

An update to the CRM1 cargo/NES database NESdb

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The nuclear export receptor CRM1 transports hundreds to thousands of diverse functioning macromolecules (proteins and RNAs) from the nucleus to the cytoplasm. CRM1 cargo proteins contain short 8–15 residue-long nuclear export signals (NESs) that bind directly to the NES-binding groove of CRM1. NES peptides are diverse in both sequence and structure; many match the classical $\Phi 1X_{2-3}\Phi 2X_{2-3}\Phi 3X\Phi 4$ consensus sequence pattern where Φ is Leu, Val, Ile, Phe, or Met and X is any amino acid (classes 1a–d in Table 1). However, recent structural studies of CRM1 bound to NESs with diverse sequences significantly expanded the diversity of this sequence pattern, increasing variation of the number of X residues to form different spacings between the four hydrophobic Φ residues (Fung *et al.*, 2015, 2017) (Table 1). In 2012, we published the NESdb (<http://prodata.swmed.edu/LRNes/index.php>), a database of 221 NES-containing CRM1 cargoes (Xu *et al.*, 2012). Each entry in NESdb displayed the sequence, structure, and experimental information on the putative NES sequence(s) of a previously reported CRM1 cargo. The NES entries were separated into two groups: “NESs” and “NESs in doubt.” Twenty-two of the 221 NESs were NESs in doubt entries, which included contradictory evidence that raised questions about the validity of the reported NESs. We have updated NESdb to a total of 399 entries. This letter describes the significant expansion of the database. We also highlight advances in NES identification that are important to consider for the search of NESs in potential CRM1 cargoes.

NESdb: an important resource for studying human diseases

Since the publication of NESdb in 2012, CRM1 has gained increasing importance in cancer biology. Many of its cargoes are tumor suppressor, proapoptotic, and growth regulator proteins that can confer survival advantages to cancer cells when the dysregulated proteins are aberrantly mislocalized to the cytoplasm. Inhibition of CRM1 can restore mislocalized tumor suppressor proteins and growth regulators to the nucleus, where they can potentially mediate apoptosis in response to DNA damage, to the cell's microenvironment or to chemotherapy (Lapalombella *et al.*, 2012;

Etchin *et al.*, 2013; Kim *et al.*, 2016; Wang and Liu, 2019; Wang *et al.*, 2020). A slowly reversible covalent inhibitor of CRM1 named Xpovio (Selinexor or KPT-330) has been shown to have broad antitumor activity that relies on cell type/context-dependent nuclear retention of a variety of CRM1 cargoes (e.g., p53, I κ B, NPM1, PAR-4, FOXO, p73, p27, topoisomerase II α , MDM2, mTOR), causing subsequent activation of cell-cycle arrest and apoptotic-, anti-inflammatory-, and stress-related gene expression (Lapalombella *et al.*, 2012; Etchin *et al.*, 2013). Xpovio was recently approved for use to treat relapsed or refractory multiple myeloma and refractory diffuse large B-cell lymphoma. Xpovio and next generation analog Eltanexor are also being tested in many clinical trials for a variety of malignancies. It remains unclear which of the many CRM1 cargoes are critical for cancer pathogenesis and are targeted by these CRM1 inhibitors in different cancers to enable therapeutic response.

In addition to cancer, CRM1 is also a drug target for other human diseases. CRM1-mediated export was found to be involved in neuronal damage (Kim *et al.*, 2010), and CRM1 levels are up-regulated in damaged brains in rat models and multiple sclerosis patients (Li *et al.*, 2013; Haines *et al.*, 2015). CRM1 inhibition to sequester proapoptotic and inflammatory proteins in the nucleus has therefore been explored as a strategy to treat traumatic brain injuries and inflammatory demyelination (Haines *et al.*, 2015; Tajiri *et al.*, 2016). Moreover, CRM1-mediated nuclear export is also critical in the replication of many viruses and CRM1 is thus a therapeutic target in viral diseases such as HIV, influenza, and COVID-19 (Perwitasari *et al.*, 2014; Boons *et al.*, 2015; Kashyap *et al.*, 2016; Mathew and Ghildyal, 2017; Taylor *et al.*, 2019; Gordon *et al.*, 2020; Zhou *et al.*, 2020). Xpovio is currently being tested in clinical trials for therapy in moderate or severe COVID-19 cases (NCT04349098/04534725).

All available CRM1 inhibitors inhibit the interaction of CRM1 with all NES-containing cargoes, and knowledge of key cargo targets that change the course of individual diseases is still lacking. It is therefore critical to understand CRM1-cargo/NES recognition more deeply and broadly. NES prediction remains a challenge as currently available predictors can only achieve ~50% precision (LocNES; Xu *et al.*, 2015). NESdb remains a useful resource of reported CRM1 cargoes and NESs for researchers from various biomedical disciplines to determine if their proteins of interest contain NESs and are potential CRM1 cargoes. Here, we report a major update of NESdb.

An updated NESdb

Regular curation of the published literature for potential CRM1 cargoes and NESs has been ongoing since the NESdb was launched. Since 2012, we have curated 178 new entries to the database by

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Class	Consensus ^a	Number of residues between Φs	Select cargoes that bind CRM1 using this consensus ^b (NES ID in NESdb)
1a	Φ1XXXΦ2XXΦ3XΦ4	3-2-1	PKI (5), MEK1 (11), HDAC5 ^c (61), Paxillin (136), 4E-T (233)
1b	Φ1XXΦ2XXΦ3XΦ4	2-2-1	
1c	Φ1XXXΦ2XXXΦ3XΦ4	3-3-1	SNUPN (1)
1d	Φ1XXΦ2XXXΦ3XΦ4	2-3-1	
2	Φ1XΦ2XXΦ3XΦ4	1-2-1	HIV-Rev (2), SMAD4 (18), FMRP (22)
3	Φ1XXΦ2XXXΦ3XXΦ4	2-3-2	mDia2 (77), CDC7 (197)
4	Φ1XXXΦ2XXΦ3XXXΦ4	3-2-3	X11L2 (141)
1a-R	Φ1XΦ2XXΦ3XXXΦ4	1-2-3	hRio2 (153), CPEB4 (198)
1b-R	Φ1XΦ2XXΦ3XXΦ4	1-2-2	
1c-R	Φ1XΦ2XXXΦ3XXXΦ4	1-3-3	
1d-R	Φ1XΦ2XXXΦ3XXΦ4	1-3-2	

^aΦ is Leu, Val, Ile, Phe, or Met and X is any amino acid.

^bSome NES sequences match multiple consensus patterns. CRM1-bound crystal structures of the NESs noted here reveal the correct consensus classes (Dong *et al.*, 2009; Monecke *et al.*, 2009; Guttler *et al.*, 2010; Koyama *et al.*, 2014; Fung *et al.*, 2015, 2017; Baumhardt *et al.*, 2020).

^cUses a Thr instead of Val as Φ1 (Fung *et al.*, 2017).

TABLE 1: NES consensus sequences.

using “CRM1” and “Leptomycin B” (LMB) or “NES” as search terms in Pubmed. The reported CRM1 cargo proteins in these new entries include 102 human, 17 nonhuman mammalian, 10 *Xenopus laevis*, 5 *Drosophila melanogaster*, 10 *Saccharomyces cerevisiae*, and 26 viral proteins from a variety of viruses; the remaining 8 proteins are from crabs, plants, fungus, and parasites. The new entries also include 17 new CRM1 cargoes categorized as “in doubt” because of missing or contradictory evidences related to CRM1 dependence. The format for displaying entries remains the same as described in the original publication.

Of the 178 new NESdb entries, 107 contain information of putative NESs that were experimentally tested and found to be sufficient to direct nuclear export as an isolated peptide; 38 of these experimentally tested NESs are also part of available PDB entries. The remaining 71 entries reported CRM1 dependency detected through various experimental methods, but the published works did not test or sufficiently demonstrate nuclear export activity of the putative NES peptides. New x-ray structures of NESs bound to CRM1 have been updated to their respective NESdb entries, and we will continue our efforts to include updates on new 3D structures for all entries. We have also improved the database to allow the pages to load more efficiently. NESdb will continue to be updated on a regular basis.

As we examined and compiled the 178 new NESdb entries, we noted two relevant recent advances in CRM1-NES research. First, recent structural studies of CRM1-NES complexes have significantly expanded the diversity of known NES consensus patterns. This is mostly due to the discovery of reverse NESs that are described by the consensus pattern Φ1XΦ2X₂₋₃Φ3X₂₋₃Φ4, which is the reverse of the traditional Φ1X₂₋₃Φ2X₂₋₃Φ3XΦ4 consensus pattern (classes 1a-R to 1d-R in Table 1). Crystal structures of CRM1 bound to two peptides with the reverse consensus, CPEB4^{NES} and hRio2^{NES}, showed the peptides binding the CRM1 groove in the opposite peptide direction (minus direction) compared with earlier identified NESs (plus direction) (Fung *et al.*, 2015). Some subsequently published works have reported the search for putative reverse NESs and their identification in putative CRM1 cargoes SHIP2, UBR5, CEP43, CDC27, ZO-2, and Ubx (Duffraisse *et al.*, 2020; Sendino *et al.*, 2020). Future NES searches

using these new sequence patterns will surely expand the current repertoire of NES sequences and CRM1 cargoes.

Second, only a small fraction of the publications that reported experimental validation of NESs considered the issue of NES accessibility within the full-length cargo proteins. Many reports of CRM1 cargoes/NESs, including those published between 2012 and 2020, lacked the perspective of the NES in context of the full-length cargo. Sequences that match NES consensus patterns are found in many if not most helix-containing proteins, but many of these sequences are buried in the hydrophobic cores of folded domains and thus are not accessible to bind CRM1. Simply testing nuclear export capabilities of putative NES peptides does not yield information on the full-length cargo, and mutating hydrophobic residues of the putative NESs may disrupt folded domains and artifactually change their cellular localization. Results of these types of experiments must be interpreted with caution. For example, a well-established strong NES in the MEK1 kinase, which binds CRM1 with high affinity and effectively direct nuclear export as a peptide, was found to be only weakly functional in the full-length protein, likely due to part of the NES being buried in the folded protein (Baumhardt *et al.*, 2020). Conversely, misfolding of the SOD1 protein causes exposure of a normally buried NES and subsequent CRM1-mediated export (Zhong *et al.*, 2017). It is therefore important to study NES function within the full-length cargo and to consider new mechanisms for dynamic regulation of protein localization in cells.

We are confident that researchers from many biomedical research disciplines will find the updated NESdb useful, and we urge users of the database to provide input on the entries of their interest to assist with validation of the NESs. Use of NESdb in publications should cite the original publication: Xu D, Grishin NV, Chook YM. NESdb: a database of NES-containing CRM1 cargoes. *Mol Biol Cell* 2012 Sep;23(18):3673-6 and/or this Letter.

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