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Commentary on "Novel Interaction of the Dopamine D2 Receptor and the Ca^{2+} Binding Protein S100B: Role in D2 Receptor Function"

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Running Title: Commentary on the Dopamine D2 Receptor and S100B

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Results and Discussion: 822

Abbreviations: D2-IC3, dopamine D2 receptor third cytoplasmic loop; CT-IC3, a portion of D2-IC3 comprising roughly the C-terminal half; ERK, extracellular signal-regulated kinase; HEK293, human embryonic kidney 293; IC3, receptor third cytoplasmic loop; NT-IC3, a portion of IC3 comprising roughly the N-terminal half, from K211-P290.

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Abstract

We previously proposed that the dopamine D2 receptor-interacting protein S100B binds to a putative S100B-binding motif at residues R233-L240 towards the N-terminus of the 3rd cytoplasmic loop. We used in vitro pull-down assays with FLAG-tagged fragments of the rat D2 receptor third cytoplasmic loop (D2-IC3) and in vitro-synthesized S100B to evaluate this hypothesis. Our results indicate that the putative S100B-binding motif is neither necessary nor sufficient for strong binding of S100B to D2-IC3. Instead, two residues at the junction of the 5th membrane-spanning domain and the cytoplasmic extension of that α-helical domain, K211-I212, are required for robust, calcium-sensitive binding of S100B. This is also the approximate location of previously identified determinants for the binding of arrestin and calmodulin. A D2 receptor mutation converting I212 to phenylalanine has been described in patients with a hyperkinetic movement disorder.

Significance Statement

S100B is a small calcium-binding protein that modulates signaling by the dopamine D2 receptor. Our new data suggest that our previous hypothesis about the involvement of an S100B-binding motif is incorrect, and that an important determinant of S100B binding includes a residue that is mutated in patients with a hyperkinetic movement disorder.

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Introduction

Binding of the calcium-binding protein S100B to the third cytoplasmic loop (IC3) of the dopamine D2 receptor was reported by this laboratory in 2008 (Liu *et al.*, 2008). We presented the following evidence for a functional and physical interaction between these two proteins: 1) S100B was identified in an unbiased screen of a rat brain cDNA library using D2-IC3 as bait in a bacterial two-hybrid assay, 2) binding of S100B to D2-IC3, but not the IC3 of the dopamine D3 receptor, was confirmed in an in-vitro pull-down assay, 3) S100B co-precipitates with the D2 receptor when co-expressed in human embryonic kidney 293 (HEK293) cells, and also co-precipitates with the native D2 receptor in rat neostriatal homogenates, 4) S100B immunoreactivity is present in rat neostriatal neuronal cultures, and shows substantial overlap with D2 receptor immunoreactivity, and 5) overexpression of S100B in HEK293 cells potentiates D2 receptor activation of ERK1/2 and inhibition of forskolin-stimulated cyclic AMP accumulation. We also identified a potential S100B-binding motif (Ivanenkov *et al.*, 1995) at D2 receptor residues R233-L240 (**Fig. 1**)(Liu *et al.*, 2008). Subsequently, another group made the important observation that S100B binding to D2-IC3 is greatly enhanced in the presence of Ca^{2+} and involved the C-terminus of D2-IC3 (Dempsey and Shaw, 2011).

Here we present data suggesting that the putative S100B-binding motif plays at most a minor role in the binding of S100B to D2-IC3. Instead, residues K211-I212, at the junction of the 5th membrane-spanning domain and the cytoplasmic extension of that α-helical domain (**Fig. 1**), are required for robust binding to D2-IC3. This region of the receptor also contains determinants for the binding of calmodulin (Liu *et al.*, 2007) and arrestin (Lan *et al.*, 2009). These results are particularly salient in light of the recent discovery of a *DRD2* mutation in patients with a hyperkinetic movement disorder, a mutation that changes I212 to phenylalanine and that dramatically alters D2 receptor signaling (van der Weijden *et al.*, 2020; Rodriguez-Contreras *et al.*, 2021).

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Materials and Methods

In vitro FLAG-tagged Dopamine D2 Receptor-IC3 Pull-down Assay. For construction of the FLAG fusion protein, the third cytoplasmic loop of the rat dopamine D2L receptor (D2-IC3), amino acids 211-371, was PCR-amplified and cloned into pT7-FLAG-3 (Sigma-Aldrich, St. Louise, MO). All FLAG-tagged D2-IC3 truncation mutants were generated using the QuikChange Lightening mutagenesis kit (Stratagene, La Jolla, CA). FLAG fusion proteins were induced with IPTG (Isopropyl β-D-1-thiogalactopyranoside) in BL21 competent cells and purified from bacterial cell lysate by using Anti-FLAG M2 affinity gel (Millipore Sigma, St. Louis, MO). Rat S100B was PCR cloned into a Novagen IPTG-inducible pET-30a (+) vector (Millipore Sigma, St. Louis, MO) and purified by Ni-NTA Purification System (Invitrogen, Carlsbad, CA). Pre-bound, washed FLAG-tagged protein beads were incubated with 1-2 μg of purified S100B for 1h at room temperature followed by wash and elution steps, SDS-PAGE, and immunoblotting. Coomassie-stained SDS-PAGE gels were used to confirm that approximately equal amounts of the various FLAG-tagged constructs were used in a given experiment. Immunoblotting was done using rabbit anti-bovine S100B antibody (Cat. S0052-10A, US Biological, Salem MA) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Thermo Fisher Scientific, Waltham, MA). Immunodetection was accomplished using a SuperSignal™ West Pico chemiluminescent kit (Thermo Fisher Scientific, Waltham, MA). The intensity of bands was quantified using Gel Doc EQ System (Bio-Rad, Hercules, CA) or ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018).

Results and Discussion

Using in vitro FLAG pull-down assay with purified S100B, we found that S100B bound robustly to FLAG-tagged constructs encoding the entirety of D2-IC3 or only the N-terminal half of IC3 (NT-IC3; K211-P290), and bound much more weakly to a construct encoding the C-terminal half of D2-IC3 (E291-K371; **Fig. 2**). Furthermore, binding was higher in the presence

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of added Ca^{2+}, as described previously (Dempsey and Shaw, 2011). $CaCl_2$ (2 mM) was included in all subsequent experiments. In the Coomassie-stained gels that were used to equalize the amounts of the various of FLAG-D2-IC3 constructs that were added to the pull-down assays, we noticed a nonspecific band that was very close to the apparent molecular weight of FLAG-D2-IC3 (**Fig. 2**, middle panel). Some mutants were also constructed from NT-IC3 (e.g., Δ211-215 NT-IC3 in **Fig. 3**) to separate the specific and nonspecific bands.

We previously hypothesized that S100B binds to a putative S100B-binding motif (Ivanenkov *et al.*, 1995) at R233-L240 in NT-IC3 (Liu *et al.*, 2008). Here we tested that hypothesis using two mutants, one in which the entire motif was deleted, and one in which the two positively charged residues were mutated to alanines (**Fig. 3**, bottom panel). Neither mutation had any discernable effect on the binding of S100B (**Fig. 3**, top panel). In contrast, deletion of the 5 residues K211-V215 greatly decreased S100B binding. We conclude from this that the presumed S100B-binding motif plays at most a minor role in S100B binding to D2-IC3, but that important determinants of binding are located in the N-terminus of D2-IC3 at the junction of the membrane-spanning and cytoplasmic portions of the 5^{th} α-helical domain.

To further localize S100B binding determinants, we compared the construct carrying the K211-V215 deletion with constructs in which either K211-I212 or Y213-V215 were mutated to alanine. The K211A,I212A mutant bound S100B as poorly as the K211-V215 deletion mutant, whereas S100B binding to the Y213A,I214A,V215A mutant was indistinguishable from wild type D2-IC3 (**Fig. 4**). Thus, S100B binding to D2-IC3 has a strong requirement for one or both of K211 and I212.

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The strong S100B binding to NT-IC3, compared to CT-IC3, that we describe here contrasts with the work of Shaw and colleagues (Dempsey and Shaw, 2011; Wang *et al.*, 2019). That group used peptide arrays and NMR spectroscopy to localize S100B binding to the C-terminus of D2-IC3, in particular to residues S288-Q345 of the rat short D2 receptor splice variant, $D2_S$. This fragment corresponds to residues 317-374 of rat $D2_L$ (**Fig. 1**). We cannot fully explain the difference between their results and ours. Their D2-IC3 construct began with R217, which omits the most critical residues identified by this work; thus, the low binding to N-terminal peptides that they observed is consistent with the low binding that we observed to the ΔKIYIV deletion mutant of D2-IC3 (**Fig. 3 and 4**). With regard to CT-IC3, our construct omitted the final three residues of S288-Q345, which might have decreased binding if those are critical residues. On the other hand, it is possible that the weaker band shown for CT-IC3 (**Fig. 2**), compared to the robust binding to NT-IC3 that includes K211-215, represents the binding characterized by Shaw and colleagues.

It is important to note that the entirety of the work from our laboratory and the laboratory of Shaw and colleagues identifying the S100B binding site(s) used receptor fragments in solution. Under these conditions, D2-IC3 and the adjoining transmembrane residues at the cytoplasmic face of the membrane likely do not have the same tertiary structure as they do in the intact membrane-located receptor. Although our studies indicated that S100B-binding enhances D2 receptor signaling (Liu *et al.*, 2008), additional work is needed to characterize more fully the nature of and functional consequences of S100B binding to the site identified in this study. Such work is complicated by the number of proteins that bind to the same part of the receptor (Liu *et al.*, 2007; Lan *et al.*, 2009), and by the observation that mutations in this region can affect

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receptor tertiary structure independently of effects on the binding of receptor-interacting proteins (Rodriguez-Contreras *et al.*, 2021).

Patients with a novel inherited movement disorder that has choreatic and dystonic features carry a mutation that changes I212 to phenylalanine (van der Weijden *et al.*, 2020). $I212^{5.61}$, according to the Ballesteros-Weinstein number scheme (Ballesteros and Weinstein, 1995), appears to be important for normal function of the receptor. The same position 5.61 is one of the top mutationally intolerant positions in the β_2-adrenoceptor at the receptor:G protein interface (Jones *et al.*, 2020), while phenylanine substitution at this position in the dopamine D2 receptor causes decreased arrestin recruitment and enhanced constitutive activation of G proteins and G protein-mediated signaling (van der Weijden *et al.*, 2020; Rodriguez-Contreras *et al.*, 2021). The data presented here show that S100B joins arrestin (Lan *et al.*, 2009) and calmodulin (Liu *et al.*, 2007) as D2 receptor-interacting proteins whose binding to the receptor is decreased by small mutations that include $I212^{5.61}$.

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Authorship Contributions:

Participated in research design: Lee and Neve.

Conducted experiments: Lee.

Performed data analysis: Lee, Rodriguez-Contreras, and Neve.

Wrote or contributed to the writing of the manuscript: Lee, Rodriguez-Contreras, and Neve.

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Footnotes

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The authors declare no conflicts of interest.

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Figure Legends

Fig. 1. Rat D2-IC3 and adjoining membrane-spanning domains. The cartoon depicts the membrane-spanning regions and intracellular extensions of the 5th and 6th α-helical domains of the rat dopamine $D2_L$ receptor, along with the connecting IC3. K211 and I212 are depicted in red, the green residues mark the putative S100B-binding motif previously identified by us (Liu *et al.*, 2008), and the blue residues indicate the C-terminal fragment described by Dempsey and Shaw (Dempsey and Shaw, 2011).

Fig. 2. A FLAG pull-down assay was used to characterize the binding of S100B to D2-IC3 (K211-A371). The upper panel depicts S100B immunoblots (IB) in eluates from two pull-down assays using the indicated portions of FLAG-tagged D2-IC3 or FLAG alone, in the absence or presence of 2 mM (top IB) or 4 mM (bottom IB) $CaCl_2$. The middle panel is a Coomassie-stained (CS) gel showing the FLAG-tagged proteins used in the pull-down assay (indicated by angle brackets), as well as a nonspecific protein that is present in all samples and that migrates with approximately the same apparent MW as full-length FLAG-D2-IC3. The four lanes of the CS gel depict receptor fragment inputs for lanes 1-4 (and 5-8), respectively, of the immunoblot. The constructs are depicted schematically in the bottom panel; green indicates strong S100B binding, whereas red indicates little or no detectable binding.

Fig. 3. Determinants of S100B binding to D2 receptor IC3.The lower panel shows the FLAG-tagged fragments that were tested; red indicates little or no detectable S100B binding, whereas green indicates robust binding. The entire putative S100B motif was deleted in Δ233-240 (bottom), and the approximate location of the two charged residues within the motif that were mutated in R233A, K237A D2 IC3 is marked with arrows. The top panel shows an immunoblot representative of 3 independent experiments. Mean ± SD was 102 ± 47% of control for Δ233-240 and 108 ± 44% for R233A,K237A. An eluate from full-length D2-IC3 is not depicted in this immunoblot. Some lanes with eluates from D2-IC3 mutants not described in this commentary were excised from the immunoblot image. The middle panel is a Coomassie-stained (CS) gel

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showing the FLAG-tagged proteins used in the pull-down assay (indicated by angle brackets), as well as a nonspecific protein that is present in all samples and that migrates with approximately the same apparent MW as full-length FLAG-D2-IC3. In lanes 3 and 4, the nonspecific protein and FLAG-D2-IC3 appear as single bands. Lanes 1-4 correspond to the four lanes of the immunoblot. Some lanes with FLAG-D2-IC3 mutants not described in this commentary were excised from the CS gel image.

Fig. 4. Alanine-substitution of two residues greatly decreased of S100B to D2 receptor IC3. The top panel shows an immunoblot (IB) representative of 7 independent experiments in which binding of purified S100B to FLAG-tagged D2-IC3 constructs was tested. The middle panel is a Coomassie-stained (CS) gel showing the FLAG-tagged proteins used in the pull-down assay. The bottom panel shows the mean ± SD of results from all experiments, expressed as a percentage of wild type D2-IC3, where ΔKIYIV is a deletion mutant of D2-IC3, KI_AA is a D2-IC3 mutant in which K211 and I212 were replaced with alanines, and YIV_AAA is a mutant in which Y213-V215 were replaced with alanines. Each IB and CS gel lane is stacked over the quantitative results for its corresponding construct as indicated by labels in the bottom panel. *P = 0.018 (ΔKIYIV) and 0.021 (KI_AA) compared to D2-IC3 by Dunnett's multiple comparisons test after repeated measures one-way ANOVA (P = 0.0058).

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Figure 1

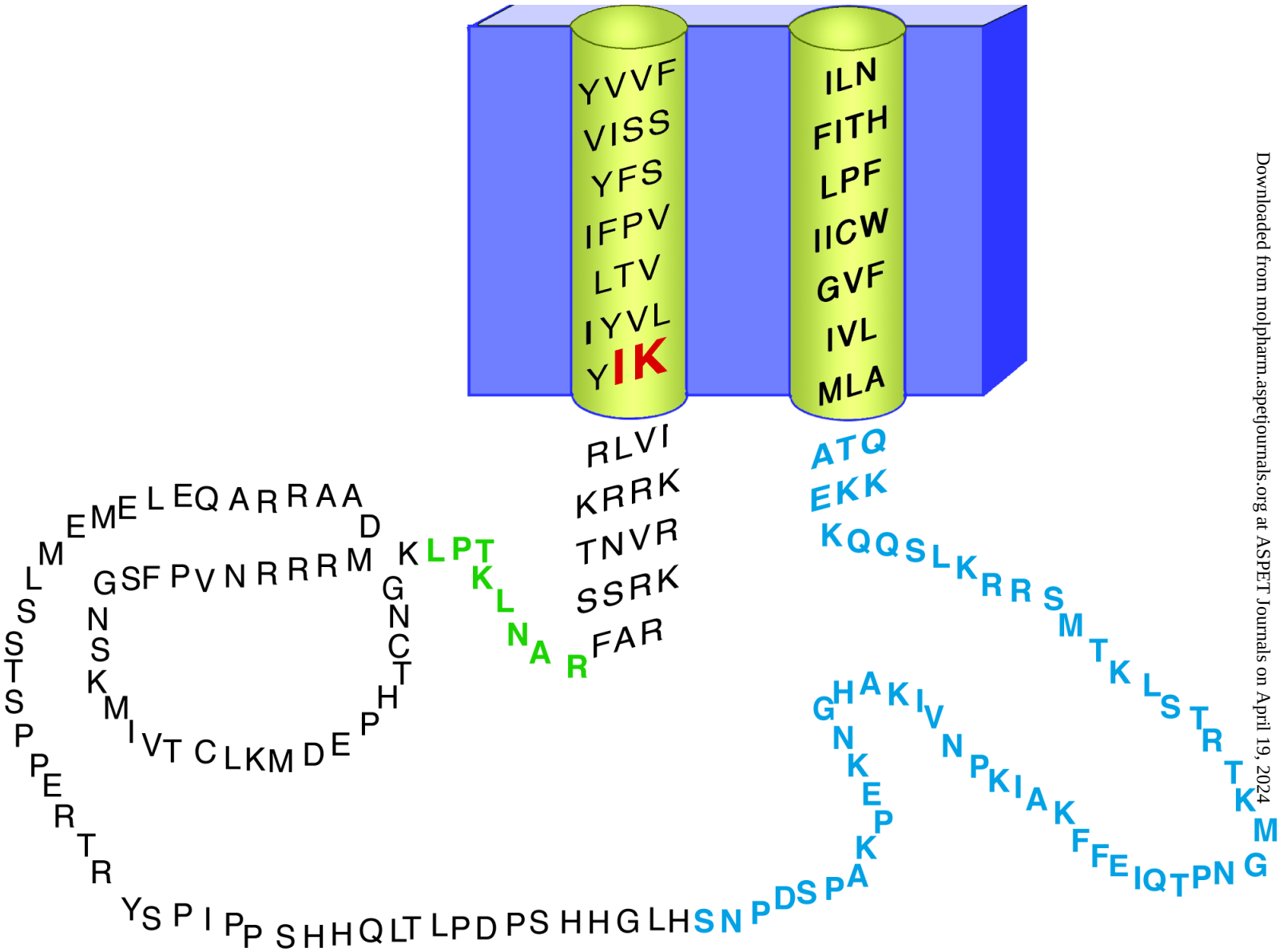

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Figure 2

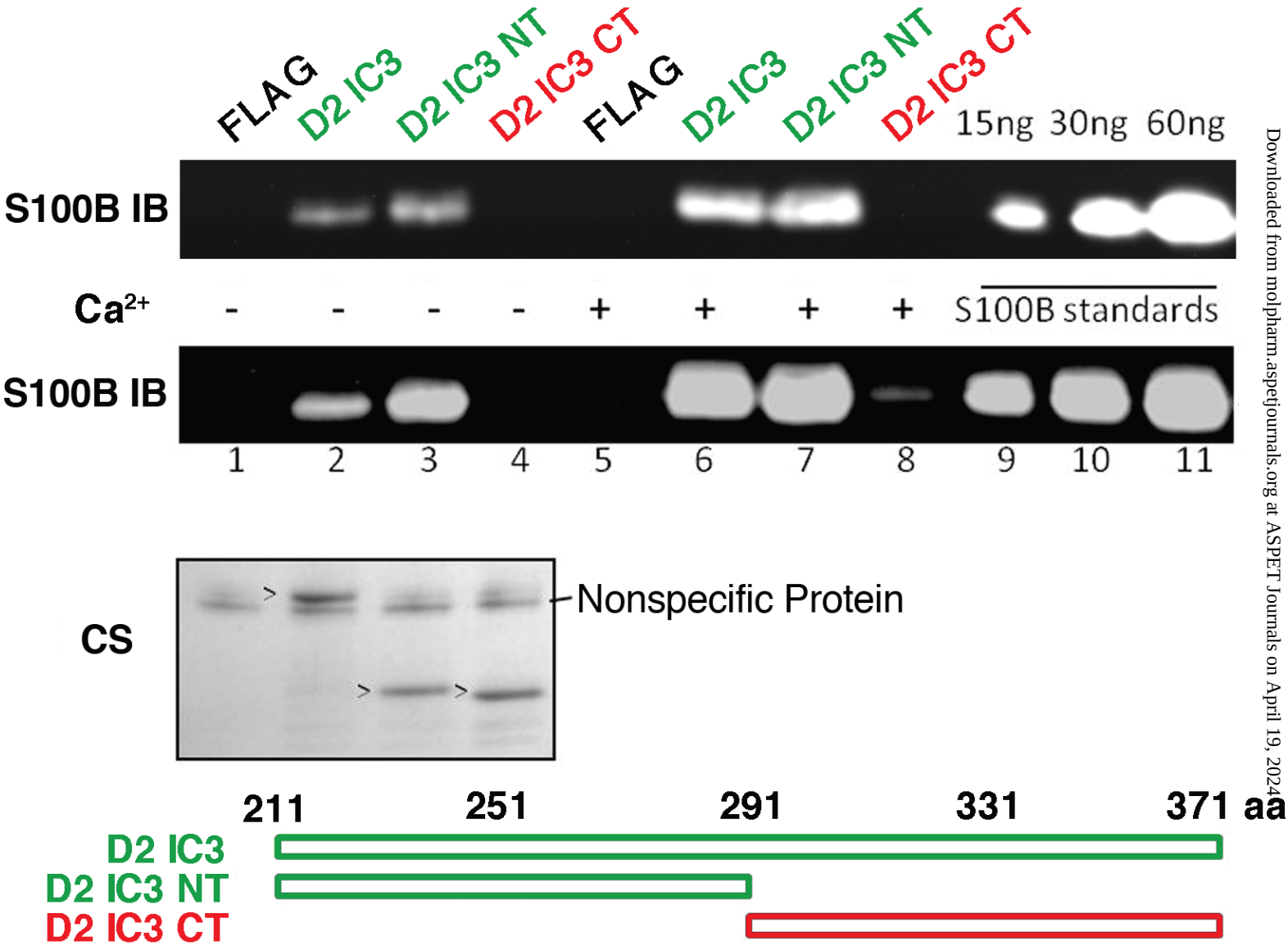

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Figure 3

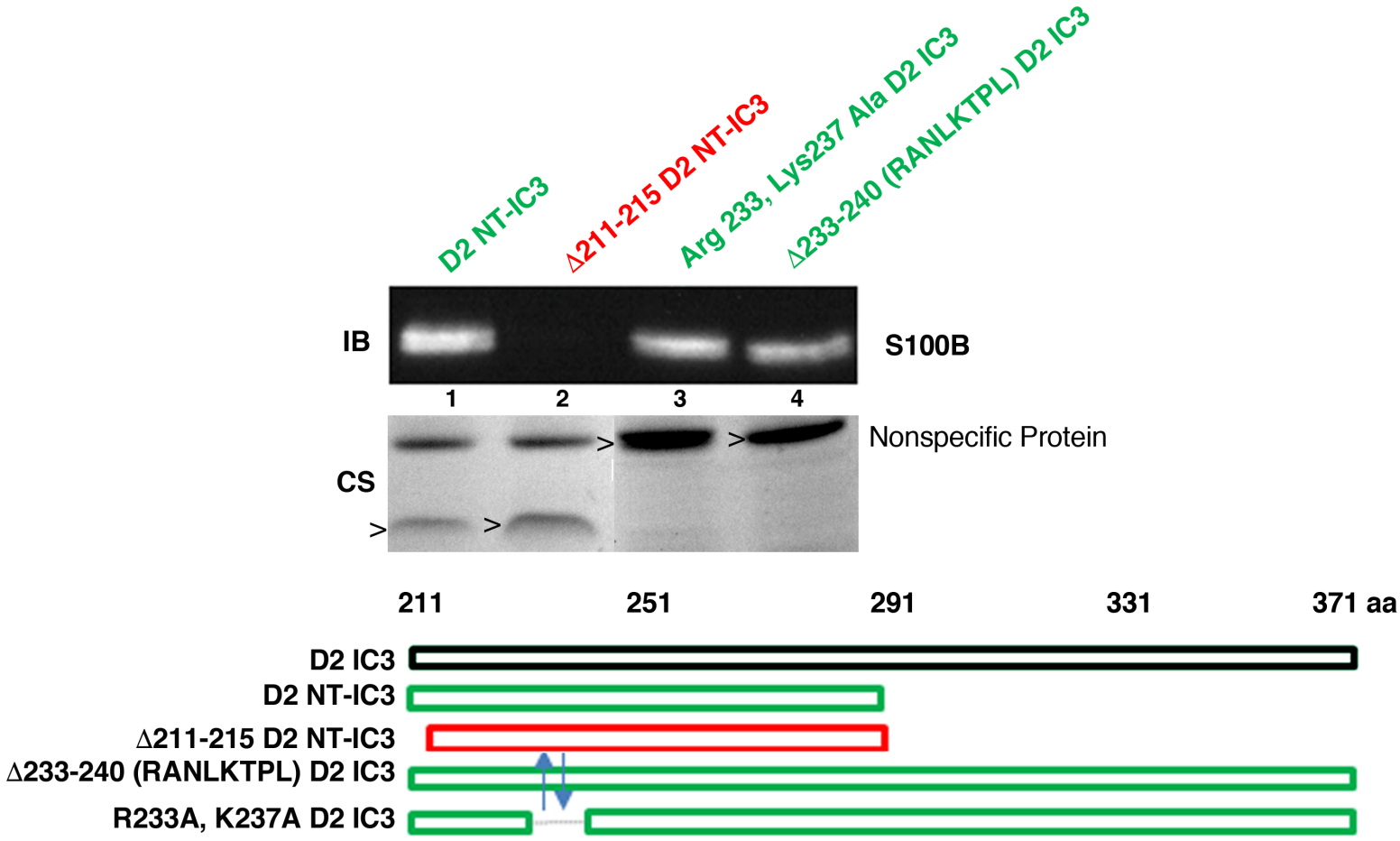

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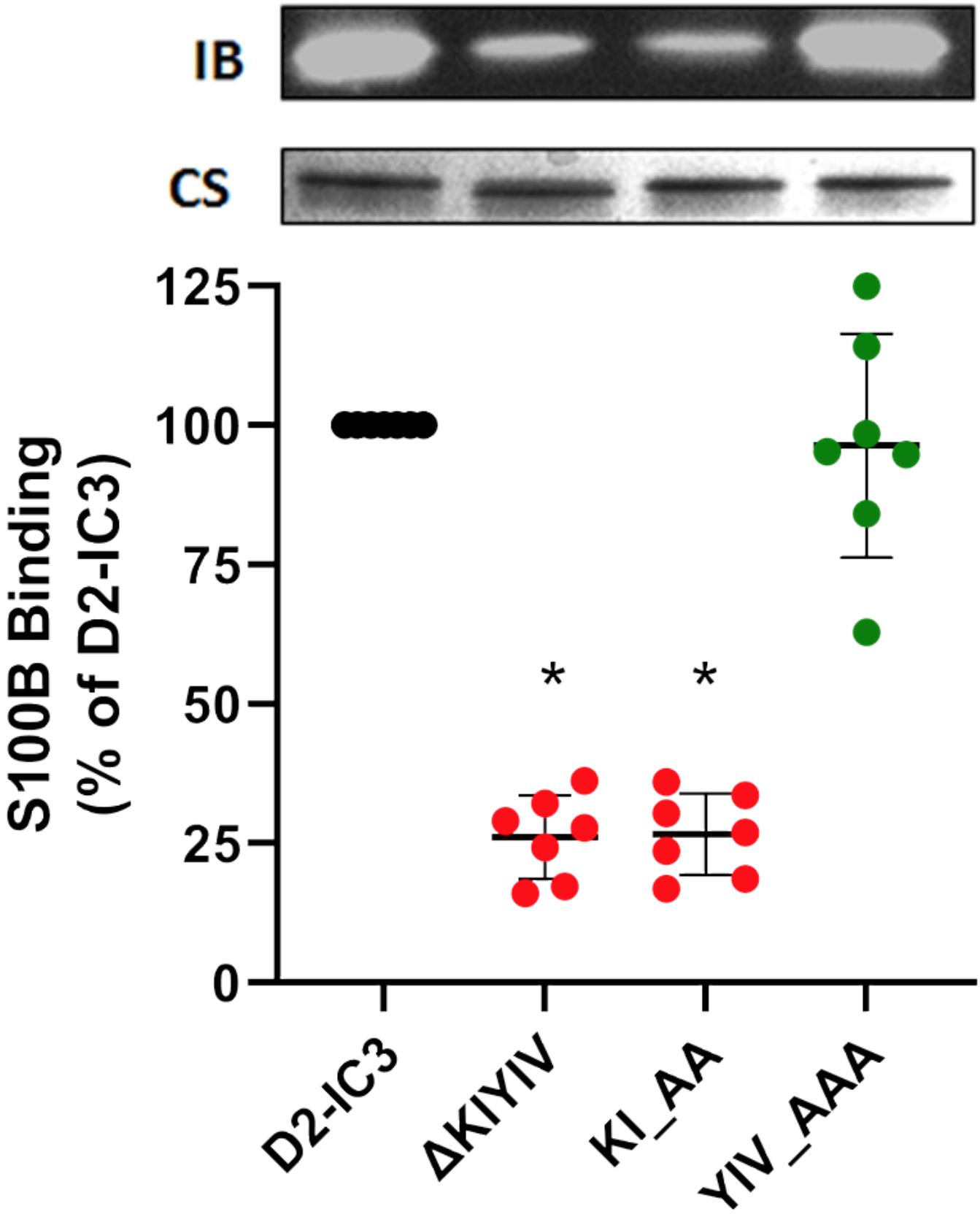
Figure 4



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