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*Biochem J.* Author manuscript; available in PMC 2014 June 10.

Published in final edited form as:
*Biochem J*. 2011 July 15; 437(2): 313–322. doi:10.1042/BJ20110226.

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# Cleavage of Notch1 by granzyme B disables its transcriptional activity

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## Abstract

Granzyme-mediated cell death is the main pathway for cytotoxic lymphocytes to kill virus-infected and tumour cells. A major player in this process is GrB (granzyme B), which triggers apoptosis in both caspase-dependent and caspase-independent pathways. A caspase-independent substrate of GrB is the highly conserved transmembrane receptor Notch1. The GrB cleavage sites in Notch1 and functional consequences of Notch1 cleavage by GrB were unknown. In the present study, we confirmed that Notch1 is a direct and caspase-independent substrate of GrB. We demonstrate that GrB cleaved the intracellular Notch1 domain at least twice at two distinct aspartic acids, $Asp^{1860}$ and $Asp^{1961}$. GrB cleavage of Notch1 can occur in all subcellular compartments, during maturation of the receptor, at the membrane, and in the nucleus. GrB also displayed perforin-independent functions by cleaving the extracellular domain of Notch1. Overall, cleavage of Notch1 by GrB resulted in a loss of transcriptional activity, independent of Notch1 activation. We conclude that GrB disables Notch1 function, probably resulting in anti-cellular proliferation and cell death signals.

### Keywords

cleavage; granzyme B; Notch signalling; proteolysis; $\gamma$-secretase; serine protease

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## INTRODUCTION

The immune system uses two types of cells as its primary defense against tumour cells and viral pathogens, i.e. NK (natural killer) cells and CTLs (cytotoxic T-lymphocytes). Both types of cytotoxic cells harbour cytotoxic granules that are released upon target cell recognition. These granules contain serine proteases, also called granzymes, and the pore-

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**AUTHOR CONTRIBUTION:** Marc Vooijs, Geert van Tetering, Niels Bovenschen and Paul J van Diest conceived and designed the experiments. Geert van Tetering and Jan Meeldijk performed the experiments. Geert van Tetering analysed the data. Geert van Tetering and Marc Vooijs wrote the manuscript.

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forming protein perforin. Although perforin pores facilitate the entry of granzymes into the target cell, the latter trigger apoptosis by cleaving critical death substrates [1].

Of the five human granzymes, GrB (granzyme B) is the most potent granzyme in inducing apoptosis [2]. GrB initiates target cell apoptosis in two ways. First, it cleaves the BH3 domain protein Bid, thereby activating the Bax pathway that leads to mitochondrial cytochrome *c* release. This leads to the formation of the Apaf1 apoptosome, resulting in the activation of pro-caspase 9/3 and subsequent DNA fragmentation and cell death. Secondly, GrB triggers apoptosis by directly cleaving and activating pro-caspase 3 [2]. Caspase activity can be efficiently and irreversibly inhibited by specific synthetic peptides, such as Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone) [3].

Besides activating caspases, GrB also has substrates that contribute to a caspase-independent cell death. One of these substrates of GrB is the intracellular domain of Notch1, a type I transmembrane receptor [4]. The Notch1 receptor is part of a highly conserved signalling pathway essential in controlling spatial patterning, morphogenesis and cellular homoeostasis in embryos and adults. Notch receptors (N1 to N4) are transmembrane glycoproteins that transduce signals by binding to membrane-bound ligands (i.e. Delta, Jagged) on adjacent cells. During maturation and transport, Notch1 is cleaved in the Golgi by a furin-like convertase (S1), resulting in a heterodimeric receptor. Upon ligand binding at the cell surface, Notch receptors undergo two successive proteolytic cleavages: an ectodomain cleavage (S2), followed by intramembrane proteolysis by $\gamma$-secretase (S3; Figure 1A) [5]. This process releases the NICD (Notch intracellular domain), which translocates to the nucleus and binds the transcription factor CSL [CBF1/suppressor of Hairless/ Lag-1; RBP-J$\kappa$ (recombination signal-binding protein 1 for J$\kappa$) in mice], participating in a complex activating its target genes [6-10]. Notch proteolysis is controlled by conformational changes in the extracellular domain induced by ligand binding or cancer-prone oncogenic mutations [11]. Increasing evidence suggests that additional proteases are capable of activating or inactivating Notch receptor signalling; however, the significance of this remains unclear.

Notch1 was identified as a GrB substrate in an *in vitro* proteomics screen [4]. *In vitro* validation studies demonstrated Notch1 is cleaved by GrB primarily at a single unidentified site, yet more fragments are observed at higher GrB concentrations, indicative of multiple cleavages. Experiments exposing cell lysates to purified GrB showed this cleavage occurs in cells in a caspase-independent manner. Moreover, NK-cell-mediated killing of intact K562 cells resulted in a similar cleavage of Notch1 as seen *in vitro* and in cell lysates. This indicated physiological granzyme levels are sufficient to cleave Notch1 in a caspase-independent manner. Although Loeb et al. [4] identified Notch1 as a physiological GrB substrate, several questions remain regarding the mechanism and the consequence of GrB cleavage of Notch1. In the present study, we addressed where in the cell Notch1 is cleaved by GrB and at which cleavage sites, and its effect on Notch1 signalling activity. We show that Notch1 is cleaved by GrB at multiple sites *in vitro* and in living cells independent of its activation. Importantly, GrB cleavage occurs in all subcellular compartments and results in a loss of Notch1 transcriptional activity.

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## MATERIALS AND METHODS

### Cell lines

HeLa FRT cells were generated by transfecting the FRT-LacZeo plasmid according to the manufacturer's instructions (Invitrogen). Single integration was determined using Southern blotting (results not shown). pcDNA5-FRT-Notch1 constructs were stably integrated into the FRT site after selection of HeLa cells with 200 μg/ml Hygromycin B. The selected single integrant cells were maintained at 200 μg/ml Hygromycin B.

### GrB production and purification

Active recombinant human GrB and inactive control GrB-SA were expressed in *Pichia pastoris* and purified by cation-exchange chromatography as described previously [12,13]. GrB preparations were dialysed against TBS (Tris-buffered saline; 50 mM Tris/HCl, pH 7.4, and 150 mM NaCl) and stored at −80°C. GrB, but not GrB-SA, was active as determined by the small synthetic chromogenic substrate IETD-pNA (Ile-Glu-Thr-Asp–*p*-nitroanilide; Bachem) (results not shown).

### Plasmids and vectors

All mNotch1 plasmids were initially cloned into pCS2+ 6Myc as described previously [14]. Notch1 full-length, LNR, NEXT and NICD constructs were constructed as described previously (shown in Figure 1), HA–N1FL-6MT (where HA is haemagglutinin and N1FL is full-length Notch1) was a gift from R. Kopan (Washington University, St. Louis, U.S.A.). Mutations in Notch molecules were introduced by PCR-directed cloning. For *in vitro* transcription, all mNotch1 variants were cloned in the pSensor backbone (Promega), replacing the luciferase. For stable expression in FRT cell lines, Notch was subcloned into the pcDNA5 vector (Invitrogen). A promoter fragment containing 12× CSL synthetic binding sites in tandem (provided by S. Boyle, Washington University, St. Louis, MO, U.S.A.) was subcloned into pGL4.24 (Promega) and was used for Notch transcription assays. For normalization of transcription assays, a CMV-driven Gluc (*Gaussia* luciferase; NEB) tag was used. N-terminally *Gaussia* luciferase-tagged N1FL was created using PCR-directed cloning from the pGluc-Basic (NEB).

### *In vitro* translation

Notch1 was produced *in vitro* using the TNT® SP6 Coupled Wheat Germ Extract System (Promega) in combination with FluoroTect™ GreenLys *in vitro* Translation Labeling System (Promega). Gels were analysed on a Typhoon scanner (GE Healthcare).

### GrB assay

Cells were pre-treated with 100 μM Z-VAD-FMK in serum-free medium for 1 h. Cells were washed twice with PBS before incubation with the GrB mix, which consisted of the perforin analogue SLO (streptolysin O; 5 μg/ml), Z-VAD-FMK (100 μM), GrB or GrB-SA (diluted in TBS). The mix was added to the cells for 30 min at 37°C, after which the cells were washed with serum-free medium and lysed by the addition of 17 μl 4× Laemmli buffer to 50 μl serum-free medium. In case of *γ*-secretase inhibition, cells were treated with DBZ

(dibenzazepine; 200 nM) for 16 h before GrB incubation. For inhibition of ER–Golgi transport, brefeldin A (1 mg/ml stock in methanol) was used at a concentration of 2 μg/ml for 4 h at 37°C. GrB activity was blocked using up to 300 nM of the inhibitor Ac-IETD-CHO (*N*-acetyl-Ile-Glu-Thr-Asp aldehyde; Merck). The caspase-3/7 GLO assay (Promega) was used according to the manufacturer's instructions.

### Western blotting

Samples were boiled for 5 min at 95°C and run on an SDS/PAGE gel (8%), blotted onto a PVDF membrane, and incubated with antibodies overnight at 4°C. Antibodies used were anti-Myc (9E10) 1:5000, anti-cleaved Notch ($Val^{1744}$, Cell Signaling) 1:1000, anti-cleaved caspase 3 ($Asp^{175}$, Cell Signaling) 1:1000, anti-HA (12CA5) and anti-actin (MP Biomedicals) 1:10000.

### Transcription assays

HeLa cells were transfected with a pGL4.24 12× CSL Firefly luciferase reporter, pBasic-CMV. *Gaussia* luciferase was used as a transfection control. Cell samples were lysed using Passive Lysis Buffer and media was collected, spun down and diluted in Passive Lysis Buffer before analysis with the Dual luciferase kit (Promega) on a luminometer. Firefly values were corrected for the secreted *Gaussia* luciferase values, resulting in relative light units (RLU).

### Cell preparation for Grb incubation

HeLa FRT Notch1 wild-type cells were grown in a T165 flask until confluency. Cells were washed 3 times with 20 ml PBS, then incubated with 14 ml hypotonic PBS (5% PBS in $ddH_2O$) for 15 min at room temperature (25 °C). Cells were washed with vesiculation buffer (2 ml of 5 M NaCl, 50 μl of 1 M $MgCl_2$, 500 μlof 1 M KCl and 709 mg of $Na_2HPO_4$, pH 8.8) and incubated with 10 ml of vesiculation buffer for 90 min at 37°C. The buffer was collected and centrifuged at 250 ***g*** for 5 min at 4°C to get rid of cell debris. The supernatant was centrifuged at 100000 ***g*** at 4°C for 1 h. The pellet was resuspended in serum-free medium and divided over 3 wells of a 96-well plate for GrB incubation.

### Apoptosis assay

Cells were grown in 96 well plates. At 8 h after GrB incubation cells were trypsinized and centrifuged at 1500 rev./min for 5 min. Cells were resuspended in 50 μl Annexin V-binding buffer (140 mM NaCl, 4 mM KCl, 0.75 mM $MgCl_2$, 10 mM Hepes and 1.5 mM $CaCl_2$, pH 7.4) containing Annexin-V-Fluos (Roche) and 1 μg/ml propidium iodide and incubated for 15 min at room temperature. Before FACS analysis, samples were diluted with 150 μl Annexin V-binding buffer.

## RESULTS

### Notch1 is directly cleaved in vitro by GrB

To investigate cleavage of Notch1 by GrB in more detail, we produced a Notch1 protein mimicking the membrane-bound form that lacks most of the extracellular domain (termed



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NEXT), containing a C-terminal Myc epitope tag (Figure 1). NEXT proteins are constitutively active and a substrate for $\gamma$-secretase [15,16]. NEXT-6Myc recombinant protein was produced *in vitro* and visualized by SDS/PAGE after incorporation using fluorescently labelled lysine residues. *In vitro* translated NEXT proteins were incubated with increasing concentrations of a catalytically active GrB and a control mutant GrB-SA, which attenuates cleavage activity. GrB cleavage of Notch1 was completely inhibited using a GrB-specific inhibitor, ruling out possible contaminants present in the *P. pastoris* culture (see Supplementary Figure S1). We observed the appearance of several cleavage products with increasing concentrations of GrB within 60 min of incubation (Figure 2A). Even at the lowest concentration tested (5 nM of GrB) a cleavage product with an estimated size of 55 kDa was observed. With higher concentrations of GrB, an additional cleavage product of around 45 kDa was detected. At the highest concentrations, the GrB-SA also generated the 55 kDa cleavage product; however, to a much lesser extent than the wild-type GrB. Under these conditions, no 45 kDa product was observed with the GrB-SA, whereas full conversion from the 55 kDa precursor had occurred with wild-type GrB. Others have previously reported that the single SA mutation attenuated but does not completely abrogate granzyme activity *in vitro* [17,18]. To further investigate GrB cleavage products, we made use of the C-terminal Myc epitope. Myc immunoblotting revealed that both the 55 and the 45 kDa could be readily detected, supporting the notion that the 45 kDa product was produced from the 55 kDa precursor (Figure 2B). To see whether sequences within the transmembrane or extracellular domains were required for GrB cleavage, we also investigated whether the non-membrane bound form, called NICD (mimicking the $\gamma$-secretase cleaved form of Notch1), was also a substrate for GrB. *In vitro* produced NICD1 proteins were also efficiently cleaved by GrB, producing similar cleavage products of 55 and 45 kDa respectively (Figures 2C and 2D). These results indicate that the intracellular domain of Notch1 is a direct substrate of GrB *in vitro*.

### GrB cleaves Notch1 *in vivo* independent of caspases

To address if GrB also cleaves Notch1 under physiological conditions, we produced HeLa cells stably expressing a full-length constitutively active Notch1 receptor carrying a mutation (L1594P) similar to those found in human acute T-cell leukemias [19]. N1L1594P HeLa cells show ligand-independent Notch1 cleavage and activity as shown by immunoblotting and transcriptional reporter assays, which is fully blocked by incubation with the $\gamma$-secretase inhibitor DBZ (see Supplementary Figure S2).

Next, we incubated GrB with N1L1594P cells, in combination with SLO acting as a pore-forming protein allowing GrB to enter the cell (Figure 3A). Immunoblotting revealed a dose-dependent appearance of a 55 kDa fragment reaching a maximum with 450 nM of GrB. This 55 kDa fragment appeared similar to the 55 kDa fragment observed by *in vitro* cleavage of Notch1 by GrB. Notably at lower concentrations of GrB, little if no cleavage occurred compared with 600 nM of the catalytic site mutant GrB-SA. Interestingly, whereas *in vitro* cleavage by GrB led to the consecutive cleavage of the 55 kDa precursor to produce a smaller 45 kDa cleavage product, this was not readily observed in the Notch1-expressing stable cell line exposed to GrB; only upon enrichment could the fragment could be detected (see Figure 5B). Importantly, Notch1 cleavage could only be detected in the presence of

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both GrB and SLO, indicating that cellular uptake of GrB was required for cleavage (see Supplementary Figure S3).

To address whether the cleavage of Notch1 *in vivo* is caused by GrB through activation of a caspase-dependent pathway, we incubated cells with GrB and the cell-permeable pan-caspase inhibitor Z-VAD-FMK, which irreversibly binds to the catalytic site of caspases and inactivates its proteolytic activity including autoproteolysis. Incubation of GrB with HeLa N1L1594P cells caused a dose-dependent increase in the appearance of the 55 kDa cleavage product largely independent of caspase activity (Figure 3B, upper panel). Under these conditions, Z-VAD-FMK efficiently blocked caspase autoproteolysis as shown by immunoblotting for activated caspase-3 (Figure 3B, lower panel) and by a caspase-3/7 profluorescence substrate assay (see Supplementary Figure S4). Finally, Z-VAD-FMK blocked GrB-induced apoptosis as measured by annexin V/propidium iodide labelling and flow cytometric analysis (see Supplementary Figure S4). Thus GrB cleavage of Notch1 *in vivo* does not depend on caspase-dependent cleavage.

To address whether prolonged GrB activity led to a full conversion of the 55 kDa fragment and the formation of additional cleavage products, we incubated HeLa N1L1594P with 300 nM GrB for 30 min, after which cells were washed and incubated again for various amounts of time. We observed robust cleavage and production of the 55 kDa fragment after 30 min of GrB incubation. Continued incubation for up to 120 min reduced the presence of the 55 kDa cleavage product (Figure 3C). Furthermore, this reduction was accompanied by a concomitant reduction of the Notch1 full-length unprocessed and S1-cleaved Notch1 TMIC isoforms. By Myc immunoblotting, we could not detect the formation of the 45 kDa product nor of any other additional cleavage products generated from the precursor fragment within 2 h after GrB addition (Figure 3C). Taken together, these results indicate that GrB cleaves Notch1 *in vivo* independent of caspases.

## GrB cleavage is $\gamma$-secretase independent

Notch activity requires proteolytic processing by a metalloprotease and $\gamma$-secretase [15]. To address whether GrB-induced cleavage is dependent on $\gamma$-secretase activity, we pre-incubated HeLa N1L1594P cells with the $\gamma$-secretase inhibitor DBZ, after which GrB (with Z-VAD-FMK) was added for 30 min and cell extracts were analysed by immunoblotting. Whereas DBZ efficiently blocks cleavage of Notch1 at $Val^{1744}$, it did not influence GrB cleavage. Both in the presence and absence of DBZ, the 55 kDa cleavage product was readily observed (Figure 4). These data point to a role for GrB in cleavage of the membrane-bound Notch1 isoforms as well as the S3/$\gamma$-secretase cleaved NICD. This is supported by the observation that the full-length uncleaved Notch1 isoform also appears to be a GrB substrate (Figures 3C and 4).

## GrB cleaves Notch in all subcellular compartments

The previous experiments support a role for GrB cleavage of Notch1 independent of $\gamma$-secretase. Our results indicate that both the truncated constitutively active and full-length unprocessed forms of Notch1 are GrB substrates. To investigate in which subcellular compartments Notch is cleaved by GrB, we determined cleavage patterns in a step-by-step

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manner. First, to address whether full-length unprocessed Notch1 is a GrB substrate, we treated HeLa N1L1594P cells with brefeldin A, an inhibitor of anterograde ER-Golgi transport. Brefeldin A treatment blocked Notch S1 processing as expected [20,21], leading to an accumulation of the full-length unprocessed form of Notch at the expense of TMIC (Figure 5A). Upon incubation of these cells with GrB, cleavage of the unprocessed full-length precursor into the 55 kDa fragment was observed.

Next, we determined if the cell surface-bound signalling-competent Notch receptor is also a substrate for GrB. To address this, we enriched for plasma membrane-bound Notch1 receptors from cells using an osmotic based vesiculation process [22], whereby vesicles bud off the plasma membrane containing surface molecules. To circumvent the presence of S2 and S3 Notch-processing fragments in these vesicles, we used HeLa cells expressing the wild-type Notch1 receptor and all preparations contained the $\gamma$-secretase inhibitor. We incubated isolated vesicles directly with GrB and addressed whether membrane-bound Notch is a substrate. A clear enrichment of membrane-bound Notch1 (TMIC) and very little unprocessed Notch1 on the membrane was observed as expected [23]. Notch cleavage products of 55 kDa and 45 kDa were readily detected in vesicles exposed to GrB but not to GrB-SA. Furthermore, a loss of the unprocessed full-length isoform was observed as well (Figure 5B). These results indicate that the membrane S1-cleaved signalling-competent Notch 1 receptor is also a GrB substrate.

Both our *in vitro* and *in vivo* data suggest that NICD1 may be a direct GrB substrate (Figure 2). To address if NICD1 is a direct substrate in living cells, HeLa cells transfected with NICD1 were treated with GrB and lysates were analysed on immunoblots. In the absence of active GrB, NICD1 was expressed at the expected molecular mass as determined by C- and N-terminal antibodies (Figure 5C). Upon incubation with GrB, the expected 55 kDa processing fragment was observed by Myc immunoblotting. This indicates that the membrane-cleaved activated Notch1 isoform NICD is also a GrB substrate.

So far, our results have shown that all intracellular forms of Notch are subject to GrB cleavage, but recent observations point to a role of extracellular activity of GrB which is perforin-independent [24-26]. Therefore, we investigated whether GrB can induce cleavage in the mature cell surface-exposed NECD (Notch extracellular domain) as well. We expressed a full-length Notch1 protein fused to *Gaussia* luciferase at the extracellular N-terminus and with a 6Myc epitope tag at the intracellular C-terminus (Gluc-Notch1 wild-type). Release of *Gaussia* luciferase in the medium can be measured by a sensitive enzymatic assay directly on cell culture medium. HeLa cells were transfected with Gluc-Notch1 wild-type together with the multimerized 12× CSL binding site containing the Notch reporter plasmid driving Firefly luciferase expression. The fusion of *Gaussia* luciferase to the Notch receptor did not induce activation of the receptor (results not shown). These cells were incubated with GrB in the absence of SLO and *Gaussia* luciferase was measured in the medium 8 h later. A 3-fold significant increase in *Gaussia* luciferase in the medium was detected when cells were incubated with 300 nM GrB but not with GrB-SA (Figure 5D). Although GrB induced release of *Gaussia* luciferase into the medium, this did not lead to an increase in Notch transcriptional activity as measured by the 12× CSL Notch reporter assay.

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To determine whether GrB is capable of cleaving Notch in the non-matured intracellularly located NECD, we expressed an N-terminally HA-tagged and C-terminally Myc-tagged Notch molecule (HA-Notch1-6MT) in HeLa cells. After treatment of these cells with GrB in the presence of SLO, we analysed the N-terminus on HA immunoblots. As expected, Myc immunoblots showed the GrB induced expected 55 kDa fragment, in addition HA blots showed a concomitant reduction in size from the full-length unprocessed Notch1 isoform of approximately 55 kDa (Figure 5E). Interestingly, we did not observe additional cleavage products, suggesting that NECD is not a direct substrate for GrB.

### Identification of GrB cleavage sites in Notch

There are five predicted GrB cleavage sites with an aspartic acid residue in the P1 position within the Notch1 intracellular domain (Figure 6A) [4,27]. To identify the main cleavage site of GrB in Notch, we performed mutation analysis on the three conserved aspartic acid residues most proximal to the membrane (VLP**D**$^{1823}$, VDA**D**$^{1860}$ and VIS**D**$^{1902}$). Either or both of the mutations D1823Q and D1902E did not result in an abrogation of Notch cleavage since both 55 and 45 kDa fragments could be observed *in vitro* (Supplementary Figure S5). The aspartic acid mutant D1860A, however, revealed a blockade in Notch processing demonstrated by the accumulation of the full-length unprocessed NEXT molecule as well as a loss of the 55 kDa fragment (Figure 6B). The 45 kDa fragment, however, could be detected. This indicates that the 55 kDa fragment results from a cleavage at VDAD$^{1860}$. Interestingly, a shift in cleavage was observed, resulting in a 65 kDa fragment, reflecting cleavage at the more proximal aspartic acid residue VLPD$^{1823}$. By combining the D1860A and D1823Q mutations, we observed an accumulation of full-length NEXT and a loss of the 65 and 55 kDa fragments respectively. However, the 45 kDa fragment could still be observed, indicating GrB is capable of cleaving this site directly, independently of cleavage at Asp$^{1860}$, albeit less efficient. Only after mutating D1961A was production of the 45 kDa fragment blocked and only full-length NEXT protein could be observed (Figure 6B). Myc immunoblotting revealed that, in addition to the 55 and 45 kDa fragments, also the larger 65 kDa fragment was observed (results not shown).

In order to determine whether the cleavage sites identified *in vitro* correspond to the *in vivo* situation, we transfected HeLa cells with truncated Notch1 LNR constructs containing the same cleavage site mutations. As observed *in vitro,* mutation of D1823Q and D1902E did not abrogate Notch cleavage in cells (results not shown); however, the single D1860A mutant shows an accumulation of the full-length Notch precursor and TMIC, and the appearance of the 65 kDa fragment similar to *in vitro* cleavage of recombinant substrates (Figure 6C). Mutating both D1823Q and D1860A completely blocked cleavage in cells as well. In this cell-based assay we could detect the 45 kDa fragment in cells in the double/ triple mutant, indicating cleavage can take place at this site *in vitro* and *in vivo* independent of a proximal cleavage. The additional mutation at D1961A resulted in a complete loss of cleavage as seen in the *in vitro* results (Figure 6C). These results indicate that the main GrB cleavage sites in Notch1 are the aspartic acid residues Asp$^{1860}$ and Asp$^{1961}$.

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### GrB cleavage disables Notch activity

To address whether GrB cleavage affects Notch activation, we performed gene reporter assays to measure transcriptional response upon Notch1 activation in the presence or absence of GrB. HeLa N1L1594P cells were transfected with a multimerized 12× CSL binding site reporter driving firefly luciferase expression pre-incubated with DBZ and Z-VAD-FMK, after which they were exposed to GrB for 30 min, washed and allowed to recover for an additional 4 h, when extracts were analysed for reporter gene luciferase activity. As expected, DBZ reduced reporter gene activity up to 95% in HeLa N1L1594P cells that normally have high constitutive Notch activation (Figure 7). Exposure of cells to 300 nM of GrB for 30 min significantly reduced transcriptional output after 4 h, suggesting that GrB cleavage of Notch1 reduces Notch1 signalling activity. Post incubation of GrB-treated cells for longer time-points resulted in a non-specific inhibition of reporter activity due to SLO alone (results not shown).

These results demonstrate that GrB cleavage of Notch leads to the inactivation of Notch1 by interfering with the ability of NICD to activate gene transcription.

## DISCUSSION

GrB is essential for NK cell- and CTL-induced cell death in pathogen-infected and tumour cells. This cell death is mainly mediated by the action of granzymes packaged in cytotoxic granules that are released into the target cell upon recognition. Previously, using NK-mediated cytotoxicity, Loeb et al. [4] showed that physiological levels of GrB are able to cleave Notch1 in a caspase-independent manner. It cleaves Notch1 into at least a single fragment at an unidentified site. In the present study, we confirmed that GrB cleaves the Notch1 receptor irrespective of caspase activation. Proteolyis of Notch by GrB results in at least two fragments *in vitro,* cleaving after $Asp^{1860}$ and $Asp^{1961}$. Cell-based assays show GrB cleaves Notch at the same sites only in the presence of the pore-forming protein SLO, demonstrating GrB cellular entry is required to cleave Notch. Furthermore, we show that GrB can cleave Notch receptors irrespective of its activity and location; it cleaves the immature Notch precursor, the cell-surface-bound receptor as well as the membrane-cleaved activated NICD. Notch1 cleavage by GrB inactivates the signalling cascade directly by blocking transcriptional activity.

Notch receptors are GrB substrates irrespective of their activation status. Our results indicate that GrB can cleave Notch at multiple levels, during maturation, at the cell surface and after release from the membrane in the nucleus. Previous results indicate nuclear access of GrB to be primarily caspase-dependent [28]. In the present study, making use of caspase inhibitors during the course of GrB treatment, we observe GrB cleavage of NICD, suggesting that GrB may also enter the nucleus and cleave Notch independent of caspase activity. Cleavage of the Notch effector NICD by GrB leads to direct inactivation of transcriptional activity. However, cleavage of the precursor and membrane-bound forms of the receptor results in a lower number of intact Notch molecules at the cell surface able to receive a signal, thereby decreasing signalling capacity indirectly. Thus GrB can inactivate Notch signalling at all levels, either directly or indirectly.

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The optimal substrate cleavage site of GrB is P4 I/VXXD-P1 (where X is any amino acid) with critical isoleucine/valine and aspartic acid residues at P4 and P1 respectively. Considering the Notch1 proteins used in the present study, this leaves five possible GrB scissile bonds, at VLPD$^{1823}$, VDAD$^{1860}$, VISD$^{1902}$, VSAD$^{1961}$ and VLLD$^{2071}$, all of which are conserved between mouse and human Notch1. We systematically mutated the P1 aspartic acids and found that mutation of both Asp$^{1823}$ and Asp$^{1902}$ in Notch still resulted in cleavage by GrB, similar to wild-type controls *in vitro* and *in vivo* (results not shown). Only upon mutation of Asp$^{1860}$ was a cleavage defect observed both *in vitro* and *in vivo*. Strikingly, this induced a shift in cleavage *in vitro* to VLPD$^{1823}$, resulting in a 65 kDa fragment which disappeared upon additional mutation of Asp$^{1823}$. This could indicate that cleavage takes place at two residues simultaneously. However, experiments using the single Asp$^{1823}$ mutant ruled out the possibility of subsequent cleavage starting at Asp$^{1823}$ (results not shown). The D1823Q/D1860A double mutant still showed the 45 kDa cleavage fragment to be present, demonstrating GrB is able to cleave this site directly, although less efficiently. Mutation of Asp$^{1961}$ showed a loss of the smaller 45 kDa fragment, revealing the second cleavage site of GrB. Strikingly, *in vivo* the 45 kDa fragment is also produced, but detection was more difficult for reasons unknown. Only upon high Notch expression or enrichment of Notch molecules could the 45 kDa fragment be detected in living cells. Detection of the 45 kDa fragment may also be hampered because of processing by other proteases or degradation by the proteasome.

GrB cleavage sites at Asp$^{1860}$ and Asp$^{1961}$ both resulted in a loss of the RAM (RBP-J$\kappa$-associated molecule) domain, although part of the ankyrin repeats were maintained. The RAM domain is required for NICD binding to CSL, as well as inducing derepression of CSL target genes [29], thus loss results in a block of transcription. The CDC10/ankyrin repeats, responsible for CSL binding [30], can activate Notch signalling in the absence of a RAM domain [31-33]. However, this is several-fold lower than wild-type NICD1. Since part of the ankyrin repeats was removed by the second cleavage at Asp$^{1961}$, this may further attenuate Notch1 transcriptional activity. Thus both cleavage scenarios lead to a loss of transcriptional activity, thereby effectively blocking transcriptional activation.

Loeb et al. [4] suggested that cleavage of Notch1 would lead to an increase in apoptosis in tumour cells. This coincides with our findings that cleavage of Notch1 results in a block of transcriptional activity. Inhibition of apoptosis by Notch1 appears to occur via several mechanisms. Notch1 has been shown to protect cells from apoptosis acting via JNK (c-Jun N-terminal kinase) activation [34], by inhibiting p53 [35-37] or the PKB (protein kinase B)/Akt pathway [37,38]. Breast epithelial cells are protected against apoptosis due to the induction of PKB/Akt signaling in response to Notch1 activation [39]. In addition, downregulation of Notch1 in pancreatic cells leads to increased apoptosis [40]. In tumour cells, NICD1 can prevent the degradation of XIAP (X-linked inhibitor of apoptosis protein) thereby increasing cell survival [41]. Loss of Notch1 signaling in either of these cases would lead to a loss of anti-apoptotic signals, which may eventually lead to cell death. This supports the hypothesis that GrB cleavage may attenuate Notch1-mediated cell survival and thereby promote apoptotic potential in tumour cells.

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T-cell proliferation and maturation is a highly Notch1-dependent process, resulting in various types of T-cells. In a subset of T-cells, mature Th2 cells, cell death induced by GrB is caspase independent [42]. Consistently, genetic loss or inhibition of GrB leads to Th2 proliferation. Since Th2 cells depend on Notch1 activation for their proliferation [43], this suggests that GrB could trigger cell death by downregulating Notch1 signalling by directly cleaving the receptor. This would ensure control over Th2 lymphocyte numbers and activity after executing their function in the immune response, thereby tightly controlling cell numbersto avoid an excessive number of activated T-cells. However, in CTLs, Notch1 is thought to positively regulate the expression of GrB and perforin by directly binding to their promoters [44]. One could see the GrB cleavage of Notch1 as an activating cleavage, thereby inducing an auto-regulatory loop by upregulating GrB and perforin. However, we have clearly shown that cleavage of Notch1 by GrB leads to inactivation of signalling, making it very unlikely that within the CTLs there is such a positive auto-regulatory loop. The function of GrB cleaving Notch1 within T-cells therefore remains obscure.

The plasma of healthy individuals contains low levels of GrB, whereas patients suffering from viral infections, inflammation, or auto-immune diseases show a several-fold increase in GrB levels [24,45,46]. This could be due to leakage out of the immunological synapse, explaining the higher GrB levels in patients since the number of immune reactions is increased. However, recently it was found that GrB is actively secreted into the extracellular milieu [26]. Moreover, perforin-independent functions of GrB have been reported [24-26]. In the present study we show that the extracellular domain of Notch1 receptor is a substrate for cleavage by GrB in the absence of perforin. We observed a significant 3-fold increase in GrB-induced Notch1 shedding; however, without the induction of transcriptional activation. This suggests that the cleavage must take place at the N-terminal end upstream of the NRR domain, since removal of this domain leads to activation of Notch. Ligand binding to Notch is thought to induce a substantial conformational change of the NRR leading to consecutive S2 and S3 cleavage, producing NICD [16,47,48]. The GrB-induced cleavage of NECD, however, does not lead to such a conformational change sufficient for receptor activation. However, this cleavage could shed off the ligand-binding domain, leaving the receptor inactive at the cell surface unable to signal. In this way, GrB can inactivate the receptor indirectly, resulting in an increased pro-apoptotic environment. Interestingly, there is a good correlation between the cleavage fragments observed using N- and C-terminal epitope tags after GrB cleavage. Whereas Myc immunoblotting detects the appearance of the 55 kDa cleavage product, the N-terminal HA tag detects a similar reduction in the size of the full-length precursor. Importantly, no other cleavage events were observed, suggesting that the *Gaussia* luciferase release in the medium seen after GrB exposure of cells is an indirect effect of GrB. Thus the NECD is cleaved by a protease which is activated by GrB. It will be interesting to identify the proteolytic activity induced by GrB responsible for this NECD cleavage.

Collectively, our results demonstrate that Notch1 is an efficient direct substrate for GrB. Cleavage of Notch1 leads to inactivation of signalling both by the action of intracellular as well as extracellular GrB. Direct cleavage of active NICD, but also downregulating total numbers of Notch1 precursors and receptors at the membrane, decreases signalling capacity.

This downregulates anti-apoptotic and pro-proliferative signals achieving a faster and more efficient apoptotic response. This example clearly shows an important caspase-independent function of granzymes. Since tumour cells recognized by CTLs will not only encounter secreted GrB, but a mixture of granzymes [49], it will be of interest to determine the caspase-independent spectrum of substrates of the granzymes altogether to gain more insight into the cytotoxic killing mechanisms involved in cancer.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank S. Boyle and R. Kopan (Washington University, Saint Louis, MO, U.S.A.) for reagents.

**FUNDING**

This study was supported by the Dutch Cancer Society (NKB-KWF) [grant numbers UU-2009-4302 (to N.B.) and UU2006-3623 (to M.V.)], the Netherlands Organization for Scientific Research (NWO) [grant number 916.66.044 (to N.B.)] and by the European Research Council under the European Community Seventh Framework Programme [grant number FP7/2007-2013/ERC Grant 208259 (to M.V.)].

## Abbreviations used

| | |
|---|---|
| **CSL** | CBF1/suppressor of Hairless/Lag-1 |
| **CTL** | cytotoxic T-lymphocyte |
| **DBZ** | dibenzazepine |
| **GrB** | granzyme B |
| **HA** | haemagglutinin |
| **N1FL** | full-length Notch1 |
| **NECD** | Notch extracellular domain |
| **NICD** | Notch intracellular domain |
| **NK** | natural killer |
| **RAM** | RBP-J$\kappa$ (recombination signal-binding protein 1 for J$\kappa$)-associated molecule |
| **RBP-J$\kappa$** | recombination signal-binding protein 1 for J$\kappa$ |
| **SLO** | streptolysin O |
| **TBS** | Tris-buffered saline |
| **Z-VAD-FMK** | benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone |

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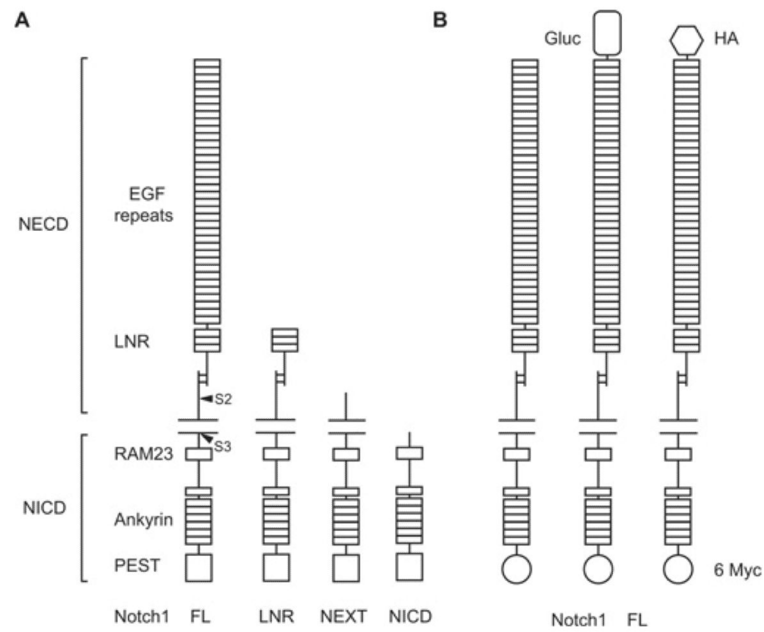


**Figure 1. Notch constructs**

(**A**) Schematic overview of Notch1 constructs used in the present study and (**B**) location of C- and N-terminal epitope tags.



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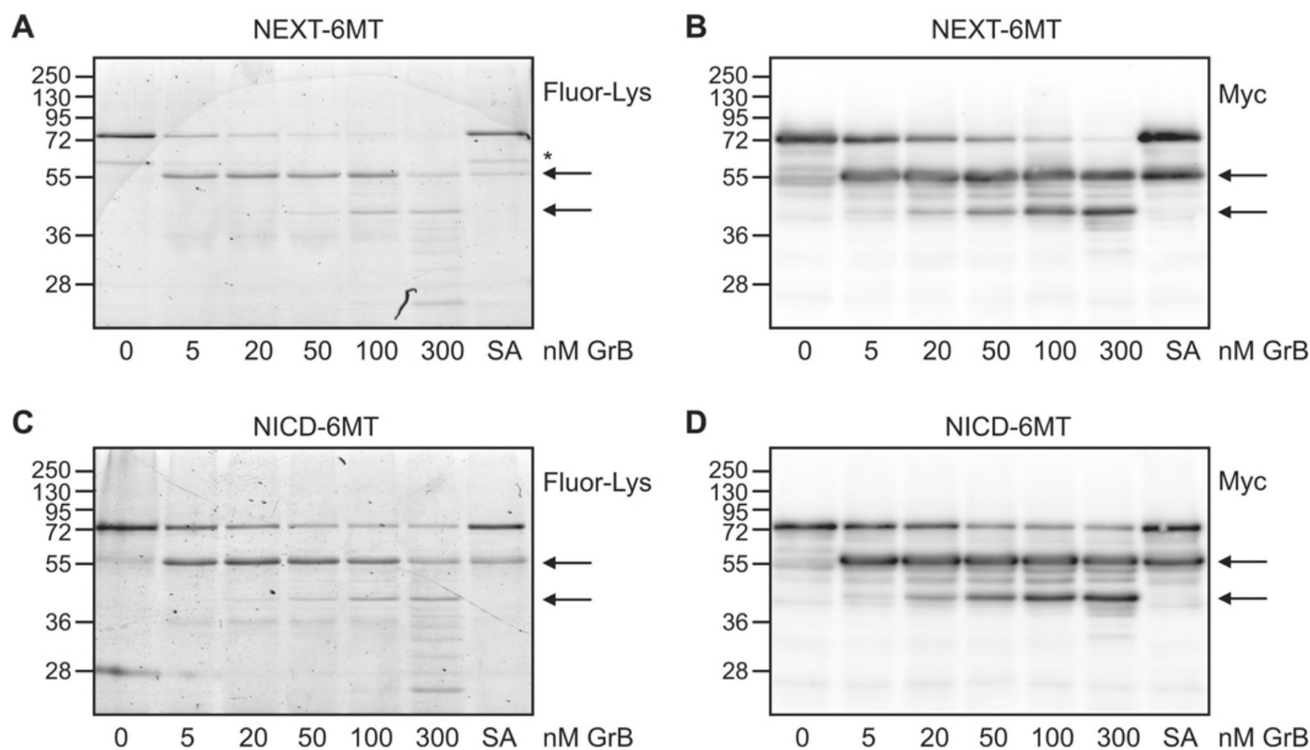


**Figure 2. *In vitro* cleavage of Notch1 by GrB in a dose-dependent manner**

(**A**) SDS/PAGE analysis showing *in vitro* produced NEXT-6Myc proteins with incorporated fluorescently labelled lysine residues are cleaved upon GrB incubation into 55 kDa and 45 kDa fragments as indicated by arrows. *, an alternative translation product. (**B**) Immunoblotting shows both the 55 kDa and 45 kDa fragments are C-terminally 6Myc tagged. (**C**) Similar to NEXT, the shorter NICD construct is also a substrate of GrB resulting in two fragments of 55 and 45 kDa. (**D**) Similar to NEXT, these fragments are also C-terminally tagged with 6Myc. Molecular mass markers are indicated in kDa.

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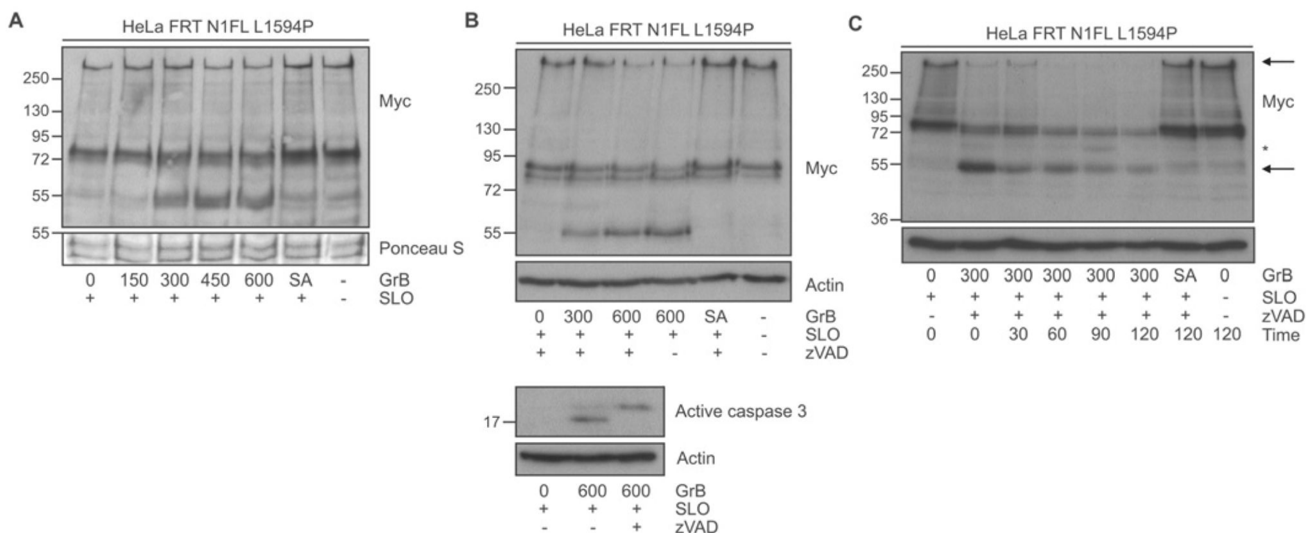


**Figure 3. *In vivo* cleavage of Notch1 by GrB**

(**A**) N1L1594P HeLa cells incubated with various concentrations of GrB show a 55 kDa cleavage fragment derived from Notch1 as determined by Myc immunoblots. Ponceau S staining of the same immunoblot serves as a loading control. (**B**) Upper panel: N1L1594P HeLa cells incubated with the caspase inhibitor Z-VAD-FMK (zVAD) show cleavage of Notch1 upon GrB treatment. Lower panel: Z-VAD-FMK inhibits the autoproteolysis of caspase 3 (p17) as determined with activated caspase 3 immunoblot. Actin staining of the same immunoblots serves as a loading control. (**C**) After GrB incubation, N1L1594P HeLa cells were additionally incubated for 2 h. This prolonged incubation demonstrated a decrease in the 55 kDa fragment, yet no 45 kDa fragments are observed. In addition, a loss of the full-length and TMIC isoforms of Notch is observed (arrows). *, an aspecific product. Molecular mass markers are indicated in kDa.

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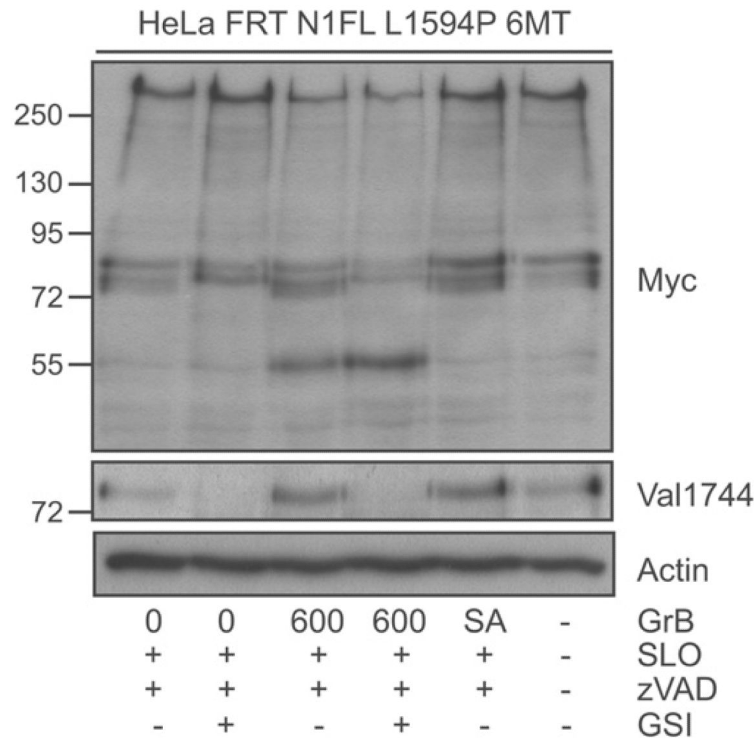


**Figure 4. Cleavage of Notch by GrB is independent of activation**

N1L1594P HeLa cells were treated with the $\gamma$-secretase inhibitor (GSI) DBZ, inhibiting the activating cleavage of Notch1. Upon GrB incubation, 55 kDa fragments could still be observed on the Myc immunoblot. The Val$^{1744}$ immunoblot stains Notch S3 cleavage products. Molecular mass markers are indicated in kDa.

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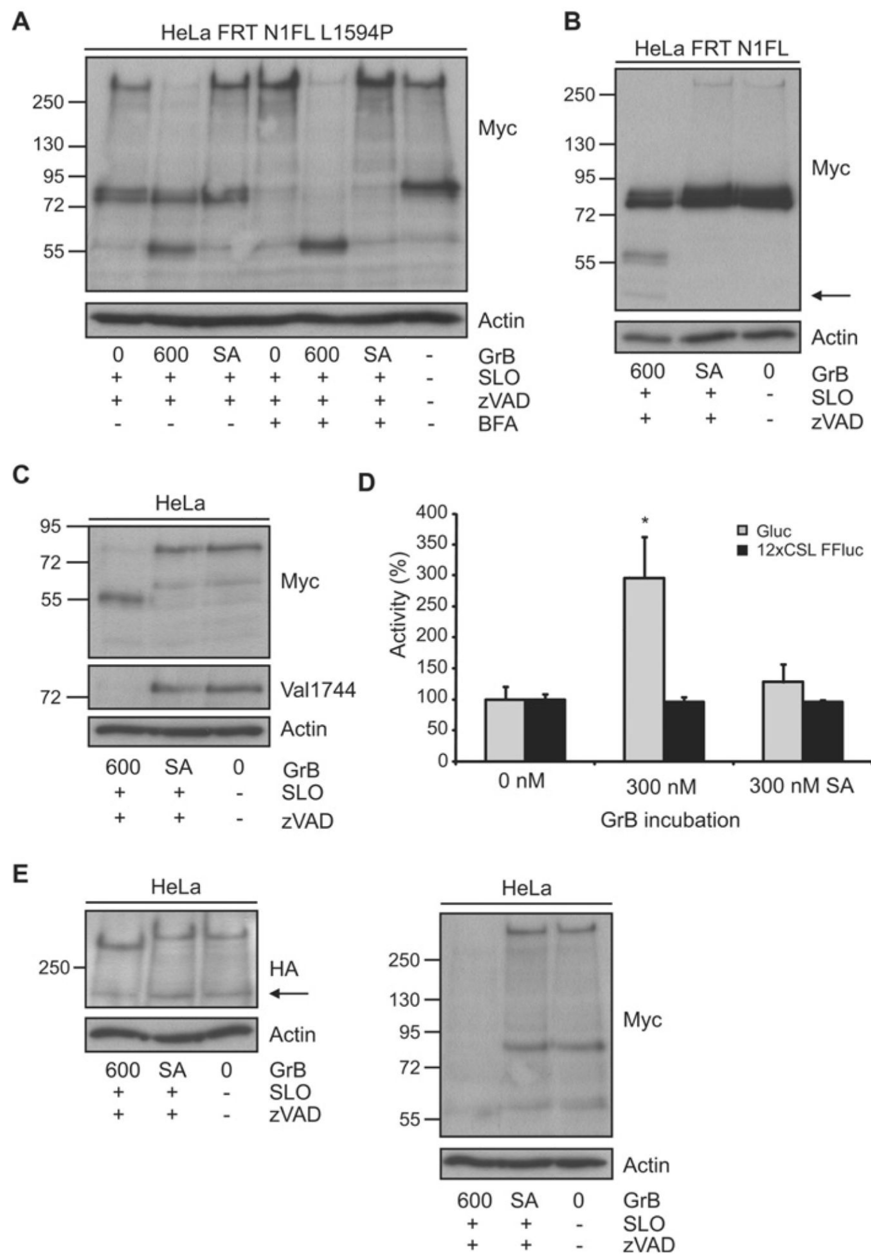


**Figure 5. Intracellular and extracellular GrB cleavage of Notch**

GrB action in different subcellular locations was analysed. (**A**) Brefeldin A (BFA)-treated cells, blocking ER to Golgi transport, show cleavage of Notch upon GrB incubation. (**B**) Fractions enriched for plasma membrane-bound Notch1 show cleavage upon GrB treatment. Arrow indicates the 45 kDa fragment. (**C**) In HeLa cells expressing activated Notch molecules, GrB induces efficient cleavage. (**D**) HeLa cells expressing N-terminally tagged Gluc-Notch1 was incubated with GrB in the absence of perforin. Gaussia luciferase activity was measured in the medium, and the 12× CSL-driven Notch activity was determined from lysates. A significant (*P = 0.0495) increase was observed upon 300 nM GrB, which did not lead to Notch activation. The graph is representative of at least two independent experiments

in triplicate, and the P-value was calculated using a Kruskall-Wallis non-parametric test. (**E**) Expression of N-terminally tagged HA–Notch-FL-6Myc indicated the NECD was not a direct substrate of GrB. Arrow indicates HA-tagged NECD. Molecular mass markers are indicated in kDa.

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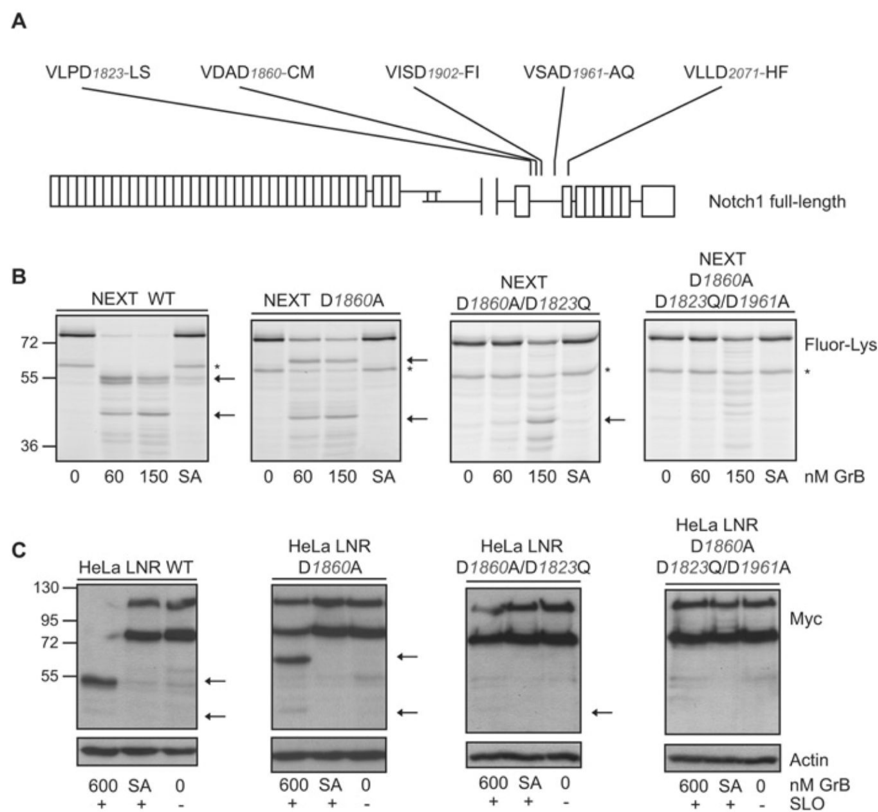


**Figure 6. Cleavage occurs at Asp$^{1860}$ and Asp$^{1961}$**

(**A**) The diagram shows the five predicted cleavage sites in the intracellular part of the Notch1 receptor. (**B**) In vitro mutation analysis reveals that wild-type Notch1 NEXT-6Myc shows the two cleavage fragments, whereas mutation of VDAD$^{1860}$ shows a loss of the 55 kDa fragment, yet a new 65 kDa fragment appears, which upon additional mutation of VLPD$^{1823}$ is lost, maintaining the 45 kDa fragment. Only upon an additional third mutation of VSAD$^{1961}$ is a complete loss seen in Notch cleavage upon GrB treatment. *, an alternative translation product. (**C**) This could be confirmed in vivo by GrB incubation of HeLa cells expressing Notch1 LNR-6Myc carrying the various mutations as determined by Myc immunoblotting. Actin staining of the same immunoblots serves as a loading control. Molecular mass markers are indicated in kDa.

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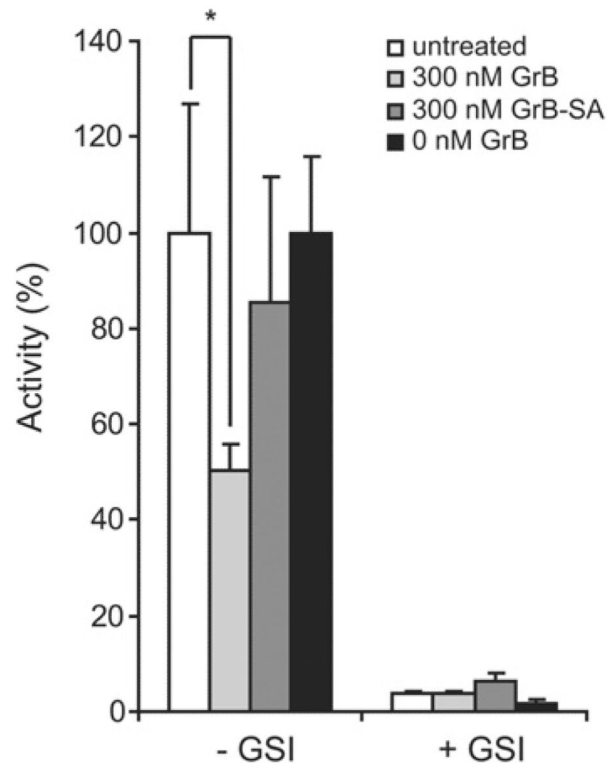


**Figure 7. GrB cleavage results in a block of transcription**

Transcriptional Notch1 activation assay shows a significant (*$P$ = 0.0495) loss of activity upon GrB treatment. The graph is representative of at least two independent experiments in triplicate, and the *P*-value was calculated using a Kruskall-Wallis non-parametric test. GSI, $\gamma$-secretase inhibitor DBZ.