

# A multifunctional, multi-pathway intracellular localization signal in Huntingtin

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Nuclear accumulation of the polyglutamine-expanded mutant huntingtin protein remains one of the most predictive cell biological phenotypes of Huntington's disease (HD) progression in patient brain samples and mouse models of the disease. Yet, the relationship between huntingtin nuclear import, neuronal dysfunction and toxicity is not fully understood and it remains unclear whether nuclear accumulation is required for disease onset. Here, we discuss several studies that have guided current understanding of this subject, and highlight our recent data detailing the discovery of a karyopherin  $\beta 1/\beta 2$ -type nuclear localization signal near the N-terminus of huntingtin. This signal can function through multiple pathways of nuclear import, and may also be responsible for huntingtin import into the primary cilium. This work represents a significant step forward in our knowledge of the regulatory pathways that govern huntingtin nuclear accumulation and will allow direct examination of both normal and mutant huntingtin nuclear function. This work also suggests a re-examination of the cell biology of any protein that contains a multi-pathway nuclear localization signal. The possibility of targeting huntingtin nuclear import therapeutically and the potential impacts of such a strategy for the treatment of HD are also discussed.

The adult-onset neurodegenerative disorder Huntington's Disease (HD) is caused by a polyglutamine repeat expansion mutation near the N-terminus of the 350 kDa protein, huntingtin.<sup>1,2</sup> Despite the widespread expression of huntingtin in all cells, neurons within the striatum and cerebral cortex are selectively vulnerable to the toxicity of mutant huntingtin.<sup>3</sup> Wild-type huntingtin has been implicated in a range of diverse cellular processes, such as vesicular transport, transcriptional regulation, synaptic function, actin remodeling and the cell stress response.<sup>4-9</sup> In healthy neurons, huntingtin localizes primarily to the cytoplasm; however, particularly in response to cell stress, nuclear entry is also observed.<sup>10,11</sup> Throughout the progression of HD, shuttling of huntingtin to and from the nuclear compartment becomes disrupted, leading to accumulation of mutant huntingtin in the nuclei of striatal and cortical neurons.<sup>12,13</sup> The synchrony of this

event with disease onset, and its specificity for affected cell types, has prompted speculation that nuclear translocation is a critical and a possibly causal step in disease pathogenesis.

We have recently reported the characterization of the first definitive nuclear localization signal (NLS) within huntingtin.<sup>14</sup> This non-classical, proline-tyrosine, or PY-NLS, found between amino acids 174–207, possesses a unique structured region that is required for recognition of the sequence by the import receptors karyopherin  $\beta 1$  and  $\beta 2$  (also known as importin  $\beta 1$  and transportin). We have named this structured region the “intervening sequence” (IVS) due to its location between the consensus epitopes of the NLS that are comprised of a basic region, a downstream single arginine and the proline-tyrosine.<sup>15</sup> This type of NLS is not unique to huntingtin, and is seen in the mRNA export factor, NXF1. The NXF1 PY-NLS can function through four karyopherin family pathways, including karyopherin  $\beta 1$  and  $\beta 2$ , and does have a long stretch of residues between the NLS epitopes.<sup>16</sup> In isolation, the IVS is capable of localizing to the cytoplasm, suggesting that it may regulate the activity of the NLS by targeting huntingtin to an insoluble phase. Our hope is that this data will guide the development of genetic tools for future exploration and proof of principle of the pathogenic link between huntingtin nuclear import and toxicity.

The earliest transgenic mouse model of HD, termed R6/2, expresses only a short amino-terminal fragment of huntingtin (1–81 amino acids).<sup>17</sup> This region contains the polyglutamine tract, and produces an extremely severe pathological phenotype, far greater than is observed in full-length huntingtin mouse models. In patients, polyglutamine tract length is strongly correlated with disease onset, with a greater number of repeats being associated with earlier development of symptoms.<sup>18</sup> This is also true for R6/2 mice – up to a particular threshold. At repeat lengths beyond 200, the life expectancy of the R6/2 mice shows a paradoxical improvement, reversing the previous trend.<sup>19,20</sup> This phenomenon may be related to the way the small, 1–81 amino acid (< 10KDa) fragment enters the nucleus. The diffusion limit of the nuclear pore complex (NPC) is roughly defined, but has been noted as approximately 50–60 kDa, with larger proteins requiring a nuclear localization signal to enter by a mechanism of facilitated diffusion.<sup>21</sup> The 1–81 fragment falls far below this threshold, granting it unrestricted access to the nuclear compartment. If nuclear translocation of mutant huntingtin is detrimental, this free and unregulated diffusion could be responsible for the severity of the R6/2 model. Thus, a slowed

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or inhibited diffusion, as would be expected with the addition of an extremely long polyglutamine tract, could manifest as reduced pathogenicity in a small fragment context. Consistent with this hypothesis, immunohistochemistry of brain samples from mice bearing longer repeats show a reduction in the formation of nuclear aggregates, and an increase in those that form in the cytoplasm, indicating a potential loss of ability of the huntingtin fragments to traverse the nuclear pore.<sup>20</sup> In larger fragment or full-length models, huntingtin is capable of nuclear entry via the PY-NLS sequence and not by simple diffusion alone. With this added regulation there is greater potential to control the timing of nuclear entry, thus delaying the onset of cellular pathology. Severe toxicity of HD mouse models is limited to the 1–81 R6/2 or N171<sup>22</sup> (1–171 fragment) models. Intriguingly, the huntingtin NLS starts at residue 174.

Consistent with its ability to shuttle from one compartment to the other, huntingtin is also equipped with two CRM1-dependent nuclear export signals: one toward the carboxyl-terminus of the protein,<sup>23</sup> and one at the extreme amino-terminus within the N17 regulatory domain (Maiuri et al., in press). In addition to binding to CRM1, N17 also acts as an endoplasmic reticulum (ER) targeting signal that prevents huntingtin from accumulating in the nucleus under normal cellular conditions.<sup>24</sup> During cell stress, N17 becomes phosphorylated at serines 13 and 16 causing release of huntingtin from the ER and enhanced nuclear entry.<sup>24,25</sup> Thus, it is unclear if the nuclear accumulation of huntingtin observed in degenerating neurons is simply a consequence of persistent cell stress, or a toxic process in itself. A bacterial artificial chromosome full-length huntingtin transgenic model (BACHD) has been developed that is modified at serines 13 and 16 to mimic constitutive phosphorylation (S-D mutation) or constitutive phospho-resistance (S-A mutation).<sup>26</sup> Although phosphorylation (or phospho-mimicry) of huntingtin at N17 is predicted to enhance the protein's nuclear translocation, in this model, the S-D mutation was protective. Consistent with these genetic experiments, treatments that increase the phosphorylation of N17 in full-length mouse models, such as the ganglioside GM1, show striking phenotypic reversal.<sup>27</sup> Thus, the relationship between nuclear import and mutant huntingtin toxicity remains unclear. Rather than nuclear translocation, the phosphoN17-status or sub-nuclear localization of huntingtin to discreet chromatin-dependent puncta may confer toxicity. Alternatively, it may be overly simplistic to view either the nucleus or the cytoplasm as the sole site of toxicity. Instead, the primary issue may be an inability to shuttle efficiently between the two compartments, causing a breakdown in cellular signaling. The stress response switch mediated by huntingtin may therefore be stuck in an on or off state, losing its ability to dynamically switch between the two. The appealing aspect of this hypothesis is that it implies that this huntingtin function is more important as the human brain ages, during which time metabolic stresses are known to increase, along with decreasing mitochondrial efficiency.<sup>28</sup>

Elucidation of the mechanisms of huntingtin nuclear and cytoplasmic shuttling will shed further light on whether limiting nuclear accumulation has therapeutic potential. Many nuclear

import pathways are not valid drug targets, due to lack of specificity in NLS-import factor interactions; however, the huntingtin NLS has a unique structured IVS that could be exploited as a binding region for small molecules. Still, this avenue should be pursued with caution. It was recently reported that huntingtin has a role in ciliogenesis,<sup>29</sup> and our lab has discovered that huntingtin is capable of entering the body of the primary cilium (Maiuri et al., in press).

Cilial entry and nuclear entry have a surprising amount in common. Proteins that form the nuclear pore complex create a permeability or diffusion barrier at the base of the primary cilium, and the import receptor karyopherin  $\beta$ 2 has been found to mediate cilial entry through recognition of PY-NLS sequences.<sup>30,31</sup> Like the nuclear pore complex, some nucleoporins, or nups, can be found at the cilial permeability barrier,<sup>30,32</sup> even though the cilial barrier is not membrane-based. Thus, multi-pathway localization signals may exist in proteins that use the nucleoporin and Ran GTP/GDP gradient to traffic in and out of the cilium as well as the nucleus. Therefore, although disrupting mutant huntingtin nuclear entry may combat the toxic effects that cause HD, it could also inhibit the cilial function of the protein.

The primary cilium is a non-motile singular organelle in neurons, used as an extra-synaptic antenna for soluble signaling through G-protein coupled receptors (GPCRs).<sup>33</sup> While vesicles are not found within the cilium, vesicular motor proteins and complexes exist within the cilium to move internalized GPCRs into the cell. The function of huntingtin in the cilium may be similar to its role in vesicular trafficking. Huntingtin acts as a scaffolding protein that connects vesicles to kinesin, as well as to the p150 glued subunit of dynactin, an activator of dynein.<sup>34,35</sup> These microtubule motor proteins are also required for intraflagellar transport along the axoneme of the cilium, which is composed of tubulin bundles.<sup>36</sup>

Our work to date indicates that the steady-state localization of huntingtin is either at trafficking early endosomes, or the endoplasmic reticulum.<sup>24</sup> Huntingtin only releases from the ER during cell stress that involves low ATP, where the soluble protein then translocates to the nucleus and cilium. When mutant huntingtin is present, even heterozygously, cells are unable to recover from stresses that involve an ATP-capping response at the actin cytoskeleton within the nucleus.<sup>8</sup> ATP is also utilized by motor proteins in the primary cilium. We hypothesize that the transient localization of huntingtin to the nucleus and cilium may be required for an optimal cell stress response in neurons.

It is still not clearly understood why some proteins have multiple nuclear transport signals, and why some signals can use multiple pathways of nuclear, and now cilial entry. These karyopherin/importin proteins are now known to have other important cell biological functions aside from just carrying cargo to or from the nucleus.<sup>37,38</sup> As the knowledge of the biology of these import factors increases, so then will the role of these multifunctional, multipathway transport signals in proteins.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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