A Multidimensional Characterization of E3 Ubiquitin Ligase and Substrate Interaction Network

**Comprehensive and comparative E3 interaction collection**

| Positive | Negative | Indirect | Non-ubiquitination | Interactor |
|----------|----------|----------|--------------------|------------|
| E3       | Substrate| E3       | Random protein     | E3         | E3          |

**Multidimensional association features**

- mRNA
- Protein
- Expression correlations
- Network connection
- Pathway overlap

**Identify E3 - substrate landscape by ensemble prediction**

\[ \text{Prob} = \sum w_i \cdot \text{Prob}_i \]

Prob > th

**Novel Discoveries**

**HIGHLIGHTS**

- Systematically describe E3 ligase and substrate association patterns
- Build a computational model for predicting E3 ligase and substrate interactions
- Predict and validate promising substrates for F box family E3 ligases
- Construct a valuable data resource on potential E3-substrate interactions
A Multidimensional Characterization of E3 Ubiquitin Ligase and Substrate Interaction Network

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SUMMARY

E3 ubiquitin ligases (E3s) play a critical role in molecular and cellular mechanisms. However, a large number of E3-substrate interactions (ESIs) remain unrevealed. Here, we integrated the increasing omics data with biological knowledge to characterize and identify ESIs. Multidimensional features were computed to obtain the association patterns of ESIs, and an ensemble prediction model was constructed to identify ESIs. Comparison with non-ESI cases revealed the specific association patterns of ESIs, which provided meaningful insights into ESI interpretation. Reliability of the prediction model was confirmed from various perspectives. Notably, our evaluations on leucine-rich repeat family of F box (FBXL) family were consistent with a proteomic study, and several substrates for SKP2 and an orphan E3 FBXL6 were experimentally verified. Moreover, a cancer hallmark ESI landscape was studied. Taken together, our study catches a glimpse at the omics-driven ESI association patterns and provides a valuable resource (http://www.esinet.dicp.ac.cn/home.php) to assist ubiquitination research.

INTRODUCTION

Protein ubiquitination refers to ubiquitin conjugation at a target substrate through three enzymes, including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3), and mainly results in degradation of a specific substrate (Scheffner et al., 1995). It is one of the most prevalent post-translational modifications in eukaryotic cells (Suresh et al., 2016). Dysregulations of ubiquitination will induce serious diseases, such as cancer (Hoeller and Dikic, 2009; Mari and Gelmann, 2005). During protein ubiquitination, E3s play a key role by specifically recognizing the target substrates. Given the specificity and diversity, E3s are regarded as potential therapeutic targets in cancer (Bassermann et al., 2014). However, apart from several well-defined E3s, e.g., MDM2 (Rayburn et al., 2005) and NEDD4 (Ye et al., 2014), most of them remain poorly characterized, making it a great challenge to fully understand the ubiquitination system.

Typically, substrates for E3s are discovered by biochemical experiments (e.g., two-hybrid screen or co-immunoprecipitation) in a case-by-case manner (Chan et al., 2012; Maddika et al., 2011), which are commonly time- and resource-consuming. Recently, some high-throughput methods (Tan et al., 2013; Yamamoto et al., 2012) have been utilized on ESI recognition. However, the screened results are still mixed with plenty of false discoveries, and proteome-wide ESI identifications are still far behind. It is indispensable to build effective computational methods to assist ESI recognition. A platform of UbiBrowser (Li et al., 2017) has been constructed to predict ESIs, providing references for deciphering a proteome-wide ESI network. However, it mainly depends on mechanism-agnostic enrichment of pairwise protein features among known E3-substrate pairs, overlooking the expression or functional correlations between E3s and substrates. In contrast to the limited knowledge of E3-substrate interactome, extensive omics data are available, owing to the efforts on collection and organization of high-throughput biological data by public repositories, like The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/) (Cancer Genome Atlas Research Network et al., 2013) and Clinical Proteomic Technology Assessment for Cancer (CPTAC, https://cptac-data-portal.georgetown.edu/cptacPublic/) (Edwards et al., 2015). They have been widely employed in functional description of cancer-driving factors or interactions (Xue et al., 2017; Ye et al., 2016). Association-centric heuristic methods are commonly the optimal option for omics-based analysis (Stuart et al., 2003; Wang et al., 2016), and functional related or interacting components can be identified. However,
given the reversibility and dynamics of ubiquitination, it is still unclear whether E3-substrate interaction (ESI) can be correctly described by omics-based associations. In addition, progressively accumulated biological knowledge, like the annotated pathways in Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2017) and experimentally verified protein-protein interaction (PPI) records in BioGrid (Chatr-Aryamontri et al., 2017), provide an alternative way to create mechanistic descriptions on ESIs.

Here, we put forward to characterize and predict ESIs in an integrative way. It turns out that integrating omics (especially proteomics data) with network or pathway information can help distinguish ESIs from various negative categories (even indirect regulating relations). A case study on leucine-rich repeat family of F box (FBXL) proteins demonstrated that our evaluations outperformed both the UbiBrowser and a proteomics-based approach. Moreover, we portrayed a cancer-oriented ESI landscape by identifying potential ESIs for cancer hallmark proteins. In addition, to facilitate the utility of our model, both confirmed and potential ESIs along with their association features were distributed on a website (http://www.esinet.dicp.ac.cn/home.php).

RESULTS

Workflow of a Comparative Characterization on ESIs

We developed a computational model for both describing and predicting ESIs by integrating omics with network and pathway (Figure 1, see also Transparent Methods). First, previously reported ESIs were collected as a positive reference dataset (PRD, Table S1), whereas three negative reference datasets (NRDs) covering randomly combined E3s and proteins ("E3-random," Table S2), non-ESI PPIs ("Other PPIs," Table S3), and pairwise E3-indirect regulatory proteins ("E3-Indirect," Table S4) were prepared as control (Figure 1A). For both PRD and NRDs, multidimensional association features between two proteins were calculated by integrating omics (Figure 1B), networks (Figure 1C), and pathways (Figure 1D). Then, specific ESI association patterns were recognized by comparing ESIs with three categories of NRDs. Finally, a prediction model was constructed through a weighted ensemble of three types of random forest (RF) classifiers trained with different categories of NRDs. See also Tables S1, S2, S3, and S4.

Constructing an ESI Network

Initially, an ESI network composed of 1,806 previously reported ESIs involving 300 E3s and 1,089 substrates was constructed to describe the complex relations between E3s and substrates (Figure 2A). Similar to other biological networks (Goymer, 2008; Rolland et al., 2014), it exhibited an approximate scale-free topology (Barabasi, 2009) (linear model fitting R2 index = 0.78). Most hubs in the ESI network were E3s; they had significantly higher (p value = 4.07 x 10^-7 based on t test) degrees than substrates, suggesting that a large number of substrates can be recognized by the same E3s. However, when mapping onto the global PPI network, such difference disappeared (p value = 0.9634 by t test); E3s and substrates exhibited similar degree distribution and their degrees were much higher than those on the ESI network (Figure 2B), emphasizing that the impacts rendered by ubiquitination may be spread to various processes by non-ESI interactions. Besides, a cancer hallmark subgraph (Figure 2C) was extracted. On this subgraph, nodes belong to genes recorded in the “Catalogue of somatic mutations in cancer” (COSMIC) (Forbes et al., 2017) and edges are annotated with the ubiquitinated consequences of substrates. It suggests that a large fraction of the reported ESIs will lead to degradation of substrates. Some E3s (e.g., BRCA1 and KEAP1) are tumor suppressors; their mutations might induce the accumulation of carcinogenic substrates (e.g., AKT, IKK-β, and JAK2), which will promote the development of cancer and affect key signaling pathways, such as AKT or nuclear factor (NF)-κB signaling pathway, confirming that ubiquitination can affect cancer development and progression in alternative ways. To be more comprehensive, further refining of the ESI network is indispensable.
Omics-Based Associations Alone Are Not Capable of ESI Recognition

Proteomics data offer the most direct resource to capture the expression relevance between E3s and substrates, given that ESIs often result in substrate degradation. In addition, as ubiquitination is also involved in transcription regulation (Hammond-Martel et al., 2012), transcriptomics data were also employed to estimate transcriptional associations (see Transparent Methods). Notably unexpected, E3s and substrates were not negatively correlated in both mRNA and protein levels (Figure 3A, median levels for ESIs were all near zero). However, when compared with NRDs, ESIs exhibited differences in both omics: ESIs showed higher correlations than “E3-random” cases on average (Figure 3A). “Other PPIs” were more mutually correlated than ESIs; indirect regulations were similar with ESIs in terms of proteomics, but differences emerged in terms of transcriptomics. Still, more features are required because the slight differences make it difficult to separate ESIs from other possibilities.

Figure 2. ESI Network

(A) An ESI network. E3s (red) and their corresponding substrates (yellow) are connected as edges, where green nodes stand for proteins acting as both E3s and substrates.

(B) Degree distribution of E3s (red bars) and substrates (blue bars) on the ESI network (horizontal axis) and the global PPI network (vertical axis). Centers of boxes represent median values. Bottom and top bounds of boxes represent 25th and 75th percentiles. Whiskers mark 1.5 times of the interquartile range.

(C) Cancer hallmark ESI subgraph. Node borders were colored according to their functions in cancer; blue stands for tumor suppressor gene (TSG), red stands for oncogene, orange represents genes with both functions, whereas the functions of gray-border ones are still unclear. The edge color represents the ubiquitinated consequence of substrates; blue stands for degradation, whereas red refers to activity change or other non-degradation effects. Nodes are labeled with the corresponding gene symbols of proteins.

See also Table S1.
E3s and Substrates Share Neighbors on PPI Network but Lack Connections on Co-expression Networks

Oms-based associations simply investigated on the “one-to-one” expression relations, ignoring the fact that cascades of other proteins also participate in the ubiquitination process. Consequently, three network systems were applied to measure the relations between E3s and substrates systematically (see Transparent Methods). As a result, E3s and NRDs showed significantly different distributions on the network-based associations (Figure 3B). No matter on which kind of network, the average correlations of E3s were significantly (p value<0.01) less than “Other PPIs.” On the PPI network, E3s and substrates are more likely to be connected by common neighbors than “E3-random” or “E3-indirect” (Figure 3B, CNR.PPI). However, this tendency was changed on the co-expression network; especially on the protein co-expression network (Figure 3B, CNR.CXNP), the E3s were less connected compared with all other NRDs, even in the random cases, reflecting that the E3s interrupt the expression correlations despite the probably transient and dynamic properties. When the co-expression networks and PPI network were integrated, the profiles (Figure 3B, CCR.PPI and CCP.PPI) was similar with that based on PPI network. The network-based associations can assist to differentiate the substrates of E3s from indirect or random situations in a relatively more accurate manner than the omics-based associations.

Pathway-Based Associations Improve ESI Discriminability and Provide Hypothesis on Their Upstream or Downstream Processes

As a special kind of bionetworks, a pathway refers to a cascade of molecular interactions with interdependent functions. A total of 306 human pathways in KEGG (Kanehisa et al., 2017) were considered as intermediary to examine the functional associations between E3s and substrates (see Transparent Methods). With regard to mRNA-based pathway associations (i.e., WCR, WCRS), E3s only exhibited modest higher associations than “E3-random” and “E3-indirect” (Figure 3C); however, permutation tests on these associations indicated that specificities (i.e., WCRP, WCRSP) of the pathway-based associations of E3s were much higher than those of the others (Figure 3D). It suggests that although E3s were not highly correlated or co-expressed with their substrates compared with random or indirect regulatory cases in mRNA level (Figures 3A and 3B), they may be closely related with the other members of the pathways their substrates belong to, or vice versa. Proteomic level presented different tendency; E3s exhibited lower associations than all three NRDs on average, but the specificities were higher than “E3-random” and “E3-Indirect.” “Other PPIs” exhibited both considerably higher pathway associations and permutation scores than E3s. It is probably a consequence of the degradation effect or dynamic nature of E3s. Moreover, the pathway-based associations promoted to discriminate E3s from NRDs; some of them (Figure 3E, WCP, WCR, WCPP, WCPSP) even rendered better performances than network-based features, especially in distinguishing E3s from “Other PPIs.”

The pathway-based associations can also capture which pathway was highly correlated with an ESI (see Transparent Methods). For example, pathways, like cell cycle (Shabbeer et al., 2013; Zheng et al., 2016), p53 signaling pathway (Li et al., 2012; Zhang et al., 2014), and transforming growth factor-β signaling pathway (Gen et al., 2017), all of which have been confirmed as ubiquitination-mediated pathways, were highly correlated with the E3s in proteomics, and a large fraction of substrates can be located on these pathways (Figure S1), similar results were observed in the transcriptomics-based pathway associations.
Pan-Cancer Analysis Show Concordance on the E3-Substrate Association Patterns

The analyses above utilized omics data from Breast Invasive Carcinoma (BRCA) in TCGA. To be more comprehensive, we asked whether the ESI association patterns were consistent across different cancers. We recalculated the features based on additional 10 cancers. Parallel analyses based on distinct cancers manifested that the differential trends between ESIs and NRDs on most available features were consistent with those shown in BRCA (Figures 3A–3D and S3A–S3J). The concordance also indicates that the multi-omics data in TCGA can be applicable to gain insights into biological mechanism in a more universal manner, even though they are originally cancer oriented.

Classification Models Based on the Multidimensional Associations Perform Well in Discriminating ESIs from NRDs

The considerable differences between ESIs and NRDs in the multidimensional association space prompt us to construct a classification model wherein three types of reverse-feature-reduction based random forest classifiers (RFE-RFs) were combined to identify ESIs (Figure 4A, see also Transparent Methods). The performance was quantified by the area under the receiver operating characteristic curve (AUROC). The classification model showed a satisfying and stable performance (Figures 4B–4D), especially when “E3-random” and “Other PPIs” categories (average AUROCs around 0.87 and 0.85, respectively) were taken as negatives, whereas the performance was somewhat declined for ”E3-Indirect” (average AUROCs around 0.78). The observation indicated it is more difficult to separate true substrates from indirect regulatory proteins than other negative cases. For different types of RFE-RFs, features like CNR.PPI, WCR, and WCP (Figure 4E) were consistently selected for all classifiers, further confirming the importance of network and pathway-based associations.

Moreover, ESIs were predicted with significantly higher probabilities than all three types of NRDs (Figure 4F). Both crossover and independent validations show that our model can help separate true and false ESIs. Although false-positives still existed, the misclassification rate was significantly reduced by inclusion of three negative categories. In general, random cases are always chosen as negative controls in interactome prediction, based on which the classifier generated much more false-positives (24% of “E3-Random,” 83% of “Other PPIs,” and 62% of “E3-Indirect” were misclassified as ESIs, Figure 4H). Similar conditions were observed when only “Other PPIs” or “E3-Indirect” was taken as the control. However, when three types of classifiers were integrated, the false discovery rate was significantly reduced, with 73% ESIs being accurately predicted under the threshold of 0.48 (Figures 4G and 4H), whereas only 15% “E3-Random,” 31% “Other PPIs,” and 30% “E3-Indirect” being misclassified.

We also trained the models by omics data from other cancers (Table S5). We observed that models trained based on ovarian serous cystadenocarcinoma(OV) and BRCA, where both transcriptomics and proteomics data were available, obtained better performance than others with only transcriptomics data, confirming the importance of multi-omics integration. As data of BRCA were more comprehensive than OV (only 155 ESIs can be assigned with both omics), we mainly utilized data from BRCA in the following study.

Prediction and Validation on Potential Substrates of FBXL Family

To further estimate the model quality, we applied it on the FBXL proteins, of which certain post-transcriptional modifications are often required for the substrates (Skaar et al., 2013). In particular, to avoid circularity of training and predicting samples, we reconstructed the classification model (Figure S4) by removing known ESIs of FBXLs from the training process. Both known and potential substrates were identified by our
model. Considering 89 confirmed ESIs of FBXLs, 39 pairs that can be assigned with the multidimensional features were calculable by our model, and 27 of the 39 (69%) cases were correctly recalled, whereas only 13 among the 39 cases were predicted by UbiBrowser (Table S6).

Multiple FBXLs were predicted to target on substrates belonging to the pathways of cell cycle, ribosome biogenesis in eukaryotes, ubiquitin-mediated proteolysis, and spliceosome (Figures 5A and 5B). The mostly influenced pathway is the cell cycle pathway, where multiple known substrates like MYC, SMAD4, and CCNE1 were correctly linked to SKP2, and other unreported ones like CCNA2 and CDK1 were predicted to be recognized by FBXL3, FBXL6, and FBXL10, indicating the potential cross-regulatory mechanism on the cell cycle pathways by FBXLs. This is consistent with the fact that F box proteins play key roles in cell cycle regulation (Zheng et al., 2016).

To test on the quality of our model, we compared our predictions to a proteomics-based experimental study (Tan et al., 2013), where a well-designed parallel adaptor capture (PAC) proteomic method was...
FBXL6 expression by transducing shRNA_FBXL6 significantly inhibited polyubiquitylation of VDAC2 (Figures 6D and 6E). In contrast, knockdown of SKP2 and FBXL6 significantly decreased the stability of CCNA2 protein (Figures 6F and S5E). Also, we examined CCNA2 protein levels in the presence of cycloheximide. As expected, SKP2 and FBXL6 significantly increased both endogenous substrates including CDK4, CCNA2, HSP90A1, HSPD1, and VDAC2 were validated (Figures 6B and S5B). For FBXL6, an orphan E3 without any known substrates, its interactions with five predicted substrates were retrieved. Among them, 79 pairs were predicted as high-confidence (p > 0.75, Figure 7A) cases for cancer hallmark proteins, where 22 of them were previously revealed and another 57 were predicted by these E3s (Figure 7A). Besides, these predicted hallmark ESIs show the “multi-to-multi” relations between E3s and substrates again. This information is important for E3s that may be taken as promising therapeutic targets for cancers. It is essential to make sure that drugs targeting on certain E3s will not lead to unfavorable outcomes by disturbing unexpected ESIs for the multifunctional E3s.

Furthermore, we experimentally checked on several high-confidence substrates of SKP2 and FBXL6 (see Transparent Methods). For SKP2, its interactions with four predicted substrates were validated, where the interaction with CCNA2 was already reported by previous studies (Nakayama et al., 2000) and the other three proteins including CASP3, DDB1, and HSP90AA1 have not been discovered (Figure 6A, SSA, and SSB). For FBXL6, an orphan E3 without any known substrates, its interactions with five predicted substrates including CDK4, CCNA2, HSP90AA1, HSPD1, and VDAC2 were validated (Figures 6B and SSA–SSD). Moreover, we found that FBXL6 inhibited CCNA2 and VDAC protein expression in a dose-dependent manner (Figure 6C). In addition, FBXL6 and SKP2 significantly increased both endogenous and exogenous CCNA2 protein polyubiquitylation (Figures 6D and 6E). In contrast, knockdown of FBXL6 expression by transducing shRNA_FBXL6 significantly inhibited polyubiquitylation of VDAC2 (Figures 6F and 6E). Also, we examined CCNA2 protein levels in the presence of cycloheximide. As expected, SKP2 and FBXL6 significantly decreased the stability of CCNA2 protein (Figures 6G and 6H). These experimental results prove that our predictions can provide credible references on identification of promising ESIs.

**Inferences on Potential E3s for Cancer Hallmark Proteins**

To be more comprehensive, the ultimate prediction model (Figure S6) was reconstructed by incorporating all known ESIs into the training procedure. Finally, about 2,80,000 pairs of proteome-wide potential ESIs were inferred by our model. To investigate whether cancer hallmark proteins might be ubiquitinated by certain E3s (see Table S7 for all investigated E3s), candidate pairs with COSMIC-recorded proteins as substrates were retrieved. Among them, 79 pairs were predicted as high-confidence (p > 0.75, Figure 7A) cases for cancer hallmark proteins, where 22 of them were previously revealed and another 57 were predicted (19 of them have been reported or examined in previous literatures, Table S8). A number of crucial pathways for cancer, e.g., NF-kB signaling pathway, Notch signaling pathway, and apoptosis, were influenced by these E3s (Figure 7A). Besides, these predicted hallmark ESIs show the “multi-to-multi” relations between E3s and substrates again. This information is important for E3s that may be taken as promising therapeutic targets for cancers. It is essential to make sure that drugs targeting on certain E3s will not lead to undesirable outcomes by disturbing unexpected ESIs for the multifunctional E3s.

Besides, we observed that BRCA1, a tumor suppressor with E3 activity, was also predicted as the substrate for multiple E3s when the prediction was conducted based on data of TCGA-BRCA (Figure 7A). However, some interactions were not high-confidence ones any more in other cancers (Figure 7B), implying the assumption that predicted results for mutant substrates can be cancer type specific.
DISCUSSION

Progressive accumulation in multi-omics data (Cancer Genome Atlas Research Network et al., 2013; Edwards et al., 2015) and prior biological knowledge (Chatr-Aryamontri et al., 2017; Kanehisa et al., 2017) allow for a data-driven investigation on ESIs. Here, we aimed to construct an ESI landscape and describe the association profiles between E3s and substrates by integrating different data resources.

Our study provides a glance at the association patterns of ESIs by combining multi-omics data and biological knowledge, where three types of negative control were taken into consideration. An initial scale-free ESI network composed of 1,806 reported ESIs was constructed, where plenty of cancer hallmark genes act as hubs, and the numbers of interacting substrates for different E3s vary considerably. It may suggest a general rule that some E3s are with a broad-spectrum function and that they can regulate various types of substrates, whereas the others only have effects on certain substrates. Unexpectedly, although a large fraction of ESIs will lead to substrate degradation (Figure 2C), E3s and substrates did not show significantly negative correlations in the omics-based associations (Figure 3A), suggesting that no consistent expression relation exists for ESIs and indicating the heterogeneity of ubiquitination mechanism. Nonetheless, integrating omics with network or pathway information greatly enhanced the differences between ESIs and NRDs, and the degradation effect emerged. Notably, the pathway-based associations can help identify downstream or upstream processes such as DNA replication (Faircloth et al., 2011) and mammalian target of rapamycin signaling pathway (Xia et al., 2016). The effectiveness and consistency of omics-driven association features for ESI identification also indicate the reusability of cancer omics-resources.

Different from the models utilized by UbiBrowser (Li et al., 2017) where only one negative category was considered, our model combined three negative categories covering “E3-random,” “Other PPIs,” and “E3-Indirect.” Both crossover and independent validations have demonstrated the model’s effectiveness, and the inclusion of three negative categories has significantly reduced the false discovery rate. Moreover, the false discoveries (e.g., SKP2-CUL1, FBXL19-UBE2T, Figures 5A and SB) can be reduced further by removing E1s, E2s, and scaffold proteins in E3 complexes from candidate substrates, as most of them are already defined by studies on well-characterized E3s, like FBXW7 and MDM2. Taken together, our predictions can provide assistance on ESI recognition, but further experimental investigations are also required to confirm the interactions.

Our model also exhibits effectiveness for substrates that require certain forms of modifications before ubiquitination. Taking FBXL family as an illustration, our predictions output consistent results with a previous proteomic experimental study (Tan et al., 2013) and with a significantly improved recall (27/89 versus 5/89). When compared with UbiBrowser, our model also showed higher recall (27/89 versus 22/89), and the superiority was even higher in terms of calculable cases (27/39 versus 13/39), suggesting the advantage of our model, especially for substrates with knowledge background. Furthermore, a number of un-reported predictions of ESIs for SKP2 and FBXL6 were validated by experimental investigations. All the above imply the effectiveness of our prediction model.

In conclusion, our study provides a data-driven way to portray the ESI landscape, offering meaningful hypothesis on the latent E3-substrate association patterns, promising ESIs or even regulatory mechanism. For convenience, a website (http://www.esinet.dicp.ac.cn/home.php) is developed for browsing the multi-scaled association features of confirmed (1,806 pairs) and proteome-wide predicted (about 2,80,000 pairs) ESIs. It provides a valuable resource and assistance for further studies on protein ubiquitination.

Limitations of the Study

The data-driven prediction model’s application scope is limited by currently available data resources, especially the proteomics data that is indispensable for most prediction-dependent multidimensional features. Although we utilized a relatively comprehensive proteomics database CPTAC wherein 10,602...
proteins (about 400 E3s were included) were measured, some E3s like FBXL2, FBXL5, and FBXL22 were still not covered. We cannot calculate the multidimensional features for these items, thus lacking predictions on these E3s. As more proteomics data and network or pathway knowledge are being accumulated, we envision a continual optimization of the prediction results.

In addition, the fate of ubiquitinated substrates depends on the linkage of ubiquitin chains (Senft et al., 2018). Although most of the reported ESIs can lead to substrate degradation by K48 or K11 linkage, ubiquitination can also induce other outcomes, like activity or stability change. Our present prediction model mainly focuses on whether there is an interaction between an E3 and a substrate; however, the linkage-dependent ubiquitinated consequence was not considered. In our future studies, we will attempt to deduce the linkage information as well.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.05.033.

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AUTHOR CONTRIBUTIONS
Conceptualization, H.-l.P., D.C., and X.L.; Methodology, D.C. and X.L.; Formal analysis, D.C.; Validation, X.L.; Investigation, T.X., D.S.T., W.W., H.C., T.L., C.L., Z.N., X.L., H.Q., and H.H.; Writing – Original Draft, D.C., X.L., and H.-l.P.; Writing – Review & Editing, D.C., X.L., and H.-l.P.; Funding Acquisition, D.C. and H.-l.P.; Resources, D.C.; Data Curation, X.L.; Supervision, H.-l.P.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

A Multidimensional Characterization
of E3 Ubiquitin Ligase
and Substrate Interaction Network

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Supplemental Figures

Figure S1. E3-pathway correlations based on proteomics. Related to Figure 3. The core matrix exhibits the associations between E3s and pathways. For clarity, only the top-50 frequent pathways which can link E3s to substrates' pathways based on proteomics were listed out in the rows, and the corresponding top-50 correlated E3s were listed as columns. The left-most column shows the pathway category information. NUW annotates the Number of Ubiquitinated substrates on the pathway. wcpM refers to the mean wcp (see Materials and methods) score across the top-50 E3s for a corresponding pathway.
Figure S2. E3-pathway correlations based on transcriptomics. Related to Figure 3. The core matrix exhibits the associations between E3s and pathways. For clarity, only the top-50 frequent pathways which can link E3s to substrates’ pathways based on transcriptomics are listed out in the rows, and the corresponding top-50 correlated E3s are listed as the columns. The left-most column shows the pathway category information. "NUW" annotates the Number of Ubiquitinated substrates on the pathway. "wcrM" refers to the mean wcr (see Materials and methods) score across the top-50 E3s for a corresponding pathway.
Figure S3. Pan-cancer based multidimensional association patterns of ESI. The association features were computed based on additional 10 tumors including Ovarian Serous Cystadenocarcinoma (OV) (A), Bladder Urothelial Carcinoma (BLCA) (B), Colon Adenocarcinoma (COAD) (C), Head and Neck Squamous Cell Carcinoma (HNSC) (D), Prostate Adenocarcinoma (PRAD) (E), Kidney Renal Clear Cell Carcinoma (KIRC) (F), Kidney Renal Papillary Cell Carcinoma (KIRP) (G), Liver Hepatocellular Carcinoma (LIHC) (H), Lung Adenocarcinoma (LUAD) (I), Lung Squamous Cell Carcinoma (LUSC) (J). Centers of boxes represent median values. Bottom and top bounds of boxes represent 25th and 75th percentiles. Whiskers mark 1.5 times of the interquartile range. Dots represent points falling outside this range. *: p<0.01, Wilcoxon-test for the differences comparing to the ESI dataset.
Figure S4. Performance of classifiers trained for FBXLs. Related to Figure 5. The same ESIs were used as positive samples, while (A) "E3-random", (B) "Other PPIs", and (C) "E3-Indirect" pairs were respectively taken as negative samples, and samples with FBXL proteins were removed. The horizontal and vertical axes represent the number of selected features and the corresponding average AUROC got from a 10-fold crossover validation. For each type of RFE-RFs, 5 classifiers were constructed by replacing negative samples. (D) The best performance features for each classifier in (A), (B) and (C).
Figure S5. Experimental validations of high-confident ESIs. Related to Figure 6. (A) Validations on interactions between CCNA2 and FBXLs. HEK293T cells were transfected with SFB-tagged RFP, SKP2, FBXL6 and FBXL15. SFB-tagged proteins were purified with S protein beads and immunoblotted with antibodies against substrate of CCNA2 and FLAG. (B-D) Validations on interactions between substrates (B) Casp3-V5 was cotransfected with empty vector or SFB-SKP2 or SFB-FBXL6 into HEK293T cells. Western blotted with antibodies against FLAG and Caspase3. CDK4-V5 (C) or VDAC2 (D) were cotransfected with empty vector or SFB-FBXL6 into HEK293T cells. Western blotted with antibodies against FLAG and V5. (E) SFB-VDAC2 cotransfected with MYC-FBXL6, HA-Ub into HEK293T cells. Cells were treated with 20 μM MG132 for 4 h before collection. VDAC2 was purified with S-beads and western blotted with antibodies against HA, Myc and FLAG. All experiments have three biological replicates, and the most representative image is shown.
**Figure S6. Performance of the final classifiers.** Related to Figure 7. All calculable ESIs were included as positive samples and the same number of (A) "E3-random", (B) "Other PPIs", and (C) "E3-Indirect" pairs were respectively taken as negative samples. The horizontal and vertical axes represent the number of selected features and the corresponding average AUROC got from a 10-fold crossover validation. For each type of RFE-RFs, 5 classifiers were constructed by replacing different negative samples. (A) The best performance features for each classifier in (A), (B) and (C).
### Table S5. Performance of classifiers based on different cancers.

Related to Figure 4. NC stands for the number of calculable ESIs based on the data of certain cancer. AUC1, AUC2 and AUC3 are respectively got from RFs trained by "E3-random","Other PPIs" and "E3-Indirect" samples. T: Transcriptomics; P: Proteomics.

| Cancer | NC  | AUC1 | AUC2 | AUC3 | Omics |
|--------|-----|------|------|------|-------|
| BRCA   | 576 | 0.87 | 0.86 | 0.79 | T+P   |
| OV     | 155 | 0.91 | 0.86 | 0.78 | T+P   |
| BLCA   | 808 | 0.85 | 0.75 | 0.74 | T     |
| COAD   | 808 | 0.84 | 0.75 | 0.71 | T     |
| HNSC   | 809 | 0.85 | 0.76 | 0.70 | T     |
| LIHC   | 809 | 0.85 | 0.77 | 0.74 | T     |
| LUAD   | 808 | 0.85 | 0.78 | 0.75 | T     |
| LUSC   | 809 | 0.87 | 0.79 | 0.74 | T     |
| PRAD   | 810 | 0.85 | 0.77 | 0.74 | T     |
| KIRC   | 811 | 0.85 | 0.77 | 0.69 | T     |
| KIRP   | 811 | 0.84 | 0.77 | 0.73 | T     |
## Transparent Methods

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-flag (M2)      | Sigma-Aldrich | F3165-5MG |
| Anti-V5             | Invitrogen           | R960-25   |
| Anti-CDK4           | Cell Signaling Technology | 12790    |
| anti-caspase-3      | Cell Signaling Technology | 9662S |
| Anti-DDB1           | Proteintech         | 11380-1-AP |
| anti-cyclin A2      | Proteintech         | 18202-1-AP |
| anti-HSP90AA1       | Proteintech         | 13171-1-AP |
| anti-HSPD1          | Proteintech         | 15282-1-AP |
| anti-VDAC1/2        | Proteintech         | 10866-1-AP |
| anti-HA             | Proteintech         | 51064-2-AP |
| anti-Myc            | Proteintech         | 16286-1-AP |
| **Deposited Data**  |        |            |
| Transcriptomics data| Cancer Genome Atlas Research et al., 2013 | http://cancergenome.nih.gov/ |
| Proteomics data     | Edwards et al., 2015 | https://cptac-data-portal.georgetown.edu/cptacPublic/ |
| **Experimental Models: Cell Lines** | | |
| HEK293T             | American Type Culture Collection | HEK293T |
| **Software and Algorithms** | | |
| R                   | https://www.r-project.org | |
| Cytoscape           | Shannon et al., 2003 | http://www.cytoscape.org/ |
| R package of Random forest | Liaw, 2002 | |
| R package of caret   | Kuhn, 2008          | |
| R package of DMwR    | Torgo, 2010         | |
| **Other**           |        |            |
| E3Net               | Han et al., 2012    | http://pnet.kaist.ac.kr/e3net/ |
| hUbiquitome         | Du et al., 2011     | http://202.38.126.151/hmdd/hubibi/ |
**ESI collection**

To be as comprehensive as possible, a positive reference dataset of ESIs were mainly integrated from four databases: E3Net (Han et al., 2012), hUbiquitome (Du et al., 2011), Uniprot (The UniProt, 2017) and BioGrid (Chatr-Aryamontri et al., 2017). (1) E3Net mainly collected the ESIs from the MEDLINE literatures and UniProt by a text mining method, which extracted E3s and substrates with definite textual description on the specificities between E3s and their substrates. We extracted all of the E3-substrate pairs from E3Net as the main part of the positive dataset. (2) From another text-mining based database hUbiquitome, which collected experimentally validated protein ubiquitination cascades (E1-E2-E3-substrate cascades), we also extracted the E3-substrate interactions. (3) Integrating all the E3s recorded by E3Net, hUbiquitome as well as UbiNet (Nguyen et al., 2016), we got 589 possible E3s. To complement newly-updated ESIs, we searched the Uniprot (release-2017_06) for these E3s and extracted E3-substrate pairs which were with definite textual description that certain protein was ubiquitinated by certain E3. (4) Additional ESIs were extracted from the physical PPIs recorded in BioGrid (version: BIOGRID-MV-Physical-3.4.152) by strict filtering rules: with at least one E3, recorded as "physical interactions", recognized by "Low-Throughput" methods, modified by "Ubiquitination", and with explicit literature evidences. Besides, ESIs summarized in a previous review (Wang et al., 2014) were also included. In total, 1806 pairs of E3s and substrates (Table S1) in human were collected as the positive reference dataset.

**Control set collection**

Three categories of negative cases were prepared. The first one ("E3-random") contained 10000 pairs of randomly paired E3s and proteins (Table S2). The second one ("Other PPIs") incorporated 10000 pairs of randomly selected ubiquitination-independent PPIs from BioGrid (Table S3). The last one ("E3-Indirect") encompassed 10000 pairs of indirect E3 regulation.
relations which were randomly sampled from the two-step distant E3s and their indirectly connected proteins on the PPI network (Table S4). During sampling, known ESIs and redundant cases were excluded.

Cancer hallmark subgraph extraction
The cancer hallmark subgraph (Figure 2C) was extracted as a representative profile to show part of the ESI network. To highlight the functional mechanism of ESIs in cancer, this subgraph was confined to two requirements. Firstly, the nodes should be recorded in the COSMIC database which focuses on cancer hallmark genes. Secondly, literature investigations were conducted to obtain the ubiquitination fate of the substrate on each edge, retained edges should be annotated with explicit ubiquitination fate, either degradation or non-degradation, for corresponding ESIs (see Table S1 for specific evidences of different edges).

Omic data preparation
For transcriptomics, normalized RNA-seq data of different cancers in TCGA (Cancer Genome Atlas Research et al., 2013) were downloaded by an R package – TCGAbiolinks (Colaprico et al., 2016). For each cancer, the RNA-Seq data contained mRNA expressions of 20501 genes across tumor and normal tissues, a fraction of the tumor tissues can be matched with normal ones which were taken from the same patients. Proteomics data for BRCA and OV were downloaded from CPTAC (Edwards et al., 2015), where the protein expressions were quantified by an isobaric peptide labeling approach (iTRAQ). For BRCA, 10599 proteins were quantified, while 6160 proteins were measured for OV.

Omic-based associations
Four forms of omic-based associations were considered. PCT (A, B) / RCT (A, B) calculated the spearman correlation coefficient (SCC) between A and B based on their protein / mRNA expressions in tumor tissues. RCN (A, B) calculated the SCC between A and B based on the mRNA expressions in normal tissues. RCF (A, B) calculated the SCC between A and B regarding the fold change of mRNA-expressions in tumor tissues relative to matched normal tissues.

Network-based associations
Three networks were considered: the PPI network was composed of 302868 experimentally-obtained PPIs in BioGrid; the mRNA/protein co-expression network was constructed by connecting two genes/proteins with SCCs larger than certain threshold (selected as 0.3, since the most significant difference between PRDs and NRDs will be observed based on the threshold of 0.3). Two forms of network-based associations were computed. On one hand, the common neighbor rate (CNR) between protein a and b on network G was calculated as:
\[ \text{CNR}_G(a,b) = \frac{|\text{Neis}(a,G)| \cap |\text{Neis}(b,G)|}{\sqrt{|\text{Neis}(a,G)| \cdot |\text{Neis}(b,G)|}} \]

where \( \text{Neis}(\cdot,G) \) represented the neighbors of node \( \cdot \) on network \( G \). On the other hand, co-expression and PPI networks were integrated, and the common neighbor rate of co-expressed factors on PPI network was computed:

\[
\begin{align*}
\text{CCR}. \text{PPI}(a,b) & = \frac{|\text{Neis}(\text{HN}(a,G_{\text{CXNR}}),G_{\text{PPI}}) \cap |\text{Neis}(\text{HN}(b,G_{\text{CXNR}}),G_{\text{PPI}})|}{\sqrt{|\text{Neis}(\text{HN}(a,G_{\text{CXNR}}),G_{\text{PPI}})| \cdot |\text{Neis}(\text{HN}(b,G_{\text{CXNR}}),G_{\text{PPI}})|}} \\
\text{CCP}. \text{PPI}(a,b) & = \frac{|\text{Neis}(\text{HN}(a,G_{\text{CXNP}}),G_{\text{PPI}}) \cap |\text{Neis}(\text{HN}(b,G_{\text{CXNP}}),G_{\text{PPI}})|}{\sqrt{|\text{Neis}(\text{HN}(a,G_{\text{CXNP}}),G_{\text{PPI}})| \cdot |\text{Neis}(\text{HN}(b,G_{\text{CXNP}}),G_{\text{PPI}})|}}
\end{align*}
\]

where \( G_{\text{CXNR}}/G_{\text{CXNP}} \) denoted the mRNA/protein co-expression network, \( \text{HN}(\cdot,G) \) represented the highly co-expressed neighbors of node \( \cdot \) on network \( G \), and only the top-10 co-expressed factors were retained.

**Pathway-based associations**

Pathway-based associations were applied to estimate whether one E3 was highly correlated with certain pathway its substrates belong to, or vice-versa. The correlation between protein \( a \) and pathway \( P \) with \( N \) member genes was calculated as:

\[
\begin{align*}
wcr(a,P) & = \frac{\sum_{\text{m} \in \{P_{\text{mRNA}}\}} |\text{SCC}_{\text{mRNA}}(a,m)|}{N} \\
wcp(a,P) & = \frac{\sum_{\text{m} \in \{P_{\text{protein}}\}} |\text{SCC}_{\text{protein}}(a,m)|}{N}
\end{align*}
\]

where \( \text{m} \) is a member gene of pathway \( P \), \( wcr \) and \( wcp \) were respectively calculated from transcriptomics and proteomics data; particularly, if protein \( a \) is also a member of pathway \( P \), it will be eliminated from the pathway during calculation.

Depending on pathways of E3 and substrate respectively, four forms of pathway-based associations were generated:

\[
\begin{align*}
\text{WCR}(a,b) & = \max \{ wcr(a,P_i) \mid b \in P_i \} \\
\text{WCP}(a,b) & = \max \{ wcp(a,P_j) \mid b \in P_j \} \\
\text{WCRS}(a,b) & = \max \{ wcr(b,P_j) \mid a \in P_j \} \\
\text{WCPS}(a,b) & = \max \{ wcp(b,P_j) \mid a \in P_j \}
\end{align*}
\]

where \( \text{WCR} \) and \( \text{WCRS} \) integrated transcriptomics with pathways, \( \text{WCP} \) and \( \text{WCPS} \) integrated
proteomics with pathways.

Permutation test was applied to examine the specificity of pathway-based associations. Taken WCR \((a, b)\) as an illustration, if pathway \(P_{\text{max}}\) has the highest correlation with protein \(a\) among all known pathways of protein \(b\), we randomly permutated the member genes of \(P_{\text{max}}\) by the same number of other genes, and calculated a new \(wcr (a, P_{\text{max}}')\), repeated this process 100 times, and the ratio of decreased scores was calculated as \(WCRP (a, b)\). The higher \(WCRP (a, b)\) was, the less likely \(b\) was related with \(a\) through a pathway with the same size of \(P_{\text{max}}\) by chance. Likewise, \(WCPP, WCRSP, WCPSP\) were calculated as well.

**Feature selection**

Integration of 4 omic-based, 5 network-based and 8 pathway-based association features generated a 17-dimensional feature description for all pairwise proteins. To identify which features are more effective in distinguishing ESIs from NDRs, we used recursive feature elimination (RFE) algorithm (Guyon et al., 2002), in which each feature was ranked according to its importance for feature selection. It was carried out based on an R package of caret (Kuhn, 2008).

**Classification algorithm**

Together with the RFE feature selection, Random forest (RF) (Breiman, 2001) was applied to examine feature importance and train classifiers. This algorithm was implemented by an R package of randomForest (Liaw, 2002). Basically, three types of reverse-feature-reduction based random forest classifiers (RFE-RFs) were trained, where the positive references were the same, but the negative ones were from three different categories respectively. To encompass more negative samples, for each type of RFE-RFs, 5 classifiers were built using different negative samples.

**Cross-over validation and independent validation**

The model performance was quantified by AUROC (Lasko et al., 2005) through both 10-fold cross-over validations and an independent validation. During the 10-fold cross-over validation, to keep sample balance, the same number of ESIs and certain type of NRDs were randomly selected from the collected ESIs and NRDs as the cross-over validation set which was separated into 10 independent parts, each single part should be used as the testing dataset once, and the other 9 were used to train the model, thus the training and testing were conducted 10 times. Since the number of NRDs was extremely larger than ESIs, the cross-over validation was repeated 5 times by replacing non-redundant NRDs. Regarding three categories of NRDs, three types of classifiers were trained and tested by the cross-over validations. From the rest samples, another dataset composed of certain number of ESIs, "E3-random" pairs, "Other PPIs", and "E3-indirect" pairs was collected as the independent validation set.
**F3-Score**

F-Score (Maratea et al., 2014) is utilized to estimate the performance of a probability threshold for discriminating ESIs in predictions. It is computed as:

\[
F_\beta = \left( 1 + \beta^2 \right) \frac{\text{precision} \cdot \text{recall}}{\beta^2 \cdot \text{precision} + \text{recall}}
\]

where \( \beta \) was defined as 3 here, weighing recall higher than precision, thus \( F_3 \) was computed.

The rationale lies in that the number of possible NRDs was extremely higher than ESIs which were only partially known, emphasis on recall will avoid the overlook of novel predicted ESIs.

**Imputation methods for missing values**

Before prediction, we calculated the multi-dimensional features for candidate samples; however, due to data limitation, some samples could not be fully-described by 17 dimensional features. For a sample with missing features, if its missing ratio was less than 30%, the KNN imputation algorithm which predicted missing values according to weighted average of the K nearest neighbors was employed to fill the missing value; otherwise, the samples were omitted from predictions. The KNN imputation (distances to others were calculated based on only known features) was performed by R package of DMwR (Torgo, 2010).

**Predict novel ESIs**

Candidate ESIs were prepared before prediction. For an investigated E3, 6686 genes which were included in at least one pathway and measured in transcriptomics or proteomics were paired with the E3 as candidate ESIs; for an investigated substrate, 589 E3s that we have collected (Table S7) were paired with the substrate as candidate ESIs.

Three types of classifiers with the purpose to distinguish ESIs from different categories of NRDs were trained before prediction, and each type contained 5 parallel repetitions (each repetition was trained by replacing non-redundant NRDs within the same category). For the candidate ESIs, we calculated the 17-dimensional association features, and filled up missing values. Then, an ensemble classification model was applied on these candidates to predict the probability of being ESI as:

\[
\text{Prob}(a,b) = \frac{1}{3} \sum_{i=1}^{3} \sum_{k=1}^{5} \text{auc}_{i,k} \cdot \sum_{k=1}^{5} \text{prob}_{i,k}
\]

where \( i \) represents the type of classifiers, \( k \) represents the repetition, \( \text{auc} \) represents AUROC of a classifier, and \( \text{prob}_{i,k} \) was the predicted probability for the input pair based on the \( k \)-th classifier of type \( i \).

**Experimental antibodies**

Anti-flag (M2) (F3165-5MG, 1:3,000 dilution) monoclonal antibody was obtained from Sigma-Aldrich. Anti-V5 (R960-25, 1:1,000 dilution) monoclonal antibody was purchased from Invitrogen. Anti-CDK4 (12790, 1:1,000 dilution) and anti-caspase-3 (9662S, 1:1,000 dilution)
polyclonal antibodies were purchased from Cell Signaling Technology. Anti-DDB1 (11380-1-AP, 1:1,000 dilution), anti-cyclin A2 (18202-1-AP, 1:1,000 dilution), anti-HSP90AA1 (13171-1-AP, 1:1,000 dilution), anti-HSPD1 (15282-1-AP, 1:1,000 dilution), anti-VDAC1/2 (10866-1-AP, 1:1,000 dilution), anti-HA (51064-2-AP, 1:2,000 dilution) polyclonal antibodies were purchased from Proteintech.

**Plasmids constructs, cell culture and transfection**

All the expression vectors used in this study (including SFB-SKP2, SFB-FBXL6, SFB-FBXL15, SFB-CCNA2, SFB-VDAC2, Caspase3-V5, CDK4-V5, VDAC2-V5, Myc-SKP2 and Myc-FBXL6) were generated by polymerase chain reaction (PCR) and then subcloned into pDONR221 vector as the entry clones using Gateway Technology (Invitrogen). Subsequently, the entry clones were recombined into gateway destination vectors fused with various tags (SFB, V5 or Myc). The FBXL6 shRNAs were purchased from Dharmacon. HEK293T cells were purchased from American Type Culture Collection (ATCC) and cultured at 37°C under humidified air containing 5% CO₂ in Dulbecco’s modified Eagle’s medium (Gibco, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin antibiotics. Plasmid transfection was performed with the polyethylenimine (PEI) reagent as previously described (Longo et al., 2013).

**Tandem affinity purification of SFB-tagged protein complex**

For affinity purification experiments, HEK293T cells were harvested after transfection for 48 h and subjected to lysis in NETN buffer (100 mM NaCl, 0.5 mM EDTA, 20 mM Tris-Cl, 0.5% Nonidet P-40) with protease and phosphatase inhibitors at 4°C for 1 h. Supernatants were obtained after centrifugation (13,000 rpm) at 4°C for 15 min and then incubated with S protein beads (Millipore) overnight at 4°C. The beads were washed three times with NETN buffer at room temperature. Bound protein complex were released from the beads after boiling with 4×SDS loading buffer for 10 min and applied for Western blot analysis.

**In vivo ubiquitination assay and half-life assay**

HEK293T cells were plated in 10-cm dishes and cultured until cell confluence came up to 80%. The cells were transfected with the indicated plasmids and incubated for 36 h and then treated with MG132 (20 µM) for 5 h prior to collection. The cells were lysed in NETN buffer. Lysates were incubated with S protein beads for affinity purification as described earlier. Western blot was applied onto the pulldown protein complex for ubiquitination analysis of predicted substrates. For the half-life assay, HEK293T cells were transfected with empty vector (EV), SFB-SKP2 or SFB-FBXL6 along with CCNA2-V5. Forty-eight hours later, cells were treated with cycloheximide (CHX, 100 µM) for the indicated time points, and cell lysates were probed with CCNA2 antibody.

**Network visualization.**
Network visualization was performed with Cytoscape (Shannon et al., 2003).

**Statistical analysis.**

Differences between ESIs and NRDs on each association feature were examined by Wilcoxon-test, and differences between node degrees of E3s and substrates were examined by t-test. Statistical test and other computations were all conducted by R.

**Data and software availability.**

Most data that support our conclusion have been contained in supplemental tables or website(http://www.esinet.dicp.ac.cn/home.php). The multidimensional association information can be browsed for both confirmed and inferred ESIs on the website. Other data produced by different stages of the computational processes are also available upon request. All computational codes are available on request.

**Supplemental References**

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