Genet. Res., Camb. (2001), **78**, *pp.* 219–223.
DOI: 10.1017/S0016672301005304 Printed in the United Kingdom

Numb suppresses the negative complementation at the *Notch* locus of *Drosophila melanogaster*, suggesting a putative mechanism for negative complementation

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(*Received 2 April 2001*)

Summary

The mutant form of the intracellular asymmetrically localized Numb membrane-bound protein of *Drosophila melanogaster* suppresses the negative complementation of certain *Abruptex* (*Ax*) mutations of the *Notch* (*N*) locus encoding a transmembrane receptor protein in which the *Ax* mutations are mutations in the epidermal growth factor (EGF)-like repeats of the extracellular domain of the receptor. One model for how *Ax* mutants affect *N* function is that they are refractory to an antagonistic signal generated by an excess of *N* ligands. Genetically *numb* (*nb*) is an antagonist of *N*. In the absence of *nb*, cells follow the same fate as they would in the presence of a gain-of-function *N* allele, such as *Ax*. Numb has been shown to interact with the cytoplasmic domain of Notch. It is therefore suggested that *numb* counteracts the effect of *Abruptex* on Notch ligand binding, i.e. that Numb is an antagonist to the activation of the Notch signal generated by Notch ligands. Numb might accomplish this by interfering with the proteolytic cleavage of the Notch intracellular domain at the cell membrane. Thus, it seems possible that the mechanism of negative complementation of certain *Ax* mutants is the failure of this cleavage. Other possible mechanisms for negative complementation are also discussed.

1. Introduction

Notch is a transmembrane receptor protein that participates in a highly conserved cell-to-cell signalling pathway that regulates morphogenesis in metazoan animals (Simpson, 1994; Artavanis-Tsakonas *et al.*, 1995). Ligands of the Notch protein in *Drosophila melanogaster* include the products of the *Delta* (*Dl*, 3-66.2) and *Serrate* (*Ser*, 3-91.9) genes (reviewed by Simpson, 1994; Artavanis-Tsakonas *et al.*, 1995), and their binding sites in the Notch protein are in the epidermal growth factor (EGF) motif (Rebay *et al.*, 1991). *Delta* and *Serrate* have a positive effect on *Notch* by activating its function (Fehon *et al.*, 1991; Kooh *et al.*, 1993).

In *Drosophila melanogaster* the *Abruptex* (*Ax*, 1-3.0) mutations are a particular type of mutation occurring at the *Notch* (*N*, 1-3.0) locus. They are point mutations in the EGF-like repeats of the extracellular domain of the receptor protein (Wharton *et al.*, 1985; Kidd *et al.*, 1986). The *Ax* mutations are characterized by lack of bristles on the head and thorax and interruption of wing veins, and can be divided into recessive lethals and viable alleles. The viable alleles, for their part, can be divided into suppressors (Ax^{SoN}) and enhancers (Ax^{EoN}) of the *Notch* mutations. The enhancers and suppressors of *Notch* show an interesting type of allelic interaction, namely negative complementation; in other words, heteroallelic combinations of these viable alleles are lethal or semilethal (Ax^{28}/Ax^{E2}) (Foster, 1975; Portin, 1975). Mutations of the *Delta* gene act as suppressors of the negative complementation (Xu *et al.*, 1990). One model for how *Ax* mutants affect *N* function is that they are refractory to an antagonistic signal generated by an excess of *N* ligands (De Celis & Bray, 2000).

The Notch signal is mediated from the cell membrane to the nucleus by proteolytic cleavage of the intracellular domain from the extracellular domain and is, together with the product of the *Suppressor of Hairless* gene [*Su*(*H*), 2-50.8], moved to the nucleus, where they constitute a transcription factor (Lecourtois & Schweisguth, 1997, 1998; Kidd *et al.*,

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1998). At least in *Xenopus laevis* embryos, association with Notch intracellular domain converts *Su*(*H*) from a transcriptional repressor to a transcriptional activator of Notch target genes (Jan *et al*., 1999).

Brennan *et al*. (1999) concluded that in *Drosophila* the function of Notch requires the product of the *Suppressor of Hairless* gene. Loss of either *Notch* or *Suppressor of Hairless* function results in cells making premature and incorrect cell fate decisions, whilst increases in Notch signalling prevent cells from making these decisions. They found that the proneural clusters are not established correctly in certain *Abruptex* mutations of *Notch* and that this failure to establish the proneural cluster correctly is not due to increased Notch signalling during lateral inhibition. In addition, the overexpression of certain dominant negative Notch molecules can disrupt the initiation of proneural cluster development in a manner similar to the *Abruptex* mutants. Thus, *Abruptex* seems to antagonize the transport of Notch signal from the cell surface into the nucleus.

The *numb* (*nb*, 3-35) gene for its part encodes a membrane-associated intracellular protein that is asymmetrically localized in the cell (Rhyu *et al*., 1994; Posakony, 1994) and antagonizes Notch in the development of the central and peripheral nervous system (Spana *et al*., 1995; Campos-Ortega, 1996; Frise *et al*., 1996; Spana & Doe, 1996; Guo *et al*., 1996; Park *et al*., 1998; Wai *et al*., 1999). The known mutations of the *numb* gene are recessive embryonic lethals, because in these mutants the neurons of the peripheral nervous system of the embryo cannot acquire their correct identity (Lindsley & Zimm, 1992). In the absence of *nb*, cells follow the same fate as they would in the presence of a gain-of-function *N* allele, such as *Ax* (De Celis & Bray, 2000). Numb has been shown to interact with the cytoplasmic domain of Notch (Frise *et al*., 1996; Guo *et al*., 1996; Wakamatsu *et al*., 1999).

Here I show that *numb* suppresses negative complementation between the viable *Abruptex* mutations, suggesting that Numb counteracts the effect of Abruptex on Notch ligand binding, in other words, that Numb is an antagonist to the activation of the Notch signal generated by Notch ligands. Numb might accomplish this by interfering with the proteolytic cleavage of the Notch intracellular domain at the cell membrane. Thus, it seems possible that the mechanism of negative complementation of certain *Ax* mutants is the failure of this cleavage, even though other mechanisms are also possible.

2. Materials and methods

The suppressors of the *Notch Abruptex* mutations studied were Ax^{28} and Ax^{9B2}, and the enhancers of the Notch mutations were Ax^{71d}, Ax^{E2} and Ax^{l6172}. In the experimental crosses all the pairwise combinations on the nb^2 *pr ch Bc*/*SM6B* background of these mutations were studied for female viability as compared with their *Abruptex* brothers by crossing homozygous *Abruptex* females carrying nb^2 *pr ch Bc*/*SM6B* autosomes with the respective males (except in the case of the female sterile Ax^{9B2} allele, where Ax^{9B2}/*Basc*; nb^2 *pr ch Bc*/*SM5B* females were crossed to Ax^{9B2}/*Y* males carrying the same autosomal marker combination: *numb-2*, nb^2, 2-[35]; *purple*, *pr*, 2-54.5; *chubby*, *ch*, 2-73.8; *Black* cells, *Bc*, 2-80.6). The control crosses were otherwise identical with the experimental crosses, but the autosomes both in female and male parents were of wild type.

The crosses were made on a standard *Drosophila* medium at 25 °C.

3. Results

The initial rationale of this study was the fact that in the absence of *numb*, cells follow the same fate as they would in the presence of a gain-of-function *N* allele, such as *Ax* (De Celis & Bray, 2000). Therefore, there is the possibility that *numb* antagonizes *Ax*, and thus the study of their interaction might elucidate the mechanism of the negative complementation of certain *Ax* mutations.

In the experimental crosses all the progenies were non-purple, non-chubby and curled winged, showing that they carried one copy of the *numb* gene, the nb^2/nb^2 genotypes being lethal.

In the control crosses all the females carrying suppressor of Notch/enhancer of Notch of the *Abruptex* mutations were either lethal or semilethal (Ax^{28}/Ax^{E2}) (Table 1).

Table 1. *Results of the control crosses*

	Suppressors of Notch		Enhancers of Notch		
	28	9B2	E2	16172	71d
28	100 (248)	100 (356)	54.4 (1752)	0 (155)	0.7 (441)
9B2		100 (543)	0 (362)	0 (656)	0 (367)
E2			100 (703)	100 (1119)	100 (1905)
16172				100 (1520)	100 (2261)
71d					100 (526)

Viabilities in percentages of homo- and heteroallelic combinations of certain *Abruptex* mutations on the wild-type autosomal background were calculated by dividing the number of female progenies by the number of their *Abruptex* brothers and multiplying by 100. In parentheses are given the total number of flies.

https://doi.org/10.1017/S0016672301005304 Published online by Cambridge University Press

Table 2. *Results of the experimental crosses*

	Suppressors of Notch		Enhancers of Notch		
	28	9B2	E2	16172	71d
28	100 (623)	100 (692)	67.1 (1651)	100 (1964)	118.5 (1160)
9B2		100 (870)	85.2 (1028)	85.5 (1423)	100 (989)
E2			100 (386)	100 (1055)	100 (883)
16172				100 (602)	100 (860)
71d					100 (457)

Viabilities in percentages of homo- and heteroallelic combinations of certain *Abruptex* mutations on the nb^2 *pr ch Bc/SM6B* background calculated by dividing the number of female progenies by the number of their *Abruptex* brothers and multiplying by 100. In parentheses are given the total number of flies.

The nb^2 *pr ch Bc/SM6B* background at least partly suppressed the lethality of all the pairwise suppressor of Notch/enhancer of Notch combinations of the *Abruptex* mutations (Table 2). Five of six $Ax^{SoN}Ax^{EoN}$ crosses showed dramatically improved viability in the presence of $numb^2/+$, all going from approximately zero viability to $>85\%$ viable with $numb^2/+$. Surprisingly, one of six Ax^{SoN}/Ax^{EoN} crosses, the allelic combination Ax^{28}/Ax^{E2}, showed only very modest improvement in viability, from 54·4% to 67·1%. This could reflect the possibility that in the Ax^{28}/Ax^{E2} genotype the gain-of-function effect is initially quite weak, and therefore *numb* is not in fact even expected to antagonize this genotype as strongly as the other genotypes.

Thus, the results show that removing one copy of the *numb* gene in the second chromosome rescues the negative complementation in certain heteroallelic combinations of the *Abruptex* type mutations of the *Notch* locus. Due to the lack of biochemical data, the discussion of this interesting finding necessarily remains rather hypothetical.

4. Discussion

The lethal crisis of the negative complementation between the *Abruptex* mutations occurs at the late pupal stage (Foster, 1975; Portin, 1975). The phenocritical period is at the transition between the third instar larval and pupal stages (Portin & Sirén, 1976). On the basis of the analysis of gynandromorphs, the lethal focus is near the ventral structures of the thorax, and is a single focus (Portin, 1977). Immunolabelling of the proteins has shown that the negative interaction of *Abruptex* proteins most likely occurs within a single cell and not between cells (Fehon *et al.*, 1990).

Genetic studies have indicated that *numb* acts upstream of *Notch*, and biochemical studies have revealed that Numb can bind Notch (Guo *et al.*, 1996). For a functional assay of the action of Numb on Notch signalling, these proteins have been expressed in cultured *Drosophila* cells. Nuclear translocation of Suppressor of Hairless [Su(H)] was used as a reporter for Notch activity. It was found that Numb interfered with the ability of Notch to cause nuclear translocation of Su(H) (Frise *et al.*, 1996).

The mechanism of negative complementation at the *Notch* locus is not known, and therefore the very aim of this study is to try find hints for the elucidation of this mechanism in spite of the fact that the results of this study are rather surprising, and biochemical data are lacking.

How is it possible that the mutant membrane-bound intracellular Numb protein suppresses the negative interaction of Notch transmembrane proteins that carry point mutations at the EGF-like repeats on their extracellular domains? The best putative answer I can see is that Numb is involved in the proteolytic cleavage of the Notch receptor at the cell membrane. This proposition, however, needs careful explanation. The Notch receptor has 36 EGF-like repeats (Wharton *et al.*, 1985; Kidd *et al.*, 1986). The *Ax* mutations map to repeats 25–30 (Kelley *et al.*, 1987) while Delta binds to EGF-like repeats 11–12 (Rebay *et al.*, 1991). There could, however, be interaction between these distantly located EGF-like repeats. In fact, De Celis & Bray (2000) proposed this possibility when they explained how the *Abruptex* phenotype might arise, and on the basis of the results of Brennan *et al.* (1999) it can be concluded that *Abruptex* inhibits the transport of the Notch signal from the cell membrane into the nucleus. Numb could possibly counteract the effect of *Abruptex* on Notch ligand binding, that is, Numb may be an antagonist to the activation of the Notch signal generated by Notch ligands (Spana *et al.*, 1995; Campos-Ortega, 1996; Frise *et al.*, 1996; Spana & Doe, 1996; Guo *et al.*, 1996; Park *et al.*, 1998; Wai *et al.*, 1999). Numb might accomplish this by interfering with the proteolytic cleavage of the Notch intracellular domain at the cell membrane. In fact, the available evidence indicates that Numb acts by inhibiting nuclear accumulation of the Notch intracellular domain (Frise *et al.*, 1996; Wakamatsu *et al.*, 1999). However, it is not yet known if Numb also affects proteolytic processing of Notch. However, I would not in particular favour impairment of nuclear accumulation as a mechanism of negative complementation, even though this remains an alternative, because negative complementation most likely involves interaction of Notch receptors in the EGF-like motif of the extracellular domain. This suggestion

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naturally needs experimental evidence with biochemical methods at the cellular and molecular levels.

If the suggestion presented above is correct, it could be proposed that the mechanism of negative complementation would be failure of the proteolytic cleavage of the Notch receptor at the cell membrane due to an impairment of the interaction of the *Ax* mutant sites and ligand binding sites among the EGF-like repeats of the Notch receptor. It is worth noticing that in a recent article De Celis & Bray (2000), on the basis of immunocytochemical studies, proposed that the *Abruptex* phenotype results from blocking of the inhibitory activity of high concentrations of Notch ligands. Moreover, Kadesh (2000) in his review article suggested that the Notch extracellular domain actively inhibits terminal proteolytic events, and that the ligand serves to neutralize the extracellular domain. Thus, if now *Abruptex* mutants interfere with the ligand binding, and in this way inhibit the cleavage of the Notch receptor, the results of this study become comprehensible.

Other plausible mechanisms of the negative complementation include blocking of Notch transport to the cell surface, processing of Notch to form a heterodimer, delivery of the ligand to the cell surface, transport of the Notch signal from the cell membrane into the nucleus, and downregulation of the receptor prior to ligand binding. I, however, favour the alternative of the blocking of intracellular cleavage, since this alternative is most compatible with the results of this study and present knowledge on the genetics of the Notch signalling pathway. Unfortunately, the discussion of the interesting results of this study necessarily remain speculative. Therefore this paper is mainly a starting point of work that addresses the problem of negative complementation.

The skilful technical assistance of Tommi Korhonen, Mirja Rantanen and Anu Sironen is gratefully acknowledged. The *numb* stock was received from Yuh Numg Jan (Howard Hughes Medical Institute, University of California, San Francisco). This paper is dedicated to the memory of Doctor Aloha Hannah-Alava (died 29.1.2000) as an expression of the gratitude which I owe to her.

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