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Activated Notch undergoes DTX4-dependent bilateral endocytosis upstream of  
ADAM10 processing

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Running title: DTX4-dependent early steps of Notch activation

## 1   **Summary**

2   The Notch signaling pathway is a major regulator of cell-fate decisions, the stimulation  
3   of which is initiated through cell-cell contacts. Mammalian Notch1 is present at cell  
4   surface as a heterodimer of Notch extracellular domain associated to the  
5   transmembrane and intracellular domains. After ligand binding, Notch undergoes  
6   activating proteolysis to eventually stimulate its target genes. Combining biochemistry,  
7   immunofluorescence and living cell imaging of Notch1-expressing cells, we monitor the  
8   early steps of activation. We show that upon ligand binding, Notch and the DTX4 E3  
9   ubiquitin ligase are first clustered at the cell surface. Notch is ubiquitinated in a DTX4-  
10   dependent manner and the two subunits of Notch are internalized separately by a  
11   mechanism of bilateral endocytosis: Notch extracellular domain in the ligand-expressing  
12   cell, and the membrane-anchored fragment, together with DTX4, into the Notch cell.  
13   Eventually, the recruitment in the same intracellular compartment of Notch and of the  
14   ADAM10 metalloprotease allows activation to proceed.

15

## 1    **Introduction**

2    The Notch signalling pathway plays multiple roles in organisms ranging from worms to  
3    mammals, during development and in adulthood. Deregulation of Notch signalling is  
4    associated with many cancers, including leukaemia and solid cancers [1], but also with a  
5    number of inherited and acquired diseases [2]. Notch activation relies on the contact  
6    between neighbouring cells, which allows the binding *in trans* of a membrane-anchored  
7    ligand to an adjacent receptor. *Drosophila* has one Notch and two ligands; mammals  
8    have 4 Notch receptors and 5 ligands, yet the activation mechanisms are mostly  
9    conserved along evolution. Mammalian Notch 1 receptor consists in a transmembrane  
10    heterodimeric protein, the extracellular part of which is attached to the membrane-  
11    anchored and intracellular domains through non-covalent linkages. Upon ligand binding  
12    to Notch, the first step described so far is the so-called transendocytosis event, which  
13    creates a mechanical force that dissociates Notch heterodimer and promotes a  
14    conformational change [3-5]: Notch extracellular domain is internalized together with  
15    the ligand into the ligand-expressing cell, whereas the remaining subunit of the receptor  
16    becomes a substrate for a disintegrin and metalloprotease (ADAM) protease, ADAM10  
17    or 17, which cleaves Notch 12 amino acids above the transmembrane domain [6-10].  
18    Notch is eventually processed by the  $\gamma$ -secretase activity, and the resulting soluble  
19    intracellular domain (NICD) [11] migrates to the nucleus where it plays transcriptional  
20    and non-transcriptional functions [12]. Despite much progress in the understanding of  
21    the molecular mechanisms of this unique pathway, a number of questions remain open:  
22    what are the first events taking place into the Notch expressing cell upon activation?  
23    What are the accessory factors at these early steps? When and where is ADAM protease  
24    recruited to activated Notch?

1 One of the first genes identified in *Drosophila melanogaster* as an actor of the Notch  
2 pathway was *deltex* (*dx*, [13,14]). Mutations in the *dx* gene produced phenotypes similar  
3 to those resulting from loss-of-function mutations in the Notch receptor, suggesting that  
4 Deltex is an activator of Notch signalling. However the specific role of the Deltex protein  
5 in fly and in other organisms has remained controversial so far, since the effect of  
6 inhibiting or mutating Deltex can vary depending on context [15]. *Drosophila* Deltex has  
7 been implicated in mediating Notch early endocytosis and trafficking to the lysosomes  
8 [16-20]. In mammals, there are 5 Deltex-related proteins (DTX1-4, and a distantly  
9 related protein DTX3L). DTX3 and DTX3L are devoid of the N-terminus containing the 2  
10 WWE modules that account for direct interaction with Notch. However all DTXs have a  
11 highly conserved carboxyl-terminal E3 ubiquitin ligase domain of the RING (really  
12 interesting new gene) family [21,22]. In addition to their putative role regarding Notch  
13 signalling, few has been described concerning their involvement in other processes [23-  
14 25].

15 With the initial goal of identifying which DTX isoform plays a role in mammalian Notch  
16 signalling, we have begun to unravel the initial steps of Notch activation. We were  
17 eventually able to visualize these early stages and to revisit the current model of  
18 transendocytosis by identifying novel steps, including DTX4-dependent ubiquitylation  
19 and bilateral endocytosis. Finally, we show that ADAM10 is required after Notch  
20 internalization.

## Results

### DTX4 is involved in Notch1 activation

To monitor Notch1 activation, we made use of murine OP9 cells expressing the ligand Dll1 (OP9-Dll1), and of human U2OS cells expressing Notch1 (these cells are hereafter named N cells) (Figure 1). Although N cells were obtained after retroviral transduction [26], the level of expression of Notch1 in these cells is in a physiological range (see Figure S1 A, B). N cells were transfected with siRNAs targeting each of the DTX isoforms that contain the WWE domain accounting for interaction with Notch [22]. Notch transcriptional activity was monitored by qRT-PCR of an endogenous target (Hey1), and siRNAs were controlled in the bottom graph (Figure 1A). As expected, Hey1 transcription was stimulated when N cells were in contact with Dll1 cells, in a  $\gamma$ -secretase-dependent manner since DAPT treatment abolished activation. Silencing DTX1 or DTX2 had no effect on Notch activity, whereas inhibiting DTX4 led to a 40% decrease of Hey1 stimulation. Silencing the three DTXs had no additional effect on Hey1, suggesting that DTX4 is the major DTX necessary for Notch activation in this cell culture model. To eliminate possible off-target effects, the impact of another siRNA targeting DTX4 was monitored using a reporter system based on a synthetic Notch-responding promoter driving the expression of luciferase (CSL-LUC) (Figure 1B). We observed a similar reduction of CSL-LUC of 40%. Overexpressing murine DTX4 restored high level of Notch stimulation (Figure S1C). Additionally we generated N-derived cells, where DTX4 expression was abolished after genome editing (CRISPR/Cas9 technology). Notch stimulation was decreased by 70% in these cells (Figure 1C), although Notch expression was not affected (Figure 1C), showing that DTX4 could be facilitating but not absolutely

essential for Notch activation. Taken together, these results demonstrated that DTX4 is an important Notch positive regulator in our cell culture model.

#### **DTX4 interacts with Notch at the cell surface and in vesicles.**

We next wanted to identify the precise step in Notch activation process involving DTX4. We generated stable cell lines from N cells, expressing the DTX4-GFP or DTX4-Cherry chimera, both able to complement DTX4 KD in the transcriptional assay (Figure S1C). We observed a partial co-localization of DTX4-GFP with full-length Notch by immunofluorescence of permeabilized (Figure 1D, panels 4-6) and non-permeabilized cells (indicated as NP, panels 1-3). Both proteins were detected at the cell surface and in intracellular vesicles. To avoid any localization artefact due to fixation [27], we observed the protein distribution by live-cell imaging. As shown in Figure 1E and in Movie 1, DTX4-GFP and DTX4-Cherry exhibited a punctate pattern in living cells, probably corresponding to membrane-associated DTX4. The slightly different fluorescence patterns between GFP and Cherry chimera could be due to different expression levels, or could reflect two distinct populations of DTX4 highlighted by the two fluorophores, as it was described for the Notch receptor [28]. However overexpressing DTX4-Cherry into DTX4-GFP stable cell line revealed that both proteins were mainly co-localized (Figure 1D, panels 7-9, compare with mCherry in panels 10-12), and later results (activation movies and Figure 5) further showed that both DTX4 chimera behave the same regarding Notch activation process and therefore recapitulate DTX4 functions. We also established a stable cell line from U2OS cells, named NgfpDC, expressing DTX4-Cherry and a fusion protein between Notch and GFP. As control, GDC cells express GFP and DTX4-Cherry. On one hand, Notch was co-immunoprecipitated with DTX4 from DTX4-GFP cells (Figure 1F, lanes 1-4), on the other hand DTX4-Cherry was specifically co-

immunoprecipitated with Notch in NgfpDC cells (lanes 5-8). Therefore DTX4 is able to interact with Notch.

#### **DTX4 is necessary for a very early step of Notch activation.**

Because of DTX4 plasma and vesicular membrane localization, and of previous work involving *Drosophila* Deltex in Notch endocytosis [17,19,20], we monitored the effect of DTX4 silencing on the event described so far to be the first step of Notch activation in mammals: the transendocytosis of the Notch extracellular domain into the ligand-expressing cell [3]. N cells were transfected with siRNAs to silence endogenous targets, then co-cultured with Dll1 cells for 4 hours before immunofluorescence using antibodies recognizing the extracellular domains of Notch (HA) and of Dll1 (VSV). DTX4-silenced cells allowed much less transendocytosis of Notch into the Dll1 cells than control cells (Figure 2A, quantified in 2D). A similar effect was observed with a different siRNA targeting the UTR of DTX4 (and not DTX1) and was partially complemented by DTX4 overexpression (Figure S2A). DTX4 silencing did not impair Notch-Dll1 interaction, since HA and VSV staining on non-permeabilized cells were always co-localized at cell contacts (Figure 2B), not did it affect Notch expression at cell surface (Figure S2B). These results were in accordance with the requirement of DTX4 for Notch transcriptional activity observed in Figure 1, and showed that DTX4 is crucial for a step occurring after ligand binding to Notch but upstream transendocytosis.

#### **Clathrin-dependent endocytosis in the Notch cell contributes to transendocytosis in the ligand cell.**

Endocytosis of Dll1 is necessary to pull Notch extracellular domain into the Dll1-expressing cell [3]. We asked whether endocytosis events in the Notch-expressing cell

could additionally participate in creating the force necessary to dissociate Notch heterodimer. As for DTX4, silencing of Dynamin2 or AP2 ( $\alpha$  or  $\mu$ 2 subunits) decreased the number of transendocytosis events (Figure 2C, quantifications in 2D) without impairing Notch and Dll1 interaction (Figure 2B). Expression of a dominant negative form of Dynamin2 in N cells also induced cell surface HA-VSV co-localizations (Figure S2C). These results strongly suggested that additionally to endocytosis in Dll1 cells, both DTX4 and early endocytosis in N cells are necessary for the dissociation of Notch heterodimer.

#### **DTX4 is required prior to ADAM10 in the Notch activation process.**

The production of the transcriptionally active form of Notch requires two successive proteolytic events, realized by an ADAM protease [9,10] (mainly ADAM10 [6,29]) and by the  $\gamma$ -secretase proteolytic complex [11]. ADAM proteases are believed to act at the cell surface [30], a possibility that would imply that ADAM10 functions before Notch endocytosis. ADAM10 silencing in the Notch-expressing cells did not affect the efficiency of Notch transendocytosis into the Dll1 cell (Figure 3A), confirming that ADAM10 acts after Notch heterodimer tearing [3], and therefore suggesting that ADAM10 could act downstream Notch endocytosis. ADAM10 requirement in our co-culture model was confirmed since Notch-driven luciferase activity was inhibited both by transfecting ADAM10-targeting siRNAs and by using a selective inhibitor of ADAM10 activity, GI 254023X (hereafter named GI, Figure 3B). Interestingly we observed cumulative effects when inhibiting DTX4 and ADAM10 in the luciferase assay (lanes 5-8). Two models could explain these results: either DTX4 is necessary before ADAM10, or DTX4 acts in an alternative activation pathway independent from ADAM10. To discriminate between these two possibilities, we treated the co-cultured cells with increasing concentrations

1 of GI (Figure 3C). When GI concentration was maximal, Notch reporter activity was  
2 dramatically reduced, showing that Notch activation was fully dependent on ADAM10  
3 activity. Inhibition of DTX4 did not have any additional effect. If DTX4 and ADAM10  
4 were participating in two separate paths, one should have expected to have a constant  
5 ratio between GI-dependent and DTX4-dependent inhibition and therefore to obtain  
6 parallel curves giving the variation in luciferase activity as a function of the GI  
7 concentration. Since we obtained converging curves, we concluded that DTX4 and  
8 ADAM10 work in the same activation pathway. Therefore and in accordance with  
9 transendocytosis results, DTX4 acts upstream ADAM10 on Notch.

#### 11 **DTX4 is involved in Notch ubiquitylation at the outset of activation**

12 Since using co-culture with Dll1-expressing cells precludes any time course analysis of  
13 Notch activation, we made use of soluble Dll1-Fc [31]. The pre-clustered Dll1-Fc  
14 (hereafter named Dll-Fc), coated on plastic, activates Notch to the same extent as Dll1-  
15 expressing cells, and with the same sensitivity to DTX4 silencing and ADAM10 inhibition  
16 (Figure 3B). Therefore DTX4 function is not dependent on the presence of Dll1 cells, and  
17 on a first pulling force provided by endocytosis in these cells. Next we incubated NDC  
18 (expressing HA-Notch and DTX4-Cherry) or N cells (expressing HA-Notch) with Dll-Fc  
19 for various period of time at 37°C, and monitored Dll-Fc, DTX4 and Notch localization.  
20 Notch binding to Dll-Fc induced clustering of the receptor (compare panels 10-12 to 4-6  
21 in Figure 4A) and DTX4-Cherry relocalization to the Dll-Fc-positive spots (panels 7-9  
22 and 1-3). Thus upon Dll-Fc binding, the heterodimeric Notch receptor was clustered  
23 together with DTX4 in spots that could correspond either to plasma membrane domains  
24 or endocytic vesicles. Co-localization of Notch, Dll-Fc and DTX4 in NDC cells,  
25 permeabilized or not, (Figure S3A) showed that Notch extracellular domain and Dll-Fc

1 were present at the cell surface in these structures. In NC cells (i.e. cells expressing HA-  
2 Notch and mCherry), there was no enrichment of mCherry in the Notch clusters,  
3 showing that DTX4 accounted for DTX4-Cherry clustering (Figure S3B). Moreover GI or  
4 DAPT treatments did not affect the formation of Dll-Fc/Notch clusters and the  
5 recruitment of DTX4 (Figure S3C). Finally we obtained the same clustering and co-  
6 localization of Notch, DTX4 and Dll-Fc with the NgfpDC cells, where both Notch and  
7 DTX4 are fluorescent (Figure S3D). Therefore DTX4 is recruited (or enriched) to cell  
8 surface Dll-Fc-bound Notch, in accordance with an early function in the activation  
9 process.

10 We next monitored Notch ubiquitylation upon Dll-Fc treatment. The overall  
11 ubiquitylation level associated to Notch dramatically increased after 30 minutes of Dll-  
12 Fc incubation in NgfpDC cells (Figure 4B, lanes 9-10), and even more after Dyn2  
13 silencing (lane 12 and quantification in the table below), in accordance with  
14 ubiquitylation taking place before endocytosis. In contrast, Notch ubiquitylation was  
15 diminished when silencing both DTX4 and Dyn2 (lane 11). Ubiquitylation of full-length  
16 human Notch1 in N cells was similarly dependent on DTX4 and stabilized by Dyn2  
17 inhibition (Figure S4A). Finally, expressing His-tagged ubiquitin in N cells and purifying  
18 ubiquitylated products under denaturing conditions [26] confirmed that Notch was  
19 directly modified by ubiquitin in a DTX4-dependent manner (Figure S4B). Taken  
20 together, these results strongly suggested that Notch and DTX4 clustering upon Dll-Fc  
21 activation induces DTX4-dependent Notch ubiquitylation before its internalization.

## 22 23 **Visualizing Notch activation in living cells**

24 We verified that Notch-GFP activation in NgfpDC cells followed a normal process: after  
25 culture on coated Dll-Fc, the  $\gamma$ -secretase processed Notch was generated (Figure 5A, lane

2, S3 band), and was no longer detected in the presence of DAPT (lane 3); instead the S2 species (GFP blot) corresponding to the ADAM cleavage product was accumulated. In addition, Dll-Fc treatment on NgfpDC cells induced clustering of Notch-GFP and DTX4-Cherry (Figure S3D), and Notch ubiquitylation (Figure 4C), just as in N or NDC cells. We thus used the NgfpDC cells to perform live imaging of Notch activation. Notch clustering occurred rapidly after adding Dll-Fc into the culture medium (in less than one minute) and extended along the whole plasma membrane over time (Movie 2 and Figure 5B). DTX4 followed Notch along this process (see enlargements in 5B, blue arrows). Surprisingly, the GFP signal evolved over time, and we observed moving vesicles in the cells that probably detached from the membrane clusters by endocytosis (Figure 5B, yellow arrows). About 30-60 minutes after Dll-Fc addition, these moving vesicles (positive for Notch and DTX4) were dominant over static, membrane-attached clusters (Movies 3 and 4, Figure 5C). DAPT or GI treatment had no effect (Movies 5, 6). These movies were consistent with clustering of Notch and DTX4 being a prerequisite for their endocytosis and suggested that DTX4 escorts Notch over all the first steps of activation. Since DTX4-Cherry fluorescence was hardly detected, we also used the DTX4-GFP cells and observed the DTX4 clustering and movements into vesicles with very similar kinetics as in NgfpDC cells after Dll-Fc addition (Movie 7).

## **ADAM10 acts on Notch after internalization**

In order to visualize the spatio-temporal recruitment of endogenous ADAM10 to Notch, we performed Notch activation by adding Dll-Fc in the presence of a well-characterized monoclonal antibody recognizing ADAM10 extracellular domain [32] to follow the fate of the protease, and with GI to prevent Notch cleavage. Notch and DTX4 clustering have begun after 15 minutes of stimulation (Figure 6A, panels 1-4, yellow arrows), whereas

1 ADAM10 co-localized neither with Notch nor DTX4. ADAM10-labeled endocytic vesicles  
2 were detected after 30 to 60 minutes, and most of them were positive for Notch and  
3 DTX4 (white arrows in Figure 6A). Notch and ADAM10-positive vesicles were not  
4 detected in non-permeabilized cells (Figure S5A), confirming that they are intracellular.  
5 Thus most Notch and DTX4-positive clusters that were negative for ADAM10 remained  
6 probably at the cell surface (yellow arrows in 6A). This result suggested that ADAM10  
7 recruitment to activated Notch does not occur at cell surface but rather after  
8 internalization of the receptor (together with DTX4) and of ADAM10. Thus we wondered  
9 whether ADAM10 recruitment to Notch required endocytosis of both proteins. We  
10 treated the cells in the presence or absence of Dll-Fc and anti-ADAM10 antibody with  
11 Dynasore, a cell-permeable inhibitor of Dynamin-dependent endocytosis [33]. In the  
12 presence of Dynasore (Figure 6B, panels 8-10), Dll-Fc-induced clustering of Notch still  
13 occurred, but Notch was mostly retained at the cell periphery, and Notch-ADAM10 co-  
14 localization dropped (compare panels 3-5 and 8-10, quantifications in Figure 6C),  
15 although ADAM10 internalization still occurred. Transfecting N cells with siRNAs  
16 targeting DTX4, Dyn2 or AP2 confirmed these observations, since Notch-ADAM10 co-  
17 localization percentage also dropped (Figure S5B). These results suggested that Notch  
18 could meet ADAM10 following DTX4 action and endocytosis. Since Notch and ADAM10  
19 co-localization was not a direct way of measuring Notch processing by ADAM10, we  
20 monitored ADAM10 cleavage of Notch in western blot after activating NgfpDC cells on  
21 coated Dll-Fc for 7 hours. Dll-Fc-dependent activation resulted in the production of S3,  
22 the  $\gamma$ -secretase processed form of Notch (Figure 6D, lane 3), however in the presence of  
23 DAPT we observed instead the stabilization of S2, the ADAM10 cleavage product (lane 4).  
24 In the presence of Dynasore, S3 and S2 products were barely detected (lanes 5, 6 and  
25 quantification below), in accordance with Dynamin-dependent endocytosis of Notch

1 being required upstream ADAM10 cleavage. Since long Dynasore treatment affected cell  
2 shape, we checked that Notch was still expressed at the cell surface and regularly  
3 matured by biotinylation and purification of cell surface proteins (Figure S5C). All  
4 together, these results strongly suggested that ADAM10 could fulfil its function on the  
5 receptor in the post-endocytic vesicles where activated Notch and ADAM10 co-localize.  
6 To eventually confirm that these first steps of Notch activation are the same, whether  
7 the receptor is activated by Dll-Fc or by cell-cell contact, we co-cultured NgfpDC with  
8 OP9-Dll1 cells for 4 hours, in the conditions of transendocytosis observation (Figure 7A,  
9 panels 2-8). We observed a significant accumulation of Notch and DTX4-Cherry together  
10 with the ligand at the cell-cell contacts, as well as internal vesicles positive for Notch and  
11 DTX4 (panel 2 and enlargements). This was reminiscent of the clusters in the presence  
12 of Dll-Fc and of the cell aggregation described by other [34-37]. When incubating the  
13 anti-ADAM10 antibody to the co-culture for an additional 30 minutes, we observed  
14 some co-localization of ADAM10 with Notch, but only when cells were permeabilized  
15 after fixation (compare panels 6-8 to 3-5 in Figure 7A), confirming that indeed Notch co-  
16 localized with ADAM10 after endocytosis of both proteins.

## Discussion

### Revisiting the course of events of Notch activation

Using two ways of activating Notch (Dll1-expressing cells and Dll-Fc), we monitored the fate of the two fragments of Notch1 heterodimer. Our work highlights new steps in Notch activation, schematized in Figure 7B: the re-localization of ligand-bound receptor at the cell surface together with DTX4 (boxes 1 and 2), Notch ubiquitylation in a DTX4-dependent manner (box 2), and subsequent endocytosis of the receptor (box 3). These events are prerequisite for the transendocytosis of Notch extracellular domain into the ligand-expressing cell. In addition, our results strongly suggest that ADAM10 is not required at the cell surface for Notch shedding, but instead in endocytic vesicles (box 3). In addition to co-culture of Notch1 and of Dll1-expressing cells, we have used pre-clustered Dll-Fc ligand to mimic the first events of Notch activation. We believe that this approach is valid since under both conditions Notch activation process has the same need for DTX4 and endocytic events before ADAM10 recruitment, and the same sensitivity to processing enzymes inhibitors. In both conditions, we first observed a clustering of Notch at the cell surface, next Notch detachment into vesicles that form even when inhibiting ADAM10 or  $\gamma$ -secretase, suggesting that Notch is not processed yet in these endocytic vesicles. The time course of these events is in accordance with published work showing cluster movements of ligand-bound Notch [37,38]. The Notch and DTX4-containing moving vesicles that we observed in our movies mostly after 30 minutes of activation appear to be very rapid, with average maximal speeds of about 1  $\mu\text{m}/\text{sec}$ , and they travel over long distances, in accordance with movements along microtubules [39]. Nevertheless it is possible that Dll-Fc in-solution treatment only recapitulates the first steps of Notch activation (until Notch meets ADAM10-positive

vesicles), since Dll-Fc, instead of being retained outside the Notch cells by Dll1-expressing cell or plastic coating, could remain bound and be unable to participate in Notch heterodimer dissociation. This could explain the accumulation of moving vesicles along time in our movies and the lack of nuclear GFP staining.

## **Roles of DTX4 in Notch signaling**

Inhibiting endogenous DTX4 expression by siRNA or by genome editing decreased Notch-dependent transcriptional activation significantly although not totally. In addition, biochemistry, immunofluorescence and *in vivo* imaging show that DTX4 acts early in Notch activation. Therefore our results do not demonstrate an obligate role for DTX4 in the Notch pathway, but strongly suggest that DTX4 is important to effectively activate Notch in our cell model. It is possible that in different cell or tissue contexts, other DTX isoforms are required for the same function. What is the molecular basis of such specificity will need further investigations.

We show here that DTX4 co-localizes and co-immunoprecipitates with Notch, even in the absence of activation, consistent with possible additional functions in the degradation of non-activated Notch, such as recruitment of arrestins to Notch [40,41]. Upon Dll1 binding, Notch becomes ubiquitinated in a DTX4-dependent manner, suggesting that DTX4 E3 ubiquitin ligase activity is turned on by an unknown mechanism that may depend on the clustering induced by the ligand. We also show that Notch ubiquitination precedes Notch internalization, since Dynamin2 silencing stabilizes Notch ubiquitinated species. DTX4-dependent ubiquitination of Notch, or/and receptor clustering at the plasma membrane could locally concentrate endocytic adaptors and initiate endocytosis. As DTX4 is able to interact with Grb2 [42], it could also indirectly activate Dynamin's GTPase activity and oligomerization, eventually favouring endocytic

vesicles pinching off [43]. Our results also reveal that DTX4 remains associated with Notch after endocytosis at least until ADAM10 recruitment. In accordance with our previous work suggesting that activated Notch is associated with DTX1 and the deubiquitinase eIF3f upstream  $\gamma$ -secretase processing [26], DTX4 could have this additional role of recruiting eIF3f to activated Notch after endocytosis.

### **Prerequisite and concurrent events to transendocytosis**

Our results confirm and complete previous work on transendocytosis of Notch [3,44]. It was recently shown that signal-sending cells supply sufficient force to induce metalloprotease sensitivity in the Notch NRR (Negative Regulatory Region) and to allow Notch activation [5]. However, as underlined by the authors, their *in vitro* experiments might not recapitulate all the *in vivo* parameters, including membrane microenvironment, interactions of the receptor with other factors or ADAM availability. In addition only a very recent study addressed whether endocytic events take place in Notch cells upon stimulation [37]. Although it shows that contact with cells expressing the ligands induced internalization and intracellular trafficking of Notch1, the conclusion on transendocytosis is divergent from our observations, since we rather demonstrate that clathrin-dependent endocytosis of the activated receptor contributes to transendocytosis. Therefore Notch conformational change necessary to open up ADAM cleavage site does not rely only on the mechanical strength provided by ligand and Notch extracellular domain co-internalization [3], but instead requires a bilateral action provided on one side by ligand internalization (transendocytosis strictly speaking), and on the other side by the internalization of the transmembrane subunit of the activated receptor. The respective requirement/importance of each event may

depend on each tissue context, which defines *in vivo* specific mechanical properties, and need to be more extensively studied.

#### **ADAM10 does not act on Notch at the cell surface**

The involvement of ADAM proteases in Notch signalling has been described 15 years ago [9,10], and confirmed by the fact that mice, worms, and flies lacking ADAM10 resemble Notch1-deficient phenotypes [8,45,46]. Although ADAM metalloproteases are believed to generally allow ectodomain shedding of their substrates at the cell surface [47,48], the cleavage of Notch by ADAM10 was largely diminished when impairing Notch internalization. Additionally, we were unable to detect cell surface co-localization of Notch and ADAM10, whether Notch was activated by Dll-Fc or by Dll1-expressing cell. These results are in perfect accordance with the requirement of ADAM10 after Notch early endocytosis, and imply that ADAM10 and Notch are first internalized separately, next meet after fusion of vesicles. Chen et al. have proposed recently that instead of being spatially and temporally separated, the  $\alpha$ - and  $\gamma$ -secretases, which act on various substrates including APP and Notch, are physically linked in high molecular weight complexes that facilitate sequential processing of substrates [49]. If so, APP and Notch should be targeted by different complexes, at the cell surface for APP, and in the endocytic pathway for Notch, in accordance with our data for ADAM10 and with previous results for the  $\gamma$ -secretase [26,50-54]. We have recently shown that ADAM10 associate with several members of a subgroup of tetraspanins referred to as TspanC8, which differentially impact its ability to mediate Notch signalling [55]. It will be interesting to determine whether these differences are linked to differences in ADAM10 trafficking. All these observations suggest the existence of distinctly composed

- 1 multisecretase complexes that might have specialized abilities on specific substrates, as
- 2 well as specific working places in the cells.
- 3
- 4

## **Materials and Methods**

### **Plasmids, Reagents, antibodies**

Notch-GFP construct was obtained by inserting the GFP fragment, obtained by PCR amplification, in frame at the ECoRV and XbaI sites of murine Notch1 [57]. GFP was inserted at amino acid 2097 of murine Notch, after the 6th ankyrin domain and replaced the C-terminus (including the PEST domain). DTX4-Cherry and DTX4-GFP constructs were obtained by inserting murine DTX4 at the HindIII-EcoRI sites of pmCherry-N1 and pEGFP-N1 vectors (Clontech) respectively. DAPT was from Calbiochem, GI 254023X (GI) and Dynasore from Sigma. The rabbit polyclonal anti-Notch1 antibody, as well as the mAb directed to human ADAM10 (11G2, IgG1) have been previously described [32,58]. Commercial antibodies used in this study were as follows: monoclonal anti-HA (HA11 from Covance, 16B12, Alexa Fluor conjugated from Life technologies), rabbit polyclonal anti-mCherry (Clontech, DsRed), rabbit polyclonal to cleaved Notch1 and mouse anti-Ubiquitin from Cell Signaling (#2421 and #3936 respectively), monoclonal anti-VSV P5D4 Cy3-conjugated from Sigma. Alexa Fluor conjugated secondary antibodies were from Life technologies.

### **Cell Culture, Transfections, and siRNAs**

OP9 cells expressing the human Notch ligand Dll1 (OP9-Dll1) and the human osteosarcoma cell line U2OS expressing human Notch1 (here named N cells) have been previously described [26]. NDC, NC cells and DTX4-GFP cells were obtained by transfecting N cells with DTX4-Cherry, mCherry and DTX4-GFP vectors respectively, NgfpDC (and GDC cells) were established by transfecting U2OS cells successively with vectors expressing DTX4-Cherry and Notch-GFP (or GFP). Stable cells were sorted by Flow-cytometry. NDC, NgfpDC and DTX4-GFP clonal populations were obtained by additional limiting dilution.

siRNAs were transfected using jetPRIME transfection reagent (Polyplus Transfection) or Lipofectamine RNAimax (Invitrogen) for immunofluorescence, DNA vectors were transfected with Fugene HD (Promega), according to manufacturer's instructions.

The siRNA sequences were DTX1 (5' CAGAGAGAACCCAGAGUUAdTdT-3'), DTX2 (5'-AGGGAAAGAUGGAGGUAAUU[dT][dT]-3'), DTX4 targeting the UTR (5'-CAGUAGGGAUCUUGAAUUU[dT][dT]-3') were purchased from Sigma-Aldrich. The siRNA DTX4 targeting the coding sequence (5'-GGAUCGACCUCACUCCAUC-3') was obtained from Thermo-Scientific.

#### **Generation of N DTX4 KO cells.**

CRISPR/Cas9-mediated gene editing was performed in N cells to obtain DTX4 gene knockout cells. Guide RNAs were identified using Crispor online tool. Two guide RNAs (5'TTCCATGTCGTAGGGCGTCC, and 5'G TTCAGAGCCAGGCACTAGT), targeting sequences in the second exon and in the second intron of hDTX4 gene respectively, were cloned into the sgRNA(MS2) plasmid (Addgene). These plasmids were transfected together with the human codon optimized Cas9-expression vector (pSpCas9, Addgene) into the N cells, 3 days later cells were cloned by limiting dilution. Clones where the intervening DNA between the two targeted guide sites has been deleted on both alleles were selected by PCR analysis of genomic DNA (primers: 5' TGCAAACCAAGATGTCCCGA, and 5'TCCCTGTACCCAAAATGCCC). Two clones were obtained, with similar results regarding Notch activation.

#### **Activation assays**

Co-culture of Notch-expressing cells with Dll1-expressing cells: Notch-expressing cells were seeded at 15 or 20,000 cells/cm<sup>2</sup>, transfected with siRNAs 8 or 18 hours later, and if necessary with DNA vectors the next day. 24 hours later, cells were co-cultured with OP9 or OP9-Dll1 at 25,000 cells/cm<sup>2</sup> for 18 hours for Notch activity assays. For

transendocytosis assay, both cell types were trypsinized, mixed and seeded at 20,000 cells/cm<sup>2</sup> (each) for 4 hours.

Activation by Dll-Fc: Dll-Fc was obtained from 3-days conditioned medium of HEK293 cells expressing the fusion protein (provided by Dr. Weinmaster, [31]). Pre-clustering was either in solution with 13 mg/ml of goat anti-human Fc antibody (Sigma) for 1 hour at 4°C, or on plates coated with the antibody. For immunostaining or live imaging, pre-clustered Dll-Fc was diluted twice with DMEM, and then used to replace cell culture medium on cells grown in complete medium for 24h on coverslips. When indicated, cells were pre-incubated for 1 hour with GI or DAPT before activation, and anti-ADAM10 mAb 11G2, GI or DAPT were added to the mixture.

### **Analysis of Notch activity**

Quantitative Real-Time PCR: Total RNA extraction was performed using the RNeasy Mini Kit purchased from Qiagen. Reverse transcription was done using the Qiagen Omniscript Reverse Transcription Kit. Quantitative PCR was then performed using the Takyon™ No Rox SYBR MasterMix dTTP Blue kit from Eurogentec and analyzed using a CFX96™ real-time PCR detection system under the CFX Manager™ Software (Bio-Rad). Gene's expressions were reported to hypoxanthine-guanine phosphoribosyltransferase (HPRT) or to the ubiquitin-conjugating enzyme UBCH5. Oligonucleotides were all purchased from Eurogentec and sequences are listed in the following table:

hHPRT for	5'-TAATTGGTGGAGATGATCTCTCAAC-3'
hHPRT rev	5'-TGCCTGACCAAGGAAAGC-3'
hUBCH5 for	5'- TGAAGAGAATCCACAAGGAATTGA-3'
hUBCH5 rev	5'- CAACAGGACCTGCTGAACACTG-3'
hHey1 for	5'-GCCTCCTATAGCAGAAAGGTGA-3'

hHey1 rev	5'-GCAGCTGGTCAGATGGATTTC-3'
hDTX1 for	5'-CTGTTCTCCCATCCTCCCTA-3'
hDTX1 rev	5'-CCTTTGGCTCTCAAAATTGG-3'
hDTX2 for	5'-CCTGCAAAACCATCTATGGAG-3'
hDTX2 rev	5'-GCGACATCTGGAACCGTAAT-3'
hDTX4 for	5'-AGCACCAGCAGCCTAGTTTC-3'
hDTX4 rev	5'-GCATCTGACGAGGTTTGTCTC-3'
hADAM10 for	5'-AAACACCAGCGTGCCAAAAG-3'
hADAM10 rev	5'-CCCTCTTCATTTCGTAGGTTGAAA-3'

1

2 Luciferase assay: Cells were transfected with the CSL-LUC reporter and Renilla plasmid  
3 24 hours before coculture. Cell lysates were prepared using a Dual luciferase reporter  
4 assay according to the manufacturer's instructions, and the activities of firefly and  
5 renilla luciferases were determined using a luminometer Centro XS (Berthold).

## 6 **Ubiquitylation assays**

7 Notch-GFP from NgfpDC cells was immunoprecipitated using GFP-trap® coupled to  
8 agarose beads, according to the manufacturer's instructions (Chromotek). For His-Ub-  
9 containing products purification, cells were lysed in 8 M urea, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 10 mM  
10 Tris pH 8, 1% Triton X-100, 20 mM Imidazole at room temperature. His-Ub conjugates  
11 were purified on Ni-charged chelating Sepharose beads (Pharmacia). WCE and  
12 precipitates were analyzed by western blotting. Image acquisition was performed using  
13 myECLimager and quantification with myImageAnalysis Software (Thermo Scientific).

## 14 **Flow cytometry, Immunostaining, microscopy, and image analysis**

15 For flow cytometry analysis, N cells were harvested and suspended in cold PBS 48h after  
16 transfection with the siRNAs. Cells were fixed in 4% paraformaldehyde in PBS,

immediately labeled with anti-HA or anti-ADAM10 antibodies at 4°C before Al-488-labeled secondary antibodies. Cell parameters were monitored using a CyAn flow cytometer (Beckman Coulter), and analyzed using FlowJo software. For immunofluorescence, cells were grown on glass coverslips. Cells were fixed with 4% paraformaldehyde, permeabilized with PBS containing 0.2% Triton X-100 for 5 min before the incubation with appropriate antibodies. Cells preparations were mounted in Mowiol 488 supplemented with DABCO (Sigma). Images were acquired using an AxioImager microscope with ApoTome system with a 63x magnification and AxioVision software (Carl Zeiss MicroImaging Inc., Le Pecq, France) for figures 2, 3, 4, figure 2 supplements 1 and 3, figure 4 supplements. Post-acquisition image analysis was performed in Icy software [59]. The undecimated wavelet transform Spot Detector plugin and the Colocalizer protocol were used to quantify the number of vesicles and the colocalized vesicles respectively. For figures 1D, 6, 7 and figure 6 supplement 1, image acquisition was performed using a confocal inverted Microscope LSM700 under Zen 2010 software (Carl Zeiss MicroImaging Inc.), and deconvolution was done with Huygens professional software. For live imaging, cells were grown on glass bottom microwell dishes (MatTek corp., Ashland, MA), and acquisition was done in an incubation chamber at 37°C under 5% CO<sub>2</sub> using a spinning-disk UltraView VOX inverted microscope, equipped with a Yokagawa CSUX1 spinning-disk, and two cameras to do dual-color simultaneous imaging, controlled by Volocity software (PerkinElmer, Waltham, MA). The GFP and Cherry signals were always acquired simultaneously; z was fixed along the acquisition. Green and red fluorescence were excited with 488-nm and 561-nm diode lasers and collected by a dual “green – infra-red” 500 – 550 nm & 680 – 750 nm filter.

## **Statistical analysis**

1 Statistical analysis was performed using Prism 6.0 (GraphPad) by one-way ANOVA  
2 followed by the Bonferroni's multiple comparisons test. Statistically significant  
3 differences are indicated with asterisks: \*\*\*\* $p < 0.0001$ , \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ ,  
4 n.s.  $p > 0.05$ .

## 7 **Author Contributions**

8 PC and CB conducted the experiments; ER and CB designed the experiments and wrote  
9 the paper.

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## Figure Legends

### Figure 1: DTX4 is a membrane-associated positive regulator of Notch signaling

**A, B.** N cells were transfected with siRNAs (NT= non-targeting, two different siRNAs targeting DTX4 were used in A and B) before co-culture with OP9-Dll1 or control OP9 cells. In A, RNAs were extracted and human Hey 1 and DTXs were quantified by qRT-PCR. Data are normalized to UBCH5B and represented as mean  $\pm$  standard error of the mean (SEM) of triplicates. A is representative of at least 3 independent experiments. In B, N cells were transfected with both CSL-LUC (Notch reporter) and TK-renilla (internal control) 24h before co-culture. The reporter activation is the ratio between Firefly and Renilla luciferase activities. Data represent the mean with SEM over 5 experiments, where 100% is the stimulation in control conditions. **C.** As in B, except that the N cells were either WT, or genome-edited to impair DTX4 expression (N DTX4 KO). Data represent the mean with SEM over 3 experiments, each condition performed twice in triplicates in each experiment. Below N and N DTX4 KO WCE are analysed by WB. White lane indicates that intervening lanes have been spliced out. **D.** DTX4-GFP-expressing N cells were fixed, permeabilized (P) or not (NP) before incubation with anti-HA antibody and appropriate secondary antibody (panels 1-6). The same cells were transfected with expression vectors encoding for DTX4-Cherry (panels 7-9) or mCherry alone (panels 10-12). Hoechst labeling is in blue. Scale bar in the first panel indicates 10  $\mu$ m. Insets correspond to 3-fold enlargements of the boxed regions. Arrows indicate co-localized vesicles. **E.** Confocal images extracted from living cells movies of N cells expressing DTX4-GFP (1), mCherry (2), DTX4-Cherry (3). **F.** Extracts (WCE) or immunoprecipitates (IP GFP) from various cell lines analysed by Western Blot with the indicated antibodies. TM-IC is the mature form of Notch, resulting from Furin cleavage. Lanes 1-4, grey

1 indicates the remaining Notch signal after the second incubation with anti-GFP antibody.

2 The apparent molecular masses (kDa) are indicated.

3 **Figure 2:** Transendocytosis is affected by events occurring in Notch expressing cells.

4 **A.** N cells transfected with the indicated siRNAs were co-cultured with OP9-Dll1 for 4  
5 hours before immunofluorescence using fluorescent anti-HA (green) and anti-VSV (red).

6 In the 4th row overexposed red labeling shows the outline of Dll1-expressing cells. **B, C.**

7 As in A, except that cells were permeabilized (C) or not (B) before immunofluorescence.

8 Stars indicate the nuclei of the murine OP9-Dll1 cells. Scale bars in first panels indicate

9 10  $\mu$ m. **D.** The percentage of co-localizations was determined using the Colocalizer

10 protocol under Icy software, as the ratio between HA and VSV positive spots over the

11 total VSV spots. Equal acquisition settings were used within each experiment. The

12 graphs represent mean with SEM from 5 independent experiments (100 to 160 cells

13 from 30 to 60 fields). Statistically significant differences are compared to the NT conditions.

14 **Figure 3:** ADAM10 acts downstream DTX4 and transendocytosis in the Notch pathway.

15 **A.** Transendocytosis when ADAM10 is silenced was measured as in Figure 2. In the  
16 second graph, data analysed from 3 independent experiments (more than 60 cells from

17 30 fields) by an unpaired t-test showed no significant difference (n.s.) and are

18 represented as mean with SEM. The third graph shows the normalized expression of

19 ADAM10/HPRT RNAs quantified by qRT-PCR. **B.** CSL-LUC activity was obtained as in

20 Figure 1, either after co-culture with Dll1-expressing cells, or after culture on Dll-Fc-

21 coated plates. Treatments with DAPT or GI (sub-optimal concentration) were applied all

22 along co-culture. No stimulation was in the absence of Dll1. Error bars are the SD of

23 triplicates of an experiment representative of 3. **C.** Curves giving the variation in

24 luciferase activity as a function of the GI concentration, depending on the transfected

25 siRNAs. 100% is Luciferase activity in the absence of GI in control conditions (si NT).

**Figure 4:** First events in the Notch-expressing cells upon activation by Dll-Fc.

**A.** NDC (panels 1-3, 7-9) or N cells (4-6, 10-12) were incubated with Dll-Fc for 0 (1-6) or 30 minutes (7-12) at 37°C, then fixed, permeabilized and incubated with anti-Fc (green) and anti-HA antibodies (in N cells, red), and appropriate secondary antibodies. In NDC cells, red labelling corresponds to DTX4-Cherry protein. Hoechst labelling is in blue. Insets correspond to 3-fold enlargements of the boxed regions; arrows indicate examples of co-localized vesicles. Scale bars in first panels indicate 10  $\mu$ m. **B.** GDC or NgfpDC cells were transfected with the indicated siRNAs before activation with Dll-Fc for 30 minutes at 37°C in the presence of GI, then harvested. Extracts (WCE) were immunoprecipitated with GFP-trap and analyzed by western blotting with the indicated antibodies. White lane indicates that intervening lanes have been spliced out. On the left are the MWs in kDa. The bracket indicates Notch-derived ubiquitylated products; TM-IC is the mature form of Notch. The table gives the quantification of the ubiquitylated/total immunoprecipitated Notch reported to control conditions, and the extinction of exogenous DTX4 by the siRNAs (means from two experiments).

**Figure 5:** Live imaging of Notch-GFP activation.

**A.** NgfpDC cells were co-cultured for 18 hours in the presence or not of DAPT with coated Dll-Fc when indicated, cell extracts were analysed by Western blot with anti-GFP or V1744 antibody (specific for the activated,  $\gamma$ -secretase processed form of Notch, named S3). p300 is the proform of Notch (not furin-processed), TM-IC is the non-activated, mature form of Notch, S2 and S3 result from ADAM and  $\gamma$ -secretase processing respectively. \* indicates the 80 kDa marker. **B.** Sequential confocal images from Movie 2, showing the activation process after Dll-Fc addition during 40 minutes. Right are shown 3-fold enlargements of the squared boxes at each time point, with Notch-GFP in green and DTX4-Cherry in red. Blue arrows indicate immobile clusters, yellow arrows mobile

vesicles. **C.** Sequential confocal images (4 s apart) of NgfpDC cells, 60 minutes after the addition of Dll-Fc, extracted from Movie 3. Scale bars indicate 10  $\mu$ m. The tracking of 4 rapidly moving vesicles (4 colors) is indicated in green, as well as their position on each image by arrowheads.

**Figure 6:** ADAM10 is recruited to Notch after internalization.

**A.** NgfpDC cells were incubated at 37°C with GI, Dll-Fc and an antibody recognizing ADAM10 extracellular domain for 15 (1-4), 30 (5-8) or 60 minutes (9-12), then fixed, permeabilized and incubated with a secondary antibody labelling anti-ADAM10. Insets correspond to 3-fold enlargements of the boxed regions; yellow arrows indicate examples of Notch and DTX4 co-localized vesicles, devoid of ADAM10 staining, white arrows point to vesicles with the three proteins. **B.** NgfpDC cells were incubated at 37°C with GI and anti-ADAM10 extracellular domain for 60 minutes, in the presence or absence of Dll-Fc and Dynasore (Dyna) as indicated. Cells were then fixed, permeabilized and incubated with a secondary antibody labelling anti-ADAM10. Insets correspond to 3-fold enlargements of the boxed regions; white arrows point to vesicles with the three proteins in 3-5. In A and B, the single channel pictures are grey scale black and white images. **C.** The percentage of Notch-ADAM10 co-localization was determined using the Colocalizer protocol under Icy software, as the ratio between HA- and ADAM10-positive spots over the total HA spots. Equal acquisition settings were used within each experiment. The graphs represent mean with SEM from 2 independent experiments (respectively 342, 245 and 143 cells for the three conditions). Statistically significant differences are compared to the NT conditions. **D.** NgfpDC cells were cultured for 7 hours on coated Dll-Fc in the presence or not of DAPT and Dynasore, as indicated. Cell extracts were analysed by Western blot with anti-GFP, V1744 and tubulin antibodies. TM-IC (S1) is the non-activated, mature form of Notch, S2 and S3 result from ADAM and  $\gamma$ -secretase

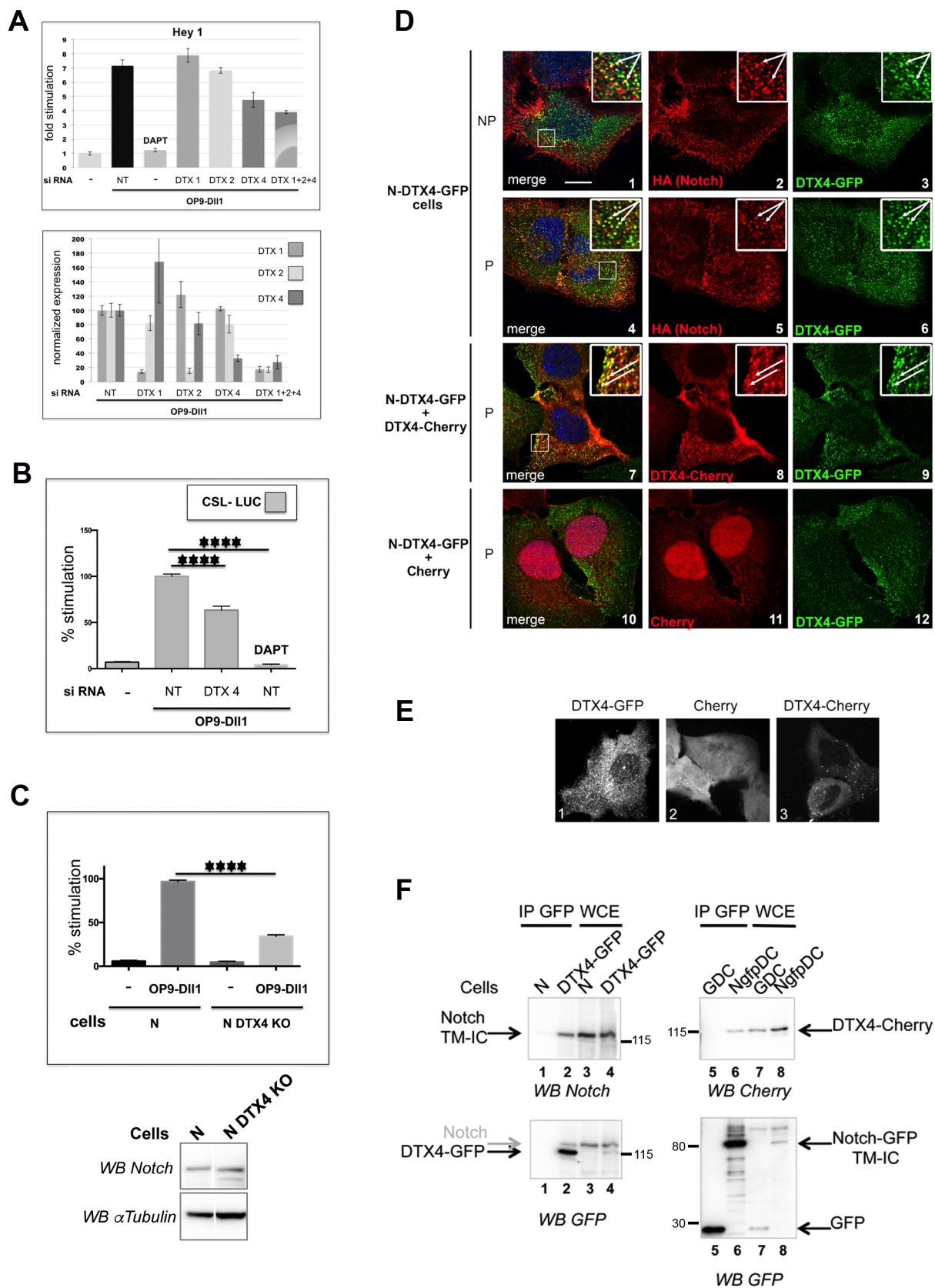
processing respectively. The apparent molecular masses (kDa) are indicated. Below is the quantification of S2 percentage, as the ratio of S2 over (S1 + S2) in the presence of DAPT. The graph represents means with SEM from 6 independent experiments, with statistical analysis by unpaired t test.

**Figure 7: ADAM10 recruitment to Notch in co-culture.**

**A.** NgfpDC cells were co-cultured with OP9-Dll1 for 4 hours, next anti-ADAM10 was added for 30 min (panels 3-8) before fixation and immunofluorescence. Cells were permeabilized (P) or not (NP). Secondary antibodies are coupled to Alexa 647 (blue in panel 2, red in 3-8). Panel 2, a star indicates the position of the closest murine Dll1-expressing cell of the Notch-expressing cell (N), anti-VSV was used to label Dll1. Insets on the right correspond to the boxed regions, green arrows indicate a probable endocytic vesicle, positive for Notch and DTX4 but devoid of VSV-staining. In 3-8, insets correspond to 3-fold enlargements of the boxed regions; white arrows indicate Notch and ADAM10 positive vesicles. Scale bars indicate 10  $\mu$ m.

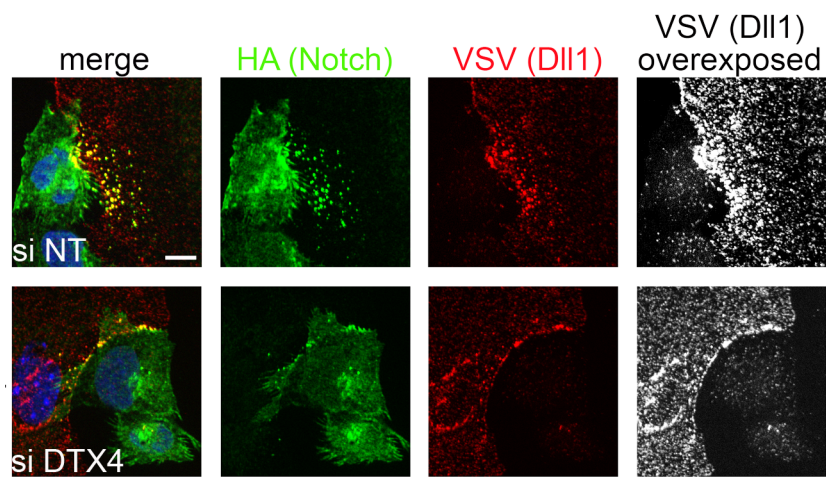
**B.** Model depicting the early steps of Notch activation. Upon Dll1 binding (1), Notch (green) and DTX4 (blue) are clustered, DTX4-dependent ubiquitylation (orange) of Notch leads to the recruitment of the endocytic machinery, including Dyn2 and AP2 complex (2), allowing bilateral endocytosis and eventually Notch heterodimer dissociation (3). ADAM10 is internalized and acts next on Notch in endocytic vesicles (3).

**Figure 1**



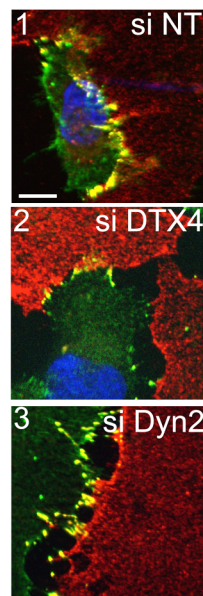
**Figure 2**

**A**



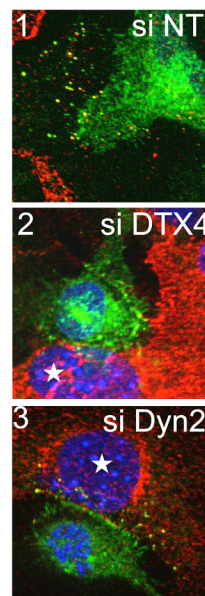
**B**

*non-permeabilized*



**C**

*permeabilized*



**D**

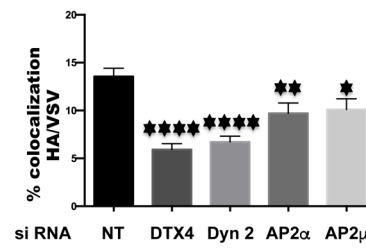


Figure 3

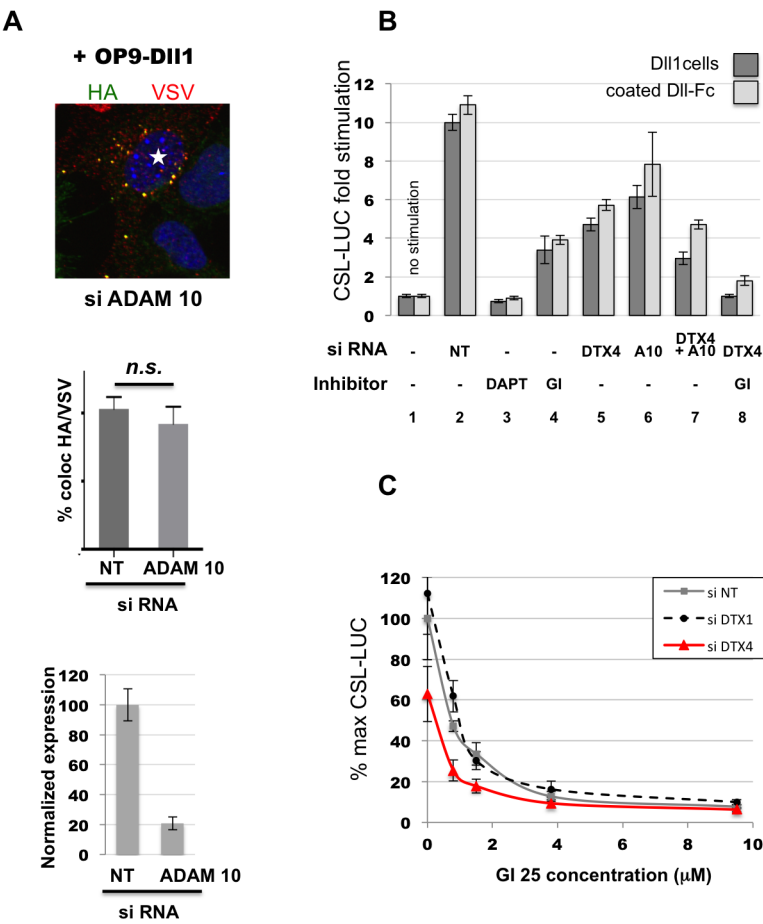


Figure 4

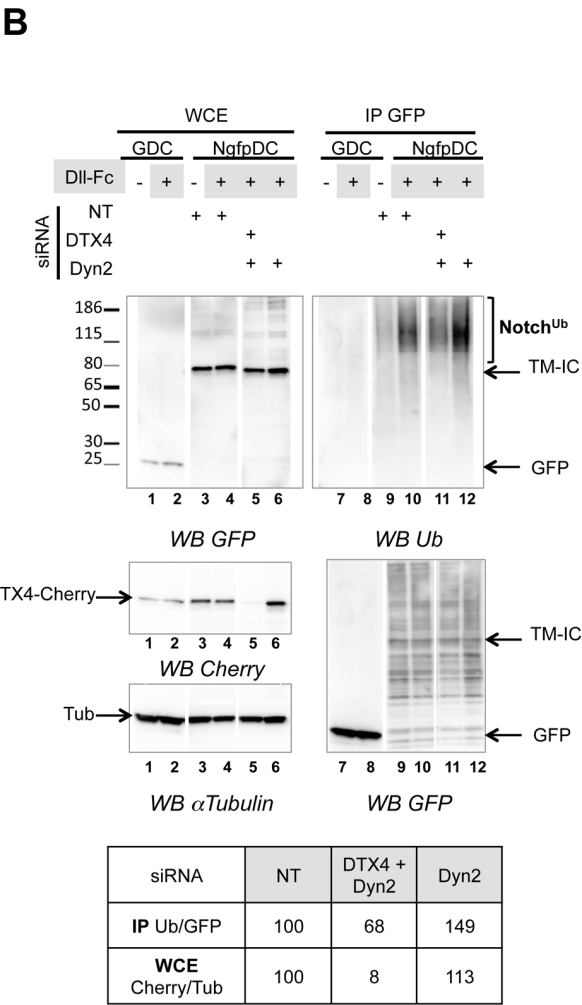
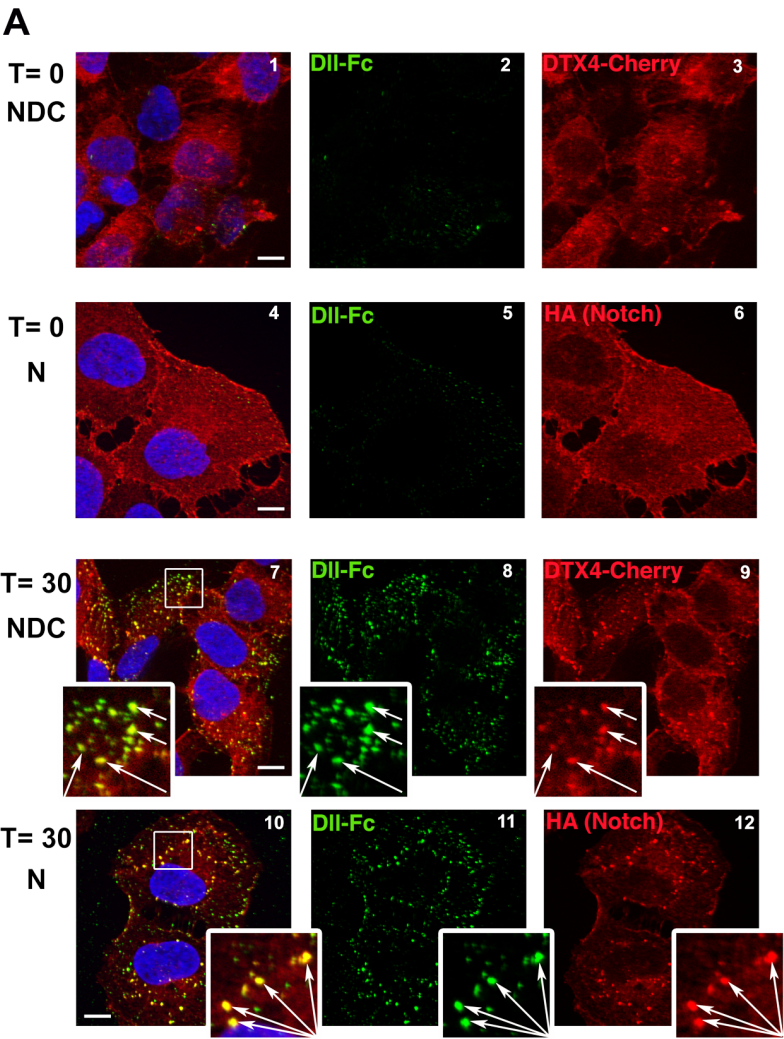
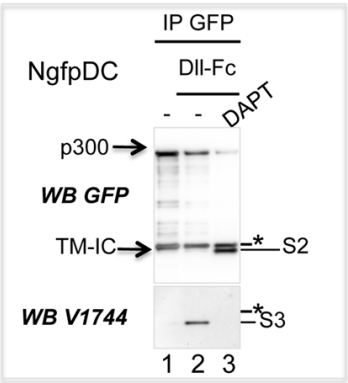
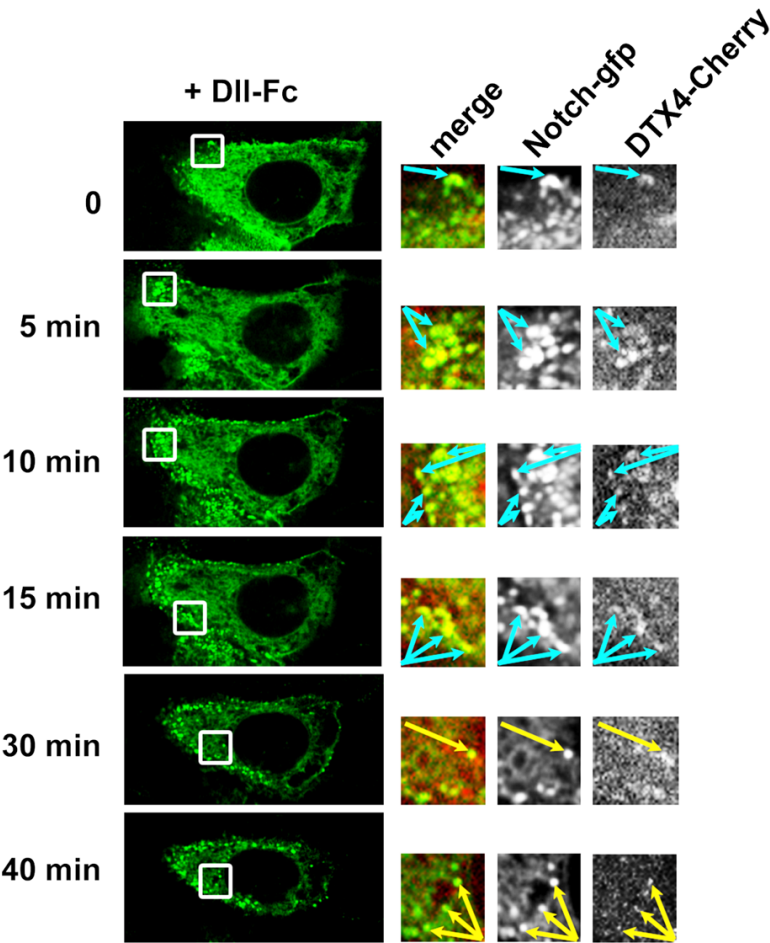


Figure 5

A



B



C

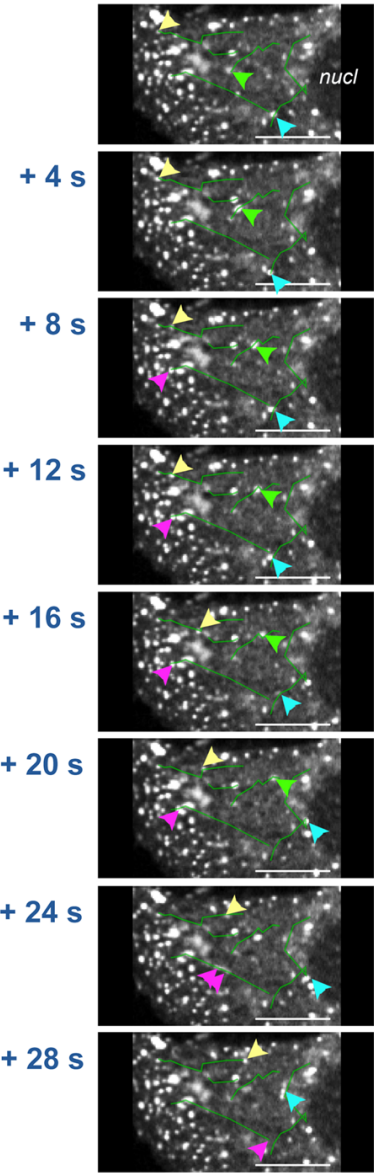
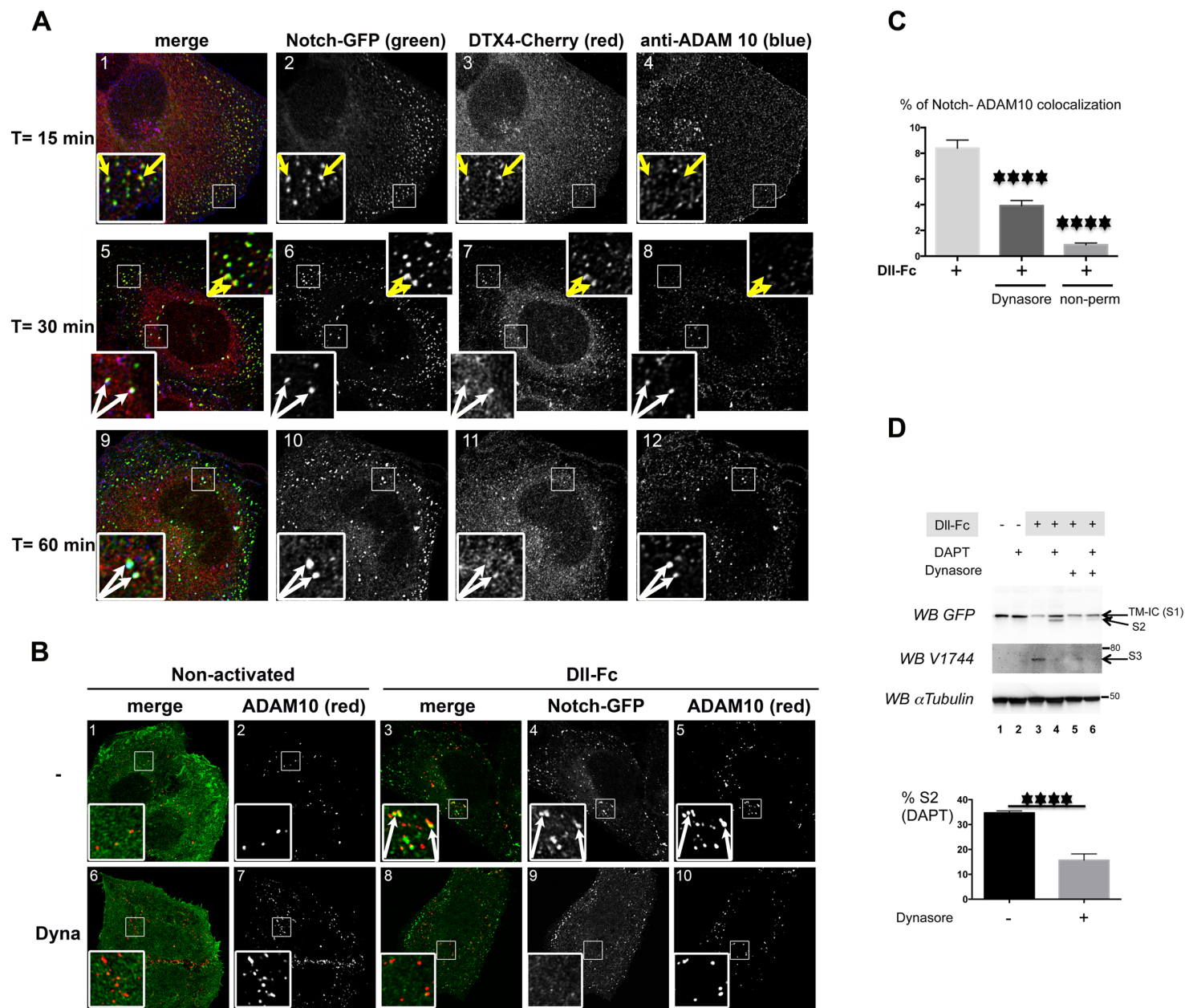
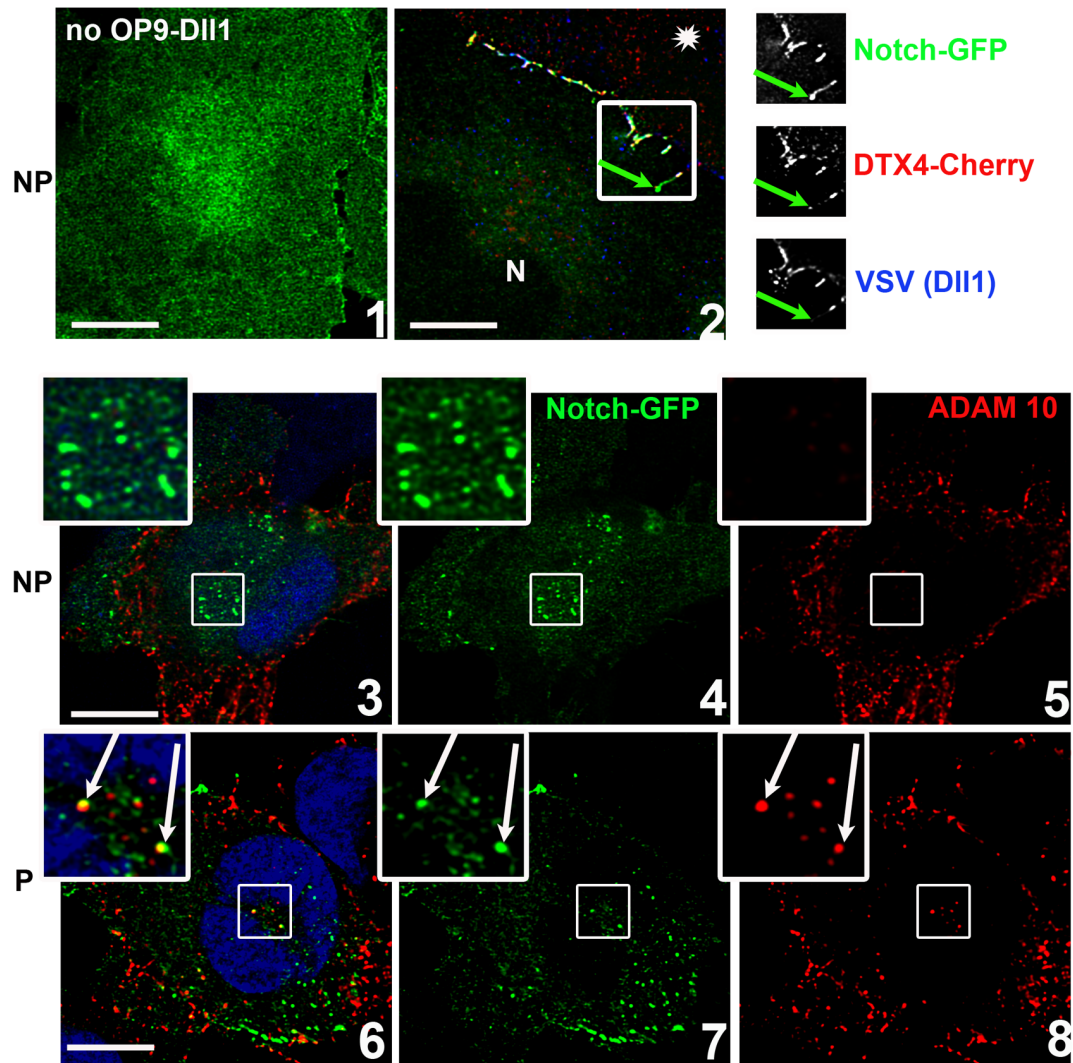


Figure 6



# Figure 7

## A



## B

