Identification of Critical Target Protein for Cystic Fibrosis using Systems Biology Network Approach

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Abstract: *The most critical step in drug discovery process is target identification. Loss of function disease such as cystic fibrosis is caused by impairment of one protein and target identification of such diseases becomes a more tedious task. Protein interactions can provide an insight of other proteins which could be targeted to affect the regulation of particular protein involved in disease. The present study was taken to identify the critical protein which could be targeted to affect the regulation of CFTR (Cystic Fibrosis Transmembrane Regulator) which is mutated in Cystic Fibrosis using a systems biology network approach involving STRING and ClusterONE plugin of Cytoscape. Five proteins namely PDZK1, SLC9A3R1, CFTR, CANX and HSPA8 were identified to be the critical proteins which could affect the regulation of CFTR. Calnexin (CANX) and HSPA8/HSC70 were also present in Cluster 2 obtained by ClusterONE which contains proteins responsible for degradation of misfolded protein. Finally HSPA8/HSC70 was selected to be a probable critical target protein as it has been reported that abolishing the interaction of F508del-CFTR with calnexin (CAS treatment) has no major (positive or negative) effect on ERAD of F508del-CFTR. Thus, systems biology approach may hold great promise to identify probable therapeutic targets.*

Keywords: *Systems biology, CFTR, Cytoscape, Protein-protein interactions, Potential therapeutic target.*

Introduction

Defect in protein trafficking has been recognized as an important mechanism for a growing number of inherited human diseases [1]. Cystic fibrosis (CF), an autosomal recessive genetic disorder affecting most critically the lungs, and also the pancreas, liver, and intestine is characterized by abnormal transport of chloride and sodium across the epithelium, leading to thick, viscous secretions [2]. CF is caused by a mutation in the gene for the protein cystic fibrosis transmembrane conductance regulator (CFTR). CFTR regulates the movement of chloride and sodium ions across epithelial membranes, such as the alveolar epithelia located in the lungs and most mutations in CF patients result in swift intracellular degradation of the CFTR protein [3].

The majority of CF cases are caused by nucleotide deletions (ΔF508del CFTR; >75%), resulting in CFTR misfolding, or insertion of premature termination codons (~10%), leading to unstable mRNA and reduced levels of truncated dysfunctional CFTR [4]. However, in many cases, the trafficking-defective mutant protein retains residual function if it can be rescued to its final destination [5]. The retention of a misfolded protein in the endoplasmic reticulum (ER) is linked to the activity of the cell quality control system [6]. This retention

generally causes the absence of the client CFTR protein at its target site, preventing its physiological function and leading to abnormal cell homeostasis and functions [5].

Newer proteomic approaches have shown that CFTR interacts with many other intracellular proteins, and the pathophysiologic relevance of these interactions has not been fully elucidated. However, this understanding is important for therapeutic interventions, since drugs that induce chloride secretion but do not affect other CFTR functions may not address all relevant aspects of CF pathophysiology [7].

Lung disease, which results from chronic infection and inflammation, is the most common reason of death in the CF population and thus its treatment is a significant area of CF therapy. In the CF lung, activation of the nuclear factor (NF)-κB signaling pathway leads to enhanced production of pro-inflammatory mediators, including interleukin (IL)-8. Drugs are being developed to treat various proteins involved in this inflammatory cycle which are still their clinical trials [8]. Gene therapy trials are on-going which aim to deliver functional CFTR genes to the epithelial cells of the CF airway. But in phase 1 studies, gene expression was not detected. The UK Cystic Fibrosis Gene Therapy Consortium is currently performing a phase 1/2 safety study using pGM169/GL67A. These systems utilizing liposomes to promote the aerosolized delivery of a DNA plasmid containing the CFTR gene are also being tested for their efficiency.

Ensuing the cloning of the CFTR gene in 1989 [9], the focus has now shifted towards the identification of candidate critical target proteins and indicators of disease severity. Restoration of normal CFTR function is considered to be the foremost therapeutic area as this will target the fundamental cause of CF disease rather than just considering the symptoms. Various proteins and protein degradation products have been discovered as candidate biomarkers of experimental outcome, such as neutrophil elastase and IL-8 [10], degradation of lung surfactant protein SP-A [11], urinary desmosine [12] and proline-glycine-proline [13]. However, none of these markers has proved adequately robust for routine acceptance in clinical trials for cystic fibrosis [14].

Systems Biology approach aims to find ways to condense and focus varied types of molecular data into meaningful perceptions [15]. For cystic fibrosis, the most immediate goal is to translate protein-protein interactions involved in the disease in a way that turns these perceptions into effective therapeutics targets. Systems biology approach, with combination of computational, experimental and observational enquiry, is highly significant to drug discovery and the optimization of medical treatment regimens for individual patients [16].

Protein-protein interactions (PPIs) are involved in most of the essential processes that occur in living organisms, such as cellular communication, immunological response, and gene expression control. Systematic description of these interactions aids elucidation of targeted drug design. Thus, targeting PPI of therapeutic interest with small-molecule compounds is becoming the holy grail of drug discovery [17]. The present study was undertaken to identify the critical protein which could be targeted to affect the regulation of CFTR which is mutated in CF using a systems biology approach.

Materials and methods

The physical interactions concerning the mutant protein CFTR and other proteins which are involved in the pathophysiology of disease were collected using STRING. After the

partitioning of the initial network into sub-graphs and intersecting the networks; we identified the critica protein, which could be targeted as drug target.

- **Databases**

To construct the network, CFTR protein was taken as input protein in STRING database [18] to find its direct and functional partners. The network was expanded to find the genes which functionally interact with CFTR and can be part of other pathways related to prognosis of cystic fibrosis. This online database, Search Tool for the Retrieval of Interacting Genes (STRING), provides uniquely comprehensive coverage and ease of access to both experimental as well as predicted interaction information. Interactions in STRING are provided with a confidence score, and accessory information such as protein domains and 3D structures are made available, all within a stable and consistent identifier space.

- **Network clustering and merging**

Cytoscape is an open source free platform for visualizing molecular networks and integrating with gene expression profiles. Additional features are available as plugins. Plugins are available for network and molecular profiling analyses, new layouts, additional file format support and connection with databases and searching in large networks [19].

The division of the resulting PPI network into overlapping sub-graphs of highly interconnected nodes was performed using the plugin ClusterONE (Clustering with Overlapping Neighbourhood Expansion) of Cytoscape. The algorithm behind the plugin ClusterONE works by "growing" dense regions out of small seeds guided by a quality function. The quality of a group is assessed by the number of internal edges divided by the number of edges connecting nodes of the group. Starting from a single seed, the algorithm extends the group step by step with new edges if they increase the quality of a group. An edge can be removed when its removal increases the quality of its group. The process stops when it is not possible to increase the quality of the groups by adding or removing another edge. Finally, sub-graphs smaller than 3 or having a density (number of edges within the cluster divided by the number of theoretically possible edges), less than 0.45 and a *p*-value under 0.05, are discarded. The density of 0.45 is chosen to get several overlapping sub-graphs having good quality. The p-value of one cluster is computed a posteriori with the one-sided Mann-Whitney U test performed on the number of intra edges and external boundary edges. An intra-edge is between a cluster node and a node within the cluster. An external boundary edge is between a cluster node and a node outside the cluster [20].

The four PPI networks involved in folding-maturation, membrane expression, degradation and inflammation of CFTR were intersected using Merge network plugin of Cytoscape to find the proteins which were common in all four pathways or may be points of connections amongst these pathways and thus may be considered as probable drug targets for cystic fibrosis.

Results and discussion

Network and systems biology offer a novel way of approaching drug discovery by developing models that consider the global physiological environment of protein targets, and the effects of modifying them, without losing the key molecular details. Protein and genetic interaction maps can reveal the overall physical and functional landscape of a biological system [21].

To identify protein target(s) which interact with the protein CFTR, protein-protein interaction (PPI) network was obtained using STRING database (Fig. 1) using CFTR as the input protein.

The network consisted of 71 nodes and 812 edges. The network was expanded up to seventy one nodes as more expanding of network resulted in increase in the number of nonspecific interactions, which would affect the analysis and specificity of the PPI network.

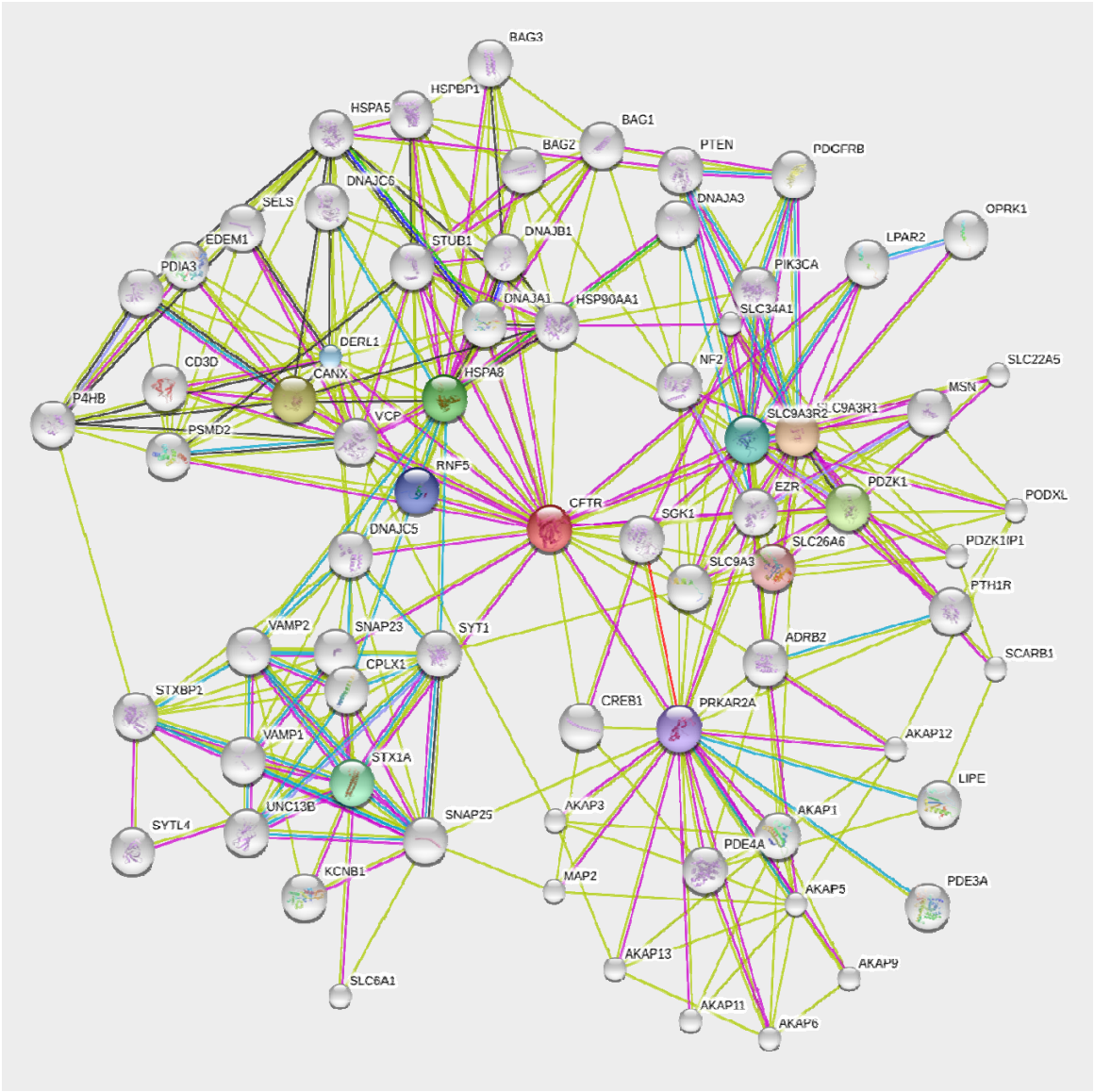

Fig. 1 PPI network of proteins interacting directly and functionally with CFTR obtained from STRING.

As cystic fibrosis is mainly concerned with misfolding of protein, the study of proteins involved in folding and maturation, membrane expression, degradation of CFTR and inflammation associated with cystic fibrosis could be used to short-list the probable drug targets which can be targeted to treat cystic fibrosis. Therefore, four other PPIs networks involved in folding-maturation, membrane expression, degradation and inflammation of CFTR were generated using STRING (Figs. 2-5).

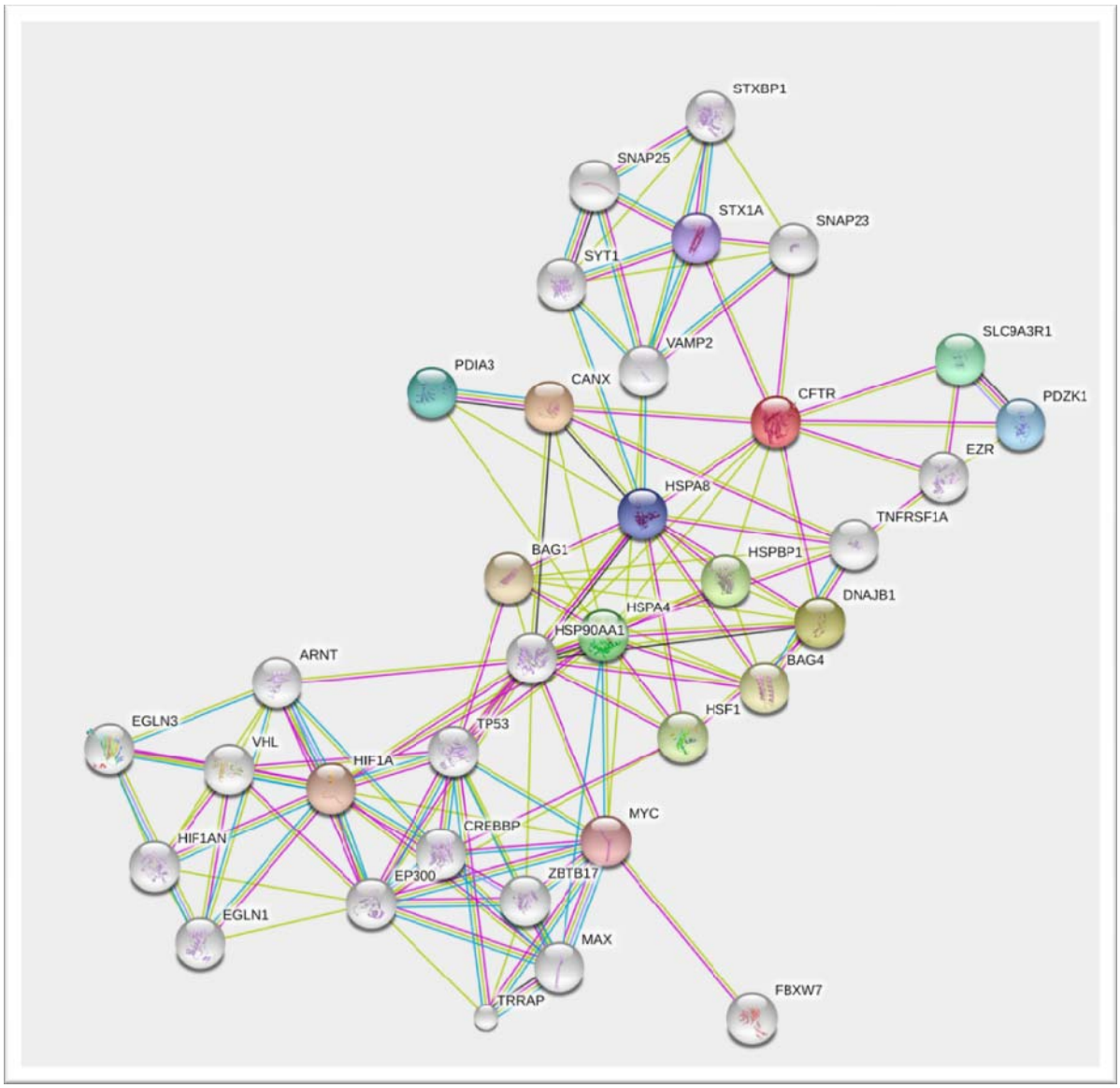

Fig. 2 PPI network of proteins involved in folding of CFTR generated in STRING

Six proteins namely CFTR, HSP70, Hsp40, Hdj-1, HspBP1 [22] and Calnexin [23], which have been reported to be involved in folding and maturation of the CFTR were used to obtain PPI network from the STRING database. A total of thirty five proteins were identified as the interacting partners of the main proteins which are involved in CFTR folding and maturation (Fig. 2).

Rab-4, Rab-11A [24], Ezrin [25] and EBP50 [26], that have been reported to be involved in CFTR membrane expression were taken as input proteins in the STRING database to find functional associations of these proteins and the network was expanded to find the PPIs. Eighty one proteins were found to be the interacting partners of the main proteins involved in CFTR membrane expression (Fig. 3).

Sec61 [30], were taken as input proteins to form the third network in the STRING database and the network was expanded and 44 proteins were recognized as the relating partners of the main proteins which are involved in CFTR degradation (Fig. 4).

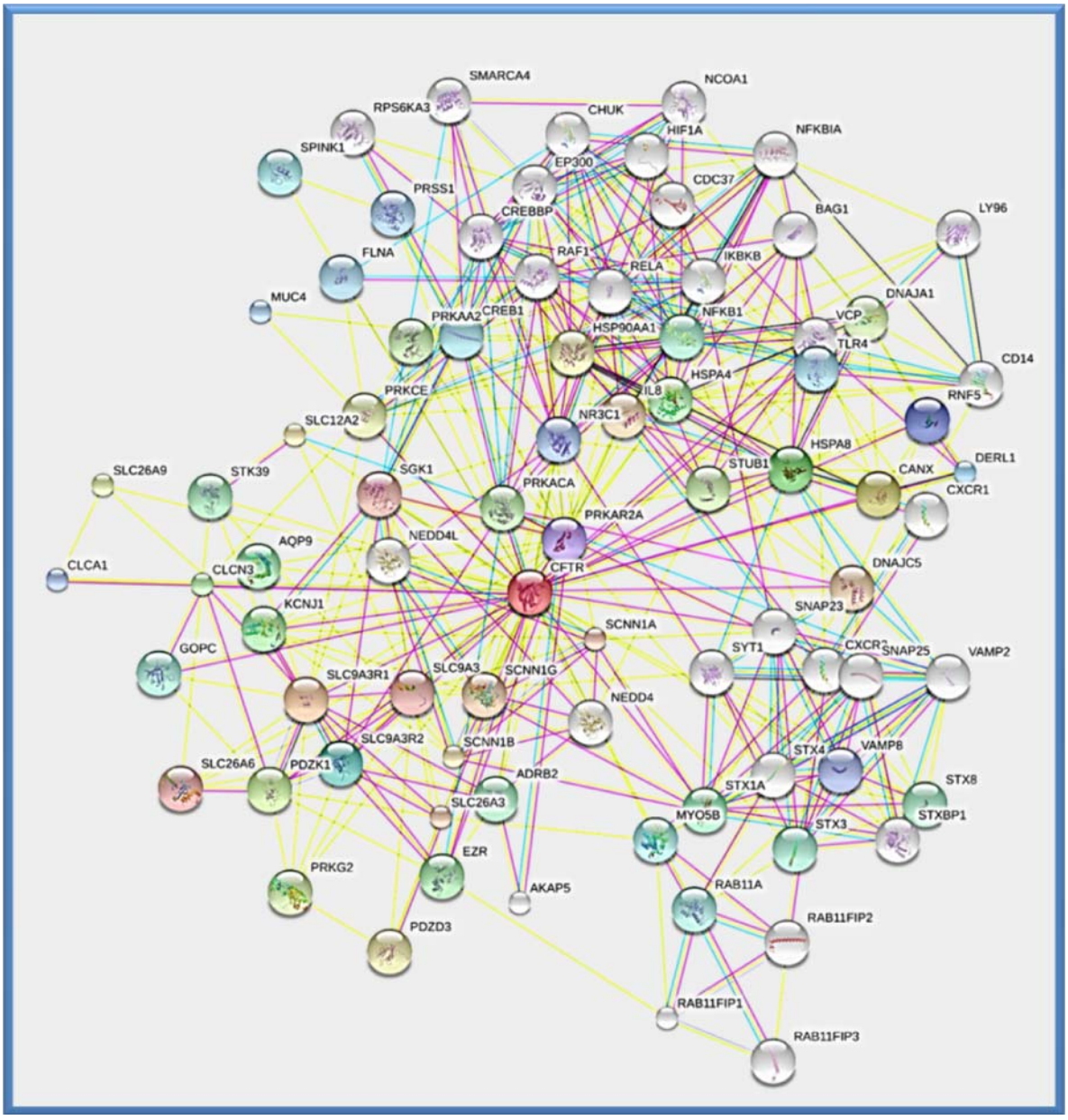

Fig. 3 PPI network of proteins involved in membrane expression of CFTR generated in STRING

Interleukin family proteins i.e., IL-1, IL-8, IL-6 and CFTR were reported by Bonfield et al. [31] to be involved in CFTR degradation. These were taken as input to build the fourth network in the STRING database and 59 proteins were identified as the interacting partners of the main proteins which are involved in CFTR inflammation (Fig. 5).

As the PPI network involving interactions of CFTR returned by STRING was too large to yield any meaningful information, therefore it was necessary to divide it into highly connected sub-graphs that represent functional modules, or protein sub-complexes. So, the network was divided into overlapping sub-graphs of highly interconnected nodes with the Cytoscape plugin ClusterONE.

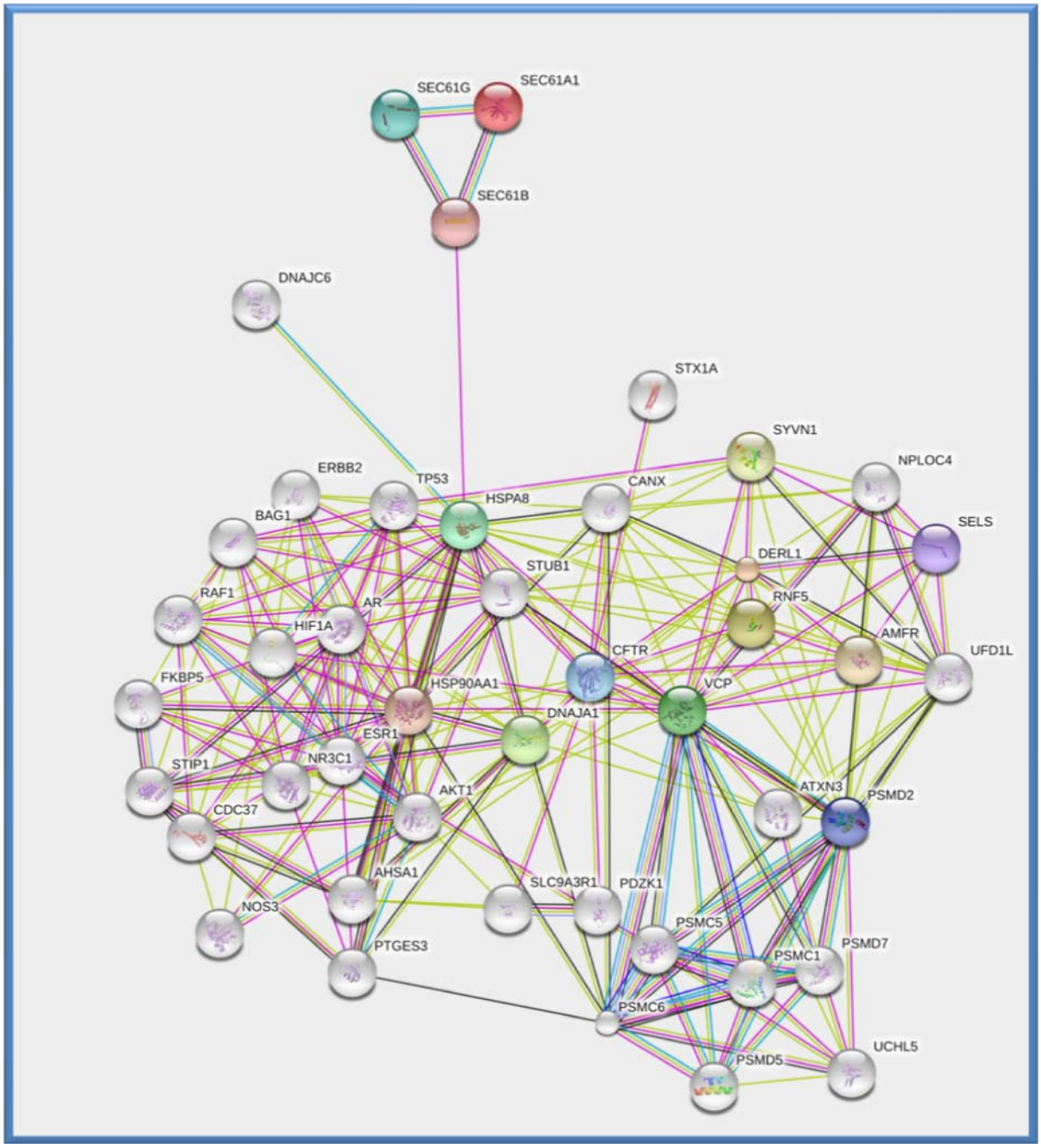

Fig. 4 PPI network of proteins involved in degradation of CFTR generated in STRING

The CFTR network was sub-divided into 6 sub-graphs (Clusters 1 to 6) (Table 1) by ClusterONE, of which five have *p*-values under 0.05 and good quality scores (Table 2). In a PPI network, sub-graphs of highly interconnected proteins can be considered as protein complexes or functional modules. Five clusters namely Cluster 1, 2, 3, 4 and 5 (Table 2) which have quality scores greater than 0.5 and density greater than 0.25 were selected for further analysis. Cluster 1 consisted of proteins mainly involved in the activation of various channels, membrane stabilization and membrane expression of CFTR. Cluster 2 contains proteins that interact with misfolded CFTR protein. These proteins are responsible for targeting the misfolded protein to degradation pathway.

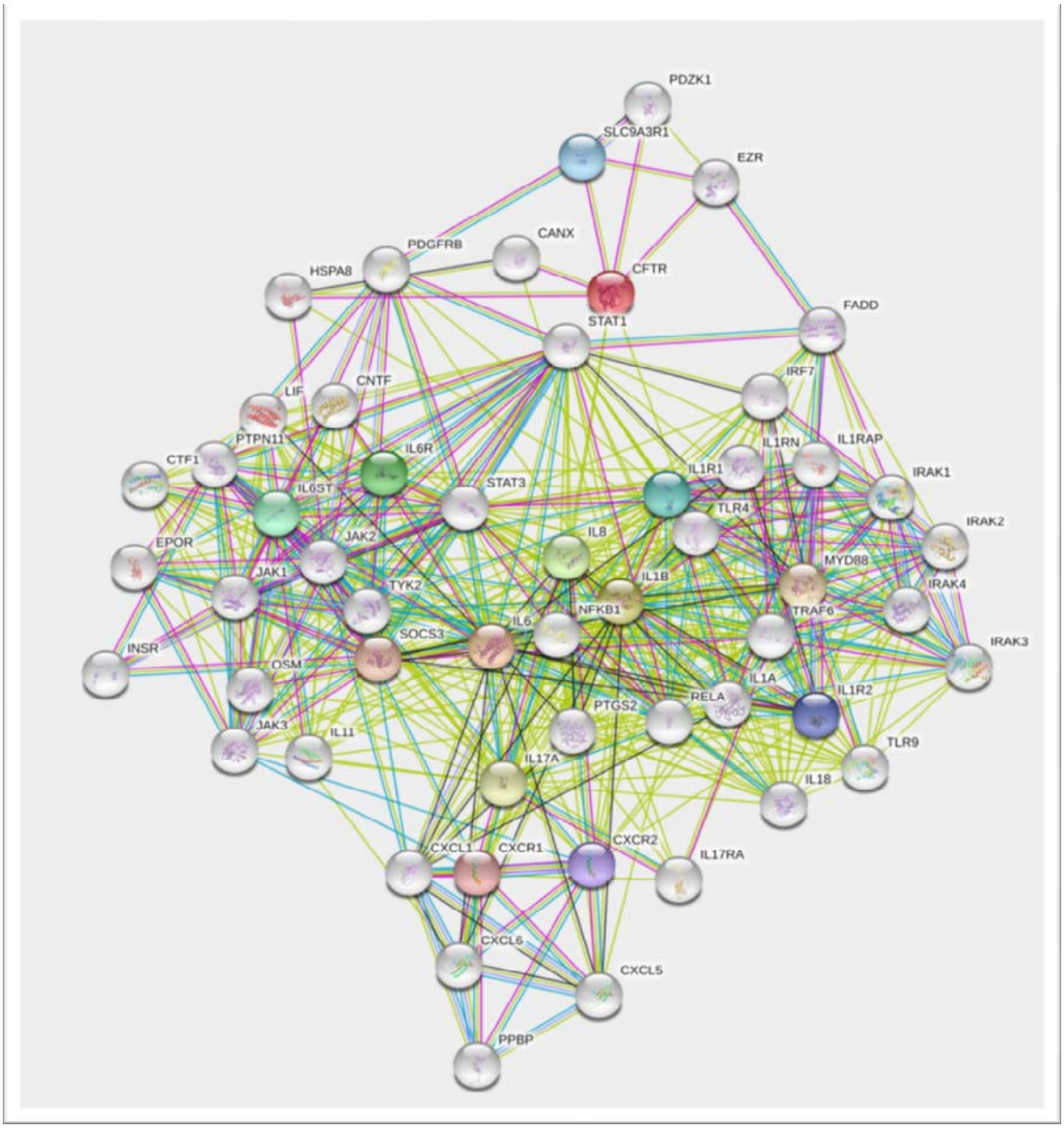

Fig. 5 PPI network of proteins involved in inflammation of CFTR generated in STRING

Cluster 3 and 4 mainly consisted of adaptor proteins which help in assembly of subunits of various complexes and proteins involved in transport through endoplasmic reticulum and degradation of misfolded proteins, respectively. So it can be hypothesized that some of the degradation proteins are in Cluster 4 along with Cluster 2 because they might have more associations with the proteins present in endoplasmic reticulum which are responsible for protein transport. This may suggest that there are various barriers to differentiate between properly folded and misfolded proteins when they are transported through ER to their destinations. Cluster 5 consisted of proteins responsible for trafficking across the Golgi. Out of all the clusters returned by ClusterONE, Cluster 2 contained proteins responsible for degradation of misfolded protein. From the proteins of Cluster 2 those proteins may be selected whose inhibition could prevent the degradation of the misfolded protein, so that misfolded protein could reach the membrane and activity of CFTR could be enhanced resulting in retention of the activity of the CFTR channel.

Table 1. Sub-graphs of CFTR network obtained from STRING analyzed using Cytoscape plugin ClusterONE

Cluster	Protein members of the sub-graphs
1	OPRK1 LPAR2 SLC9A3R2 EZR AKAP12 PODXL ADRB2 PTH1R SLC26A6 SGK1 PIK3CA PTEN SLC9A3 PDZK1IP1 NF2 MSN PDZK1 SLC34A1 SLC22A5 SLC9A3R1 PDGFRB SCARB1
2	BAG2 DNAJA1 VCP DNAJC6 RNF5 PDIA3 P4HB PSMD2 HSPA5 STUB1 HSPA8 DERL1 HSP90AA1 BAG1 HSPBP1 DNAJB1 BAG3 CD3D SELS CANX EDEM1 CFTR
3	AKAP3 PDE4A AKAP13 PRKAR2A AKAP1 MAP2 CREB1 AKAP11 AKAP5 AKAP12 ADRB2 AKAP6 AKAP9 PDE3A LIPE
4	VCP PDIA3 P4HB PSMD2 HSPA5 DERL1 CD3D SELS CANX EDEM1
5	MAP2 CREB1 SGK1
6	DNAJC5 DNAJC6 VAMP1 STXBP1 VAMP2 KCNB1 UNC13B CPLX1 DNAJA3 SYTL4 SNAP25 SYT1 SLC6A1 STX1A SNAP23 HSPA8

Table 2. Characteristics of the CFTR PPI sub-networks obtained with Cytoscape plugin ClusterONE

Cluster	Nodes	Density	Quality	*p*-value
1	22	0.84	0.703	2.047×10^{-7}
2	22	1.186	0.772	1.689×10^{-7}
3	15	0.914	0.65	1.457×10^{-4}
4	10	2.022	0.562	0.002
5	3	1.66	0.152	0.975
6	16	1.317	0.693	3.067×10^{-5}

To find the proteins which were common in all four pathways or may be points of connection amongst these pathways, the four PPI networks involved in folding and maturation, membrane expression, degradation and inflammation of CFTR were intersected. Using the Merge networks plugin of Cytoscape, the following network combinations were generated (Fig. 6):

- Intersection of membrane expression and folding with maturation network;
- Intersection of membrane expression with inflammation network;
- Intersection of membrane expression with degradation network;
- Intersection of folding with degradation network;
- Intersection of folding with inflammation network;
- Intersection of inflammation response with degradation network.

It was observed that five proteins were common in all the six intersected networks. These were PDZK1, SLC9A3R1, CFTR, CANX and HSPA8/HSC70. Out of these calnexin (CANX) and HSPA8/HSC70 were also present in Cluster 2, which contains proteins responsible for degradation of misfolded protein. One of the proteins responsible for rescuing CFTR was Calnexin. CalnexinRNAi studies by [32], suggest that abrogating the interaction of F508del-CFTR with calnexin (CAS treatment) has no major (positive or negative) effect on ERAD (Endoplasmic-reticulum-associated protein degradation) of F508del-CFTR, and therefore may not be suitable for therapeutic intervention.

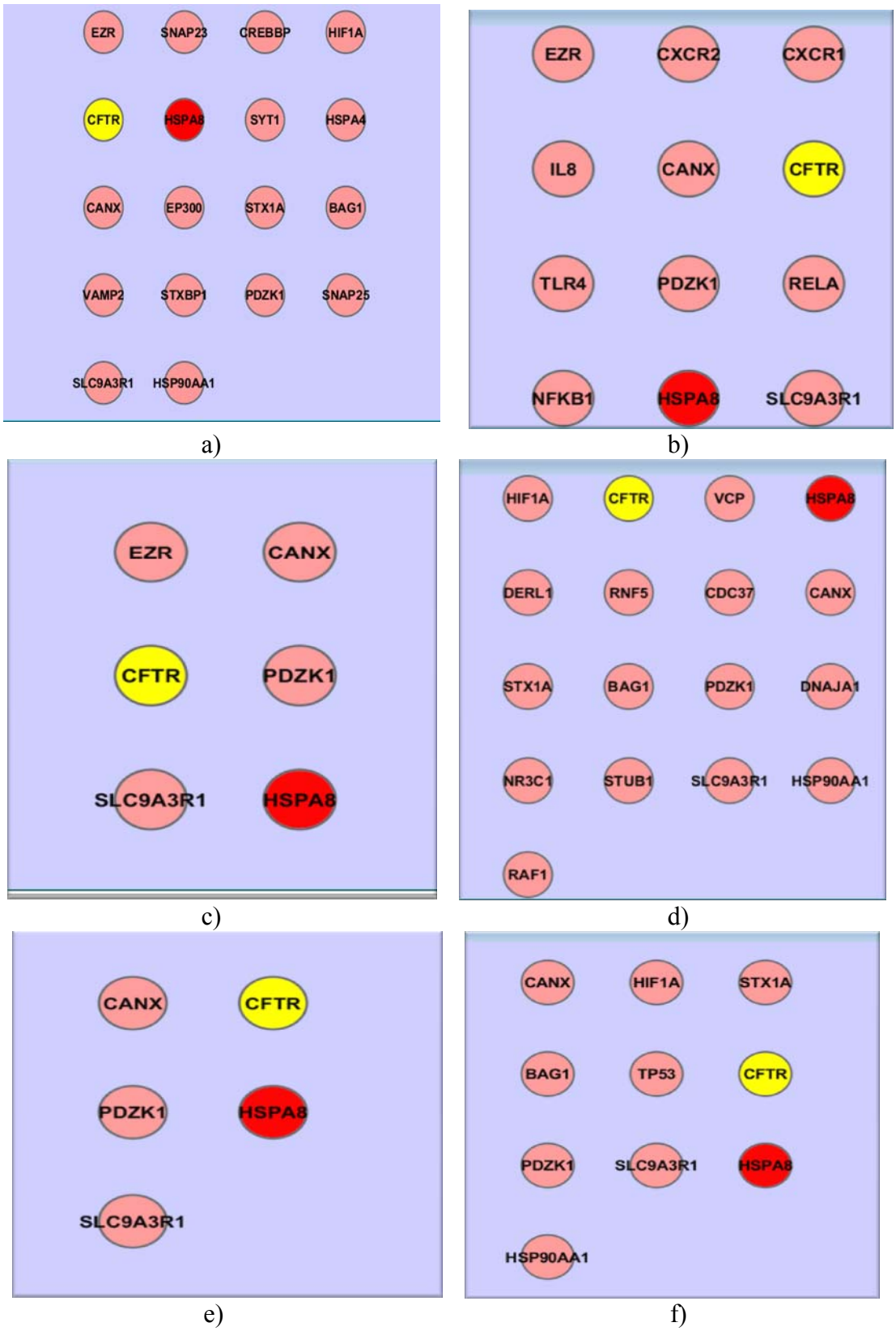

Fig. 6 Intersected Networks obtained through Merge network plugin of cytoscape: a) intersection of membrane expression and folding with maturation network; b) intersection of membrane expression with inflammation response network; c) intersection of membrane expression with degradation network; d) intersection of folding with degradation network; e) intersection of folding within flammation network; f) intersection of inlammation response with degradation network

The other protein which could be considered as possible therapeutic intervention target is HSPA8/HSC70. Proper folding of CFTR in the endoplasmic reticulum is vastly dependent on a molecular chaperone HSPA8/HSC70. HSPA8/HSC70 protein is constitutively expressed in many cell types and has numerous functions such as protein folding, protein translocation, degradation of misfolded proteins, and regulation of assembly and disassembly of protein complexes [33].

F508del-CFTR interacts with HSPA8/HSC70, CHIP that recognizes the C-terminus of HSPA8/HSC70 and has the ubiquitin ligase activity, targets the mutant for proteasomal degradation by promoting its ubiquitination. Therefore, CHIP acts as a protein that converts HSPA8/HSC70 from a protein-folding factor to a degradation factor in the ERQC (endoplasmic reticulum quality control) system [5]. Importantly, F508del-CFTR rescued on the plasma membrane has a chloride channel function, although the rescued mutant is less stable than the wild-type protein and thus small molecules that block degradation of the mutant have potential applications in cystic fibrosis therapeutics [7]. It has been suggested that inhibition of HSPA8/HSC70 activity can rescue defective cellular processing of mutant CFTR [33-34], therefore could be probable target for therapeutic intervention.

Thus using systems biology approach we have been able to identify HSPA8/HSC70 as a potential drug target for cystic fibrosis which is a critical molecular chaperon involved in cell signalling survival and protein homeostasis. Although the targeting of the core mediator of the protein homeostasis can be quite challenging due to its widespread cellular roles, but it can be further studied as the drug target based on the success of targeting other molecular chaperons such as HSP90 for cancer [35].

Conclusion

Protein network approach holds the promise of providing a theoretical framework for studying the wealth of biological data being generated on the potential drug targets and considers the global physiological environment of protein targets. Using this approach we have been able to identify a potential drug target (HSPA8/HSC70) for therapeutic intervention of cystic fibrosis.

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