NOTCH1 S435 in the middle of binding interface with DLL4. Potential regulation of folding and quality control of Notch as well as Notch-ligand interactions in combination with EGF8 or EGF12 | ND | NOTCH1 NOTCH3 | [30,31] |
| **POGLUT3**  | O- glucosyltransferase | Mouse: Notch1: S435 on EGF1 Mouse: Notch3: S414 on EGF10 | | | | | |
| **FRINGE: lunatic, manic, radical** | O-fucosylated EGF-repeats of Notch | | | | | | |
| **FRINGE: lunatic, manic, radical** | β1,3 N-acetylglucosaminyltransferase | | | | | | |
| **FRINGE: lunatic, manic, radical** | O-fucosylated EGF-repeats of Notch | | | | | | |
| **FRINGE: lunatic, manic, radical** | Glucosidase xylosyltransferase | | | | | | |
| **FRINGE: lunatic, manic, radical** | O-glucosyl EGF-repeats of Notch | | | | | | |
| **FRINGE: lunatic, manic, radical** | Xylosidase xylosyltransferase | | | | | | |
| **FRINGE: lunatic, manic, radical** | Xylosidase xylosyltransferase | | | | | | |

(continued on next page)
| PTM            | Protein Function | Discovered sites | Corresponding sites in human | Effect on Notch signaling | Implicated in cancer/disease | Notch pathway component | Reference(s) |
|----------------|------------------|------------------|-----------------------------|--------------------------|-----------------------------|-------------------------|---------------|
| EOGT1          | EGF-domain specific O-linked N-acetylglucosamine transferase | Mouse (consensus seq.): EGF2, 3, 7, 8, 9, 10, 11, 14, 15, 17, 19, 20, 21, 23, 26, 27, 28, 29, 35 | S534, S609, S647, S722, S759, S797, S951, S1027, S1065, S1189, S1273 | Potentiates Dll1 and Dll4-mediated Notch interactions. Important for vascular development. EGF2, 10, 17 and 20 in mouse suggested important for DLL1/4-NOTCH1 interactions | Adams-Oliver syndrome | NOTCH1 | [68–72, 77, 79, 80, 222] |
| OST            | Oligosaccharyltransferase (N-glycan) | Human (consensus seq.): N41 (EGF1), N959 (EGF26), N1179 (EGF30), N1241 (EGF32), N1489 (LNR1), N1587 (12 aa C-terminal of LNR3) | Same in humans | ND | ND | NOTCH1 | [9, 12] |
| Hydroxylation  | FIH              | Asparaginyl hydroxylase | Mouse: N1945, N2012 | Human: N1955, N2022 | NA mutants of the hydroxylated asparagines inhibit N1-ICD transcriptional activity | ND | NOTCH1 | [82, 84] |
|                | FIH              | Asparaginyl hydroxylase | Mouse: N1902, N1969 | Human: N1904, N1971 | ND | ND | NOTCH2 | [83] |
|                | FIH              | Asparaginyl hydroxylase | Mouse: N1867, N1934 | Human: N1866, N1933 | ND | ND | NOTCH3 | [83] |
| Methylation    | CARM1/PRMT4      | Methyltransferase | Mouse: R2253, R2362, R2303, R2317, R2361 | Human: R2263, R2272, R2313, R2327, R2372 | Drives punctuated Notch-response and ubiquitin-mediated proteasomal degradation of N1-ICD | ND | NOTCH1 | [105] |
| ND             | Methyltransferase | Human: R2174 | Human: R2174 | ND | Detected in colon cancer cells | ND | NOTCH3 | [106] |
| ND             | Methyltransferase | Mouse: R1850 | Human: 1871 | ND | ND | NOTCH1 | [106] |
| Phosphorylation | AKT              | Ser/Thr kinase   | ND | ND | Downregulate Notch nuclear localization and transcription | ND | NOTCH1 | [118] |
|                | AKT              | Ser/Thr kinase   | Human NOTCH4: S1495, S1847, S1865, S1917 | Human NOTCH4: S1495, S1847, S1865, S1917 | Serve as binding site for 14–3-3 which sequesters N4-ICD in the cytoplasm | ND | NOTCH4 | [119] |
|                | CDK8             | Ser/Thr kinase   | Xenopus laevis: S2482, S2485, S2507 | Human: S2513, S2516, S2538 | Enhances FBXW7/SEL-10 dependent N1-ICD polyubiquitination and degradation. | ND | NOTCH1 | [110] |
| Cyclin C — CDK1 | Ser/Thr kinase   | Human in vitro: T1461, T1963, S2183, T2511, S2513, S2516, S2524 | Human in vitro: T1461, T1963, S2183, T2511, S2513, S2516, S2524 | Enhances FBXW7 dependent N1-ICD polyubiquitination and degradation. | T cell-acute lymphoblastic leukemia | NOTCH1 | [111, 112, 290] |
| CK2            | Ser/Thr kinase   | Human in vitro: T1461, T1486, T1963, S2183, T2511, S2513, S2516, S2524 | Human in vitro: T1461, T1963, S2183, T2511, S2513, S2516, S2524 | Enhances FBXW7 dependent N1-ICD polyubiquitination and degradation. | ND | NOTCH1 | [113, 276] |

(continued on next page)
| PTM         | Protein Function | Discovered sites | Corresponding sites in human | Effect on Notch signaling | Implicated in cancer/disease | Notch pathway component | Reference(s) |
|------------|------------------|------------------|-----------------------------|--------------------------|-----------------------------|--------------------------|--------------|
| DYRK1A     | Ser/Thr kinase   | Mouse: T1887, S1890 Human: S1900, T1897 | ND                          | Decreases binding of N1-ICD to Notch-Mastermind-CSL ternary complex. Reduces capacity to maintain transcription | ND                          | NOTCH1       | [126]        |
| G-CSF      | Ser/Thr kinase   | Human: 2078 Notch1 Mouse: T1851, T2122, T2124 Notch2 Mouse: T2069, S2071, T2075, S2094 Notch2 Human: T2068, S2070, T2074, S2093 | Human: 2078 Notch1 Mouse: T1861, T2132, T2134 Notch2 Human: T2064, S2070, T2074, S2093 | Protects from proteasomal degradation and/or negatively regulates NOTCH1/N1-ICD. Inhibits transcriptional activity of NOTCH2. | ND                          | NOTCH1       | [125]        |
| GSK3β      | Ser/Thr kinase   | Mouse: T1851, T2122, T2124 Notch2 Human: T2069, S2071, T2075, S2094 Notch2 Human: T2068, S2070, T2074, S2093 | Human: 2078 Notch1 Mouse: T1861, T2132, T2134 Notch2 Human: T2064, S2070, T2074, S2093 | Protects from proteasomal degradation and/or negatively regulates NOTCH1/N1-ICD. Inhibits transcriptional activity of NOTCH2. | ND                          | NOTCH1/2      | [121–123]    |
| ILK        | Ser/Thr kinase   | Mouse: S2173 | Human: S2183 | Enhances FBXW7 dependent N1-ICD polyubiquitination and degradation. | ND                          | NOTCH1       | [114]        |
| NLK        | Ser/Thr kinase   | Xenopus laevis: S2118, S2132, S2137, S2194, S2211, S2217, S2222 | Human: S2121, S2136, S2142, S2198, S2215, S2221, S2226 | Inhibits formation of transcriptionally active ternary complex. | ND                          | NOTCH1       | [116]        |
| PIM        | Ser/Thr kinase   | Mouse: S2152 | Human: S2162 | Enhances N1-ICD nuclear localization and transactivation. Regulates NOTCH1 trafficking | Breast cancer, prostate cancer | NOTCH1       | [117]        |
| PKCζ       | Ser/Thr kinase   | Mouse: S1791 Human: S1801 | Human: Y2074, Y2116, Y2145, Y1938 | Inhibits MAML binding, decreases N1-ICD half-life | ND                          | NOTCH1       | [115]        |
| SRC        | Tyrosine kinase  | Human: Y2074, Y2116, Y2145, Y1938 | Human: Y2074, Y2116, Y2145, Y1938 | Inhibits MAML binding, decreases N1-ICD half-life | ND                          | NOTCH1       | [115]        |
| Unknown    | Tyrosine kinase  | Drosophila: Y1850, Y1860, Y2097 Mouse: Y2064 | Human: Y2074 | Mediates non-canonical Notch/Abl signaling | ND                          | dNotch, NOTCH1 | [127,223]    |
| EYA1       | Threonine       | Mouse: Notch1: T2122 | Human: T2132 | Increases stability of N1-ICD | ND                          | NOTCH1       | [124]        |
| ND         |                  | Mouse: S1781, T1851 Human: S1791, T1861 | Human: S1791, T1861 | Human: S1791, T1861 | ND                          | NOTCH1       | [224–226]    |
| ND         |                  | Mouse: T1718, S1724, S1780, T1803, S1805, T1809, T1811, S1837, S1842, S1843, S1846, T2069, S2071, T2075, S2079, S2082, S2091, S2094, T2098, S2116, T2298, Y2342, T2387, S2390, Y2472 | Human: S1780, T1716, S1722, S1778, T1802, S1804, T1805, S1836, S1841, S1842, S1845, S1854, S1855, T1860, S1864, S1868, T1873, T1876, S2070, S2078, S2081, S2090, T2097, S2115, T2296, Y2340, T2385, S2388, Y2470 | Human: S1780, T1716, S1722, S1778, T1802, S1804, T1805, S1836, S1841, S1842, S1845, S1854, S1855, T1860, S1864, S1868, T1873, T1876, S2070, S2078, S2081, S2090, T2097, S2115, T2296, Y2340, T2385, S2388, Y2470 | ND                          | NOTCH2       | [135,136,223,227,229-241] |
| PTM         | Protein Function                  | Discovered sites                      | Corresponding sites in human | Effect on Notch signaling | Implicated in cancer/disease | Notch pathway component | Reference(s) |
|-------------|-----------------------------------|---------------------------------------|------------------------------|---------------------------|------------------------------|-------------------------|--------------|
| MAPK p38    | Ser/Thr kinase                    | Human: T339                           | Human: T339                  | Required for full ligand activity | ND                          | CSL                     | [172]        |
| MAPK        | Ser/Thr kinase                    | Mouse in vitro: T638, S693, S696       | Human: T339                  | Induces Ubiquitination and degradation of CSL/RBPJx | ND                          | CSL                     | [173]        |
| Unknown     | Ser/Thr kinase                    | Mouse in vivo: T426                    | Human: T352                  | May slow down activator complex formation | ND                          | CSL                     | [174]        |
| Sumoylation | ND                                | E3 ligase                             | Human: K1780                 | Represses expression of Notch target genes | ND                          | NOTCH1                  | [130]        |
| Ubiquitination | Mindbomb 1,2                | E3 ligase                            | ND                           | Required for Notch activation, DLL internalization, MB1 KO mice are not viable, Mib2 is dispensable for mouse development, but can compensate for loss of Mib1 in zebrafish | ND                          | DLL1,2,3,4 JAGGED1,2 | [169–171,242–291] |
| Skeletrophin/ Mib2 | E3 ligase                  | Drosophila: K742                      | ND                           | JAGGED2 internalization | ND                          | JAGGED2                | [243]        |
| Neuralized 1,2 | E3 ligase                  | Drosophila: K742                      | ND                           | Required for Notch activation in Drosophila/Near 1:Neur2 double knock out mice are viable. Regulates DLL1 trafficking Promotes ligand-independent Notch receptor endocytic trafficking and activation in Drosophila. NOTCH1 proteasome degradation | ND                          | dDelta, JAGGED1, DLL1 | [244–249]   |
| Deltex 1–4  | E3 ligase                        | ND                                    | ND                           | Notch loss of function wing phenotypes in Drosophila, Promotes Notch receptor endocytosis before cleavage by γ-secretase in mammals Mediates lysosomal degradation of non-activated Notch receptors and NOTCH1 proteasomal degradation | ND                          | dNotch, NOTCH1         | [250–252,292] |
| Nedd4       | E3 ligase                        | PPSY motif                            | ND                           | Mediates lysosomal degradation of non-activated Notch receptors and NOTCH1 proteasomal degradation | ND                          | NOTCH1                  | [140,141,256] |
| Suppress of Deltex /ITCH/ AIP4 | E3 ligase                 | ND                                    | ND                           | T cell-acute lymphoblastic leukemia | ND                          | NOTCH1                  | [146–148,151–257] |
| FBXW7/SEL-10/ Archipelago | E3 ligase              | Mouse: Phosphodegron at T2486 Human: Phosphodegron at T2511 | ND                           | Induces Ubiquitination and degradation of N1- and N4-ICD, Notch proteasomal degradation. NOTCH1 ligand independent lysosomal degradation Increases NUMB degradation and thereby Notch activity, NOTCH1 stability. | ND                          | NOTCH1, NOTCH4       | [154,155,259] |
| Cbl         | E3 ligase                        | ND                                    | ND                           | NOTCH1 ligand independent lysosomal degradation | ND                          | NOTCH1                  | [258]        |
| Mdm2        | E3 ligase                        | ND                                    | ND                           | Increases NUMB degradation and thereby Notch activity, NOTCH1 stability. | ND                          | Numb, NOTCH1, NOTCH4 | [154,155,259] |
| USP12       | Deubiquitinating enzyme (DUB)    | ND                                    | ND                           | Promotes Notch degradation | ND                          | NOTCH1                  | [145]        |
| eIF3f       | DUB                              | ND                                    | ND                           | Promotes Notch activation | ND                          | NOTCH1                  | [260]        |

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Research into PTMs of Notch is a developing field of study with the potential to shed new light on the pathway’s still poorly understood multitude of context-dependent phenotypic effects. Advanced developments in mass spectrometry (MS) with high sensitivity, now allows for global profiling of the proteome. In recent years analysis of PTMs have generated hope in therapy with the identification of several PTM-linked biomarkers for different diseases, including several types of cancer [5,6]. In this review we aim to summarize PTMs of the Notch pathway and present clues to the wide implications of these on the status and function of the Notch as well as the potential therapeutic targetability of the pathway. Indeed, much of the still unexplained Notch pleiotropism is believed to lie in the PTM-mediated spatio-temporal control of Notch signaling. However, what impact each modification has on the output of Notch signaling in different cell contexts and epigenetic states, and whether it is to fine-tune signal strength or to mediate larger signaling crosstalk is still largely unknown and still being unraveled.

2. PTMs of the Notch receptors

Notch receptors are single-pass transmembrane proteins expressed at the plasma membrane. Synthesis of the monomeric Notch-receptors takes place in the endoplasmic reticulum (ER) followed by cleavage in the trans-golgi at S1 [7]. The two components subsequently form a non-covalent heterodimer representing the active Notch-receptor, which is then transported to the cell surface. The Notch extracellular domain (NECD) is modified by glycans (or sugar residues) [8,9] and the intracellular domain is modified by a multitude of other PTMs [3]. In the last few decades many individual sites have been identified as potential targets of PTMs as shown in the list of identified sites in Table 1. Nevertheless, the various PTM sites of Notch do not modulate the signaling output in a vacuum. Instead, the different modifications present on the receptor, work together to determine the final output. In this sense, the different modifications can be seen as a computing unit where the input of all modifications together generates a specific output based on their combinatorial effect (Fig. 1). The PTMs can therefore be seen as modifiers of signaling which convert an activated signal to a specific output. In the following sections we dig deeper into each individual type of PTM and its impact on Notch function.

2.1. Glycosylation of the NECD

During synthesis and processing in the ER, the NECD domain is modified by O-linked glycans [10,11]. These are sugar modifications of hydroxy groups on specific serines or threonines that alter the structure of the Notch receptor and its specificity for different ligands. There are three types of O-glycosylation of EGF repeats of Notch: O-fucosylation, O-glucosylation and O-GlcNAcylation [12]. In addition, an O-GalNAc glycan modification has been shown to modify Notch outside of the EGF-repeats near the S2 cleavage site [13].

In mammals the Notch receptors consists of up to 36 EGF-like repeats in their extracellular domain. The EGF repeats are approximately 40 amino acid long sequences, including six conserved cystein residues, which form three disulfide bridges [1]. The O-linked glycans can be added at specific consensus sequences between these cysteins. O-glucosylation by PGLUT1 can occur between the first and the second cysteine residues, O-fucosylation by POFUT1 can occur between the second and the third cysteines and O-GlcNAcylation by EOGT between the fifth and sixth conserved cysteine residues [12]. The Notch receptors contain the most O-fucose and O-glucose consensus sites, in a comparison to other proteins with EGF-like repeats [14,15]. The O-linked glycans can be further modified by other sugars, notably: O-fucose by GlcNAc (Fringe enzymes) [8,16,17], and O-glucose by xyloses (xylosyltransferases) [18,26].

The role of glycosylation has been studied with detail in Drosophila melanogaster, partly because of the simplicity of having only one Notch...
residues to form a glucose-xylose disaccharide or a glucose-xylose-xylose trisaccharide can further elongate the O-glucose on Notch EGF-repeats. The first xylose is added by Glucoside xylosyltransferases, which in mammals are catalyzed by GXYLT1 and GXYLT2. The second xylose can be added by a xylosyltransferase termed XXYLTI in mammals [18,26]. Rana et al. showed that all glucosylated EGF-repeats could form the O-glucose trisaccharide, consisting of Glc-Xyl-Xyl. The trisaccharide was formed at high stoichiometries and was the most common form of O-glucose modification in their samples in all cases except on EGF27 [15]. It has been reported that xylose extensions of Drosophila melanogaster Notch glycans inhibit Notch signaling, although xylose modifications in flies are limited to only a few EGF repeats [27]. Downregulation of the Drosophila melanogaster glucoside xylosyltransferase was shown to increase Notch activity while overexpression of the human GXYLT1 decreased Notch activation in developing fly wings by reducing cell surface expression of Notch [27]. It is therefore thought that xylose modifications could interfere with endocytosis of Notch receptors, but more work still needs to be done in this area. Interestingly, the retaining glycosyltransferase XXYLTI can greatly change the conformation of the EGF repeats of the acceptor substrate and mutations in XXYLTI have been linked to several different cancers, notably squamous cell carcinomas [28]. Matsumoto and colleagues have also recently shown that the dixylose modification of O-glucose can play redundant roles with the single O-fucose modification in Notch trafficking and activation. It was therefore proposed that previous studies focusing on knock out or mutations of individual glycans may have led to misinterpretation or an underestimation of the function of glycans in Notch signaling [29].

Recent structural studies showed that serine 435 at EGF11 of NOTCH1, a previously unidentified O-glucose site, forms interactions with the DSL domain of DLL4 [30]. Interestingly, S435 on EGF11 is not part of a PGLUT1 consensus motif, even though the crystal structure of the modified DLL4-NOTCH1 complex showed that the EGF11-DLL interface is a major point for ligand binding, with O-glucosylated S435 being a key part of the interface [30]. Even more recently work from the Halitschke lab showed that S435 is indeed glucosylated in vitro [31], not by PGLUT1, but by previously identified PGLUT1 homologs named KDELC1 and KDELC2 with previously unknown functions [23]. The identification of these enzymes modifying S435 on EGF11 of NOTCH1 as well as the corresponding site S414 on EGF10 of NOTCH3 led the authors to rename these enzymes, to PGLUT2 and PGLUT3 [31]. Both PGLUT2 and PGLUT3 were able to add an O-glucose modification on S435 of NOTCH1 and corresponding site S414 of NOTCH3 in their in vitro studies, but with different specificity in a way that PGLUT2 slightly prefers EGF10 of Notch3, while PGLUT3 has a strong preference for EGF11 of NOTCH1. A mutation of S435 alone did not have significant impact on Notch activity but mutations of EGF8 and S435 together significantly decreased NOTCH1 at the cell surface while EGF12 and S435 reduced activation by DLL1, showing that the modification at EGF11 is an additional step of fine-tuning for Notch folding and activation [31]. The proposed consensus site based on the new data for PGLUT2/PGLUT3 modification is C1-X-N-G-S-F-X-C4.

2.1.1. O-glucosylation by PGLUT1

The O-glucosylation of Notch receptors occurs at C1-X-S-X-(P/A)-C2 (where X is any amino acid) and is mediated by O-glucosyltransferases called Rumi in Drosophila melanogaster and PGLUT1 in mammals [10,15,22,23] (see Fig. 2).

Mice lacking Poglut1 display embryonic lethality with phenotypes linked to Notch, such as defects in vascular remodeling, cardiogenesis and somitogenesis. Loss of Rumi/PGLUT1 is essential for Notch function in both Drosophila melanogaster and mice, and Poglut1 mutants also show defects in trafficking of the receptor [10,24]. Loss of POGlut1 in mice has also displayed phenotypes that are not linked to Notch activity, such as defects in neural tube development, thereby implying that not all effects of the loss of PGLUT1 are mediated through Notch [10,22]. Furthermore, loss of PGLUT1 in mice also causes the accumulation of the apical transmembrane protein CRUMBS2 (CRB2) in the ER, resulting in defects in gastrulation with earlier embryonic defects than those from Notch [25]. Two xylose

Fig. 1. The multitude of post-translational modifications on the Notch receptor determines the output of the signal.

The effects of many single PTMs are already well known, but the interplay between different modifications is mostly lacking within the field. Some of the main modifications of Notch and their key effects are illustrated above, but to truly understand Notch output, the crosstalk between PTMs and which ratios of different modifications produce certain outcomes.

receptor, two ligands and one Fringe protein [19]. Mammals are more complex with four different Notch receptors (NOTCH1–4) and five ligands (JAGGED1–2, DLL1, 3–4) as well as three mammalian Fringe homologs: Lunatic Fringe (LFNG), Radical Fringe (RFNG) and Manic Fringe (MFNG) [20]. There are also reports of N-linked glycan modifications of Notch in mammals, and experiments in mutant Chinese Hamster Ovary (CHO) cell lines have so far indicated that complex-type N-glycans are not essential for Notch signaling [9,21]. The locations of these N-linked and O-linked glycans can be found in Table 1.

2.1.1. O-glucosylation by PGLUT1

The O-glucosylation of Notch receptors occurs at C1-X-S-X-(P/A)-C2 (where X is any amino acid) and is mediated by O-glucosyltransferases called Rumi in Drosophila melanogaster and PGLUT1 in mammals [10,15,22,23] (see Fig. 2).

Mice lacking Poglut1 display embryonic lethality with phenotypes linked to Notch, such as defects in vascular remodeling, cardiogenesis and somitogenesis. Loss of Rumi/PGLUT1 is essential for Notch function in both Drosophila melanogaster and mice, and Poglut1 mutants also show defects in trafficking of the receptor [10,24]. Loss of POGLUT1 in mice has also displayed phenotypes that are not linked to Notch activity, such as defects in neural tube development, thereby implying that not all effects of the loss of PGLUT1 are mediated through Notch [10,22]. Furthermore, loss of PGLUT1 in mice also causes the accumulation of the apical transmembrane protein CRUMBS2 (CRB2) in the ER, resulting in defects in gastrulation with earlier embryonic defects than those from Notch [25]. Two xylose
target [33]. The effect of Fringes on the fucose residues depend on their catalytic activity, and elimination of the catalytic domain interferes with modification of the Notch receptor [8,16]. In vitro studies have shown that the Fringe modifications alter ligand binding [34–36].

In Drosophila melanogaster Fringe modifies the O-fucose of a Notch receptor to potentiate Delta-mediated signaling while inhibiting the Jagged equivalent Serrate-mediated signaling [8,16,37]. The role of Fringes in mammals is similar, but slightly more complicated. Consistent with their function in Drosophila melanogaster, modification by Fringes in mammals always seems to potentiate Dll signaling and for the most part is reported to inhibit Jagged signaling [16,21]. There are exceptions, however, as Radical fringe has been shown to also increase JAGGED1 signaling to NOTCH1 [36,38,39]. In all cases relating to NOTCH1 receptor studies, both MFNG and LFNG promoted DLL1- NOTCH1 signaling while decreasing JAGGED1-NOTCH1 signaling [36]. In signaling to NOTCH2 on the other hand, LFNG also leads to enhanced JAGGED1 signaling in addition to DLL1 signaling [40]. In a recent comparison of the three Fringes in NOTCH1 signaling assays, Kakuda and Haltiwaenger showed that out of 17 EGF-like repeats which were modified by O-fucose, only a handful were further elongated by Fringes [38]. The different Fringe homologs selectively modified different fucose sites, showing that modifications of fucose sites at EGF 6 and EGF 36 are the ones that inhibit JAGGED1 to Notch signaling. RFNG only modifies three EGF repeats: EGF8, EGF12 and EGF26. The RFNG modification of EGF12 was shown to be the major site that increases Jagged to Notch signaling. All three fringes increase Notch activity from DLL1, and the key EGF repeats seem to be EGF8 and EGF12 with the fucose of EGF12 extending the interaction to the ligands [38]. The fucose modification at EGF12 was also shown to bind directly to ligands, in addition to O-glucose on EGF11, based on the co-crystal structure of DLL4 and NOTCH1 mentioned above in Section 2.1.1 [30]. Further structural binding studies, using high affinity ligands, have demonstrated that JAGGED1 binds EGF8–12 on NOTCH1 with high affinity, while measurements with DLL4 showed that even though it does bind to EGF8–12 of NOTCH1, the majority of the affinity comes from EGF11 and 12 [41]. Molecular force measurements showed that Notch receptor-ligand interactions form so called catch bonds, where tensile forces can increase the time of the bond interactions and induce activation under low affinity conditions. As JAGGED1 and DLL4 have different thresholds for activation, the catch bond formation under low affinity conditions was shown to be important for viability and fertility of mice [48–50]. LFNG mutations have also been reported to cause spondylocostal dysostosis in humans, which gives rise to abnormalities similar to those in mice lacking LFNG [51]. A lack of LFNG has also been found to lead to basal-like breast cancer [52]. In another study, modulation of Notch receptors by LFNG had a tumor-suppressive function in prostate cancer [66]. On the contrary, expression of MFNG has been implied to have an oncogenic role in claudin-low breast cancer [53]. It was recently shown that all Fringes are required to modulate Notch activity during B- and T-cell differentiation [54]. After the Fringe enzymes add the N-acetylgalactosamine (GlCNac) to an O-fucose sugar, the subsequent addition of a galactose and sialic acid residues can occur, forming a tetrasaccharide [9]. The analysis of O-fucose function based on Polut1 knock out has been complicated by the fact that POFUT1 also has crucial functions related to Notch signaling that are independent of its O-fucosyltransferase function. POFUT1 has been shown to work as a chaperone needed for correct Notch protein folding, independently of its O-fucosyltransferase function [55,56]. Overexpression of Ofut1, the Drosophila melanogaster homologue of POFUT1, has also been shown to promote Notch endocytosis nonautonomously [57]. Based on a mutant of Ofut1 lacking the fucosyltransferase activity (Ofut1R245A), but retaining the chaperone activity, that was able to rescue the loss of wild type Ofut1 during embryonic neurogenesis in flies, it was deduced that the actual O-fucosylation of Notch does not have a key function on its own. Furthermore, the phenotype of Ofut1R245A was the same as Fringe mutant clones indicating that the single monofucosylation of Notch, without GlCNac added by Fringe, is not necessary for proper function of Notch [55]. More recent evidence, however, illustrates that the function may not be quite that simple. By using a knock-in version of the mutant Ofut1, lacking O-fucosyltransferase activity, Ofut1R245A knock-in [44], Ishio and co-workers could show that the monofucosylation by Ofut in Drosophila melanogaster has a clear role for Notch signaling during embryonic development in a temperature specific manner, where the loss of monosaccharide modification by Ofut1 leads to the neurogenic phenotype in embryos at 30 °C but not at 25 °C, with no differences in the protein levels of Ofut1-mutants between the different temperatures and in a Fringe-independent manner [58]. With the same setup they also showed that monofucosylation is required for correct localisation of Notch at 30 °C. Even more interestingly, by using a double mutant of Ofut1R245A and a null mutation of the glucosyltransferase rumi called rumi44, they further showed that the O-glucose modification of Notch could function redundantly to the monofucosylation during development, and not only at 30 °C. Similarly, these double mutants hindering O-fucose and O-glucose monosaccharides resulted in less Notch at the cell surface and instead accumulated in intracellular compartments whereas either Ofut1R245A knock in or rumi44 cells had no effects on Notch at the cell membrane, indicating a redundant role also in trafficking of...
Notch to the cell membrane [58]. Even more recently it was shown that O-fucose functions redundantly with both monosaccharide glucose and the already mentioned diolysolated O-glucose. The monosaccharide O-glucose was required for the transport of Notch from the apical plasma membrane to adherens junctions while the diolysate of O-glucose regulated Notch export from the ER. Loss of these could, however, both be compensated for by monosaccharide O-fucose [29]. These studies highlight the importance of analysis that deciphers the function of several glycans and other PTMs as a combination on output. Focusing on one site at a time may be overlooking key pieces of information about the true function of the modification.

Dysfunctional glycosylation has been shown to lead to developmental disorders and diseases in humans. There have been numerous studies reporting an overexpression of glycosyltransferases in various cancer types. POFUT1 has been found overexpressed in brain tumors, colorectal cancers, oral squamous cell carcinoma, and hepatocellular carcinoma [59–62]. In a microarray screening of glioblastomas POFUT1 was found overexpressed [59]. In another study, POFUT1 was introduced as a potential diagnostic marker for human oral cancer [62]. Heterozygous mutations of both POFUT1 and POGULT1 have also been identified in the rare skin disease Dowling-Degos Disease (DDD) in mice heterozygous for either mutation [73]. The broad consensus sequence of C5-X-X-X-X-(T/S)-G-X2–3-C6 [72] with the ones in mammals [71], Ogawa and Okajima present a paring the modified sequences identified in Drosophila [73]. The consensus sequence C5-X-X-X-(F/W/Y)-(T/S)-G-X-X-C6 [72], although linked GlcNAc between the fifth and the sixth conserved cysteine at the specific O2.1.3. O-GlcNAcylation by EOGT

Notch has also been shown to be modified by the EGFR domain specific O-GlcNAc transferase (EOGT) [67–70]. EOGT can add a O-linked GlcNAc between the fifth and the sixth conserved cysteine at the consensus sequence C5-X-X-X-(F/W/Y)-(T/S)-G-X-X-C6 [71], although this sequence could yet be further specified in the future. When comparing the modified sequences identified in Drosophila melanogaster [72] with the ones in mammals [71], Ogawa and Okajima present a broad consensus sequence of C5-X-X-X-(F/W/Y)-(T/S)-G-X-X-C6 [73]. The functional effects of O-GlcNAc on Notch signaling are not immediately clear in Drosophila melanogaster as EOGT mutants lack obvious Notch phenotypes [70]. Harvey and colleagues only found 5 sites modified by O-GlcNAc in a MS analysis, out of 18 predicted Drosophila melanogaster consensus sites [72]. Unlike in flies, O-GlcNAc is further modified by additional glycans (galactose and sialic acid) in mammals, implying that O-GlcNAc could be distinct between flies and mammals [38,71]. Specifically, Ogawa and colleagues showed (with highly variable stoichiometries) monosaccharide O-GlcNAc modifications on EGF14, EGF27 and EGF28, mono- or disaccharide O-GlcNAc-Gal on EGF11, EGF15, EGF21, EGF23 and EGF35, as well as mono-, di- or trisaccharide form O-GlcNAc-Gal NeuAc on EGF2, EGF10 and EGF20 [71]. T673 on EGF17 of Notch2 might also be modified by O-GlcNAc [67]. Mutations in human EOGT and Notch components have been linked to Adams-Oliver syndrome, which is a rare congenital disorder characterized by missing or underdeveloped distal limbs, combined with abnormal skin development of the scalp that may extend to abnormalities in the skull bone. Sometimes Adams-Oliver syndrome is accompanied by blood vessel and heart defects [74–78]. All EOGT mutations found in Adams-Oliver syndrome affect the enzyme activity of EOGT in distinct ways [77,79]. It was also recently shown that binding of DLL1 and DLL4 to NOTCH1 was reduced in EOGT-deficient mice while JAG1-NOTCH1 signaling was not affected [80]. The majority of O-GlcNAcylated was related to EGF2, EGF10, EGF17 and EGF20, although their individual contributions were not specifically determined. In the same study loss of EOGT reduced Notch signaling and was shown to regulate angiogenesis and vessel integrity in the mouse retina [80].

2.2. Hydroxylation of NICD

Hydroxylation is the addition of a hydroxyl group to amino acid side chains, most often proline and this occurs frequently under hypoxic conditions. Hypoxia-inducible factor (HIF) is a target for hydroxylation by both prolyl hydroxylases (PHD) and the asparaginyl hydroxylase Factor Inhibiting HIF (FIH) (reviewed in [81]). FIH also hydroxylates conserved asparagines on the ankyrin repeat domain (ANK) of N1-ICD, N2-ICD and N3-ICD. In contrast, the N4-ICD-ANK lacks the necessary binding motif and the second asparagine residue and is not a FIH substrate [82–84]. FIH overexpression negatively regulates Notch1 signaling [4,82,84]. Surprisingly, this effect is independent of FIH enzymatic activity [82,84]. One function of the FIH-N1-ICD interaction appears to be modulation of HIF-1 signaling and the hypoxia response as FIH has higher affinity for N1-ICD than HIF-1α [83,84]. N1-ICD promotes HIF signaling through competition for FIH binding and subsequent decreased HIF asparagine hydroxylation [82,84]. N1-ICD hydroxylation decreases FIH affinity for N1-ICD and may fine tune the N1-ICD/HIF competition [82,84]. Based on proteomics screens, asparaginyl hydroxylation by FIH is common for ANK-containing proteins [85].

2.3. Acetylation of NICD

Acetylation involves the transfer of acetyl groups from acetyl coenzyme A to protein lysine residues regulating the protein function and stability. Several acetyltransferases have been shown to associate with the Notch intracellular domain or the CSL-NICD-MAML ternary complex. They are not only involved in regulating Notch transcriptional activity through histone acetylation and consequent effects on chromatin, but also by targeting the individual components of the ternary complex itself. The (histone) acetyltransferases p300, p300/CREB-binding protein-associated factor (PCAF) and GCN5 have been shown to interact with and directly acetylate certain Notch ICDs [86,87] whereas the (histone) deacetylases sirtuin 1 (SIRT1) and histone deacetylase 1 (HDAC1) have been found to deacetylate them [88,89], p300 and/or PCAF acetylate numerous lysines in the mammalian N1-ICD and many of these sites are deacetylated by the NAD+ dependent deacetylase SIRT1. Acetylation attenuates ubiquitination and subsequently degradation of the N1-ICD [88,90]. MAML1 may have a central role in promoting the acetylation through recruitment of p300 to the N1-ICD and stimulation of autoacetylation and acetyltransferase activity of p300 [90–93]. The acetylation-dependent stabilization of N1-ICD is counteracted by SIRT1 activity. Loss of SIRT1 function promotes anti-angiogenic DLL4-Notch signaling in endothelial cells [88,94–96]. Accordingly, in zebrafish and mouse retinas decreased Sirt1 activity leads to an attenuation of vascular growth which is rescued by treatment with the γ-secretase inhibitor DAPT [88]. Inhibitory effects of SIRT1 function on N1-ICD level, localization and activity has additionally been reported in murine neural stem and progenitor cells [97], Treg cells [98] and macrophages [99] as well as in neonatal rat cardiomyocytes [100]. In Ewing’s sarcoma cells NOTCH1 has been shown to repress SIRT1 levels through HEY1 in a negative feedback loop [101].

Interestingly, in Drosophila melanogaster Sirt1 activity on the contrary promotes Notch signaling and the phenotype of Sirt1 mutant flies is similar to flies with a weak loss of Notch function. However, it is not certain if this is due to an effect on the acetylation status of Notch or e.g. Su(H) which has also been identified as a Sirt1 deacetylation target in Drosophila melanogaster [102]. The NOTCH3 ICD is acetylated by p300, and deacetylated by HDAC1 [89]. By contrast to the stabilizing effect of p300-mediated acetylation on N1-ICD, acetylation of N3-ICD promotes its ubiquitination and degradation [89]. Furthermore, non-acetylatable lysine-to-arginine mutants of the identified N3-ICD acetylation sites promote proliferation in T-cells whereas treatment with the (histone) deacetylase inhibitor (HDACi) trichostatin A (TSA) has the opposite effect [89]. Importantly, in NOTCH3-overexpressing transgenic mice which typically develop T-cell acute lymphoblastic leukemia (T-ALL), inhibition of N3-ICD deacetylation via TSA treatment prevents T-ALL formation [89]. Similarly, in urothelial cancer cells another HDACi, suberylanilide hydroxamic acid (SAHA), has been shown to also
increase Notch3 ubiquitination and degradation as well as inhibit proliferation [103]. On the other hand, a recent study attributed the effect of TSA on NOTCH3 signaling in T-ALL cells to increased acetylation of α-tubulin and consequent lysosomal trafficking of full length NOTCH3 [104].

2.4. Methylation of NICD

Methylation refers to the transfer of a methyl group to lysine or arginine residues in proteins, and beyond maintaining epigenetic control and impacting histone proteins, methylation also regulates non-histone protein expression and stability. The first methylation events modulating NICD function have only recently been discovered. Methyltransferase coactivator-associated arginine methyltransferase 1 (CARM1), a member of the protein arginine methyltransferase (PRMT) family, methylates N1-ICD on five conserved arginine residues within the C-terminal transactivation domain (TAD) [105]. Methylated N1-ICD was found to occur primarily in the cell nucleus [105]. CARM1 was also shown to physically interact with both N1-ICD and CSL and to be present at Notch target gene enhancer elements together with N1-ICD [105]. Interestingly, methylation on one hand stimulates Notch transcriptional activity and on the other increases ubiquitin-mediated degradation of the N1-ICD [105]. Based on mathematical modeling N1-ICD methylation regulates the duration and strength of its activity and mediates a full, but transient activation [105]. This example demonstrates the complexity of PTMs and the need to decipher functional consequences of specific PTMs in detail. Additionally, MS screen of methylated proteins identified NOTCH3 as a target of arginine methylation in HCT116 colon cancer cells [106].

2.5. Phosphorylation of NICD

Phosphorylation is the addition of phosphate groups from ATP to specific serine, threonine, and tyrosine residues. It is one of the key mechanisms for tight dynamic regulation of protein activity in eukaryotic cells as one third of all eukaryotic proteins undergo reversible phosphorylation. Indeed, Notch is included in the phospho-protein family containing a myriad of paralog-specific phosphorylation sites impacting on both activity and stability. So far, both the membrane-tethered furin S2-cleaved NEXT as well as NICD have been shown to undergo phosphorylation while the full-length Notch does so only to a lesser extent [107]. This could also be a consequence of challenges in identifying sites on full-length Notch. Subsequently many kinases have already been identified as acting on the Notch pathway (reviewed in [3,108]). However, within the Notch field notation of phosphorylation sites varies and is inconsistent with protein sequence data from UniProt [109]. This is mostly due to the use of the 2556 aa long sequence conflict variant NOTCH1 sequence instead of the canonical 2555 aa NOTCH1 in the UniProt database. In this review we have corrected phosphosite locations to match the canonical 2555 aa NOTCH1 UniProt-protein sequence.

2.5.1. Cyclin-dependent kinases (CDKs)

A classical example of Notch-targeted phosphorylation is that of cyclin-dependent kinase 8 (CDK8) in the article by Fryer et al., where in N1-ICD isolated from the African clawed frog *Xenopus laevis* in vitro assays show CDK8 binding directly to MAML and phosphorylating N1-ICD at S2482, S2485, and S2507 of the PEST domain (corresponding to S2513, S2516, and S2538 in human) leading to enhanced PEST-dependent degradation of N1-ICD by FBXW7/SEL10 ubiquitin ligase [110]. However, the impact of CDK-mediated phosphorylation on N1-ICD has evolved considerably after the emergence of Cyclin C as an arbiter of CDK-driven phosphorylation of Notch [111]. In their study, Li et al. unveiled the tumor suppressor Cyclin C as a potent activator of CDK3, 8 and 19 with a myriad of targets identified both in *vivo* and in *vitro* on different domains of Notch corroborating the evidence of CDK-phosphorylation mediated control of N1-ICD turnover. The sites identified in *vivo* were T1861, T1963, S2183, T2511, T2513, S2516, and S2524. The in vivo sites concluded S1801, S1856, T1861, S2121, S2198, S2202, S2205, S2211, S2215, T2483, S2486, S2492, T2497, T2511, S2513, S2516, S2521, S2522, S2523, S2527, and S2530 [111]. From these S2513 and S2516 align with the previously found sites S2482 and 2485 on *Xenopus laevis* [110]. Furthermore, also CDK1 and CDK2 have been shown to phosphorylate S2513 leading to FBXW7-mediated degradation of N1-ICD, impacting the somite segmentation clock [112].

2.5.2. CK2, ILK, SRC, and NLK

Many kinases also directly control transcriptional activity. Casein kinase 2 (CK2) initially targets N1-ICD on S1900 subsequently revealing another site for CK2 at T1897 [113]. The collective effect of phosphorylation at both sites leads to decreased binding of the N1-ICD-Mastermind-CSL complex thus lowering transcriptional activity [113]. Based on sequence conservation the authors hypothesize that also NOTCH2 may be similarly regulated by CK2. Likewise, Integrin-signalizing is modulating Notch as integrin-linked kinase (ILK) phosphorylates N1-ICD at 2173 in mouse (corresponding to S2183 in human) thus inhibiting transcriptional activity and leading to FBXW7-mediated proteasomal degradation [114]. Recently also SRC kinases, acting downstream of Integrins, have been identified to phosphorylate N1-ICD on several tyrosine residues in the ANK domain (Y2074, Y2116, Y2145, Y1938), leading to decreased MAML recruitment and N1-ICD half-life [115]. Interestingly, by sequence alignment the authors also find the phosphorylation sites conserved across many species and present in several Notch paralogs. In a similar fashion in *Xenopus laevis*, Nemo like kinase (NLK) phosphorylates N1-ICD C-terminally of the ANK domain potentially on seven sites at S2118, S2132, S2137, S2194, S2211, S2217, and S2222 decreasing transcriptional activity by interfering with formation of the transcriptionally active ternary complex [116]. These sites in human correspond to S2121, S2136, S2142, S2198, S2215, S2221, and S2226. The authors found the inhibition of transcription to roughly correspond to the number of residues phosphorylated. By contrast, in the same study N3-ICD activity was found to be increased by NLK phosphorylation [116].

2.5.3. PIM, AKT, and PKC zeta

PIM-kinases have been found to mediate phosphorylation of N1-ICD at the second NLS (S2152 in mouse, S2162 in human) thus increasing nuclear localization and transcriptional activity [117]. Moreover, in the same study, also N3-ICD was discovered as a target of PIM-mediated phosphorylation. By contrast, AKT-mediated phosphorylation of N1-ICD has been shown to inhibit proper nuclear localization and transcriptional activity [118]. Similarly on NOTCH4, four distinct AKT phosphorylation sites (S1495, S1847, S1865, and S1917) were found to mediate N4-ICD association with 14-3-3 thus restricting nuclear translocation or triggered Notch receptor recycling [119]. Surprisingly, despite the similarities in output between N1-ICD and N4-ICD upon Akt-mediated phosphorylation, only S1847 on N4-ICD is conserved on N1-ICD. Phosphorylation also impacts receptor recycling as shown by Sjöqvist et al., where PKC zeta was observed to phosphorylate NOTCH1 as both membrane bound NEXT and full-length NOTCH1 receptor form at S1791 in mouse (S1801 in human). This phosphorylation was found to regulate Notch endocytosis which depending on activation state, either enhanced N1-ICD formation or triggered Notch receptor recycling [120].

2.5.4. GSK3-α/β and the dephosphatase EYA1

Findings regarding the effects of glycogen synthase kinase 3 α/β (GSK3-α/β) on Notch activity have revealed conflicting results. In one study, GSK-3β/Shaggy has been shown to increase Notch3 ubiquitination and degradation as well as inhibit proliferation [103]. On the other hand, a recent study attributed the effect of TSA on NOTCH3 signaling in T-ALL cells to increased acetylation of α-tubulin and consequent lysosomal trafficking of full length NOTCH3 [104].
group reported GSK3α/β as a negative regulator of NOTCH1/N1-ICD in mouse embryonic fibroblasts and HEK 293 cells with suggested phosphorylation sites in mouse at T1851, T2122, and T2124 (corresponds to T1861, T2132, and T2134 in human) [122]. Supportive of this, GSK-3β has also been described to target N2-ICD at four specific sites C-terminally of the ANK domain (T2068, S2070, T2074, and S2093) thus negatively regulating the transcriptional activity of N2-ICD [123].

Recently the phosphatase EYA1, previously shown to be important for craniofacial morphogenesis was found to be acting on the Notch pathway through dephosphorylation of T2122 on mouse NOTCH1 (corresponding to T2132 in human) [124]. The proposed mechanism involves dephosphorylation-mediated mitigation of FBXW7-activity on N1-ICD and thus lowered ubiquitination and the subsequent proteasomal degradation leading to enhanced N1-ICD stability [124].

2.5.5. G-CSF, DYRK1A, and ABL

Similarly, the serine-threonine rich region at 2067–2099 of NOTCH2 has also been reported to contain at least three phosphorylation sites including S2078 for Granulocyte colony-stimulating factor (G-CSF) [125]. Phosphorylation at these sites, on cleaved N2-ICD is shown to negatively regulate myeloid differentiation in 32D cells [125].

The Down-syndrome associated kinase (DYRK1A) is co-expressed with Notch in many tissues during embryonic development. Fernandez-Martinez et al. have shown DYRK1A to phosphorylate NOTCH1 on up to 18 different Serines and Threonines in the Ankyrin-domain thereby attenuating Notch-mediated transcription [126]. Also AKT has been shown to phosphorylate NOTCH1 leading to down-regulated Notch-dependent transcription [118], however, as with DYRK1A the exact sites remain obscure.

During development in Drosophila melanogaster, the Notch protein undergoes tyrosine-phosphorylation on three sites (Y1850, Y1860, Y2097) yielding a phosphorylated population selectively associated with ABL-kinase components Disabled and Trio [127]. Despite the phosphorylation event not being required for complex formation, the authors hypothesize the importance of the phosphorylation in mediating downstream Notch/Abl signaling during axon patterning [127].

2.5.6. Sites targeted by unknown kinases

Several more phosphorylation sites have been discovered in phosphoproteomics studies with yet undetermined kinases acting on them. A complete list of discovered phosphosites is presented in Table 1, as well as reviewed in Borggrefe at al. [3]. It is clear that although mapping of the phosphorylation sites on NOTCH1 has yielded significant headway, the marathon to identify all the players phosphorylating the other Notch paralogs is only beginning. As a harbinger of cell fate decisions exhibiting both context and temporal dependency understanding dynamic Notch phosphorylation at different times in different physiological situations will be of crucial importance in the future.

2.6. Sumoylation of NICD

The PTM mediated by small-ubiquitin like modifier proteins (SUMO) regulates a wide variety of cellular functions including transcription, macromolecular assembly, chromatin organization, protein homeostasis, signal transduction, trafficking and DNA repair [128]. Sumoylation has already been shown to have a key role in nuclear function [129].

The roles of sumoylation within the Notch pathway, however, have only started to be uncovered in the last few years as we and others have shown that SUMO regulates critical aspects of Notch activity. We recently showed that heat stress and proteotoxic stress induce direct interaction between SUMO and N1-ICD in the nucleus and represses the expression of the classical Notch1 target genes HES1,HEY1 and HEY2 [130]. However, it had already previously been indicated over a decade ago that sumoylation inhibits one or more components in the LIN-12/Notch signaling pathway in Caenorhabditis elegans [131]. More indications of SUMO modification regulating Notch signaling was obtained when it was shown that the LIM domain protein KyoT2, which negatively regulates Notch signaling by interacting with RBPI, is a target protein for SUMO in a protein inhibitor of activated STAT1 (PIAS1)-catalyzed reaction [132]. The growth of NOTCH1-activated breast epithelial cells decreases by mitigation of global sumoylation by inhibition of the E1-activating complex SAE1/UBA2 and knockdown of the E2-conjugating enzyme UBC9 [133]. It has also been shown that N1-ICD sumoylation is increased in mesenchymal stem cells (MSCs) and HEK293T cells with stabilized hypoxia-inducible factor 1 alpha (HIF-1α) expression [134].

A role and mechanism for NOTCH1 sumoylation has been proposed to occur in the endothelium. According to recent data, deletion of endothelial sentrin-specific protease 1 (SENP1), which is the primary protease in sumoylation, enhances endothelial Notch sumoylation and prolongs NOTCH1 signaling, which suppresses vascular endothelial growth factor (VEGF) receptor signaling and angiogenesis. Thus, by targeting endothelial Notch signaling, sumoylation negatively regulates angiogenesis [228]. PIAS1 catalyzes the oxidative stress-induced sumoylation of the Notch signaling factor hairy and enhancer of split 1 (HES1), which represses the expression of GADD45α and consequently enhances cell survival [137]. Interestingly, a SUMO proteomics strategy has recently indicated NOTCH2 to undergo conjugation by SUMO at K1353 and NOTCH3 at K2061 [138]. The physiological functions of these sumoylations are still to be discovered.

2.7. Ubiquitination of NICD

Ubiquitination adds ubiquitin groups to lysine residues modulating cellular localization and protein stability, with polyubiquitination being the standard tag for degradation by proteasomes. The lifespan of Notch is fairly short and its degradation occurs predominantly through poly-ubiquitin conjugation. Although ubiquitination of Notch does not always cause repressed Notch levels, ubiquitination-mediated proteasomal degradation of Notch is crucial to fine-tune the half-life of NICD. Failure in preventing prolonged Notch signaling causes serious diseases [139].

Many different ubiquitin E3 ligases have been discovered to ubiquitinate NICD. Ubiquitin conjugation of the membrane-tethered NOTCH1 by Itch [140] is facilitated by the mammalian protein NUMB, which also promotes the degradation of N1-ICD [141]. Recently it was shown that Shootin1 enhances the activity of the Notch pathway by interacting with LINX1/2 and stimulating NUMB ubiquitination, or by forming a complex with Itch and debilitating NICD ubiquitination [142]. Ligand activated NOTCH1 is prior to ADAM10 processing ubiquitinated by the E3 ubiquitin ligase DTX4 resulting in the endocytosis of the NOTCH1 extracellular domain by the ligand expressing cell, whereas the membrane attached fragment of Notch and Dtx4 are internalized by the NOTCH1 expressing cell [143]. In Caenorhabditis elegans, the E3 ubiquitin ligase Ubiquitin protein ligase E3 component n-recognition 5 (UBR5) negatively regulates GLP-1 and LIN12 activity, the two Notch receptors expressed by Caenorhabditis elegans [144]. The Ubiquitin-specific Protease 12 (USP12) is a negative regulator of Notch signaling as USP12 directly targets Notch and directs it to lysosomal degradation. Accordingly, USP12 silencing leads to less lysosomal degradation of Notch and consequently to a greater number of Notch at the cell membrane and consequently to higher activity of Notch [145].

Phosphorylation of the C-terminal PEST domain of N1-ICD has been shown to lead to subsequent ubiquitination by F-box/WD40 domain-containing protein 7 (FBXW7/SEL-10), which negatively regulates Notch signaling by targeting Notch for proteasome-mediated degradation [146–148]. Flaws in the N1-ICD-FBXW7 interaction are associated with leukemia and many solid cancers [149]. For instance, in T-cell leukemia (ATL) FBXW7 mutant proteins are defective to interact with
N1-ICD resulting in increased N1-ICD levels and prolonged NOTCH1 signaling [150]. Interestingly, FBXW7 gene mutations in leukemic cells do not only enhance the signaling efficiency of NOTCH1, but also mediate resistance to γ-secretase inhibitors [151]. Recently it was shown that also the E3 ubiquitin ligase ring finger protein 8 (RNF8) ubiquitinates N1-ICD resulting classically in the degradation of N1-ICD. In addition, low expression of full-length RNF8 correlates with bad prognosis for breast cancer patients [152].

However, ubiquitination does not always result in decreased N1-ICD levels. Ubiquitination of N1-ICD by the RING ubiquitin ligase RNF4 stabilizes and enhances the transcriptional activity of N1-ICD [153]. Similarly, N1-ICD ubiquitination by murine double minute 2 (MDM2) leads to activation and stimulation of N1-ICD transcriptional activity [154]. MDM2 also ubiquitinates N4-ICD, which stimulates an N4-CD-Trp53 interaction, which in turn represses N4-ICD-induced anchorage-independent growth in mammary epithelial cells [155].

Ubiquitination is balanced to some extent by deubiquitinases (DUBs). The utilization of an in vivo RNA interference (RNAi) screen led to the discovery of several DUBs that regulate Notch signaling [156]. The loss of the deubiquitinating protein Ubiquitin carboxyl-terminal hydrolase S (Usp5) leads to the upregulation of Notch during *Drosophila melanogaster* eye development [157]. HES1 is a target for the E3 ubiquitin ligase SCFFBXL14 complex resulting in HES1 proteolysis, which further enhances neuronal differentiation [158].

3. PTMs of Notch ligands

Similarly to Notch receptors, the Notch ligands also have EGF-repeats in their ECD, which can be modified by glycosylation, but the functional effects are not immediately evident [14,159]. Oft, the O-fucosylation protein crucial for correct Notch receptor activation in *Drosophila melanogaster* does not seem to be required for signal sending cells expressing Notch ligands [160]. Signal assays from mice lacking Pofut1 also only show a need for POFUT1 in the signal receiving cells [161]. DLL1 harboring mutations in the O-fucosylation consensus sequences still activates Notch signaling, but does show some additional intracellular accumulation [162]. By contrast, recent evidence shows Fringe elongation of fucosylated sites on DLL3 affecting somitogenesis in mice. Here DLL3-mutants lacking the POFUT1 target site are unable to rescue the somitogenesis defects of mice lacking DLL3 [163]. *Drosophila melanogaster* Delta and Serrate have also been shown to be targets for O-GlcNAcylation by EOGT [68,164]. DLL1 has been shown to be phosphorylated at its intracellular domain. Three sites in mouse at T638, S693, and S696 were detected in *vitro* (two sites corresponding to S694 and S697 in human) while only S693 was found to be phosphorylated in *vivo* [165]. Interestingly, phosphorylation of S693 and S696 occurred sequentially and although the phosphorylations were required for full ligand activity in *vitro*, the authors found them dispensable for normal embryonic development in mice. All DSL ligands are potential targets of ubiquitination by E3 ligases and all mammalian Notch ligands have been reported to undergo ubiquitination (see Table 1) [166,171]. Two notable E3 ligase families affecting endocytosis of Notch ligands through monoubiquitination have been documented in multiple different species, Neuralized (NEUR1–2 in mammals) and Mind Bomb (Mib) 1 and 2 [166,296]. The ubiquitin E3 ligase Neuralized (Neur) interacts with the Notch ligand Delta1 and represses its levels in Xenopus laevis [167] and *Drosophila melanogaster* [168]. NEUR1 and NEUR2 are dispensable for normal development in mice, even in *Neur1/2* double knockout mutants [169]. Removal of Mib1 results in embryonic lethality with Notch phenotypes [170]. Mib2 is not required for normal mouse development [171], but Mib2 in zebrafish has been able to partially rescue neurogenic and vasculogenic phenotypes of *Mib1* knockouts [297]. For reviews on Notch ligand ubiquitination and its effects on ligand endocytosis, see [166,293,294].

4. PTMs of the Notch transcriptional complex (CSL and MAML)

4.1. Acetylation, phosphorylation and ubiquitination of CSL

In *Drosophila melanogaster*, The Suppressor of Hairless (Su(H)), the homologue of mammalian CSL has been shown to be acetylated in *vivo* and to be at least partially deacetylated by the *Drosophila melanogaster* Sirt1 [102]. Sirt1 activity in *Drosophila melanogaster* has a stimulatory effect on Notch signaling, but it is uncertain if this is due to deacetylation of Su(H) or effects on other components of the Notch transcription complex [102]. Similarly phosphorylation of human CSL by the MAPK p38 at T339 induces ubiquitination and degradation of the CSL protein [172].

However, the phosphorylation of Su(H) can also modulate Su(H) activity in *Drosophila melanogaster*, depending on the phosphorylated residue. MAPK-mediated phosphorylation has been documented to occur both in *vivo* and in *vitro* at the beginning of the C-terminal domain (CTD) on T426 (corresponding to T352 in humans) slowing down the activator complex formation, possibly favoring the formation of the repressor complex and thus inhibiting transcriptional activity [173]. The authors also hypothesize this phosphorylation to be plausible in humans. Similarly another site in the beta-trefoil domain (BTD), on S269 (corresponding to S195 in humans) was also discovered in *Drosophila melanogaster* and is shown to inhibit the binding of BTD to DNA [174].

4.2. Acetylation, phosphorylation and ubiquitination of MAML

p300 interacts with MAML1 [91,93] and acetylates it on several lysine residues in the N-terminus, most prominently on K188/K189 [175]. Another acetyltransferase, CBP, has also been shown to acetylate MAML1 [176]. MAML1 promotes autoacetylation of the N1-ICD through monoubiquitination have been documented in multiple different species, Neuralized (NEUR1–2 in mammals) and Mind Bomb (Mib) 1 and 2 [166,296]. The ubiquitin E3 ligase Neuralized (Neur) interacts with the Notch ligand Delta1 and represses its levels in Xenopus laevis [167] and *Drosophila melanogaster* [168]. NEUR1 and NEUR2 are dispensable for normal development in mice, even in *Neur1/2* double knockout mutants [169]. Removal of Mib1 results in embryonic lethality with Notch phenotypes [170]. Mib2 is not required for normal mouse development [171], but Mib2 in zebrafish has been able to partially rescue neurogenic and vasculogenic phenotypes of *Mib1* knockouts [297]. For reviews on Notch ligand ubiquitination and its effects on ligand endocytosis, see [166,293,294].
NACK through lysine (K245) corresponding to MAML1 K188/189 has an effect on the viability of oesophageal adenocarcinoma (EAC) cell lines CBP (with C464) and Notch (with DAPT) has a synergistic inhibitory effect. NACK recruits RNA polymerase II to the complex, which leads to increased transcription [176]. Interestingly, MAML2 does not contain a corresponding lysine or area based on amino acid sequence and does not bind NACK [176].

In addition, MAML induces phosphorylation of CBP/p300 via its second transcriptional activation domain (TAD2) during assembly of MAML into the Notch enhancer complex, however the direct implications of this phosphorylation on Notch signaling are unknown [91]. Likewise, NLK has been observed to phosphorylate MAML, yet also the function of this phosphorylation remains unknown [116].

MAML1 in the N1-ICD transcriptional complex is a subject for SUMO conjugation, which enhances the MAML1-HDAC7 interaction causing decreased MAML1 transcriptional activity [177]. Ubiquitin conjugation to MAML1 is inhibited by N1-ICD, but enhanced by p300, and decreased MAML1 ubiquitination causes increased transcriptional output in a Hes1 reporter assay [178].

5. Interplay between PTMs

One of the challenges of Notch-PTM research is the study of interplay between different dynamic modifications. Although direct impact of singular PTM-events has been established to influence protein-protein interactions and creating new binding sites on their own, a largely untouched aspect of post-translational modulation of Notch is the interactions between PTMs and their cumulative effects on protein function. How can the addition of a singular dynamic PTM affect the addition of another PTM in the same domain? How do the PTMs differ within the entire family of Notch paralogs? How do competitive modifiers targeting the same site affect PTM-saturation? What is the crosstalk between PTM “writers” and “erasers”? What is the effect of specific PTM-signatures? Although deciphering this complex map of interactions is still in its infancy, there is mounting evidence pointing towards a much more complex “molecular switchboard” than any modification on its own could achieve. These crosstalk mechanisms generate multiple different functional forms of a protein and provide a complex fine-tuning necessary for rapid responses within the cell. There already exists examples of this crosstalk on Notch, which will we highlight below, but there is also recent data on modification interactions from other fields that we will use to discuss future possibilities within the Notch-field.

A common form of post-translational crosstalk is when one PTM generates a signal for the addition or removal of another PTM, or when the first PTM simply allows a binding protein to carry out another modification. CK2 for example initially targets one site on N1-ICD for phosphorylation and subsequently another in a sequential manner ultimately leading to inhibited transcriptional complex activation [113]. A recent example of phosphorylation-mediated recognition of a binding protein comes from NOTCH4 phosphorylation mediating 14-3-3 association [119]. Phosphorylation can also create a signal that an E3 ligase can recognize for ubiquitin-mediated degradation. Short sequences that are targets for phosphorylation-dependent ubiquitination are called phosphorydromes. N1-ICD contains two phosphorydromes located in both of its terminal ends, although the C-terminal PEST domain contains what is considered the main phosphorydrome of Notch [179]. Many kinases are known to enable docking for FBXW7 leading to subsequent ubiquitination and degradation of proteins [108]. In the case of Notch, CDR-mediated phosphorylation of NICD is one of the enablers for FBXW7-mediated degradation [110,111].

Another form of crosstalk comes from the competitive inhibition of a single site by different PTMs. Single lysines have been shown to be targets for both ubiquitination and sumoylation. Similarly acetylation, methylation and ubiquitination of the same site have been identified in other proteins like Eras [180]. Acetylation has previously been shown to be able to stabilize proteins [181]. As such, lysines that can be modified by both acetylation and ubiquitination are likely to be competing in a form of negative crosstalk where acetylation stabilizes and ubiquitination can mark the protein for degradation.

The complex protein fine-tuning mediated by ubiquitination originates from the different ways ubiquitin chains can be generated. The ubiquitination events recognized by the proteasome for degradation depend on Lys48-linked chains, while Lys63 chains are used for various purposes including endocytotic recycling and sorting as well as activation of protein kinases [182]. In addition monoubiquitination events can regulate protein function independently [182]. Thereby the crosstalk between phosphorylation and ubiquitination can affect function in several ways starting from the fact that phosphorylation can both promote and inhibit ubiquitination.

PTMs may also function as mediators of coordinated regulation of signaling pathways. This applies in particular for SUMO conjugation. In some cases several proteins of the same pathway can be simultaneously multisumoylated, stabilizing the interaction between the modified proteins [183]. Furthermore, protein complexes may be held together by several SUMO-SUMO interacting motifs (SIMs) [184], and elimination of one SUMO site may thus have no major physiological consequences. Proteins engaged in interrelated complexes have been demonstrated to be subjects to synchronous SUMO2 modification in heat-shocked cells [185]. However, strong signaling network control may not be exclusive to SUMO-mediated regulation, but may extend to other PTMs as well, causing challenges in elucidating the physiological relevance of PTMs of a single target protein. Indeed, coordinated control of the activity of different components in the Notch signaling mechanism would constitute a dynamic, but robust cell-intrinsis system to regulate cellular responses to Notch activation.

In a more obscure form of crosstalk there is also a report on how both stem cells and cancer stem cells can be modified by the cis/trans isomerization protein prolyl-isomerase PIN1 in a way where NOTCH1 and NOTCH4 are able to escape proteasomal degradation by blocking FBXW7a-mediated ubiquitin ligation [186]. Most amino acids have a strong preference for trans peptide bond conformation, but proline with its cyclic structure can be catalyzed to form a cis bond which in the case of NOTCH1 and NOTCH4 leads to a steric hindrance for FBXW7a and in this case promotes tumor growth and metastasis in vivo [186].

While methylation of N1-ICD promotes its ubiquitination and proteasomal degradation, it also coincides with phosphorylation of N1-ICD [105]. It has also been shown that acetylation of N1-ICD by p300 inhibits its ubiquitination and vice versa, phosphorylation of N1-ICD by CDK8 in turn inhibits the acetylation and subsequently promotes ubiquitination and degradation of N1-ICD [90]. Both p300 and CDK8 are recruited to N1-ICD by MAML1 [91,93,110]. By contrast, another study has shown that acetylation promotes N3-ICD ubiquitination [89].

The dynamic regulation of phosphorylation is a complex entity on its own, and is mediated not by single actors but by a myriad of kinases and phosphatases acting in concert. Indeed, as published data shows us, different phosphorylation sites are often intertwined in complex arrangements and can sometimes overlap. The identified sites of GSK3 (T1861), ILK (2183), and NLK (S2121, S2198, S2215) all overlap with identified CDK-sites. In a similar fashion more complex relationships can form for example as the identified EYA1 phosphatase targets a GSK3β-site (T2132). These discoveries imply that the system exhibits a fair share of redundancy with the arguably same functional modification being implemented by different mediators, however, the exact biological relevance of this overlap in sites is still not clear.

In a screen that identified over 2500 proteins being targets for SUMO E3 ligase-dependent sumoylation, it was revealed that protein kinases were particularly often conjugated by SUMO, indicating crosstalk between phosphorylation and sumoylation [188]. In addition, a site-specific mapping identified over 800 sumoylated peptides that...
were co-modified with phosphorylation [138]. In another recent screen, which focused on the crosstalk between sumoylation and ubiquitination, the authors describe an improved method for enrichment of co-modified proteins. The screen identified 498 proteins which are modified by both sumoylation and ubiquitination, but the method can be applied to other combinations of PTMs [189]. The improvement in purification of proteins simultaneously modified by different PTMs could help reveal the network of Notch crosstalk as well.

6. Discussion

In this review we have glimpsed beneath the surface of the Notch-PTMome and shown that the devil is indeed in the details as many deceivingly small dynamic modifications (Fig. 3) can have dramatic effects on Notch protein output. While certain PTMs of Notch have been described only in single publications, others have been more meticulously researched (Table 1). One of the most studied Notch modifications is glycosylation by POFUT1, POGLUT1 and FRINGE, where the effects of glycosylation and the embryonic lethality of glycosylation enzyme knockouts has been corroborated between several research groups. Also, the Cdk-mediated phosphorylation of NICD and subsequent ubiquitination by FBXW7 represents one of the most robust examples of PTM-driven Notch fine-tuning. Many diseases are also linked to mutations of Notch-modulators (Table 2) and although in some cases (e.g. Dowling-Degos disease) the effects have been directly linked to Notch-modulation (POFUT1/POGLUT1/NOTCH1/DLL4), in others (e.g. Down’s Syndrome) the dysregulated genes impact a larger proportion of the proteome with Notch being just one of the targets (DYRK1A). Thus certain large scale modulators e.g. AKT likely impacts many signaling pathways including Notch, via PTMs that can have implications in different disease contexts.

The complete post-translational roadmap of Notch is however far from finished. In the case of phosphorylation, as shown in Table 1, and by Borggrefe et al. [3] many novel phosphorylation sites have already been discovered in phosphoproteomic studies although info on kinases i.e. writers acting on these sites as well as their physiological relevance and output are yet to be determined. Furthermore, several in silico proteinos finder tools are available, including The Human Protein Reference Database, where algorithms can be applied to specific protein sequences to find consensus motifs for all known kinases in specific proteins of interest. The effects of PTMs can also be increasingly studied in silico, with novel advanced protein-modeling programs. Already used in drug development, modeling software packages can predict unknown structures of proteins based on other related proteins and provide information on protein-protein docking as well as how a PTM affects the interface of the protein and its docking capabilities with other proteins [190–194]. These kinds of modeling programs can then predict what kind of molecules might block specific PTM-sites to affect inter-molecular interactions for therapeutic effects.

The current PTM data in the Notch-field across scientific publications sometimes contains unorganized and inconsistent data, which interfere with data utilization. Challenges arise with the use of multiple non-human protein species due to sequence homology mismatches, and with phosphosite discrepancies in published journals due to the use of different protein variants. A new accurate repository for Notch PTM sites, especially for human PTMs would certainly help analysis of data. We also urge the Notch field to implement the canonical 2555 aa NOTCH1, i.e. the most common polymorphic sequence variant presented by uniprot.org, for data consistency. This would allow for more meaningful utilization of the data.

Today, methods relying on MS and phospho-specific antibodies are the standard ways of detecting and analyzing PTMs. MS is the large-scale PTM tool for mapping in vitro sites, despite certain shortcomings for identifying actual physiologically relevant sites in vivo. These include the inability to determine the stoichiometry of a phosphorylation event and the fact that the increasingly higher detection sensitivity today enables detection of phosphorylated peptides at such low stoichiometry that the results may become irrelevant on a cellular scale [195]. This is especially true for in vitro phosphorylation studies that combine high kinase concentrations with long reaction times in the absence of phosphatases that are normally active in the cell. Luckily, quantitative MS is able to home in on physiologically relevant sites by also deciphering the stoichiometry of a phosphorylation reaction. When quantitative MS is not convenient or accessible, comparing the in vitro sites with in vivo data highly increases the relevance of the identified sites. Sites discovered in vitro that have been conserved through evolution also imply a functional role for the identified modification. Utilization of MS to gauge PTM levels in cancer patient samples for diagnostic and prognostic purposes has already shown potential [196]. Similarly, PTM levels in certain plasma biomarkers of glioblastoma were found changed using MS analysis, a finding that could provide diagnostic value in the future [197].

Despite these advances in PTM analyses, an obvious need still exists to both develop and utilize new methods in enrichment and analysis of

### Table 2

Diseases associated with mutation of Notch-modifiers. Overview of diseases linked to mutations of Notch PTM writers or erasers. Although in certain diseases, PTM-modifier mutations link directly to Notch, e.g. POFUT1/POGLUT1 in Dowling-Degos disease (DDD), in others the impact on proteome function is broader, e.g. DYRK1A in Down’s Syndrome, where Notch is only one of the many proteins affected.

| Gene       | PTM on Notch | Mutation | Disease                                                                 | Reference                        |
|------------|--------------|----------|------------------------------------------------------------------------|----------------------------------|
| AKT1       | Phosphorylation | Hyperactivation | Proteus syndrome                                                        | [265]                            |
| CBP        | Acetylation   | Loss of function | Robinstein-Taybi syndrome                                               | [266]                            |
| CK2        | Phosphorylation | Loss of function | Intellectual disability and neurodevelopmental disorders               | [267]                            |
| Cyclin C   | Phosphorylation  | Loss of function  | T-ALL (T-cell acute lymphoblastic leukemia)                              | [111]                            |
| CDK8       | Phosphorylation | Loss of function | Syndromic developmental disorder                                        | [268]                            |
| DYRK1A     | Phosphorylation | Overexpression | Intellectual disability, especially in Down’s Syndrome                 | [126,269]                        |
| E3 ligases | Ubiquitination | Loss of function | Fanconi’s anemia, neurological diseases including ALS, Alzheimer’s, multiple sclerosis, autism etc. (reviewed in [270]) | [270,271]                        |
| EGOT       | O-GlcNacylation | Loss of function | Adams-Oliver syndrome                                                   | [79,222]                         |
| EYA1       | Dephosphorylation | Loss of function | Branchio-oto and branchio-oto-renal syndrome, congenital cataracts, ocular anterior segment anomalies | [272]                            |
| FBXW7      | Ubiquitination | Loss of function | T-ALL (T-cell acute lymphoblastic leukemia)                              | [273]                            |
| G-CSF      | Phosphorylation | Loss of function | Severe congenital neutropenia (SCN), acute myelogenous leukemia (AML)   | [280,281,286]                    |
| GSK3β      | Phosphorylation | Hyperactivation | Psychiatric diseases (bipolar disorder, depression, anxiety, schizophrenia), and neurological diseases (Alzheimer’s, Parkinson’s, multiple sclerosis) | [274,275]                        |
| Lunatic FRINGE | O-fucosylation | Loss of function | Spondylocostal dysostosis                                               | [51]                             |
| POFUT1     | O-fucosylation | Loss of function | Dowling-Degos disease (DDD), or reticular pigmented anomaly of the flexures | [64]                             |
| POGLUT1    | O-glucosylation | Loss of function | DDD                                                                    | [63]                             |
PTM crosstalk and functionality. Today, several additional tools to study combinations of PTMs are already being developed. An interesting emerging toolset is the combination of sensitive MS with computational modeling allowing for analysis of systems level dynamics. Combinatorial bioinformatics such as multiple clustering analysis methodology (MCAM) could provide data on how the whole PTM network behaves following different input stimuli off the cells [198]. MCAM has already been used to study how receptor tyrosine kinases behave following EGF stimulation. In a similar fashion Gajadhar et al., used systems biology to analyze the effects of increased HER2 expression on the RTK network by quantifying tyrosine phosphorylation at different timepoints following HER2 stimulation. Using partial least squares regression algorithms (PLSR) on the signal-response data they identified the phosphorylation sites correlating with the cell response at each time point [199]. Another interesting method comes in the form of novel signal-seeker PTM kits that have shown promise in utilizing phospho-specific antibodies conjugated to affinity beads to detect endogenous levels of different PTMs [200,201]. This toolkit allows for simultaneous detection of multiple PTMs using a single lysis system, and thus enables an easy methodology for a more comprehensive snapshot of the proteome at any given time. Furthermore, the use of PTM-specific antibodies in combination with high-resolution immunofluorescence imaging can be utilized to reveal the location of modified proteins and can thereby also hint to their function in the cell. Simultaneous improvements in live-cell imaging can also make it possible to study modification events in real time. This kind of information could help decode when the modifications happen, where and as a consequence to which stimuli.

In the realm of therapeutics the promise of PTM-specific antibodies has yet to come into fruition, although concepts already exist. Ever since the development of the first therapeutic monoclonal antibody (mAb) in 1985 (murinomab-CD3) as an anti-rejection agent for organ transplantation, mAbs have been successfully used to treat several conditions including autoimmune-diseases, cardiovascular- and infectious diseases as well as cancer (reviewed in [202]). The use of mAbs in therapeutic use for blocking of plasma membrane proteins to inhibit complete signaling pathways or parts of them is a well-established concept that has already shown potential. Examples include the CTLA-4 and PD-1 mAbs utilized in modern immunotherapy [203] or the mAb 2F1 inhibiting the Wnt pathway coreceptor LRPs [204] in the treatment of diabetic retinopathy. Indeed, one therapeutic avenue of PTM-specific mAb therapies may be to block PTM-specific signaling pathway interactions. However, as most PTMs occur intracellularly new methodology is required for targeting. Administration of so called intracellular antibodies, or intrabodies would require modification of the antibodies making them tolerant to cytosolic conditions and permeable to the cell membrane. Alternatively, a type of gene therapy could be used enabling vectors to produce the necessary antibodies within the cell [205].

The design and generation of PTM-specific antibodies on their own is however still difficult. This includes difficulties in molecular recognition of PTMs, however, recent engineering advances may allow for highly functional anti-PTM antibodies with exquisite specificity to be developed in the future (reviewed in [206]). PTM-antibodies have so far mostly been utilized in research and whether these will develop into a therapeutic modality remains to be seen. This futuristic vision would however allow for the selection of expected changes in the PTM makeup of a selected protein and the utilization of this information for the design of specific PTM-antibodies for different needs, for example to block certain PTM-specific outputs. These “PTM-epitopes” would thus enable another layer of specificity and allow for tailored therapeutic approaches.

In contrast to issues with low stoichiometries of phosphorylation sites, the O-glycans are present at high stoichiometries on NECD, which has enabled evaluation of relative levels of modification by recent mass spectral glycoproteomics [38,72]. As glycosylation regulates Notch activity through extracellular NECD-modifications it might also be more amendable to therapeutics than targeting specific intracellular modifications. To this end, Schneider et al. recently introduced synthetic GDP-fucose analogs as substrates for POFTU1, which were added onto Notch EGF-repeats. These sugar analogs were able to inhibit DLL signaling with no significant effect on signaling from JAGGED1 [207]. It has also been suggested that the recent structural insights for XXYLT1 [28], POGLUT1 [208] and POFTU1 [209] could facilitate the development of inhibitors for these enzymes [210]. As xylosyltransferases, in contrast to O-glycosyltransferases, have been shown to reduce Notch signaling, there is an opportunity to increase or reduce Notch signaling depending on enzyme target.

The technological breakthrough of CRISPR (clustered regularly interspersed short palindromic repeats)/Cas (CRISPR-associated protein) has introduced a platform for efficient and specific genome editing. The RNA-guided nuclease Cas9 can be targeted to specific sequences in the genome upstream of a NGG or PAM-sequence making genetic editing easier than ever before. Introducing double-strand breaks (DSBs) with CRISPR allows for insertion of specific mutations that knock-out a gene of interest, a method adopted widely today. Indeed in the case of Notch, knocking out specific PTM writers and/or erasers can give useful information about the effects of PTMs on certain phenotypic effects related to Notch. However, in order to pinpoint the exact site for a particular modification and for deciphering the mechanism more meticulous examination is required. Although basic mutagenesis of expression plasmids in cells in vitro will give an overview of the effects, in order to study it on the endogenous level CRISPR can again be utilized. By taking advantage of homology-directed repair (HDR) with single-stranded oligo-donor nucleotides (ssODNs) as templates its now possible to make single amino acid substitutions in the form of serine to alanine phospho-dead mutants (S → A) for probing the effects of PTMs [211]. Although CRISPR/Cas9 is advancing the field of biology on a big scale, the more advanced CRISPR techniques have been suffering from low efficiency. However, as the methods and procedures for single amino-acid substitutions and knock-in insertions are improved [212-214] so will our abilities to probe the functional effects of multiple modified sites. With that said, the PTMs occurring in the cell can be very rapid and are highly dynamic in order to accommodate constantly changing signals in the cell. In the case of phosphorylation for example, an amino acid substitution can be utilized to mimic a phosphorylated form, e.g. serine to glutamate (S → E). These types of PTM-mutants where the protein is locked in a certain position mimicking phosphorylation can give rise to phenotypes similar to in a phosphorylated situation. These mimics may however not behave as their true physiological counterparts not only because of the biochemical difference in glutamate compared to phosphorylated serine but also as in vivo phosphorylations are often rapidly reversed. This is another highlight of the complexity and difficulty of accurately registering and studying such rapid and dynamic effects. Nevertheless, new emerging technologies continue to push the envelope on what is technologically possible and will in the future allow for decoding the complexity associated with Notch output and help us devise new therapeutic strategies targeting PTMs.

A largely unstudied layer of regulation of Notch is by the PTMs of the auxiliary proteins of the Notch pathway, i.e. of proteins that bind to the trimeric-complex of NICO/MAML/CSL and facilitate either gene expression or repression. Indeed, many contact-dependent signaling pathways rely on dynamic switching between activation and repression states mediated by co-activators and co-repressors leading to changes in specific chromatin and histone tail modifications. In the case for Notch certain examples of PTM-mediated co-ordinator control exist, such as that of the p21-activated kinase PAK1 that has been shown to phosphorylate SHARP thus enhancing the SHARP-mediated repression of Notch target gene expression [215]. Similarly, the Notch-repressor Nuclear hormone receptor corepressor (nCoR) is phosphorylated by CK2α [216] and this phosphorylated nCoR can bind SHARP together
with HDAC thus replacing the Notch-activating Lysine methyl-transferase 2D (KMT2D) [217]. These complicated relationships between Notch-complex cofactors and chromatin modifying proteins are outside the scope of this review, however the examples herein aid in illuminating the importance of this research area, which will hopefully lead us closer to a complete understanding of the harmonious symphony of PTMs in the Notch pathway.

7. Conclusion

PTMs add a crucial layer of fine-tuning and regulation of cellular processes. PTMs can provide an immediate response to cell cues to modulate protein function, including stability, protein-protein interactions, localization and activity. The highly dynamic nature of many PTMs makes the characterization and functional relationship between different modifications a big challenge. Furthermore the latest development of techniques have not been fully utilized within the Notch field to understand the intricate effects that PTMs can have on the cellular decisions and their contribution to pathophysiology. Advancements in enrichment methods and in high-resolution MS have led to an enormous increase in data, but the biological relevance and function of the identified sites as well as their combinatorial effects are largely unknown. Generating multiple phosphospecific antibodies by for instance using phage display libraries could show the status of many individual phosphosities during different timepoints and reveal the activation state of Notch components. In silico methods such as applying computational algorithms to combine different datasets, provides a promise for untangling the highly dynamic processes of PTM crosstalk, protein-protein interactions and their combined effect on cellular output. Combining the latest advancements in PTM identification with functional predictions of their output on function could help explain a great deal of the pleiotropism of Notch signaling.

Author contributions

D.A., C.A, K.K. and S.K.-J.L. and C.S. researched data for the article and wrote the manuscript. All authors contributed to discussion of content and reviewed the manuscript before submission.

Transparency document

The Transparency document associated with this article can be found, in online version.

Declaration of Competing Interest

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