Correlation of CRM1-NES affinity with nuclear export activity

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ABSTRACT CRM1 (Exportin1/XPO1) exports hundreds of broadly functioning protein cargoes out of the cell nucleus by binding to their classical nuclear export signals (NESs). The 8- to 15-amino-acid-long NESs contain four to five hydrophobic residues and are highly diverse in both sequence and CRM1-bound structure. Here we examine the relationship between nuclear export activities of 24 different NES peptides in cells and their CRM1-NES affinities. We found that binding affinity and nuclear export activity are linearly correlated for NESs with dissociation constants (K_d) between tens of nanomolar to tens of micromolar. NESs with K_d outside this range have significantly reduced nuclear export activities. These include two unusually tight-binding peptides, one from the nonstructural protein 2 of murine minute virus (MVM NS2) and the other a mutant of the protein kinase A inhibitor (PKI) NES. The crystal structure of CRM1-bound MVM NS2NES suggests that extraordinarily tight CRM1 binding arises from intramolecular contacts within the NES that likely stabilizes the CRM1-bound conformation in free peptides. This mechanistic understanding led to the design of two novel peptide inhibitors that bind CRM1 with picomolar affinity.

INTRODUCTION
Nuclear-cytoplasmic transport of macromolecules is largely mediated by karyopherin-β family nuclear transport receptors (Kaps; importins and exportins). Importins bind their cargoes in the cytoplasm and release them in the nucleus whereas exportins mediate the reverse process. The chromosome region maintenance 1 (CRM1) protein (also known as exportin-1 or XPO1) binds 8- to 15-residues-long nuclear export signals (NESs) in hundreds of different protein cargoes (Fornerod et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997; Thakar et al., 2013). The repertoire of the protein cargoes of CRM1 continues to grow; ~250 experimentally identified protein cargoes are recorded in NES databases ValidNESs and NESdb (Fu et al., 2012; Xu et al., 2012, 2015), and over 1000 putative CRM1 cargoes were identified in a recent proteomics study (Kirli et al., 2015). Accordingly, the diversity of NES sequences has also grown with ever-expanding NES patterns that result in many false positives when used in NES prediction. Further complexity is observed as recent structural analysis of 13 different CRM1-NES complexes revealed a large range of NES backbone conformations (Fung et al., 2017). Nevertheless, the study of NES recognition by CRM1 is important as CRM1-NES interactions are the targets of small molecule inhibitors such as Selinexor/KPT-330, Eltanexor/KPT-8602, Verdinexor/KPT-335, KPT-350 (Karyopharm), and SL801 (Stemline), which are being tested in clinical trials for a variety of cancers and inflammatory diseases or as an antiviral agent.

In addition to CRM1, the importin α/β (Impα/β) system also recognizes hundreds to thousands of broadly functioning cargoes, in this case proteins that contain the classical nuclear localization signal (cNLS). Extensive structural, biochemical, and cell biological studies of cNLS recognition by Impα preceded analogous CRM1-NES studies (Conti et al., 1998; Kobe, 1999; Marfori et al., 2011). More than 80 crystal structures of Impα-cNLS complexes are available, showing how various poly-basic monopartite and bipartite cNLS peptides interact with two binding sites on several Impα isoforms. Nuclear import activities of different cNLS peptides in cells and their affinities for Impα were measured and compared (Fanara et al., 2000; Hodel et al., 2001, 2006). These studies roughly divided cNLSs into three groups: 1) active NLSs with K_d from 1 nM to hundreds of nanomolar where nuclear import appears to correlate with Impα-cNLS affinity, 2) inactive NLSs with dissociation constants (K_d) in the...
RESULTS AND DISCUSSION

Measuring NES activity in live cells

We selected 24 different NES peptides to measure nuclear export activities in cells. The NESs were selected to sample a broad range of affinity for CRM1 in order to evaluate how wide a dynamic range of nuclear export activity versus CRM1-NES affinity can be covered. We selected 24 different NESs to measure nuclear export activities in cells, suggesting an upper limit in CRM1-mediated nuclear export of average NESs. Structure of a supertight peptide bound to CRM1 showed intramolecular contacts that were not observed in other CRM1-NES structures. The use of this unusual NES structural element led to the design of two peptide inhibitors that bind CRM1 with picomolar affinity.

Comparing NES activities in cells and CRM1-NES affinities

We plotted CRM1-NES affinity (log_{10}K_{d}) as a function of its R_{CN} or nuclear export activity. Both parameters are presented with error bars showing their respective 95% confidence limits (Figure 1C). The plot revealed a strong negative correlation between log_{10}K_{d} and R_{CN} as indicated by Pearson's r = -0.65 (p = 0.0006). Active NESs have an impressively wide range of affinities for CRM1 that span four orders of magnitude, from K_{d}s of single-digit nanomolar to tens of micromolar. The NES from the murine minute virus nonstructural protein 2 (MVM NS2\textsuperscript{NES}) has the highest affinity for CRM1 (K_{d} = 2 nM). At the other end, there are four active NESs with K_{d}s of tens of micromolar: Strado\textsuperscript{NES} (K_{d} = 10 µM), SNUP\textsuperscript{NES} (K_{d} = 13 µM), CDC7\textsuperscript{NES} (K_{d} = 20 µM), and HPV E7\textsuperscript{NES} (K_{d} = 34 µM). The measured K_{d}s of two known inactive PKI\textsuperscript{NES} mutants are 150 and 900 µM, respectively. Interestingly, two unusually tight-binding NES peptides with K_{d}s of 5 nM also have low nuclear export activity in cells. One of the supertight NES is MVM NS2\textsuperscript{NES}, which was previously designed to increase CRM1 affinity for crystallographic study (Güttler et al., 2010). When these two unusually tight-binding NESs were excluded from the analysis, the correlation coefficient between CRM1 affinity and nuclear export activity increased to a Pearson's r = -0.87 (p = 1.48 × 10^{-7}). We note that interactions between CRM1 and full-length cargo proteins may be more complicated and require further investigations that include physiological concentrations of CRM1, cargoes, Ran, and other cargo proteins. Caution is advised when translating the K_{d}s reported in this study into CRM1-full-length cargo affinities.

It is generally thought that karyopherin-NLS/NES interactions should occur within a range of affinity suitable for both binding in one cell compartment and release in the other compartment. The two inactive PKI\textsuperscript{NES} mutants PKI\textsuperscript{NES}(I47A) and PKI\textsuperscript{NES}(L42A/L45A) (Wen et al., 1995) have R_{CN} values of 0.03 and 0.01 that are consistent with their lack of activity (Table 1). The measured K_{d} of PKI\textsuperscript{NES}(I47A) for CRM1 is 150 [90, 310] µM (values in bracket represent 95% confidence interval). The next weakest CRM1 binder is the HPV E7\textsuperscript{NES} (K_{d} = 34 [22, 53] µM), which has a significantly higher R_{CN} proteins in the cell, suggesting that variation in expression level (at least across the range examined here) does not affect CRM1-mediated export of the reporter proteins.

Measuring CRM1-NES affinities

CRM1-NES binding affinities (dissociation constants or K_{d}s) were measured using a previously described differential bleaching assay, with purified CRM1 and NES proteins in the presence of excess RanGTP (Fung et al., 2015). MBP-NES fusion proteins were used as the MBP tag does not affect interactions with CRM1 (Fung et al., 2015). K_{d} values are reported with 95% confidence intervals obtained using error-surface projection method. The K_{d} values of the 24 different NESs binding to CRM1 range from low nanomolar to a hundred micromolar (Table 1; differential bleeding data shown in Supplemental Figure S3, A and B). Negative controls, PKI\textsuperscript{NES}(I47A) and PKI\textsuperscript{NES}(L42A/L45A) mutants (Wen et al., 1995), have K_{d} values >150 µM, suggesting extremely weak binding. Analysis of simulated differential bleeding data in PALMIST (Scheuermann et al., 2016) indicated that the high-affinity detection limit of our experiments is ∼1 nM K_{d} (Supplemental Figure S3C; see details under Materials and Methods). Therefore, K_{d} values of several high-affinity NESs (Super PKI\textsuperscript{NES} and MVM NS2\textsuperscript{NES}; Table 1) are beyond the limit of accurate determination. K_{d}s with undefined confidence intervals are reported as such and are used for rough comparisons.
value of 0.29. On the basis of these data points, we suggest that the lower limit of binding affinity for active nuclear export likely lies between 34 and 150 µM.

CRM1-NES interactions must also occur at affinities suitable for cargo/NES release in the cytoplasm. Therefore, there is likely an upper limit of CRM1-NES affinity for optimal nuclear export. An NES or cargo that binds CRM1 tighter than this limit will likely remain bound to CRM1 after RanGTP is hydrolyzed to RanGDP in the cytoplasm and potentially be taken back into the nucleus. Two NESs that bind CRM1 with $K_d$< 5 nM, Super PKI$_{NES}$ ($K_d$ = 4 [U, 12] nM; U cannot be defined) and MVM NS2$_{NES}$ ($K_d$ = 2 [U, 9] nM), exhibit 42–72% lower nuclear export activity than the lower affinity PKI$_{NES}$ ($K_d$ = 34 [25, 46] nM). These results suggest that the upper limit of CRM1-NES affinity for optimal nuclear export activity lies between 4 and 34 nM. Previous studies (Engelsma et al., 2008; Güttler et al., 2010) and pull-down binding data shown in Supplemental Figure S3D show that these two unusually tight-binding NESs can bind CRM1 in the absence of Ran.

**The supertight NESs can inhibit nuclear export**

A protein or peptide that binds CRM1 too tightly may outcompete bona fide cargoes. Engineered peptides like the Bimax or M9M peptides, which bind Imp$_{x}$a or Kap$_{x}$2, respectively, with picomolar affinities, are inhibitors of the karyopherins (Cansizoglu et al., 2007; Kosugi et al., 2008). We wondered whether the

![FIGURE 1: Correlation of NES export activity with binding affinity to CRM1. (A) Schematic of the workflow to quantify the ratio of cytoplasmic to nuclear mean fluorescence intensity ($R_{C/N}$). $R_{C/N}$ values are used as a measurement of NES activity in living HeLa cells. The loop in the workflow indicates that this process is repeated for at least 30 representative cells collected from at least three independent experiments (listed in Table 2) for statistical analysis. (B) Leptomycin B (LMB) sensitive nuclear export activity of EYFP$^2$-SV40$_{NLS}$-NES fusion proteins in HeLa cells. YFP (pseudocolored in yellow) and Hoechst (pseudocolored in blue) images were captured using spinning disk confocal microscope (40×). From left to right, PKI$_{NES}$, HIV Rev$_{NES}$, and CDC7$_{NES}$. Nuclear accumulation after treatment with 5 nM leptomycin B (+LMB) for 16–18 h demonstrates CRM1-dependent export. Representative images of all other NESs are shown in Supplemental Figure S1A. (C) Correlation of in vitro affinity and in vivo nuclear export activity. In vitro binding affinities of 24 NESs (includes two negative controls PKI$_{NES}$(I47A) and PKI$_{NES}$(L42A/L45A)) are plotted as a function of their nuclear export activities. Error bars represent 95% confidence intervals. Half of the vertical error bars for the binding affinities of PKI$_{NES}$(L42A/L45A), Super PKI$_{NES}$, and MVM NS2$_{NES}$ are missing because the upper or lower limits are undetermined. The Pearson’s r value of the 24 NESs is −0.65 (p = 0.0006) and is −0.87 (p = 1.48 × 10$^{-7}$) when Super PKI$_{NES}$ and MVM NS2$_{NES}$ are not included.
### Table 1: In vitro CRM1 binding affinity and in vivo activity of NESs.

| NES         | Mean $R_{\text{C/N}}$ | $R_{\text{C/N}}$ 95% confidence interval | $K_d$ (nM) 95% confidence interval |
|-------------|------------------------|----------------------------------------|-----------------------------------|
| PKI         | 1.37                   | Low: 1.33, High: 1.41                  | Low: 34, High: 46                |
| CPEB4       | 1.20                   | Low: 1.13, High: 1.27                  | Low: 800, High: 1200              |
| MEK1        | 1.17                   | Low: 1.11, High: 1.23                  | Low: 70, High: 130               |
| ADAR1       | 0.85                   | Low: 0.75, High: 0.96                  | Low: 69, High: 96                |
| hRico2      | 0.82                   | Low: 0.72, High: 0.92                  | Low: 2800, High: 4700             |
| FMRP        | 0.81                   | Low: 0.76, High: 0.86                  | Low: 2000, High: 1200             |
| Super PKI   | 0.80                   | Low: 0.64, High: 0.96                  | Low: 400, High: 1000              |
| X11L2       | 0.79                   | Low: 0.71, High: 0.87                  | Low: 1500, High: 2300             |
| NPM          | 0.72                   | Low: 0.61, High: 0.83                  | Low: 790, High: 980              |
| HIV Rev     | 0.70                   | Low: 0.62, High: 0.77                  | Low: 1180, High: 1400             |
| Pax         | 0.60                   | Low: 0.49, High: 0.70                  | Low: 700, High: 1000              |
| mDia2       | 0.59                   | Low: 0.52, High: 0.66                  | Low: 1600, High: 2700             |
| CPEB4-R     | 0.59                   | Low: 0.50, High: 0.67                  | Low: 710, High: 880               |
| p73         | 0.59                   | Low: 0.53, High: 0.65                  | Low: 2000, High: 2400             |
| HDAC5       | 0.54                   | Low: 0.48, High: 0.60                  | Low: 1600, High: 2400             |
| SMAD4       | 0.44                   | Low: 0.37, High: 0.51                  | Low: 4600, High: 5900             |
| MVM NS2     | 0.38                   | Low: 0.30, High: 0.45                  | Low: 2, High: 9                  |
| hRico2-R    | 0.36                   | Low: 0.30, High: 0.43                  | Low: 2600, High: 3300             |
| HPV E7      | 0.29                   | Low: 0.24, High: 0.33                  | Low: 34,000, High: 53,000        |
| CDC7        | 0.21                   | Low: 0.16, High: 0.26                  | Low: 20,000, High: 27,000        |
| Strad        | 0.19                   | Low: 0.13, High: 0.26                  | Low: 10,300, High: 13,000        |
| SNUPN       | 0.16                   | Low: 0.12, High: 0.20                  | Low: 12,500, High: 15,000        |
| PKI (I47A)  | 0.03                   | Low: 0.02, High: 0.04                  | Low: 150,000, High: 310,000      |
| PKI (L42A/L45A) | 0.01     | Low: 0.00, High: 0.02 | Low: 900,000, High: 300,000 |

### Crystal structure of MVM NS2 NES bound to CRM1

The structure of Super PKI NES (fused to the Snurportin-1 protein) bound to CRM1 showed the leucine in the N35L mutation site binding in the P0 pocket of the CRM1 NES-binding groove. This additional contact increases the number of anchoring hydrophobic residues from four in wild-type PKI NES to five in the supertight mutant (Güttler et al., 2010). It is unclear from the sequence of MVM NS2 NES (STVDEMTKKFGTLTIHD) why it binds CRM1 so tightly. Using the engineered CRM1-Ran-RanBP1-NES quaternary complex (Fung et al., 2015), we solved the 2.0 Å resolution crystal structure of MVM NS2 NES bound to CRM1 (Figure 3A, crystallographic statistics in Supplemental Table S1, and electron densities for the NES peptide in Figure 3B). MVM NS2 NES binds CRM1 with the common NES conformation of an N-terminal α-helix followed by a C-terminal strand. Five hydrophobic side chains of MVM NS2 NES (Val79, Met82, Phe86, Leu89, and Leu91) occupy the five hydrophobic pockets in the CRM1 groove (Figure 3A, left panel). The structures of CRM1-bound Super PKI NES and MVM NS2 NES are highly similar (C α r.m.s.d. of 0.66 Å).
0.5 Å for 85 CRM1 groove residues). However, unique to CRM1-bound MVM NS2NES are two intramolecular polar contacts involving the C-terminal strand of MVM NS2NES (Figure 3A, right panel). Thr90 of MVM NS2NES makes hydrogen bonds with both the side chain and main chain amides of MVM NS2NES His92. These intrapeptide contacts likely stabilize the configuration of the C-terminal strand, perhaps preorganizing the structural element, for the unusual high-affinity binding to CRM1. Such intramolecular NES contacts have not been observed in any other CRM1-NES structures (Dong et al., 2009; Gütllier et al., 2010; Fung et al., 2015, 2017).

**Structure-based design of novel peptide inhibitors**

To investigate the importance of the unusual intrapeptide contacts seen in CRM1-bound MVM NS2NES, we generated chimeric NES peptides where L9LTIHD93 of MVM NS2NES is fused to N-terminal helical portions of other NESs. We asked whether the L9LTIHD9 strand of MVM NS2NES, which contains the intramolecular contacts, could increase affinities of the chimeric NES peptides for CRM1. Fusion of L9LTIHD93 to the very tight-binding Super PKI NES (Kd = 4 [U, 12] nM) resulted in chimeric mutant Super PKI-NS2NES (NLEALALKAGLTIHD). Fusion to the weak-binding CDC7NES (Kd = 20 [15, 27] µM) gave chimera CDC7-NS2NES (QDLRKCLERLRGLTIHD) (Figure 3C). Differential bleaching measurement of MBP-Super PKI-NS2NES and MBP-CDC7-NS2NES binding to CRM1 gave Kd values of 7 [U,U] and 27 [U,U] pM, respectively. Both chimeric peptides have picomolar affinity beyond the limit of accurate determination with our method (Figure 3D). These results indicate that the C-terminal L9LTIHD93 residues of MVM NS2NES contribute significant binding energy for interactions with CRM1. The transfer of this MVM NS2NES segment can increase affinities of other NESs by as much as 4 orders of magnitude (Kd of CDC7-NS2NES ≪ 1 nM vs. Kd of CDC7NES = 20 µM). More importantly, the intramolecular contacts within L9LTIHD93 appear to contribute significantly to the increased affinity of the NESs. The differences between Super PKI NES and Super PKI-NS2NES occur only in non-Φ residues; LDIKNK in Super PKI NES vs. LTIHD in Super PKI-NS2NES (Φ residues in bold; Figure 3C). The three-residue change increased binding affinity from 4 nM to ≪1 nM. We show that the picomolar affinity Super PKI-NS2NES peptide is an effective CRM1 inhibitor in the cell (Supplemental Figure S1B), much like the engineered M9M peptide.
...that specifically and potently inhibits Kapβ2 (Cansizoglu et al., 2007). Finally, we also examined the effect of overexpression of Super PKI-NS2NES on cytotoxicity to T-REx-293 cells using a tetra-cycline-induced expression system (Supplemental Figure S2B). Although overexpression of Super PKI-NS2NES is cytotoxic, it does not alter the subcellular localization of endogenous CRM1 (Supplemental Figure S2C).

Conclusion

There is a strong correlation between CRM1-NES affinity and nuclear export activity for NESs that bind CRM1 with Kd in the tens of nanomolar to tens of micromolar range. The wide affinity range for NESs no longer seems to be active NESs was determined by measuring the steady-state distribution of fluorescence-tagged reporter proteins (EYFP-SV40NES-NES). HeLa cells were transiently transfected with different EYFP-SV40NES-NES constructs and live cell imaging was performed after 24 h. The ratio of cytoplasmic to nuclear YFP signal (Rcy/Cnu) was normalized by the corresponding Rcy/Cnu after 5 nM leptomycin B (LMB) treatment of 16–18 h in duplicate wells. Rcy/Cnu with LMB treatment can account for the passive export of reporter proteins by diffusion, as LMB specifically inhibits CRM1 export activity.

Live cell imaging was performed at 37°C in a 5% CO2 atmosphere using a spinning disk confocal microscope system (Nikon-Andor) with a 40 × 0.6 NA air objective and the MetaMorph software. Z-stack images were obtained in the YFP and Hoechst channels using a step size of 0.6 µm (total z size 18 µm). In addition, a single differential interference contrast (DIC) image was taken in the middle of the z-stack. Cells were randomly selected for imaging if they 1) expressed YFP at adequate levels that the signal is not saturated, 2) contained no obvious abnormality observed under DIC, and 3) were not dividing, as evidenced by Hoechst staining.

The acquired z-stack images were imported into ImageJ (version: 1.49i) for further analysis. Four nuclear z-planes spanning the middle of the nucleus (total z size 1.8 µm) were selected and merged into a single YFP (by average intensity projection) and Hoechst image (by maximum intensity projection) for analysis. Watershed segmentation was applied to Hoechst images to define the nuclear region of interest (ROI). For each nuclear ROI, a cytoplasmic “ring” ROI with a thickness of 150 nm was generated by dilating the nuclear ROI twice and performing exclusive-or (XOR) operation on these two dilated areas in ImageJ. The mean intensities in nuclear and cytoplasmic ROIs were measured and exported to Microsoft Excel, where the ratio of cytoplasmic to nuclear YFP signal (Rcy/Cnu) was calculated.

Cell culture

HeLa cells from the American Type Culture Collection were cultured in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine

MATERIALS AND METHODS

Cloning

NES sequences, along with three to five immediately adjacent residues on either ends, were cloned into different vectors used in this study (Table 2). To measure the in vivo activity and the in vitro binding affinity for CRM1, the same NES sequences were cloned into BamHI and Xhol sites of pEYFP-SV40NES and pMal-TEV vectors, respectively. For the use of peptide inhibition assay, MSM NS2NES, Super PKI-NES, and Super PKI-NS2NES were cloned into Xhol and HindIII sites of the pmKate2-C vector. All constructs were cloned using procedures as previously described (Xu et al., 2015) and verified by sequencing.

Quantitation of nuclear export activity

We developed a quantitation pipeline to measure the NES activity in live HeLa cells as illustrated in Figure 1A. The nuclear export activity of NESs was determined by measuring the steady-state distribution of fluorescence-tagged reporter proteins (EYFP-SV40NES-NES). HeLa cells were transiently transfected with different EYFP-SV40NES-NES constructs and live cell imaging was performed after 24 h. The ratio of cytoplasmic to nuclear YFP signal (Rcy/Cnu) was normalized by the corresponding Rcy/Cnu after 5 nM leptomycin B (LMB) treatment of 16–18 h in duplicate wells. Rcy/Cnu with LMB treatment can account for the passive export of reporter proteins by diffusion, as LMB specifically inhibits CRM1 export activity.

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serum (FBS; Sigma-Aldrich) and 1% antibiotic-antimycotic (Life Technologies, Thermo Fisher Scientific) at 37°C in 5% CO₂.

**Transfections**

HeLa cells that were seeded onto glass-bottom 24-well culture plates (Phenix Research Products) and grown to 50–70% confluency. Transfections were performed according to the manufacturer’s instructions with reduced DNA-to-reagent ratios of 1:1 (Lipofectamine 3000) or of 1:10 (Effectene), respectively. Cotransfection with red fluorescent protein (RFP)-NES was conducted using a transfection mixture of plasmid DNA at a reporter-to-inhibitor ratio of 1:9.

**Quantitation of binding affinity for CRM1**

The $K_d$ values are obtained by competition differential bleaching experiments using a fluorescent probe FITC-PKI$^\text{NES}$ that bleaches at distinct rates when unbound and bound to CRM1. FITC-PKI$^\text{NES}$ undergo a reproducible differential bleaching when titrated with CRM1. The bleaching rate of the fluorescent probe is dependent on the concentration of the titrant CRM1, is saturable at high concentrations of CRM1, and can be fitted to a sigmoidal curve, which suggests a two-state behavior of the probe when it is unbound or bound to CRM1 (Fung et al., 2015). Differential bleaching of the probe can be counteracted by titration of MBP-NESs. MBP-NESs compete with FITC-PKI$^\text{NES}$ for the NES-binding groove of CRM1 and the changes in bleaching rate of FITC-PKI$^\text{NES}$ will reflect the fraction of CRM1 bound to the FITC-PKI$^\text{NES}$ or to MBP-NES.

Therefore, different MBP-NESs can be titrated to compete with FITC-PKI$^\text{NES}$ for CRM1 to allow measurement of affinities of various MBP-NESs. Data were processed in PALMIST (Scheuermann et al., 2016) using averages of triplicate experiments without weighted fitting. Confidence levels at 95% were obtained by error-surface projection method. Nine binding affinity data were measured in this study while the others were measured in our previous works (Fung et al., 2015, 2017) and reanalyzed to obtain 95% confidence interval (see details in Supplemental Figure S3 and Table 1).

**Statistical analysis**

Mann–Whitney tests, $p$ values (two-tailed), 95% confidence interval error bars, and Pearson correlation coefficients in this study were computed and calculated using the SciPy module of Python.

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