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Full Length Research Paper

The effect of destrin mutations pathways on the gene expression profile

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Corneal disease is responsible for the main cause of bilateral blindness in the world, second only to cataract. As an essential actin binding protein, destrin mutation often result in cytoskeleton dynamics abnormalities in corneal epithelial surface, and thus, cause corneal epithelial cell hyperproliferation, inflammation, and angiogenesis, namely, corneal diseases. In this study, we explored the interaction and crosstalk between pathways in response to destrin mutation. As we expected, the results showed that regulation of actin cytoskeleton was the significant pathway in destrin mutation mice. Further analysis indicated that there were 28 significant pathways crosstalk to pathway of regulation of actin cytoskeleton. Importantly, three pathways, including regulation of actin cytoskeleton pathway, pathway in cancer, and B cell receptor signaling pathway were linked by inositol phosphate metabolism in crosstalk analysis of GO relationships among pathways. All of them have been demonstrated to play important roles in cytoskeleton dynamics. We deeply hope that our study could provide insights into cytoskeleton dynamics abnormalities in destrin mutation corneal disease.

Key words: ADF/cofilin, cytoskeleton dynamics, pathway crosstalk.

INTRODUCTION

Corneal disease is responsible for 6% of legal blindness in the USA and frequently the main cause of bilateral blindness in the world, second only to cataract (Ikeda et al., 2003; Li et al., 2009). Visual loss in many corneal diseases is due to changes in morphology and function of the corneal epithelial surface, including cell hyperproliferation, inflammation, and angiogenesis (Dawson and Geroski, 2009). Destrin also known as actin-depolymerisation factor (ADF), is an essential actin regulatory protein belonging to the ADF/cofilin family that binds to actin subunits in filamentous actin (F-actin), enhancing the subunit off-rate and promoting filament severing. Thus, this family of proteins is responsible for increasing the turnover of actin filament and involved in regulation of cytoskeleton dynamics (Tokuraku et al., 2001; Maciver and Hussey, 2002). Corneal disease 1 ($Dstn^{corn1}$) mice are a spontaneous mutant line that exhibit ocular surface abnormalities shortly after birth, therefore often serves as a good model to study corneal diseases. Histology of $Dstn^{corn1}$ corneas showed that the hyperplastic corneal epithelium expressed an increased level of keratin 14 and involucrin, while the level of keratin 12 was not altered (Zhang et al., 2008). The normal cornea is deficient in blood, and lymphatic vessels are used to maintain corneal transparency unless severe inflammatory or other strains cause a disruption of the antiangiogenic privilege of the cornea. It has been shown that hemangiogenesis and lymphangiogenesis in the $Dstn^{corn1}$ cornea depend on vascular endothelial growth factor receptor 3 (VEGFR3) signaling (Cursiefen et al., 2005). Recent reports have suggested that vascularization of $Dstn^{corn1}$ corneas arises from lack of soluble VEGF receptor, sflt-1, which was proposed as an essential factor for maintenance of avascularity in a normal cornea. Suppression of this endogenous sflt-1 by neutralizing antibodies, ribonucleic acid (RNA) interference or Cre-lox-mediated gene disruption abolishes corneal avascularity in mice, but recombinant sflt-1 administration restores corneal avascularity in corn1 and Pax6+/− mice (Ambati et al., 2006).

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Genome-wide screening of differentially expressed genes in the cornea of Dstn^{corn1} mice reveal that a large portion of genes associated with cytoskeletal dynamics were up-regulated expression (Verdoni et al., 2008) and almost half of these genes are targets of the serum response factor (SRF), an essential regulator of the actin cytoskeleton (Miano et al., 2007; Miano, 2008). The conditional ablation of SRF in the corneal epithelium of a diseased Dstn^{corn1} cornea results in the rescue of the epithelial cell hyper-proliferation, inflammation, and neovascularization phenotypes (Verdoni et al., 2010). These results indicate that there is an underlying interaction relationship among these differentially expressed genes. Given the complex nature of biological systems, pathways often need to function in a coordinated fashion in order to produce appropriate physiological responses to both internal and external stimuli (Li et al., 2008). Therefore, we performed the research on the protein-protein interaction (PPI) network, significant pathway, and crosstalk between pathways based on our previous work, and we hope to make an important theoretical foundation for understanding of mechanisms of destrin in corneal diseases.

MATERIALS AND METHODS

Data sources

We downloaded all the pathways from KEGG (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa, 2002) and PPI datasets from MINT (Ceol et al., 2010) database, which contain the mouse PPI datasets from IntAct (Aranda et al., 2010), BIOGRID (Stark et al., 2011) and HPRD (Keshava-Prasad et al., 2009). Then an ensemble PPI network was constructed by integrating two aforementioned existing PPI databases in mouse. A total of 65851 unique PPI pairs were collected from 10951 unique proteins Involved.

We extracted the gene expression profile data on Dstn mutations with normal wild-type from Verdoni et al. (2008), which was deposited in NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/) database (ID: GSE9743). All mouse procedures were performed in accordance with the protocols approved by the Animal Care and Use Committee at the University of Wisconsin-Madison, and conformed to the ARVO statement for the use of animals in Ophthalmic and Vision Research and APS's Guiding Principals in the Care and Use of Animals. The Dstn mutations and wild type sample that contain 6 replications in each group were compared. The limma method (Smyth, 2004) was used to identify differentially expressed genes (DEGs). Background intensities were adjusted and the original expression datasets from all conditions were processed into expression estimates using the Robust Multi-array Analysis (RMA) method with the default settings implemented in R (version 2.12.1), and then construct the linear model. The DEGs only with the fold, change value larger than 1.5 and *P* less than 0.05 were selected.

Pathway crosstalk analysis

Here, the crosstalk pathways are defined as those pathways which have the overlapping genes and edges with each other. The overlapping genes imply both of the two pathways included, and the overlapping edges imply both of the two pathways included the PPI interaction edges.

To determine the co-expressed significance of a gene pair in disease cases, we used the Pearson's correlation coefficient (PCC) test to calculate the *P*. Map those p-values to the nodes and edges in the PPI network collected from MINT. The following formula is used to define a function as the combination of statistical significance of an interaction by a scoring scheme. The detail description could be seen in Liu et al. (2010).

$$S(e) = f(diff(x), cor(x, y), diff(y))$$

$$= -2\sum_{i=1}^{k} \log_e(p_i)$$

The diff(x) and diff(y) are differential expression assessments of gene x and gene y, respectively. cor(x, y) represents their correlation between gene x and gene y, *f* is a general data integration method that can handle multiple data sources differing in statistical power. Where k = 3, p1 and p2 are the *P* of differential expression of two nodes, p3 is the *P* of their co-expression.

Significant pathways analysis

$$Sp = \sum_{e \in P} S(e)$$

The frequency of scores that are larger than Sp is used as the significance *p*-value of pathway P to describe its importance. We also use the DAVID (Huang and Lempicki, 2008) for the pathway enrichment analysis with the $P < 0.05$ input the DEGs dataset.

Pathway crosstalk analysis

The detailed analysis of crosstalk of relationships among pathways is then investigated, especially that with overlap of two significant pathway analysis results. To define the interaction significance between pathways, we summarize all the scores of edges [S(e)] of all non-empty overlaps. Specifically, the interaction score between two pathways is estimated by their overlapping status of weighted pathways in the following formula:

$$C(pi, pj) = \sum_{e \in \mathrm{Oij}} S(e)$$

Where Pi and Pj are two pathways, and Oij is their overlapping.
To estimate the significance of the overlapping between the different pathways, we random sample 10^5 times of the same size two pathways in the edges of pathway network and calculate their overlapping scores. The frequency larger than C, is regarded as the interaction significance *P*. At last, the crosstalk with $P < 0.001$ was considered as the significant pathway crosstalk.

Significant GO enrichment analysis in each pathway

The functional enrichment among proteins in one pathway is defined as:

$$P = 1 - \sum_{i=0}^{k-1} \frac{\binom{f}{i}\binom{n-f}{m-i}}{\binom{n}{m}}$$

Table 1. Significant pathway analysis.

ID	Node	Edge	Size	Score	*P*	Description
mmu04623	3	49	64	869.37	0	Cytosolic DNA-sensing pathway
mmu05020	7	55	35	795.18	0	Prion diseases
mmu00052	10	115	29	1706.70	0	Galactose metabolism
mmu03450	11	150	13	1692.31	0	Non-homologous end-joining
mmu04114	11	31	114	739.21	0	Oocyte meiosis
mmu00100	12	60	18	1101.54	0	Steroid biosynthesis
mmu00500	15	131	48	1630.65	0	Starch and sucrose metabolism
mmu00630	19	187	19	2076.68	0	Glyoxylate and dicarboxylate metabolism
mmu04621	19	74	58	1190.49	0	NOD-like receptor signaling pathway
mmu04330	27	208	51	2758.92	0	Notch signaling pathway
mmu00970	34	473	65	6169.44	0	Aminoacyl-tRNA biosynthesis
mmu05220	34	273	74	3175.71	0	Chronic myeloid leukemia
mmu04622	47	1196	69	12312.19	0	RIG-I-like receptor signaling pathway
mmu03008	48	825	91	9438.96	0	Ribosome biogenesis in eukaryotes
mmu04662	55	831	77	8634.24	0	B cell receptor signaling pathway
mmu04810	65	318	218	3823.94	0	Regulation of actin cytoskeleton
mmu04722	79	1170	132	13045.08	0	Neurotrophin signaling pathway
mmu04110	97	883	128	11079.05	0	Cell cycle
mmu04010	128	521	269	5473.65	0	MAPK signaling pathway
mmu04510	151	1901	200	19541.14	0	Focal adhesion
mmu01100	360	1207	1202	12580.05	0	Metabolic pathways
mmu00900	8	73	15	899.86	1.00E-05	Terpenoid backbone biosynthesis
mmu04145	77	712	179	7202.11	1.00E-05	Phagosome
mmu04380	79	718	118	7221.60	6.00E-05	Osteoclast differentiation
mmu03430	21	336	22	3504.59	9.00E-05	Mismatch repair
mmu05200	107	422	326	4274.35	0.00049	Pathways in cancer
mmu04612	13	72	81	828.29	5.00E-04	Antigen processing and presentation
mmu05133	5	9	74	138.45	0.00057	Pertussis
mmu04070	2	11	78	160.22	0.00085	Phosphatidylinositol signaling system
mmu04666	7	23	92	297.94	0.00098	Fc gamma R-mediated phagocytosis
mmu05152	12	53	179	619.05	0.00113	Tuberculosis
mmu00562	34	181	57	1899.71	0.00136	Inositol phosphate metabolism
mmu04672	14	32	45	392.47	0.00154	Intestinal immune network for IgA production
mmu04130	26	154	35	1610.03	0.00359	SNARE interactions in vesicular transport
mmu04115	15	33	70	379.31	0.00851	p53 signaling pathway
mmu04916	7	13	101	164.38	0.00877	Melanogenesis

Where n is the number of nodes in the network, *f* is the number of proteins annotated with a particular GO function, m is the number of proteins involved in the pathway, and k is the frequency of the GO term. We identified the GO function enrichment of the pathways respectively.

RESULTS

Significant pathway analysis

In the present study, the GSE9743 was first downloaded to form GEO (http://www.ncbi.nlm.nih.gov/geo/), and then the R language was used to calculate the DEGs. Base on the expression profile, we utilized the PPI dataset and KEGG pathway to further find the significant pathways and crosstalk among them.

We used the Sp to evaluate the importance of pathways (for detail, see the methods). Total 36 pathways (Table 1) were detected with the $P < 0.01$. The limma packages were used to detect 120 DEGs (for detail, see the methods). Using the DAVID with the DEGs, several pathways were identified. However, we only found 4 significant pathways, that is, regulation of actin cytoskeleton (mmu04810) with the $P = 0.01$, leukocyte transendothelial migration (mmu04670) with the $P = 0.001$, tight junction (mmu04530) with the $P = 0.017$, and

Table 2. Crosstalk between mmu04810 and corneal related pathways.

pathID_A	pathID_B	pathID_B_name	*P*
mmu04810	mmu00052	Galactose metabolism	0
mmu04810	mmu00100	Steroid biosynthesis	0
mmu04810	mmu00500	Starch and sucrose metabolism	6.00E-05
mmu04810	mmu00970	Aminoacyl-tRNA biosynthesis	0
mmu04810	mmu01100	Metabolic pathways	0
mmu04810	mmu03008	Ribosome biogenesis in eukaryotes	0
mmu04810	mmu03450	Non-homologous end-joining	0.0018
mmu04810	mmu04070	Phosphatidylinositol signaling system	0
mmu04810	mmu04110	Cell cycle	0
mmu04810	mmu04114	Oocyte meiosis	0
mmu04810	mmu04115	p53 signaling pathway	0
mmu04810	mmu04145	Phagosome	0
mmu04810	mmu04330	Notch signaling pathway	0
mmu04810	mmu04380	Osteoclast differentiation	0.00026
mmu04810	mmu04510	Focal adhesion	0.00032
mmu04810	mmu04612	Antigen processing and presentation	1.00E-05
mmu04810	mmu04621	NOD-like receptor signaling pathway	0
mmu04810	mmu04622	RIG-I-like receptor signaling pathway	0
mmu04810	mmu04623	Cytosolic DNA-sensing pathway	0
mmu04810	mmu04666	Fc gamma R-mediated phagocytosis	0.00258
mmu04810	mmu04672	Intestinal immune network for IgA production	0.00426
mmu04810	mmu04722	Neurotrophin signaling pathway	2.00E-05
mmu04810	mmu04742	Taste transduction	0.00654
mmu04810	mmu04916	Melanogenesis	0
mmu04810	mmu05020	Prion diseases	2.00E-05
mmu04810	mmu05140	Leishmaniasis	0
mmu04810	mmu05152	Tuberculosis	0.00738
mmu04810	mmu05220	Chronic myeloid leukemia	0

Only high correlated pathways to the mmu04810 were listed in Table 2. The first two columns are the correlated pathways. The third column is the pathway name of the second column. The last column is the P of two correlated pathways.

Arrhythmogenic right ventricular cardiomyopathy (ARVC) (mmu05412) with the $P = 0.04$. Remarkably, only one overlap significant pathway that is, regulation of actin cytoskeleton (mmu04810 in Table 1), was detected in the Dstn mutations.

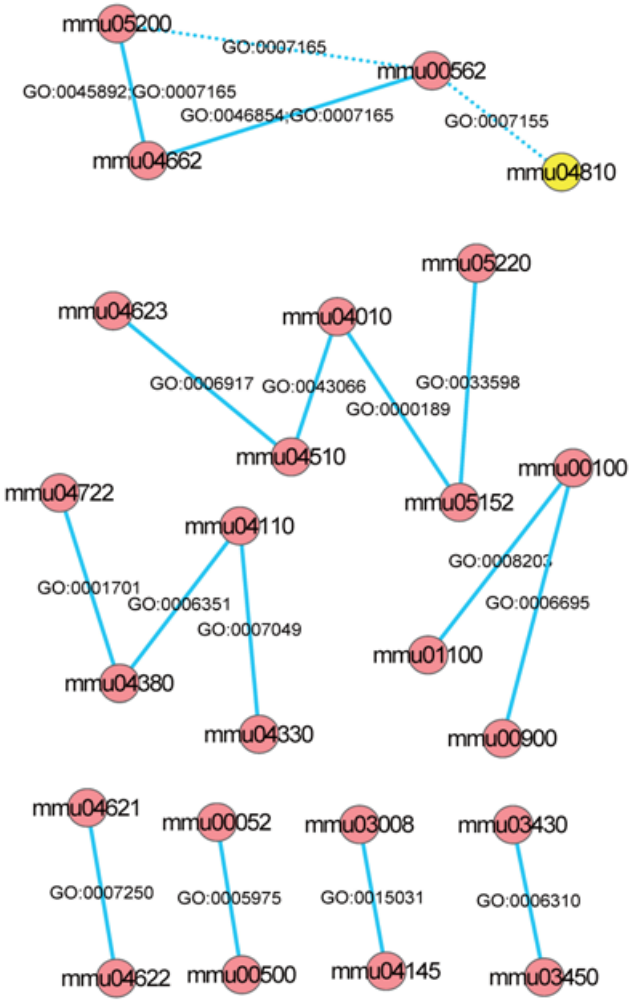
Crosstalk among the pathways

Further, we exploited the pathway crosstalk between regulation of actin cytoskeleton (mmu04810) and other significant pathways by the overlapping score. We found that these 28 significant pathways crosstalk to the mmu04810 pathway (Table 2).

Crosstalk of GO relationships among pathways

For detail analysis, the crosstalk between the significant pathways using the hypergeometric test to find the significant GO terms in each pathway with the $P < 0.05$, respectively. The results of the top five GO terms in part of the pathways are used to construct the connection among pathways. In Figure 1, regulation of actin cytoskeleton (mmu04810) is connected with the Inositol phosphate metabolism (mmu00562) through the cell adhesion (GO: 007155), through the crosstalk's $P < 0.01$.

Inositol phosphate metabolism (mmu00562), Pathways in cancer (mmu05200), and B cell receptor signaling pathway (mmu04662) were connected between each other through signal transduction (GO: 007165). We also found that significant pathways Chronic myeloid leukemia (mmu05220), Tuberculosis (mmu05152), MAPK signaling pathway (mmu04010), Focal adhesion (mmu04510), and Cytosolic DNA-sensing pathway (mu04623) are connected by the mammary gland epithelial cell proliferation (GO:0033598), MAPK import into nucleus (GO: 0000189), negative regulation of apoptosis



ID	name
mmu00052	Galactose metabolism
mmu00100	Steroid biosynthesis
mmu00500	Starch and sucrose metabolism
mmu00562	Inositol phosphate metabolism
mmu00900	Terpenoid backbone biosynthesis
mmu01100	Metabolic pathways
mmu03008	Ribosome biogenesis in eukaryotes
mmu03430	Mismatch repair
mmu03450	Non-homologous end-joining
mmu04010	MAPK signaling pathway
mmu04110	Cell cycle
mmu04115	p53 signaling pathway
mmu04330	Notch signaling pathway
mmu04380	Osteoclast differentiation
mmu04510	Focal adhesion
mmu04621	NOD-like receptor signaling pathway
mmu04622	RIG-I-like receptor signaling pathway
mmu04623	Cytosolic DNA-sensing pathway
mmu04662	B cell receptor signaling pathway
mmu04722	Neurotrophin signaling pathway
mmu04810	Regulation of actin cytoskeleton
mmu05152	Tuberculosis
mmu05200	Pathways in cancer
mmu05220	Chronic myeloid leukemia

GOID	Description
GO:0006310	DNA recombination
GO:0007155	cell adhesion
GO:0007250	activation of NF-kappaB-inducing kinase activity
GO:0006351	transcription, DNA-dependent
GO:0007049	cell cycle
GO:0006695	cholesterol biosynthetic process
GO:0008203	cholesterol metabolic process
GO:0000189	MAPK import into nucleus
GO:0007165	signal transduction
GO:0006917	induction of apoptosis
GO:0033598	mammary gland epithelial cell proliferation
GO:0046854	phosphatidylinositol phosphorylation
GO:0015031	protein transport
GO:0005975	carbohydrate metabolic process
GO:0043066	negative regulation of apoptosis
GO:0001701	in utero embryonic development
GO:0045892	negative regulation of transcription, DNA-dependent

Figure 1. Crosstalk with the overlap of top5 GO terms of pathways. Significantly enriched GO biological processes are identified in every pathway respectively. The edge of each pair of pathways represents the connection with the same GO terms. The solid lines mean the crosstalk's $P < 0.01$ and the dotted lines mean the $P > 0.01$.

(GO:0043066), and induction of apoptosis (GO:006917). From the significant GO enrichments, we predicted the crosstalk of GO biological processes during the disease development among the pathways.

DISCUSSION

Dstn^{corn1} mice exhibited an actin dynamics defect in the corneal epithelial cells, offering an *in vivo* model to investigate cellular mechanisms affected by the Dstn mutation and resultant actin dynamics abnormalities. Microarray analysis using the cornea from Dstn^{corn1} and wild-type mice demonstrated that Dstn mutations have a strong impact on the gene expression profile, especially on actin cytoskeleton regulator. Identically, as we expected, regulation of actin cytoskeleton also has been demonstrated as a significant pathway in our analysis. Further, 28 significant pathways crosstalk to the mmu04810 pathway (namely, regulation of actin cytoskeleton) were identified by the overlapping score (Table 2). Importantly, crosstalk analysis of GO relationships among pathways indicated that mmu04810 pathway (regulation of actin cytoskeleton) was indirectly

connected to mmu05200 pathway (pathways in cancer) and mmu04662 pathway (B cell receptor signaling pathway) in mmu00562 pathway-mediated manner (inositol phosphate metabolism). The results indicated that, there was an interaction relationship among these four pathways, which seemed to be in accordance with results of previous reports.

Recent studies have demonstrated that the organization and dynamics of the actin cytoskeleton could be regulated by phosphoinositides pathway at several levels, such as phosphatidylinositol-3,4,5-trisphosphate (PIP3), phosphatidylinositol-4,5-bisphosphate (PIP2), and the enzymes producing or hydrolysing these lipids. Therefore, crosstalk between regulation of actin cytoskeleton and phosphoinositides pathway is obviously present. Among different PIs, PI(4,5)P2 is the best-characterized regulator of the actin cytoskeleton. PI(4,5)P2 interacts directly with several actin-binding proteins, such as ADF/cofilin to regulate their activities (Zhao et al., 2010). Typically, PI(4,5)P2 inhibits those actin-binding proteins that promote actin filament disassembly, and activates proteins that induce actin filament assembly (Van Rheenen et al., 2007). As a consequence, PI(4,5)P2 is considered to promote the formation of actin filament structures beneath the plasma membrane and other phosphoinositide-rich membrane organelles. This is supported by a number of studies demonstrating that an increase in the plasma membrane PI(4,5)P2 levels induces actin filament assembly in mammalian cells, while sequestration of PI(4,5)P2 leads to a defect in the cortical actin cytoskeleton (Saarikangas et al., 2010).

Study has demonstrated that there is a regulatory relationship between the regulation of actin cytoskeleton and B cell receptor (BCR) signaling in Ag processing and presentation process. Bruton's tyrosine kinase (Btk) is one linker connecting BCR signaling to actin dynamics. Using xid mice and a Btk inhibitor, BCR engagement increases actin polymerization and Wiskott-Aldrich syndrome protein (another actin binding protein as ADF/cofilin) activation in a Btk-dependent manner. Concurrently, Btk-dependent increases in the levels of phosphatidylinositide-4,5-bisphos-phate and phosphory-lated vav are also observed upon BCR engagement. Thus, the BCR-triggered signaling regulates the dynamics of the actin cytoskeleton through WASP in a Btk-dependent manner (Sharma et al., 2009).

Cell hyperproliferation, inflammation and angiogenesis are biological processes central to the pathogenesis of corneal disease, as well as other conditions including tumorigenesis and chronic inflammatory disorders. Therefore, pathways in cancer may be involved in the regulation of actin cytoskeleton. In the past decade, many signaling pathways are identified associated with cancer development, such as Ras/MAPK, MAPK/ERK, TGF-β, PI3K, etc (Dreesen and Brivanlou, 2007). These pathways all have been proposed to influence the regulation of actin cytoskeleton. For example, Ras/MAPK pathway is suggested as the critical pathway involved in cytoskeleton disruption during Ras-transformation. Oncogenic Ras can specifically target the actin-based cytoskeleton, and achieve morphological transformation of the cells by down-regulation of structural components of the cytoskeleton and inhibition of ROCKI/Rho-kinase–dependent pathways (Amaj et al., 2004; Pawlak and Helfman, 2002). TGF-β via Smad and p38Mapk up-regulates expression of actin binding proteins, such as ADF/cofilin to regulate the actin cytoskeleton and cell motility in epithelial cells (Bakin et al., 2004; Moustakas and Heldin, 2008; Vardouli et al., 2005). Study demonstrated that JNK and PI3K signaling cascades would be initiated in the early stages of angiogenesis through the reorganization of the actin cytoskeleton to increase production and activation of matrix metalloproteinase-2 (MMP-2). However, JNK mainly regulates the mRNA expression of MMP-2 and MT1-MMP, whereas PI3K regulates protein levels (Ispanovic and Haas, 2006).

Conclusion

In our present work, a network-based approach was used to analyze the crosstalk among corneal related pathways. The crosstalks of pathways are found and analyzed using the PPI datasets and expression profiles. The results are consistent with our prior knowledge of actin dynamics. The crosstalk of pathways presents new alternative insights for corneal diseases. Our work shows that comprehensive and system-wide analysis provides evidence for corneal, and complements the traditional component-based approaches.

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