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Comment on *Phosphorylation adjacent to the nuclear localization signal of human dUTPase abolishes nuclear import: structural and mechanistic insights* by Róna *et al.* (2013)

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Phosphorylation can regulate nuclear targeting of classical nuclear localization signal (NLS)-bearing cargoes by modulating their affinity for the cellular transporters of the importin (IMP) superfamily, which is critical to many important biological processes (Jans *et al.*, 2000; Poon & Jans, 2005). In particular, phosphorylation within or immediately upstream of the NLS can prevent IMP recognition and nuclear import (Jans *et al.*, 1991, 1995), whereas distinct phosphorylation generally up to 30 amino acids upstream of the NLS can promote IMP recognition/nuclear import (Hübner *et al.*, 1997; Rihs & Peters, 1989). This pattern, originally described for the prototypal NLS identified from Simian Virus 40 Large T-antigen (T-ag) (Kalderon *et al.*, 1984; Rihs & Peters, 1989) was subsequently proven to apply to a plethora of proteins of viral and cellular origins that mediate important biological effects, potentially representing a target for therapeutic intervention (Alvisi *et al.*, 2005, 2011, 2008, 2013; Poon & Jans, 2005).

However, the mechanistic details of how cargo-phosphorylation really influences IMP binding remained elusive until now. In the case of phosphorylation enhancing nuclear transport events, the crystal structure of a complex of a truncated form of IMPα and a phosphorylated peptide from the prototypical NLS of SV40 T-ag was solved, but revealed no direct interaction between the phosphorylated cargo and IMPα (Fontes *et al.*, 2003), suggesting that the effect of phosphorylation in influencing IMP binding in this context might only be observed in the full-length protein (Alvisi *et al.*, 2008, 2013); clearly crystal structures of the full IMPα/β heterodimer complexed with a whole protein, rather than an NLS peptide, is ultimately required to enable understanding of how phosphorylation can indeed promote IMP recognition and nuclear import.

The study from Róna *et al.* (Róna *et al.*, 2013) in the August 2013 issue of *Acta Crystallographica Section D* provides new structural insight into the nature of the phosphorylation-regulated recognition of cargoes by IMPs. In particular, the structure of phosphomimetic and phosphonull-NLS peptides derived from human dUTPase (DUT) complexed with a truncated form of IMPα is correlated with IMP-NLS binding ability. Critically, the phosphomimetic S11E mutation altered the conformation of R15 DUT-NLS, leading to a change in the binding arrangement of the peptide in the IMPα binding site, and to a loss of interaction between the P12 and R15 NLS residues and the IMPα surface. Róna *et al.* (Róna *et al.*, 2013) conclude that this conformational change explains the *ca* tenfold lower binding affinity of the phosphomimetic peptide to the IMPα subunit, as observed in their *in vitro* experiments.

These findings are of great interest in the nuclear transport field because they provide a structural explanation of how phosphorylation might negatively affect the NLS-IMPα interaction. However, a more careful consideration of the biological relevance of the results is

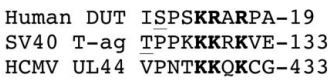

Figure 1
DUT, T-ag and UL44 NLSs all possess phosphorylation sites that negatively regulate nuclear import adjacent to the basic NLS. Phosphorylation sites are underlined, basic residues within NLS core are in bold, the single-letter amino-acid code is used. Numbers indicate amino-acid position within the indicated proteins.

justified since the present study seems to ignore the fact that other proteins exist in the cell that can bind NLSs/NLS-like sequences. In short, Róna *et al.* (2013) do not consider the possibility that phosphorylation of DUT S11 may regulate DUT's interaction with other cellular factors.

In a seminal paper, Hodel *et al.* (2001) clearly showed that only mutations diminishing the binding affinity of IMPα to its NLS bearing cargo by two orders of magnitude significantly impact on nuclear targeting (Hodel *et al.*, 2001); mutations impairing recognition by up to tenfold in fact, have little effect on nuclear accumulation (Hodel *et al.*, 2001; Harreman *et al.*, 2004). Thus, nuclear exclusion of the S11E-DUT, rather than being explicable solely in terms of reduced binding to IMPα, may in fact indicate that other factors are playing an important role in the the observed cytoplasmic localization in cells.

Cytoplasmic retention factor(s) that contribute to negatively regulate NLS activity upon phosphorylation was postulated over 20 years ago (Jans *et al.*, 1991), and more recently shown to be a key factor limiting nuclear accumulation of both SV40 T-ag (through the T124 phosphorylation site) and *Human Cytomegalovirus* processivity factor UL44 (through T427) (Fulcher *et al.*, 2010), both of which closely resemble S11 of DUT in terms of position relative to the NLS core of the respective proteins (see Fig. 1). The cytoplasmic retention factor involved in this case is BRCA-1 binding protein 2 (BRAP2) (Fulcher *et al.*, 2010).

Róna *et al.* (Róna *et al.*, 2013) should consider the possible contribution of negative regulators of nuclear targeting within eukaryotic cells, which compete with IMPs to finely tune the nuclear levels of certain cargoes. Given the modest (*ca* tenfold) reduction in IMPα binding of the entirely cytosolic S11R-DUT as compared to entirely nuclear S11A-DUT, and the fact that in light of previous work (Hodel *et al.*, 2001; Harreman *et al.*, 2004) this is unlikely to impact so strongly on nuclear targeting, it seems feasible that S11 phosphorylation may confer interaction with a factor such as BRAP2, and that this may be the mechanism responsible for the strong cytoplasmic localization.

Given the importance of structural information to our understanding of key physiopathological processes such as gene expression, cell growth and transformation, and virus–host interactions, the work of Róna *et al.* (Róna *et al.*, 2013) is of great importance. We now wait with expectation for new, highly informative crystal structures of IMPs bound to full cargoes, and arguably of even more interest will be crystal structures of negative regulators of nuclear import such as BRAP2 to phosphorylated NLS-containing cargoes.

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Factors influencing nucleo-cytoplasmic trafficking: which matter? Response to Alvisi & Jans' comment on *Phosphorylation adjacent to the nuclear localization signal of human dUTPase abolishes nuclear import: structural and mechanistic insights*

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Nucleo-cytoplasmic trafficking of proteins is tightly regulated in a number of ways (Poon & Jans, 2005; Pouton *et al.*, 2007; Sekimoto & Yoneda, 2012). Post-transcriptional modifications play a key role in these regulatory processes, and phosphorylation can either enhance or reduce nuclear accumulation (Jans, 1995; Nardozzi *et al.*, 2010). Phosphorylation in the vicinity of nuclear localization signals (NLSs) can affect the binding affinity between the cargoes and their nuclear transport receptors. A negative charge in the proximity to the positively charged NLS can diminish its recognition by importin-α, while it might have an enhancing effect if positioned further upstream of the NLS (Alvisi *et al.*, 2008; Fontes *et al.*, 2003; Harreman *et al.*, 2004; Hübner *et al.*, 1997; Kosugi *et al.*, 2008, 2009; Marfori *et al.*, 2012). Until now, however, no clear crystallographic model has been available explaining how phosphorylation inhibits cargo protein binding to importin-α. The crystallographic model presented in our study has now shed light on a structural mechanism that lies behind the phosphorylation-mediated inhibition of nuclear import (Róna *et al.*, 2013).

Recent studies have shown that the cytoplasmic retention factor BRAP2 can recognize NLSs or NLS-like sequences upon phosphorylation, and therefore can affect the localization pattern of several viral and cellular proteins (Fulcher *et al.*, 2010; Li *et al.*, 1998). However, even overexpressed BRAP2 was not able to completely block its binding partners (either endogenous or also overexpressed) from entering the nucleus, since these binding partners still remained mainly nuclear. BRAP2 therefore appears to have mainly a fine-tuning function in determining the nuclear levels of its binding partners (Fulcher *et al.*, 2010).

Nevertheless, the involvement of BRAP2 in the cytoplasmic localization of the NLS phosphorylated (S11) dUTPase (Ladner *et al.*, 1996; Róna *et al.*, 2013; Takacs *et al.*, 2009; Tinkelenberg *et al.*, 2003), as suggested by Alvisi & Jans (2014), is an interesting hypothesis but could only be answered after careful experimental testing. Our unpublished preliminary experiments aiming at identifying the binding partners of human dUTPase did not detect BRAP2 as its binding partner, although further confirmation of this result is needed. Even though the classical monopartite NLS (class 2) (Kosugi *et al.*, 2009) of dUTPase resembles some of the phospho-NLSs BRAP2 binds (ppUL44, T-ag, p53), the phosphorylatable moiety is positioned differently relative to the positively charged cluster.

Alvisi & Jans (2014) argue that mechanisms other than the phosphorylation affecting binding to importin-α may be responsible for the cytoplasmic localization profile of phosphorylated dUTPase, because the hyperphosphorylation mimicking mutation (S11E) only impairs the K_d value of the dUTPase:importin-α interaction by

tenfold. They suggest that according to the work of Hodel *et al.* (2001) and Harreman *et al.* (2004), only mutations resulting in changes of two orders of magnitude in the affinity constant would have a significant impact on nuclear translocation. Based on the available literature, we are of the opinion that this is a somewhat oversimplified interpretation of the issue. Hodel and coworkers (Hodel *et al.*, 2001) established that functional NLSs have dissociation constants for importin-α binding in the range of 10 n*M* to 1 μ*M*. This is a rather wide range of values and what most likely matters for a particular NLS is where its affinity lies within this range, rather than the magnitude of the effect of phosphorylation, *i.e.* whether the phosphorylation moves the affinity over the threshold so it falls outside the functional range. If the affinity of a certain cargo:importin-α interaction is close to the low affinity limit for a functional NLS, a small difference can have a considerable effect. On the other hand, if the K_d value is close to the high affinity limit, a much more substantial alteration of the NLS would be required to make it non-functional.

Alvisi & Jans (2014) suggest that based on the results of Harreman *et al.* (2004), a tenfold difference in binding affinity would not by itself explain the strong impact of the S11E mutation on the cellular localization observed in our study (Róna *et al.*, 2013). However, the data in the same study clearly indicates (Harreman *et al.*, 2004) that affinity differences of less than tenfold could lead to drastic changes in the localization of the cargo protein. The wild-type SV40 large T-antigen NLS derivative used in their study (SPKKKRKAE, termed SV40A7) had a K_d value for ΔIBB-importin-α of 80 n*M*, while its hyperphosphorylation mimicking mutant variant (EPKKKRKAE, termed SV40A7E) had a K_d value of 283 n*M*, as determined *in vitro* by a GFP anisotropy-based binding assay. The ability of the SV40A7 NLS to drive a GFP reporter construct to the nucleus was validated *in vivo* in yeast. However, the SV40A7E mutant was not imported into the nucleus, despite the just 3.5-fold affinity difference compared to the wild-type NLS. The NLS of Swi6 was also tested in a similar manner. The wild-type NLS (SPLKKLKID) had a K_d value of 26 n*M*, while the hyperphosphorylation mimicking mutant (EPLKKLKID) had a K_d value of 124 n*M*. The mutant NLS was not able to drive GFP accumulation into the nucleus, although the affinity difference was only 4.8-fold. The authors also used the full-length sequence of Swi6 fused to GFP to validate their results. The wild-type Swi6 protein had a K_d value of 45 n*M*, while the K_d value of the phosphorylation-mimicking mutant was 163 n*M*. The phosphorylation-mimicking mutant was not able to enter the nucleus *in vivo*, despite only a 3.5-fold difference in the K_d value compared to the wild-type Swi6, which was able to enter the nucleus in the G1 phase (Harreman *et al.*, 2004).

We believe that our data are in agreement with previous reports in the literature, and that a tenfold affinity difference between the phosphorylation-mimicking (S11E) and wild-type dUTPase for importin-α could realistically be the sole reason for the nuclear exclusion of the phosphorylation-mimicking protein. However, the data certainly allow the possibility that cytoplasmic retention factors, such as BRAP2, could contribute to the fine-tuning of the localization pattern of dUTPase. This possibility will need to be investigated experimentally.

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