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Alternative Splicing of Neurofibromatosis Type 1 Exon 23a Modulates Ras/ERK Signaling and Learning Behaviors in Mice

Karl Andreas Mader and Hua Lou

Abstract

Neurofibromin is one of the few Ras-GTP activating proteins (Ras-GAPs) expressed in the brain. Disruption of its expression leads to the detrimental disease neurofibromatosis type 1 (NF1). Many studies have revealed the crucial role of *NF1* in developing and adult tissues. However, these studies have focused on the expression of the entire *NF1* gene and largely ignored the role of an alternative splicing event that controls the Ras-GAP function of neurofibromin. The focus of this chapter is *NF1* exon 23a. This exon is located in the GAP-related domain (GRD) of neurofibromin. Its expression level, indicated by the percentage of its inclusion in the *NF1* mRNA transcripts, has a profound effect on the Ras-GAP function of neurofibromin. In this chapter, we review the expression pattern of exon 23a and the molecular mechanisms that regulate its expression. We then discuss the role of its expression in Ras/ERK signaling and learning behaviors in mice. Lastly, we propose a few directions for future studies.

Keywords: NF1, alternative splicing, exon 23a, Ras-GAP, learning behaviors, mouse

1. Introduction

Neurofibromatosis type 1 (NF1) is a genetic disorder that affects approximately 1 in 2000–4000 individuals [1]. The disease hallmark includes tumors in the nervous system, most commonly, benign peripheral nerve tumors or neurofibromas, and café-au-lait macules [1]. In addition, many NF1 patients exhibit cognitive and behavioral problems, bone abnormalities and hypertension [1].

The underlying cause of the NF1 disease is the germline mutation in one of the two alleles of the *NF1* gene on chromosome 17q11.2. This gene, spanning more than 350 kilobases (kb) of genomic DNA, codes for neurofibromin, which is one of the major GTPase activating proteins (GAPs) that down regulates the activity of Ras [2–5]. Neurofibromin attenuates Ras signaling by converting it from its active GTP-bound form to its inactive GDP-bound state via the GAP-related domain (GRD). Mutations in the *NF1* gene reduce/abolish the Ras-GAP function of neurofibromin, which leads to abnormally high cellular activity of the Ras signaling pathway [4, 5].

Proper expression of the GRD is critical to achieving the optimal Ras-GAP function of neurofibromin. In mammals, the *NF1* gene expression is highly regulated temporally and spatially. During embryonic development, *NF1* is expressed in many tissues.

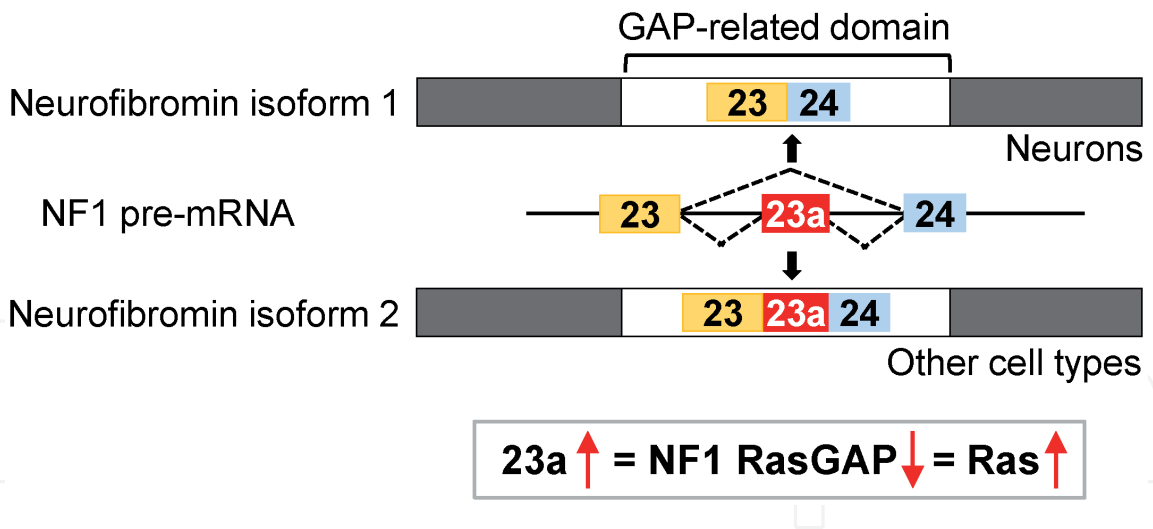


Figure 1. A diagram showing that alternative splicing of the NF1/Nf1 exon 23a can give rise two isoforms of neurofibromin. Early *in vitro* experiments demonstrated that inclusion of exon 23a leads to reduced Ras-GAP activity of neurofibromin and thus increased active Ras activity. The two protein isoforms are depicted above and below and pre-mRNA in the middle. The diagram is not drawn to scale.

However, in adults, its expression is highly enriched in the nervous system in neurons, oligodendrocytes and Schwann cells [6–8]. A recent study demonstrated that in both mouse and human brain, *NF1* expression is enriched in inhibitory neurons [9].

In addition to transcriptional regulation, the expression output of the *NF1* gene is subject to a post-transcriptional alternative splicing regulation that gives rise to two distinct NF1 transcripts: one with and the other without exon 23a (exon 31 in the current *NF1* nomenclature) [10]. Exon 23a is a 63 nucleotide in-frame cassette exon that, when translated, adds additional 21 amino acids in the neurofibromin protein [10]. The neurofibromin that does not contain the 21 amino acids is named type 1 isoform whereas the protein that contains the extra amino acids is named type 2 isoform (**Figure 1**) [10]. Because these additional amino acids are located in the GRD, it was immediately suspected when exon 23a was identified that inclusion of these amino acids would affect the Ras-GAP function of neurofibromin. As predicted, early *in vitro* analysis using truncated GRD expression plasmids with or without exon 23a demonstrated that the GRD polypeptide containing exon 23a showed up to 10 times lower Ras-GAP activity than that the GRD without exon 23a [10, 11]. For more than a decade after these initial studies, it remained unknown if expression of this alternative exon regulates the full-length neurofibromin protein in a similar fashion. Since 2002, our group has conducted extensive research to understand the biology of this alternative splicing event.

In this chapter, we will focus on exon 23a and discuss the following questions. What is the expression pattern of this exon? How is its expression regulated? How does its expression affect the Ras-GAP function of neurofibromin? How does its expression affect the signaling pathways downstream of Ras? How does disruption of its expression affect animal behaviors *in vivo*? Lastly, we will discuss the pressing remaining questions for future studies.

2. The functional role of regulated expression of NF1 exon 23a

2.1 Expression of Nf1 exon 23a in mouse

RT-PCR analysis indicated that the alternative inclusion of exon 23a is tightly regulated in tissue- and developmental stage-specific patterns. In adults, exon 23a

is predominantly skipped in the brain and testis leading to production of the type 1 NF1 isoform, while in other tissues, exon 23a is included to various extents leading to production of the type 2 isoform [6, 12, 13]. In adult mouse, exon 23a is included at 8% in the testis, 11% in the brain, 42% in the spleen, 58% in the heart, 62% in the liver, 78% in the kidney and 82% in the lung [14]. Within the brain, exon 23a is least included in hippocampus at 2–4% and slightly more included in the cortex at 10% [15]. In primary mouse cardiomyocytes, exon 23a is included at 70% [16].

During development, in the mouse brain, a switch from the isoform 2 to isoform 1 occurs during early embryonic development between day E10 and E11 [6, 13]. The biological significance of this switch has not been investigated.

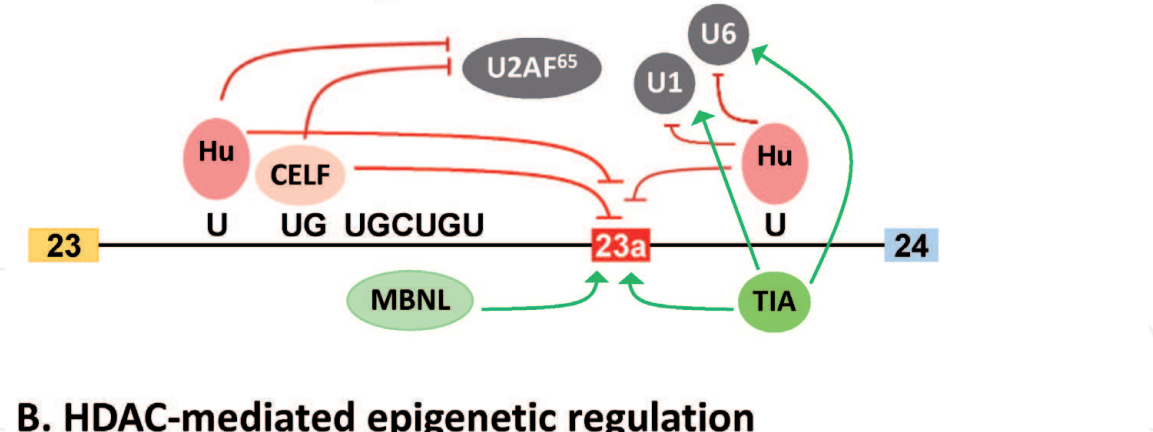
2.2 Molecular mechanisms regulating alternative splicing of exon 23a

Most of our experiments were conducted using human *NF1* sequence and human, mouse or rat cells. All of the existing evidences indicate that this alternative splicing event is conserved in mammalian cells [17]. Consistent with the widely used nomenclature, the human gene is designated as *NF1* while the mouse gene is designated as *Nf1* throughout the chapter.

The differential splicing of exon 23a is under complex control by two distinct mechanisms (**Figure 2**). The first mechanism involves several regulatory RNA-binding proteins (RBPs) which promote either its skipping or inclusion (**Figure 2A**). Two families of RBPs which promote the skipping of exon 23a have been identified, Hu proteins, also known as ELAV-like proteins, and CUG-BP1 and ETR-3 like factors (CELF). Hu proteins bind to AU-rich regions of RNA both upstream and downstream of exon 23a while CELF proteins bind to UG-rich motifs upstream of exon 23a (**Figure 2A**) [18–20]. Mechanistically, upstream of exon 23a, Hu and CELF proteins function to block the splicing factor U2AF from binding to the 3' splice site, while downstream of exon 23a, Hu proteins block splicing factors U1 and U6 snRNP complexes from binding to the 5' splicing site [18, 20]. Two additional families of RBPs, TIA-1/TIAR and muscleblind-like (MBNL) proteins, on the other hand, promote the inclusion of exon 23a (**Figure 2A**). TIA-1/TIAR proteins, in direct competition for binding with Hu proteins, bind to the U-rich sequence downstream of exon 23a, promoting the U1 and U6 snRNP binding at the 5' splice site and inclusion of the exon [18]. MBNL proteins binds to a sequence upstream of exon 23a to promote its inclusion (**Figure 2A**) [21].

The second mechanism involves epigenetic regulation of alternative splicing, at the chromatin level, through altering histone modifications and transcriptional elongation rate (**Figure 2B**). One of the models that explains the epigenetic regulation of splicing is the kinetics coupling model of transcription and splicing [22]. This model predicts that faster transcriptional elongation rate of RNAPII promotes skipping of an alternative exon, which is usually surrounded by suboptimal splicing signals, as in the case of NF1 exon 23a [23]. One of the factors regulating transcriptional elongation rate is the “openness” of chromatin modulated by histone acetylation [22]. The higher level of histone acetylation is correlated with more relaxed configuration of the chromatin, which allows RNAPII move faster during transcription. Exon 23a is subjected to this mode of regulation in two different ways as shown in **Figure 2B**. Studies using mouse primary cardiomyocytes where exon 23a is normally included at 70% demonstrated that an increase in Ca^{2+} by KCl-induced depolarization led to a significant reduction of inclusion to 10–15% through increasing histone acetylation on the body of the entire *Nf1* gene [16]. In neuronal cells, Hu proteins interact with HDAC2, a member of the histone deacetylase family, that reduces the deacetylation enzymatic activity of HDAC2 in a localized fashion [24]. In both cases, the transcriptional elongation rate is increased by histone hyperacetylation, leading to exon 23a skipping [16, 24].

A. RBP-mediated regulation



B. HDAC-mediated epigenetic regulation

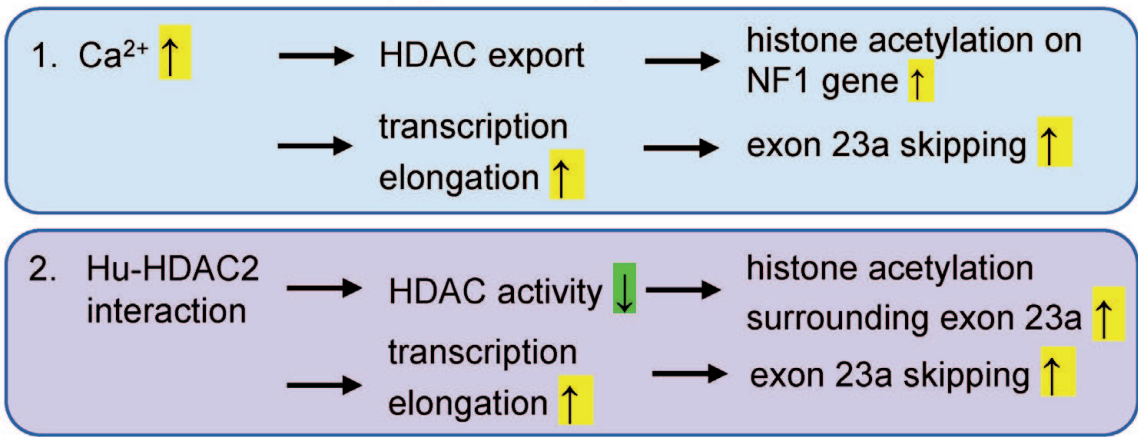


Figure 2. Regulation of alternative splicing of NF1/Nf1 exon 23a. A. RBP-mediated regulatory mechanisms. TIA and MBNL proteins promote inclusion of exon 23a by recruiting splicing factors U1 and U6 to the 5' splice site and U2AF to the 3' splice site, respectively. Hu and CELF proteins promote skipping of exon 23a by preventing these splicing factors from binding. B. Epigenetic regulatory mechanisms. Both mechanisms involve increased histone acetylation, which leads to increased transcription elongation rate causing exon 23a to be skipped. The increased histone acetylation is triggered by either nuclear export of HDAC proteins (box 1) or decreased activity of HDAC2 (box 2). The up and down arrows indicate increase and decrease, respectively.

2.3 Role of exon 23a expression in cell signaling regulation

In order to uncover the biological importance of exon 23a inclusion, our laboratory generated mutant embryonic stem (ES) cell lines through the classical gene-targeting knock-in approach [23]. We generated two contrasting mouse ES cell lines, one showing 100% exon 23a inclusion in the endogenously expressed *Nf1* gene, *Nf1*^{23aIN/23aIN}, and the other 100% exon 23a skipping, *Nf1*^{23aΔ/23aΔ} [23].

We then differentiated these ES cells into CNS-like neurons following an established protocol [25]. In this two week procedure, mouse ES cells were first grown in a non-adherent dish in the presence of retinoic acid to form cellular aggregates, which were then dissociated and plated on laminin-coated tissue culture plates in neuronal culture medium that support neural differentiation and maturation. This procedure was shown to produce pyramidal neurons with >90% homogeneity [25]. When the two mutant *Nf1* ES cell lines were differentiated into neuronal cells, they showed drastically different Ras signaling but similar cAMP activities [23]. Compared to wild type neurons (10%, exon 23a inclusion), the *Nf1*^{23aΔ/23aΔ} neurons (0% exon 23a inclusion) exhibited a slightly lower level of Ras-GTP while the *Nf1*^{23aIN/23aIN} neurons (100% exon 23a inclusion) exhibited at least three times more Ras-GTP [23]. These experiments establish that exon 23a expression affects the Ras-GAP function of the endogenously expressed neurofibromin. Interestingly,

Nf1 exon 23a expression specifically affects the phospho-ERK1/2 level downstream of Ras but not the PI3K/Akt/mTOR pathway [23].

Using the *Nf1*^{23aIN/23aIN} ES cells, we generated a mutant mouse line [14]. The *Nf1*^{23aIN/+} mouse ES cells were from the 129 background. Chimeric 129:C57Bl/6 J mice were generated by blastocyst injection of the *Nf1*^{23aIN/+} ES cells and crossed with C57Bl/6 J mice. A founding *Nf1*^{23aIN/+} mouse was obtained. The mice were then crossed for 10 generations onto the C57Bl/6 J background. In the *Nf1*^{23aIN/23aIN} mice, the *Nf1* gene only produces the isoform II neurofibromin where exon 23a is included in all cell types at 100% [14]. When the mouse brain proteins were analyzed, similar results were found as in the ES-derived neurons. While Ras-GTP level was barely detectable in the wild type mouse brain, it was significantly increased in the *Nf1*^{23aIN/23aIN} brain [14]. The pERK1/2 is six times higher in the mutant than wild type brain while the PI3K/Akt/mTOR pathway was unaltered [14]. These findings support a model in which alternative splicing of exon 23a plays a crucial role in regulating the Ras–Raf–MEK–ERK signaling pathway *in vivo*.

2.4 Role of exon 23a expression in mouse learning and memory behaviors

To explore the link between exon 23a regulation of Ras and cognitive behaviors, a battery of learning and memory tests were conducted comparing the wildtype and mutant *Nf1*^{23aIN/23aIN} mice. The results of these tests indicated clear impairments in learning and memory performance in the mutant mice [14].

To test short-term and long-term spatial memory, a T-maze and Morris water maze test were conducted, respectively. T-maze test is used to examine the short-term spatial memory. In this test, mice were placed in a T-shaped maze and allowed to explore the maze freely for 10 minutes while one of the arms was closed. Following the exploration, mice were returned to their home cage for 2 hours and then put back in the T-maze with all three arms open. Once put in the T-maze, mice were video recorded. The memory measurement was calculated as the time spent in the previously closed arm divided by the overall time spent in both arms, which was expected to be 50% by chance. The wild type mice were more likely to explore an unfamiliar lever arm than a familiar one, a preference indicative of an active short-term memory. The mutant *Nf1*^{23aIN/23aIN} mice showed an impairment of this function and failed to display any preference between lever arms, with a selection rate around 50% [14].

Morris water maze test is used to examine the long-term spatial memory. In this test, mice were trained in a small water pool in a well-lit room replete of visual cues. A hidden escape platform was placed 0.5 cm beneath the water level in a particular location in the pool. Animals were tested for three trials per day over 4 days. For these trials, mice were placed in the water and allowed to swim for 60 seconds. If mice did not find the platform during the allotted time, they were guided toward it, and held for 15 seconds on the platform. Swim time and path length were recorded. Following the final session, the platform was removed for a probe trial to test for spatial strategy and retention. During the probe test, mice were allowed to swim for 60 seconds without the possibility of escape; the percentage of time spent in the quadrant where the platform was previously located was measured. In this test, the mutant *Nf1*^{23aIN/23aIN} mice fell behind their wild type counterparts in their ability to find a hidden platform upon repeated exposure to the same conditions. Additionally, when the hidden platforms were removed, the mutant mice spent less time swimming in the region that the platform had been in previous trials [14].

The mutant *Nf1*^{23aIN/23aIN} mice also exhibited impairment in the fear associative learning test in a fear conditioning experiment [14]. In this experiment, mice were placed in a cage and given a short electric shock after being given an audio cue.

After 24 hours, their freezing response times after being placed in the same cage and being played the audio cue were measured. The mutant mice showed increased freezing times over wild type mice after being placed back within the cage that shocks had been given [14]. When this testing was repeated over time, the mutant mice showed an inability to extinguish this conditioned response as compared with the wild type mice [14].

3. Conclusions and future studies

Our studies have demonstrated that *Nf1* exon 23a expression is tightly regulated and it plays a key role in controlling Ras signaling and learning behaviors in mice. In the brain, when exon 23a inclusion is increased, e.g., as shown in the mutant *Nf1*^{23aIN/23aIN} mice, the Ras-GAP function of neurofibromin decreased, leading to an increase in pERK1/2 activity, which results in defects in learning and memory behaviors.

Given the role of Ras/ERK in many brain functions, it is reasonable to predict additional behavioral defects in the mutant *Nf1*^{23aIN/23aIN} mice. For example, regulated Ras/ERK signaling is known to modulate circadian as well as depressive behaviors [26, 27]. Future experiments can be established to examine such behaviors in the mutant mice.

The behavioral defects observed in the mutant *Nf1*^{23aIN/23aIN} mice is very interesting in light of a prior study by Costa and colleagues [28]. In this study, *Nf1* exon 23a was deleted from the *Nf1* gene in mice. These mice also suffered from learning and memory impairments. Specifically, similar long-term spatial memory defects were observed in these mice in the same Morris water maze test [28]. Without exon 23a, these mice should have very low Ras/ERK activity, opposite of our mutant mice. However, both mutant mice display the same learning defect. These results lead to a tantalizing hypothesis that both isoforms of neurofibromin are required for optimal brain functions. Even though isoform 1, the one without exon 23a, is the predominant isoform in the brain, its deletion is detrimental. Thus, it appears that the potential for this exon to be included is important for normal brain functions. Is it possible that the alternative splicing is regulated dynamically so under certain physiological conditions exon 23a is included significantly more in certain areas of the brain? Give the complex nature of this question, only exquisitely designed experiments will reveal the answer.

Lastly, the ratio of neurofibromin isoform 1 and isoform 2 in neuronal tissues in NF1 patients has never been examined. It will be interesting to study if the ratio changes in patients and if so, how does the change contribute to the disease development.

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Conflict of interest

The authors declare no conflict of interest.

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