World Academy of Science, Engineering and Technology
International Journal of Biomedical and Biological Engineering
Vol:3, No:12, 2009

In Silico Analysis of Pax6 Interacting Proteins Indicates Missing Molecular Links in Development of Brain and Associated Disease

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Abstract**—**The PAX6, a transcription factor, is essential for the morphogenesis of the eyes, brain, pituitary and pancreatic islets. In rodents, the loss of Pax6 function leads to central nervous system defects, anophthalmia, and nasal hypoplasia. The haplo-insufficiency of Pax6 causes microphthalmia, aggression and other behavioral abnormalities. It is also required in brain patterning and neuronal plasticity. In human, heterozygous mutation of Pax6 causes loss of iris [aniridia], mental retardation and glucose intolerance. The 3' deletion in Pax6 leads to autism and aniridia. The phenotypes are variable in peneterance and expressivity. However, mechanism of function and interaction of PAX6 with other proteins during development and associated disease are not clear. It is intended to explore interactors of PAX6 to elucidated biology of PAX6 function in the tissues where it is expressed and also in the central regulatory pathway. This report describes *In-silico* approaches to explore interacting proteins of PAX6. The models show several possible proteins interacting with PAX6 like MITF, SIX3, SOX2, SOX3, IPO13, TRIM, and OGT. Since the Pax6 is a critical transcriptional regulator and master control gene of eye and brain development it might be interacting with other protein involved in morphogenesis [TGIF, TGF, Ras etc]. It is also presumed that matricelluar proteins [SPARC, thrombospondin-1 and osteonectin etc] are likely to interact during transport and processing of PAX6 and are somewhere its cascade. The proteins involved in cell survival and cell proliferation can also not be ignored.

Keywords**—**Interacting Proteins, Pax6, PIP, STRING

I. INTRODUCTION

THE biological functions are governed by a set of proteins interacting with each other. They form complexes and work as a system in the cell. The knowledge about interaction network of a protein can elucidate its functions but predicting interaction of proteins with specificity is largely an unsolved problem. The probable interacting partners can be explored through computational analysis in parallel to experimental work. The validity of information is cross checked experimentally. The PAX6 is a transcriptional regulator and highly conserved and critical protein for development and maintaining functional status of eyes, brain and endocrine pancreas [1-12]. The PAX6 contains two DNA binding domains. The paired domain [PD] and a paired like homeodomain [HD] are linked by a glycine rich region.

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The transactivation domain [TD] of PAX6 is proline, serine and threonine [PST] rich at the C-terminus. It is involved in specification, regionalization and arealization of cerebral cortex [13] as well as cooperates with other proteins to develop caudal forebrain primordial and patterning of telencephalon [14-16]. Several protein co-expresses with PAX6 during development in brain, eyes and pancreas. The Emx2 and Pax6 are co-expressed and function in cooperation with Otx2 and Otx1 in the development of brain ontogenetically [17]. It also co-expresses with MSX2, SIX3 and PROX1 [18]. The proteins like Chx10, Six3, Lhx2, En-1, Prep1, and HoxB1 are also known to be co-expressed with Pax6 [29]. It is reported that PAX6 interacts with Karyopherin 13 [Kap13] through homeodomain during transport from cytoplasm into nucleus. It is also observed that Kap13 does not interact with PAX6 mutant lacking regions from 208 to 214 and 261 to 267 [19]. In the endocrine pancreas, alpha-cell-specific expression of the glucagon gene is mediated by a complex formed by three proteins namely PAX6, CDX2 and p300. It is reported that PAX6 and CDX2 are in contact with each other through the glucagon promoter region and both interact with N-terminal C/H1 domain of p300 also [20]. The PAX6 also interacts with Maf for synergistic activation of glucagon promoter [21] and development of lens fibrous cells. A protein complex involving PAX6, c-MAF and CREB along with TBP, optimizes the regulation of crystalline gene. The PAX6 is also reported to interact with TBP, c-MAF and CREB [22]. The Pax6 and retinoblastoma proteins participate in regulatory pathways controlling epithelial cell division, fiber cell elongation, and crystalline gene expression during lens development [23]. The Pax6/cVax interaction inhibits the transactivation properties of PAX6 [24]. The PAX6 and SOX2 activate delta crystalline gene and elicit lens placode development, indicating that the complex of PAX6 and SOX2 formed on specific DNA sequences is the genetic switch for initiation of lens differentiation [25]. The interaction of PAX6 with HOMER3 and DNCL1 is proposed to facilitate synaptic activation that could lead to changes in neuronal transcriptional activity. The HOMER3, TRIM11 and DNCL1 interacted with the C-terminal peptides of Pax6 [26]. The PAX6 also regulates the activity of the transcriptional promoter for matrix metalloprotein, gelatinaseB [gelB; MMP9] by binding with its promoter region through PD. It interacts with AP-2 alpha through C-terminal domains [27]. The Pax6 and microphthalmia transcription factor [Mitf] both are required for proper eye development. The PAX6 interacts with the 'Mitf' through their respective DNA-binding

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domains. Since Pax6 and 'Mitf' are known to form a protein-protein complex, they are no longer able to bind to DNA and to transactivate their target promoters [28]. Thus, the interaction leads to suppression of transactivation properties of both these molecules. The PAX6 also interacts with PAX6ΔPD [pairedless] isoform and paired-like homeodomain protein Rax and super activate PAX6-mediated transactivation from paired domain [29]. Although the role of Pax6 in brain, eye, and pancreatic islets development is known, the mechanism of its function is not clear. Thus, Pax6 regulates formation of cerebral cortex, axon guidance, differentiation of neurons from glia and neuronal migration in the cerebellum. Once in the nucleus, Smad3 interacts with the RED sub-domain of the paired domain in Pax6 and releases Pax6 from its DNA binding site. Thus, the Smad /TGFβ signaling pathway turns off Pax6 expression by preventing it from auto regulating its own promoter. However, the information related to its interacting protein network is not clear. It is also not clear how does a protein which co-express with Pax6 interact and regulate during brain development, differentiation and disease. This report presents In-Silico analysis and models representing putative interacting partners of Pax6. The Proteins, which shows high tendency of interactions, are selected for analysis. It is presumed that the interaction of Pax6 with SPARC may facilitate shuttling of Pax6 for Smad3 dependent auto-regulation. It is also expected that Pax6 influences p53-mediated neuronal morphogenesis. This report provides insight to associated proteins in the cascade or hierarchy of Pax6 transcription factor.

II. MATERIALS AND METHOD

Studies on models of interacting proteins with Pax6

Models generated by servers like PIP [PIP: Potential Interactions of Proteins [30] and STRING [32] were studied. The information was carefully analysed which indicates possibilties of missing links about mechanism of Pax6 function. The proposed interacting partners are under investigation for validation. Maximum homology and occurence view was observed through STRING. This homology and occurence view was observed among putative interacting protein in human and mouse.

Multiple sequence analysis [31] was performed between the putative interacting proteins of Pax6 in mouse and human. It produces biologically meaningfull multiple sequence alignments of divergent sequences. The evolutionary relationships was analysed by cladogram and Phylogram with the help of PHYLIP.

III. RESULTS

About 45 interacting proteins with Pax6 were observed by the model of PIP with PAX6 [Fig. 1] and analysed on the basis of score value [Table I]. It represents novel interactors with Pax6 except two (retinoblastoma 1 and TATA box binding proteins).

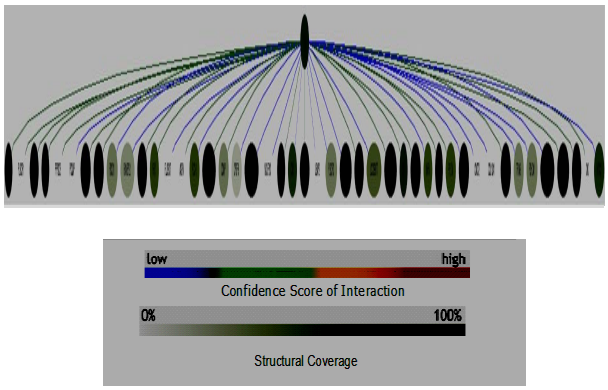

Fig. 1 The Model shows 45 putative interactors of PAX6

TABLE I

REPRESENTS PUTATIVE PARTNERS OF THE PAX6 HAVING HIGH SCORE THROUGH PIP SERVER. HIGHEST SCORE PROTEIN HAS MORE CHANCES OF INTERACTING WITH PAX6.

Protein information	Protein ID	Score
O-linked GlcNAc transferase isoform 1	NP_858058	13.38
retinoblastoma 1	NP_000312	12.86
ww domain containing E3 ubiquitin protein ligase2 isoform 1	NP_008945	12.60
nuclear transcription factor, X-box binding 1 isoform	NP_002495	12.54
cartilage oligomeric matrix protein precursor	NP_000086	12.44
protein phosphatase 2 [formerly 2A], catalytic subunit	NP_001009552	12.28
SPRY-domain-containing SOCS box protein SSB-1	NP_079382	12.02
vesicle docking protein p115	NP_003706	11.88
LIM domain kinase 1 isoform 1	NP_002305	11.77
TATA box binding protein	NP_003185	11.53
ribosomal protein L7a	NP_000963	11.52
quaking homolog, KH domain RNA binding isoform HQK-5	NP_006766	11.43
PAP associated domain containing 4	NP_776158	11.37
connective tissue growth factor	NP_001892	10.72
photoreceptor-specific nuclear receptor isoform b	NP_055064	10.59

Model by STRING indicate several puative interactors of PAX6 from human [Fig. 2]and mouse [Sey][Fig. 3].

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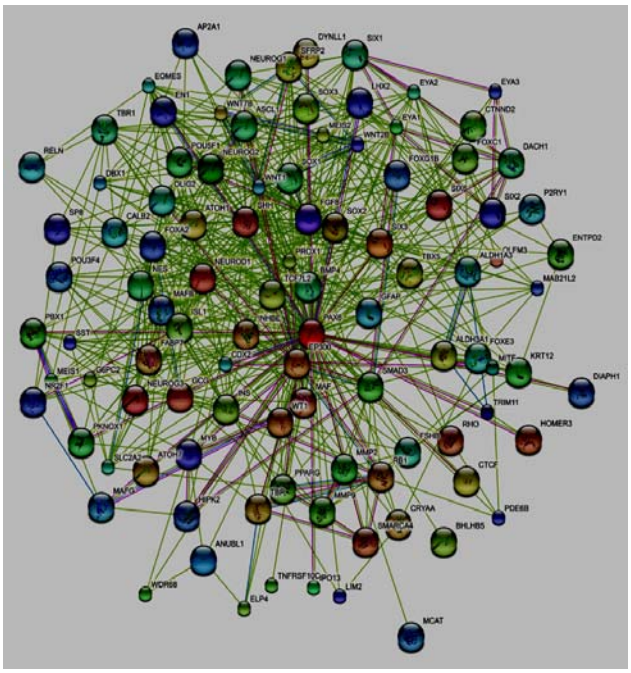

Fig. 2 The model shows 100 putative proteins which interact with PAX6 in human

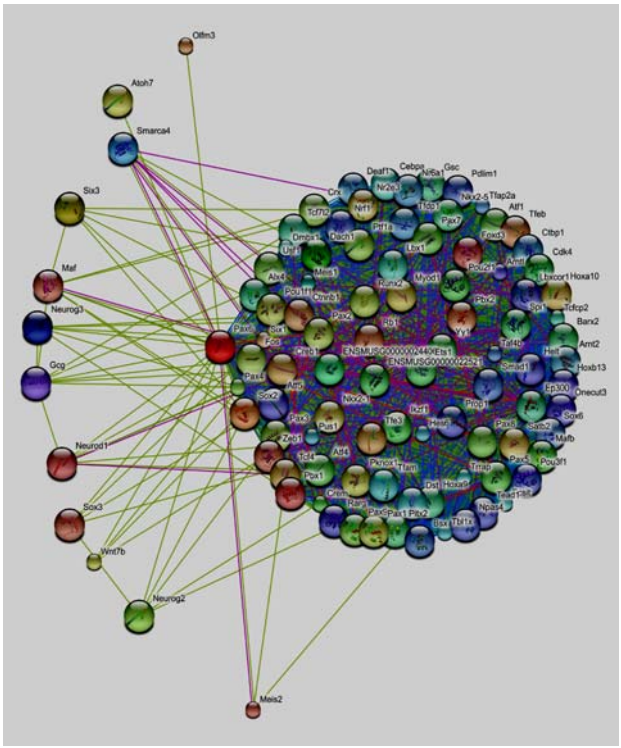

Fig. 3 The model shows 100 putative proteins interacting with Pax6 [Sey] in mouse

It was interesting to observe some novel interacting proteins of PAX6 from human and mice [Table II] which are important for central regulatory pathway associated with PAX6.

TABLE II

LISTS IMPORTANT PROTEINS WHICH INTERACT WITH PAX6 IN HUMAN AND MOUSE

Protein name	Description	Amino acids
SOX2	Transcription factor SOX-2	317
IPO13	Importin-13 or karyopherin 13	963
MITF	Micropthelmia associated transcription factor	526
SIX3	Homeobox protein SIX3	332
SOX3	Transcription factor SOX3	446
CDX2	Homeobox protein CDX2	313
TRIM11	Tripartite motif protein 11	467
CHX10	Homeobox protein CHX10	361
SMARCA4	SWI/SNF related matrix associated actin dependent regulator of chromatin subfamily A member 4	1679
HOMER3	HOMER protein homolog 3	361
RX	Retina and anterior neural fold homeobox protein	346
TBP	TATA binding protein	339
EP300	E1A-associated protein p300	2414
EN1	Homeobox protein engrailed-1	392
CTCF	Transcriptional repressor CTCF	727
EMX2	Homeobox protein EMX2	252
NEUROG3	Neurogenin 3	214
NEUROG2	Neurogenin 2	272
IPF1	Insulin promoter factor 1	283
SHH	Sonic hedgehog protein precursor	462
WT1	Wilms' tumor protein [WT33]	449
EMX1	Homeobox protein EMX1	257
NEUROD1	Neurogenic differentiation factor 1	356
OLFM3	Noelin-3 precursor [Olfactomedin-3] [Optimedin]	478
POU4F2	POU domain, class 4, transcription factor 2	409
FGF8	Fibroblast growth factor 8 precursor	244
GCG	Glucagon precursor	
WNT7B	Wnt-7b protein precursor	325
AADAC	Arylacetamide deacetylase	399
SFRP2	Secreted frizzled-related protein 2 precursor	
RHO	Rhodopsin [Opsin 2]	348
ANUBL1	AN1, ubiquitin-like, homolog	727
FABP7	Fatty acid-binding protein,	166
TBX5	T-box transcription factor TBX5	518
INS	Insulin precursor [Insulin B chain; Insulin A chain]	110
MAFG	Transcription factor MafG	162
SIX1	Homeobox protein SIX1	284
G6PC2	glucose-6-phosphatase, catalytic, 2	355
CTNND2	Catenin delta-2 [Delta-catenin]	1255
PBX1	Pre-B-cell leukemia transcription factor 1	430
MEIS2	Homeobox protein Meis2 [Meis1-related protein 1]	477

The analysis of scores obtained from STRING server (Table III) provides maximum combined score between Pax6 and Sox3 (0.998) and Pax6 Sox2 (0.997).

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TABLE III
HOMOLOGY OF PUTATIVE INTERACTORS OF PAX6 IN HUMAN THROUGH STRING

Node1	Node2	Node1 STRING ID	Node 2ST RIN G ID	Combined Score
PAX6	SMARCA4	422640	417341	0.948
PAX6	SIX3	422640	405475	0.994
PAX6	TRIM11	422640	407851	0.961
PAX6	SOX3	422640	419799	0.981
PAX6	OLFM3	422640	416068	0.951
PAX6	RHO	422640	408779	0.947
PAX6	SOX2	422640	412534	0.990
PAX6	WT1	422640	413745	0.944
PAX6	HOMER3	422640	416711	0.949
PAX6	NEUROD1	422640	408570	0.955
PAX6	SHH	422640	408946	0.955
PAX6	ANUBL1	422640	415018	0.967
PAX6	NEUROG2	422640	411673	0.981
PAX6	MAF	422640	412981	0.946
PAX6	NEUROG3	422640	404134	0.956
PAX6	IPO13	422640	420294	0.977
PAX6	GCG	422640	421288	0.954
PAX6	MITF	422640	408646	0.977

In mouse the maximum homology combined score [Table IV] was also observed in Pax6 and Sox2 (0.990) and Pax6 and Six3 (0.994).

TABLE IV
HOMOLOGY OF PUTATIVE INTERACTORS OF PAX6 IN MOUSE THROUGH STRING

Node1	Node2	Node1 STRING ID	Node2 STRING ID	combined Score
Pax6	Neurog3	535056	523885	0.955
Pax6	Neurog2	535056	517644	0.977
Pax6	Dach1	535056	527558	0.975
Pax6	Meis1	535056	527071	0.960
Pax6	Six3	535056	520745	0.988
Pax6	Pbx1	535056	526506	0.952
Pax6	Olfm3	535056	525418	0.936
Pax6	Sox2	535056	534736	0.997
Pax6	Sox3	535056	522877	0.944
Pax6	Zeb1	535056	516069	0.941
Pax6	Foxd3	535056	530958	0.949
Pax6	Maf	535056	526765	0.946
Pax6	Tfeb	535056	515947	0.936
Pax6	Meis2	535056	517298	0.947
Pax6	Neurod1	535056	520855	0.954
Pax6	Rb1	535056	515375	0.938
Pax6	Pbx2	535056	520880	0.938
Pax6	Smarca4	535056	534586	0.957

The homology and occurrence was maximum in human [Fig 4] and mouse [Fig 5] and minimum in archea for all 20 putative interactors.

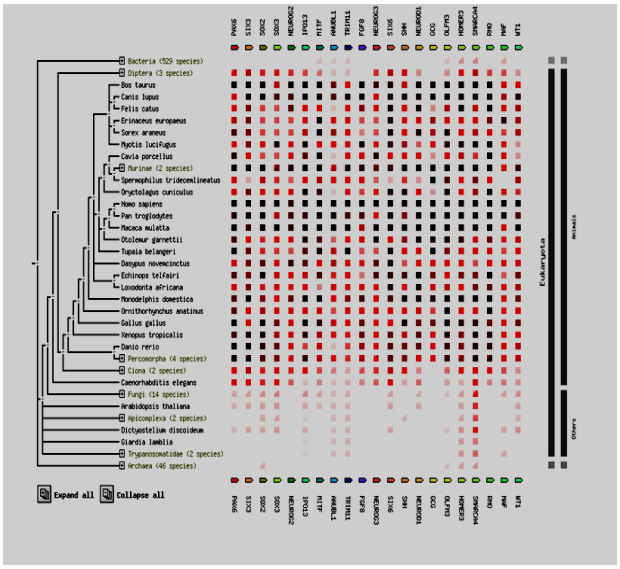

Fig. 4 It shows the occurrence view of putative interacting proteins of Pax6 in among phyla in human. The displayed 20 proteins have maximum occurrence in human.

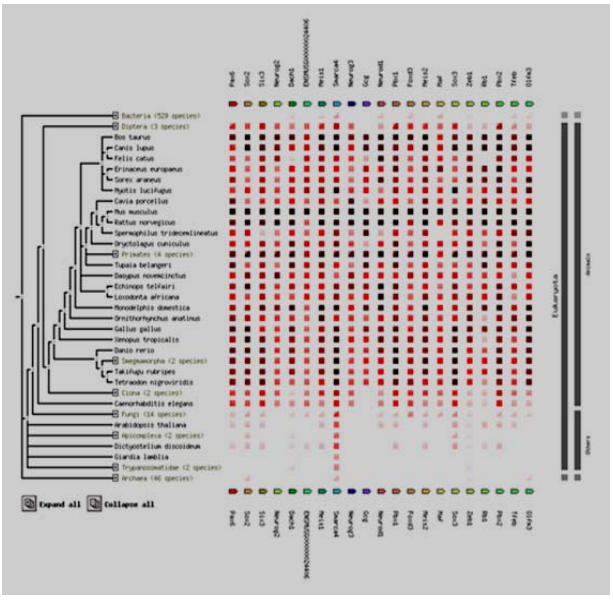

Fig. 5 It shows the occurrence view of putative interacting proteins of Pax6 in among phyla in mouse. The displayed 20 proteins have maximum occurrence in mouse.

The presumptive model describes association of proteins like LIM, OTX2, OTX1, EMX1, EMX2, EN1, and EN2 that co-express with Pax6. It is presumed that certain proteins like matri-cellular Proteins, TGIF, TGF, FGF, Neurotrophins, Ras and p53 are likely to interact with Pax6 in the cascade of its functions [Fig.6] for balanced and optimal activity.

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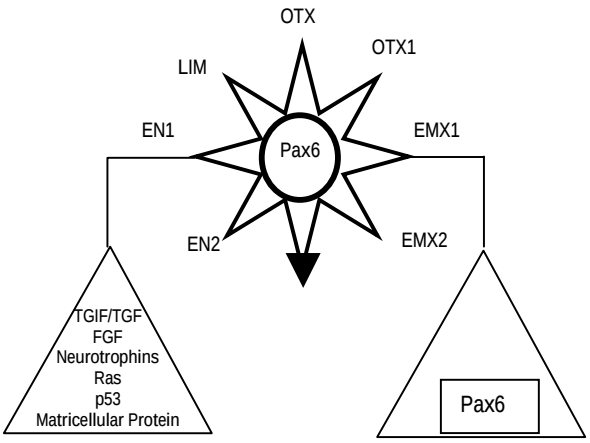


Fig. 6 Presumptive model showing proteins like LIM, OTX2, OTX1, EMX1, EMX2, EN1, EN2 that co-express with Pax6. These proteins are required to maintain functional status of brain. We presume certain proteins like matri-cellular Proteins, TGF, FGF, Neurotrophins, Ras and p53, are likely to be co express with Pax6 and interact in the cascade of their hierarchy.

Analysis of data from human and creating a phylogenic tree [Fig. 7A] and tree view [Fig. 7B] indicate that SPARC is near to MITF and p53 is near to RHO protein.

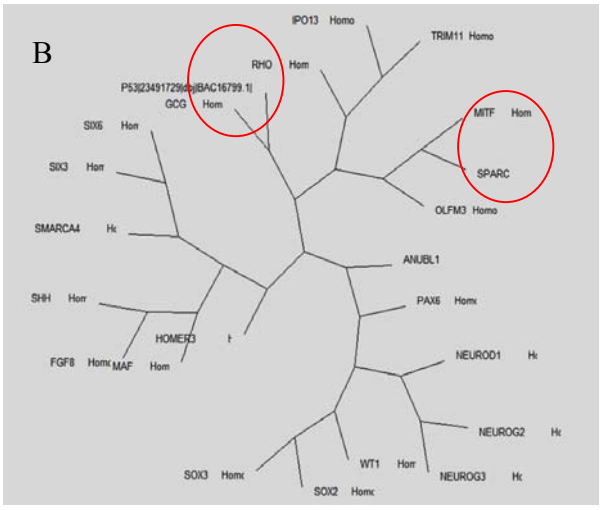
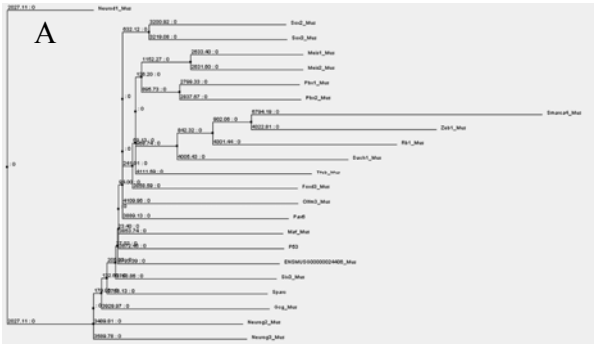


Fig. 7 It shows the neighbour joining tree [A] and tree view [B]of putative interactors of Pax6 as well as SPARC and p53 in human. It shows that our presumptive protein SPARC is nearer to MITF and p53 is nearer to RHO during evolution in human.

Analysis of data from mouse and phylogenic tree [Fig 8A] and tree view [Fig 8B] exhibit that Sparc is nearer to Zeb1 and Dach1 and p53 is nearer to Gcg and Rb1 in putative interactors of Pax6.



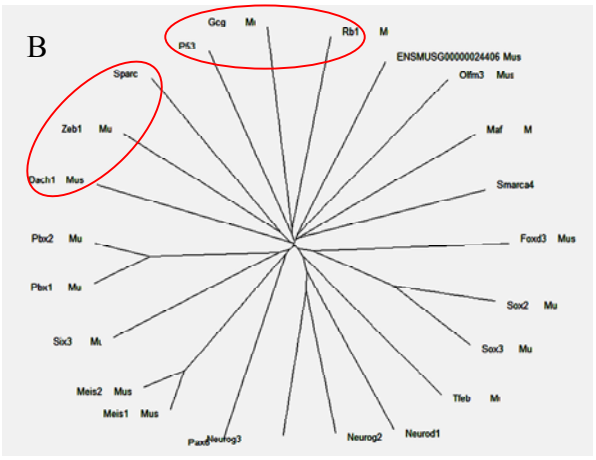


Fig. 8 It shows the neighbour joining tree [A] and tree view [B]of putative interactors of Pax6 as well as SPARC and p53 in mouse. It shows that our presumptive protein SPARC is nearer to Zeb1 and Dach1 and p53 is nearer to Gcg and Rb1 during evolution in mouse.

IV. DISCUSSION

The models by PIP and STRING suggest valuable interrelations between mouse [Figure 2] Pax6 [Sey] and human PAX6 [Figure 3]. Analysis of putative interactors of Pax6 based on score value, lowest score value of 9.20 was observed for RNA binding motif protein 12B [NP_976324] and highest score of 13.38 was found for O-linked GlcNAc transferase isoform 1 protein. The proteins with high score value exhibit more affinity of interaction with PAX6 than low score proteins [Table I]. The model based on PIP [Figure 1] does not show most of the proteins which are reported to interact with PAX6. While comparing models generated through STRING and PIP it was noticed that the model through PIP infers putative interactors for a given protein from homologous protein interaction data, even when there is no experimental data available for it. It may be due to PIP searches for homologues to proteins that have been found experimentally to interact. The results are convincing because data come from different species and based on a variety of experimental methods such as yeast-two-hybrid, X-ray crystallography, mass spectroscopy, and affinity purification.

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Once lists of homologues to each of the experimentally determined proteins have been constructed, PIP tries to identify interactions between the homologues. These putative interactions are then given confidence scores based on two factors, [i] the level of homology to proteins found experimentally to interact, and [ii] the amount of experimental data available.

The model from STRING proves better showing interactors of PAX6 in human [Figure 2] and mice [Figure 3]. Some putative partners of PAX6 which are common in human and mouse through STRING [Table II] reveal that proteins like [IPO3, SIX3, CDX2, RX, EN1, CTCF, EMX2, NEUROG3, NEUROG2, IPF1, SHH, WT1, NEUROD1, FGF8, WNT7B, TBX5, SIX1, MEIS2, MMP9, ISCL1, GBX2, EYA1, MAFG] are strong interacting partners. Among these proteins, Neurogenin-2, SIX3, MAFG, EMX2, SHH, NeuroD and FGF8 are reported to be critical for development and maintaining functional status of brain and eyes. The mutations in these proteins cause severe brain abnormalities. The neurogenin-2 [Ngn2] is a member of the neurogenin sub-family of basic helix-loop-helix [bHLH] transcription factor that play an important role in neurogenesis from migratory neural crest cells. The Ngn2 and Ngn1 are expressed in distinct progenitor populations in the central and peripheral nervous systems during mouse neurogenesis [33]. It is reported that expression of Ngn2 in the ventral spinal cord results from the modular activity of at least 3 enhancers that are active in distinct progenitor domains. The results of Ngn2 expression and enhancer activity in Pax6 mutant mice suggest that Pax6 regulates Ngn2 expression in the spinal cord by controlling distinct enhancer elements that are active at different positions along the dorso-ventral axis. It has been hypothesized that Ngn2 is both responsive to and a regulator of genetic pathways that provide positional identity and specify neuronal fates in the ventral spinal cord [34]. It is recently reported that Ngn1, a pro-neural gene that directs neuronal differentiation of progenitor cells, during development, is sufficient to convert the mesodermal cell fate of Mesenchymal Stem Cells [MSCs] in to a neuronal one. It is also stated that induction of MSCs is advantageous for the treatment of neurological dysfunction [35].

The holoprosencephaly [HPE] is a common, severe malformation of the brain that involves separation of the central nervous system into left and right halves. The analysis identified 4 different mutations in the homeodomain of SIX3 that were predicted to interfere with transcriptional activation. They were also associated with HPE. It was proposed that SIX3/HPE is essential for the development of the anterior neural plate and eyes in humans [36]. The MAF [subunit MafF, MafG, or MafK] are expressed in CNS neurons. The mafG /mafK compound mutant mice display a hypertonic motor disorder with myoclonus and abnormal responses to startle stimuli [37]. The Gbx2 is expressed in the anterior hindbrain, with a shared border at the level of the mid/hindbrain organizer. It was demonstrated that in Gbx2 -/- mutants lacks these region in the developing brain in mouse [38]. The homeodomain transcription factor EMX2 is critical for central nervous system and urogenital development. The heterozygous mutation in *EMX2* leads to absence of large portions of the cerebral hemispheres and/or replaced by cerebrospinal fluid. The mutations [de novo] were not present in the patients' parent that indicates that the EMX2 protein appears to be required for the correct formation of the human cerebral cortex [39].

The mammalian homologs of hedgehog *(hh,* Sonic [*Shh*] is expressed in the Hensen node [floor-plate of the neural tube], the early gut endoderm, the posterior of the limb buds, and throughout the notochord. It has been implicated as the key inductive signal in patterning of the ventral neural tube [41], the anterior-posterior limb axis [42], and the ventral somites [43]. The mutations in SHH results in holoprosencephaly [44]. The Basic helix-loop-helix [bHLH] proteins are transcription factors involved in determining cell type during development. The NeuroD [neurogenic differentiation], a bHLH protein, functions during neurogenesis [45]. It is widely expressed during development of mammalian brain and pancreas. It is reported that mice homozygous for a deletion of the *NeuroD* gene failed to develop a granule cell layer within the dentate gyrus, one of the principal structures of the hippocampal formation [46]. The fibroblast growth factors are secreted proteins that interact with the FGF tyrosine kinase receptors to mediate growth and development. The *Fgf8* is expressed at the junction between the midbrain, mesencephalon and anterior hindbrain metencephalon. Likewise, zebrafish embryos with reduced *Fgf8* function have an abnormal telencephalon, with striking defects at the midline [47]. The HOMER3, DNCL1&TRIM11 are reported as interactors of Pax6 and play major role in brain development and as well as age related mental disorder, Alzheimer's. The HOMER3 is a member of HOMER family of protein that is constitutively expressed in the brain and plays a role in postsynaptic signaling, axon guidance and receptor trafficking during brain development [48].

V. CONCLUSION

The proteins like LIM, OTX2, OTX1, EMX1, EMX2, EN1, EN2 that co-express with Pax6 are required to maintain functional status of brain. However, interaction of PAX6 with proteins like SPARC, TGIF, TGF, FGF, Neurotrophins, Ras and p53 which are involved in cell survival and cell proliferation cannot be ignored.

ACKNOWLEDGMENT

RT acknowledges UGC Research Fellowship in Science for meritorious students. The support from the Department of Science and Technology [SR/SO/BB-63/2006], New Delhi is gratefully acknowledged by RM.

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