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Paired-Like Homeodomain Transcription Factor 2 (PITX2): Time Behavioural Study of 3rd Order Combinations in WNT3A Stimulated HEK 293 Cells

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Paired-Like Homeodomain Transcription Factor 2 (PITX2): Time Behavioural Study of 3rd Order Combinations in WNT3A Stimulated HEK 293 Cells †

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† Time behavioural study of 3-odr PITX2 comb. in WNT3A stimulated cells

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Abstract: PITX2 (also known as pituitary homeobox 2), encodes a member of the RIEG/PITX homeobox family (a bicoid class of homeodomain proteins), that acts as a transcription factor and regulates procollagen lysyl hydroxylase gene expression. It is known to play an important role in pituitary, heart, brain, lungs, spleen, twisting of the gut and stomach, as well as the development of the eyes. Gujral and MacBeath [1] provides a quantitative, and dynamic study of WNT3A-mediated stimulation of HEK 293 cells, where they record time based expression profiles of several response genes which correlated significantly with proliferation and migration. By monitoring the dynamics of gene expression using self-organizing maps, they identified clusters of genes that exhibit similar expression dynamics and uncovered previously unrecognized positive and negative feedback loops. However, their study depicts/uses singular measurements of individual gene expression at different time snapshots/points to infer the system wide analysis of the pathway. At any particular time point, it is often the case that genes are working synergistically in combinations, even though their expression measurements are singular in nature. Here, I • enumerate and rank all 2415 PITX2 related 3rd order combinations in a forest of ${}^{71}C_3$ combinations using four different sensitivity methods; • show the conserved rankings for PITX2-X-X combinations, which point to existence of biological synergy of some of these combinations across the different sensitivity methods; and • study the behaviour of some of these combinations related to WNT3A response genes that are ranked by the machine learning search engine (Sinha [2]) in time. Patterns of combinations emerge, some of which have been tested in wet lab, while others require further wet lab analysis.

Keywords: sensitivity analysis; support vector ranking; Hilbert Schmidt Independence Criterion indices (HSIC) and Sobol indicies; WNT3A

1. Significance

Sinha [2] recently demonstrated the use of machine learning based search engine to rank/reveal gene combinations at 2nd order for the time series data by Gujral and MacBeath [1] and showed how it is possible to locate combinations of priority that might be working synergistically, using sensitivity methods and powerful support vector ranking algorithm. However, the problem explodes combinatorially with even a small set of 71 recorded genes in the study by Gujral and MacBeath [1], when one steps to explore 3rd order combinations. With the total number of ${}^{71}C_3$ (= 57155) combinations, it becomes nearly impossible for any biologist to study the system wide dynamics of any pathway. Also, the amount of time usually needed to search for and test a combination is far more than the search down by the machine learning based search engine. Here, I extend the research work by Sinha [2] to conduct a behavioral study of 3rd order PITX2 related combinations using individual gene expressions measured in time, in WNT3A stimulated HEK 293 cells.

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2. Introduction

The details of the machine learning based search engine has been recently published in Sinha [2] and deployed to explore the 2nd order combinations of genes in the data set provided by Gujral and MacBeath [1]. Nevertheless, here, I point to the fundamentals of the published work for completeness.

2.1. A Combinatorial Problem

Sensitivity analysis plays a major role in computing the strength of the influence of involved factors in any phenomena under investigation. When applied to expression profiles of various intra/extracellular factors that form an integral part of a signaling pathway, the variance and density based analysis yields a range of sensitivity indices for individual as well as various combinations of factors. These combinations denote the higher order interactions among the involved factors. Computation of higher order interactions is often time consuming but it gives a chance to explore the various combinations that might be of interest in the working mechanism of the pathway. For example, in a range of fourth order combinations among the various factors of the Wnt pathway, it would be easy to assess the influence of the destruction complex formed by APC, AXIN, CSKI and GSK3 interaction. But the effect of these combinations vary over time as measurements of fold changes and deviations in fold changes vary. So it is imperative to know how an interaction or a combination of the involved factors behave in time and Sinha [2] develops a procedure to track the behaviour by exploiting the influences of these involved factors.

2.2. A Possible Solution

In this work, after estimating the individual effects of factors for a higher order combination, the individual indices are considered as discriminative features. A combination, then, is a feature set in higher order ($\geq$2 ,i.e multivariate). With an excessively large number of factors involved in the pathway, it is difficult to search for important combinations in a wide search space over different orders. Exploiting the analogy with the issues of prioritizing webpages using ranking algorithms, for a particular order, a full set of combinations of interactions can then be prioritized based on these features using a powerful ranking algorithm via support vectors Joachims [3]. Recording the changing rankings of the combinations over time reveals how higher order interactions behave within the pathway and when an intervention might be necessary to influence the interaction within the pathway.

2.3. Paired-Like Homeodomain Transcription Factor 2 (PITX2)

PITX2, encodes a member of the RIEG/PITX homeobox family (a bicoid class of homeodomain proteins (Holland et al. [4])). A homeobox is an approximately 180 base pairs long DNA sequence, that regulates anatomical features in the early stages of embryonic development (Wikipedia contributors [5]). Mutations in a homeobox may change large-scale anatomical features of the full-grown organism. Homeobox genes (Gehring [6] and Gehring [7]) encode homeodomain protein products are transcription factors with important roles in embryonic patterning and cell differentiation, and several have been implicated in human diseases and congenital abnormalities (Boncinelli [8]).

ALL1 (human homologue of Drosophila trithorax), is directly involved in human acute leukemias associated with abnormalities at 11q23. Arakawa et al. [9] isolated a gene that is down-regulated in All1 double-knockout mouse embryonic stem (ES) cells and designated it ARP1 (also termed RIEG, PITX2, or OTLX2) belonging to the PRD class homeoboxes and pseudogenes. Logan et al. [10] show that PITX2 encodes a transcription factor expressed through-out the left lateral plate mesoderm and subsequentlyon the left side of asymmetric organs such as the heart and gut during organogenesis in the chick embryo. Misexpression of PITX2 on the right side of the embryo is sufficient to produce reversed heart looping and heart isomerisms, reversed body rotation, and reversed gut situs (Campione et al. [11], Shiratori et al. [12], Zacharias et al. [13]). Finally, different isoforms of the transcription factor: PITX2-A/B/C, each with distinct and non-overlapping functions exist.

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In this research work, I present 3rd order combinations of PITX2 with other genes, that the machine learning based search engine points to, as possible synergistic combinations that might be working in time.

3. Methods

Please refer to sections of Sinha [2] for methods, design of study and analysis of data for 2nd order combinations. The same method and design of study is used to generate results for 3rd order combinations presented in this study.

4. Time Series Data

Gujral and MacBeath [1] present a set of 71 WNT-related gene expression values for 6 different times points over a range of 24-hour period using qPCR. The changes represent the fold-change in the expression levels of genes in 200 ng/mL WNT3A-stimulated HEK 293 cells in time relative to their levels in unstimulated, serum-starved cells at 0-hour. Gujral and MacBeath [1] state that qPCR data are the means of three biological replicates. Only genes whose mean transcript levels changed by more than two-fold at one or more time points during the 24-hour time course were considered significant. Positive (negative) numbers represent up (down) -regulation. We have already covered the issues related to these data sets in detail in Sinha [14]. Readers are requested to go through them in the pointed reference. The tools of study which are used here have been published in another foundational work in Sinha [14].

5. Design of Experiment

5.1. Pipeline for Time Series Data

For the case of time series data, interactions among the contributing factors are studied by comparing triplets of fold-changes at single time points. The prodecure begins with the generation of distribution around measurements at single time points with added noise is done to estimate the indices. A distribution is generated for the fold changes at single time points. Then for every gene, there is a vector of values representing fold changes as well as deviations in fold changes for different time points and durations between time points, respectively. Next a listing of all C_k^n combinations for k number of genes from a total of n genes is generated. k is ≥ 2 and $\leq (n-1)$. Each of the combination of order k represents a unique set of interaction between the involved genetic factors. After this, the datasets are combined in a specifed format which go as input as per the requirement of a particular sensitivity analysis method. Thus for each p^{th} combination in C_k^n combinations, the dataset is prepared in the required format from the distributions for two separate cases which have been discussed above. (See .R code in mainScript-1-1.R). After the data has been transformed, vectorized programming is employed for density based sensitivity analysis and looping is employed for variance based sensitivity analysis to compute the required sensitivity indices for each of the p combinations. This procedure is done for different kinds of sensitivity analysis methods.

After the above sensitivity indices have been stored for each of the p^{th} combination, the next step in the design of experiment is conducted. Since there is only one recording of sensitivity index per combination, each combination forms a training example which is alloted a training index and the sensitivity indices of the individual genetic factors form the training example. Thus there are C_k^n training examples for k^{th} order interaction. Using this training set SVM_{learn}^{Rank} Joachims [3] is used to generate a model on default value C value of 20. In the current experiment on toy model C value has not been tunned. The training set helps in the generation of the model as the different gene combinations are numbered in order which are used as rank indices. The model is then used to generate score on the observations in the testing set using the $SVM_{classify}^{Rank}$ Joachims [3]. Note that due to availability of only one example per combination, after the model has been built, the same training data is used as test data to generates the scores. This procedure is executed for each and every sensitivity analysis method. This is followed by sorting of these scores along with the rank indices

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(i.e the training indices) already assigned to the gene combinations. The end result is a sorted order of the gene combinations based on the ranking score learned by the SVM^{Rank} algorithm. Finally, this entire procedure is computed for sensitivity indices generated for each and every fold change at time point and deviations in fold change at different durations. Observing the changing rank of a particular combination at different times and different time periods will reveal how a combination is behaving.

Note that the following is the order in which the files should be executed in R, in order, for obtaining the desired results (Note that the code will not be explained here) - • use source("mainScript-1-1.R") with arguments for Dynamic data • source("SVMRank-Results-D.R"), to rank the interactions (again this needs to be done separately for different kinds of SA methods), • use source("Combine-Time-files.R"), if computing indices separately via previous file, • source("Sort-n-Plot-D.R") to sort the interactions. Note that the sorting is chages the interaction ranking in time. Thus • use source("Interaction-Priority-Intime.R") to find the prioritized ranking of each and every interaction over the different time points and finally • use source("Print-Ranking-AND-Interaction-Rank.R") to print individual ranking of the required input factor with other interaction factors.

6. Results & Discussion

6.1. Time Series Data by Gujral and MacBeath [1]

NOTE - Ranking was assigned on scores that were sorted in DECREASING values. So, 1 was assigned to highest score and vice versa.

Results for the 3^{rd} order interactions are presented here. The results first discuss the behaviour of interactions across the snapshots of time using the computed sensitivities on fold change measurements per time snapshot. The analysis was done using 4 different sensitivity indices. Out of the ${}^{71}C_3$ combinations, I consider/present only those combinations that show a ranking within first 10,000 out of 57,155. This choice is liberal and biologists/oncologists can have a more stricter choice as per need. Two observations are made, • the ranking of a particular combination is conserved (i.e within the 10,000 range) in a particular time point or in the early phase or late phase of WNT3A stimulation, across the majority of the four sensitivity methods, which is a strict criteria of assessment or • the ranking of a particular combination is conserved across time points/phase (i.e they are within the 10,000 range) and the majority of the four sensitivity methods, which is relaxed criteria of assessment. Applying this filter helps reveal important combinations of interest that might be working synergistically at a higher order level in the cell.

Regarding technical points of implementation, the rankings were generated without scaling/normalizing the time series data provided by Gujral and MacBeath [1]. For estimating the sensitivity indices, a small gaussian distribution using the function **rnorm** that generates a vector of normally distributed random variables given a vector length n (here 9, the 10th one is the mean/recorded gene regulation itself), a population mean μ and population standard deviation σ. The syntax for using rnorm is as follows: **rnorm(n, mean, sd)**. Further, I use the **jitter** funtion to add a little bit of noise to the data. This helps to see if the generated rankings are robust or not.

6.2. Enumeration and Ranking of 2415 PITX2-X-X Combinations from Gujral and MacBeath [1]

In the supplementary section, I present four files, each containing the rankings of 3rd order combinations, that wary in time (shown for 5 time points). Each file represents the rankings computed using a particular sensitivity method. The changing rankings in time for a particular combination represents the importance of contribution/role that combination plays in the cell stimulated with WNT3A. The sensitivity methods used are Hilbert Schmidt Independence Criterion indices (HSIC) indices (with rbf and linear kernel in Da Veiga [15]) and Sobol indicies (with 2002 implementation in Saltelli [16] and martinez implementation in Martinez [17] and Baudin et al. [18]).

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6.3. Conserved Machine Learning Rankings for Tested PITX2-X-X Combinations

A total of 2415, 3rd order combinations involving PITX2 were obtained from a full set of ${}^{71}C_3$ = 57155 combinations. Further, from this selected set, using the above criteria for conserved rankings, I report/tabulate the meaningful combinations that might be working synergistically. Tables 2, 3 and 4 show the rankings for the same combinations as in table 1, but using rbf kernel for HSIC, 2002 implementation for SOBOL and martinez implementation for SOBOL, respectively. As one tallies the rankings of across these tables for a particular combination, one finds that the role of the combination of interest is conserved. This conservation points to the existence of the biological synergy, whether the combination has been tested or unexplored/untested.

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Table 1. Rankings of PITX2-X-X. A list of approximately first 125 combinations with rankings below 10,000 out of 57,155. SA - HSIC; Kernel - linear

	RANKING @ t_i USING HSIC - LINEAR										
3rd order comb.	t_1	t_3	t_6	t_{12}	t_{24}	3rd order comb.	t_1	t_3	t_6	t_{12}	t_{24}
APC-PITX2-SFRP4	14	26861	33433	10197	18249	APC-PITX2-SENP2	37	14630	40719	18877	15582
APC-PITX2-TCF7	130	34995	20653	15851	17861	PITX2-PORCN-TLE2	169	22676	35351	44094	14423
PITX2-PORCN-WNT4	177	16891	32175	32123	27627	PITX2-PORCN-SENP2	270	20159	27718	25665	18827
DVL2-JUN-PITX2	293	27343	32163	42607	21387	FZD5-PITX2-SENP2	328	35236	22541	1156	14803
APC-PITX2-PPP2CA	334	14753	2900	19245	30663	APC-PITX2-RHOU	397	38592	32191	27698	15148
PITX2-PORCN-WNT5A	460	36097	17787	53210	23094	PITX2-PORCN-FBXW4	499	12554	20396	26439	17842
DIXDC1-PITX2-SENP2	526	28707	7274	5676	35235	CTNNBIP1-JUN-PITX2	546	6389	45937	54346	11119
CCND1-FGF4-PITX2	554	54983	49474	23953	29838	PITX2-PORCN-TCF7L1	572	42893	22702	25919	13429
DIXDC1-PITX2-SFRP4	614	29602	6543	3868	43507	PITX2-PORCN-WNT2B	630	42884	44910	50269	15186
APC-PITX2-WNT4	634	38339	50192	23301	18227	FRAT1-JUN-PITX2	748	50033	38113	50412	44660
PITX2-PORCN-TLE1	813	43605	21513	22845	15786	DKK1-JUN-PITX2	913	6407	42538	52798	634
AES-AXIN1-PITX2	978	46773	17137	47484	7382	PITX2-PORCN-PPP2R1A	1003	8071	39783	44256	5481
PITX2-PORCN-RHOU	1220	38816	23756	29958	13324	CCND1-JUN-PITX2	1260	51205	42011	18763	52917
FOSL1-JUN-PITX2	1479	1306	37107	54515	12375	FZD6-PITX2-WNT2B	1504	10725	14634	54318	17213
FZD5-PITX2-SFRP4	1559	51198	27058	788	14497	PITX2-PORCN-SFRP4	1607	32189	32121	34063	21972
CCND1-CTBP1-PITX2	1644	56351	33831	12174	34580	FSHB-FZD2-PITX2	1681	25060	56319	33266	14323
PITX2-WNT3-WNT5A	1726	32979	5136	41370	17012	CCND2-LRP6-PITX2	1887	36449	16637	55064	12492
PITX2-PORCN-SFRP1	1905	39236	31192	37387	9110	AES-EP300-PITX2	2125	50901	43504	52709	17083
FZD6-PITX2-SFRP4	2136	44403	22523	5027	25162	PITX2-WNT3-WNT3A	2155	50776	27010	46543	38273
DAAM1-FOXN1-PITX2	2183	45577	23419	17738	1022	CSNK2A1-JUN-PITX2	2295	11006	48321	42767	19026
FBXW11-LRP6-PITX2	2307	48503	41757	51594	35405	CCND1-CTNNBIP1-PITX2	2315	54797	33975	42838	34145
CSNK2A1-FOXN1-PITX2	2356	10265	14777	12628	14201	FOXN1-GSK3A-PITX2	2381	48019	32781	32844	21255
AXIN1-FZD6-PITX2	2480	44926	6159	53367	9917	FRZB-GSK3A-PITX2	2482	10817	2209	668	13402
PITX2-WNT1-WNT5A	2609	5926	14837	51671	5692	BTRC-GSK3A-PITX2	2613	49655	52881	6241	50065
PITX2-SFRP4-WNT2B	2621	47714	20847	52301	32634	DAAM1-JUN-PITX2	2739	53719	47964	55644	657
CXXC4-JUN-PITX2	2757	3424	36286	40345	36376	APC-PITX2-WNT3	2778	27077	34088	34710	13819
PITX2-PORCN-PPP2CA	2847	24874	33030	43172	21878	DIXDC1-PITX2-WNT2B	2863	14619	3889	45440	40568
FBXW11-FOXN1-PITX2	2886	3843	4957	17704	23607	CSNK1D-FGF4-PITX2	3093	11417	24874	53815	30793
LRP5-PITX2-SFRP4	3210	16171	31459	8145	26086	DIXDC1-PITX2-WNT4	3214	52681	19702	7189	37575
FOSL1-GSK3A-PITX2	3246	8961	918	7353	12922	FBXW11-JUN-PITX2	3374	35385	45082	55257	41153
CCND3-PITX2-WNT3A	3484	43666	17937	5868	27350	CSNK2A1-MYC-PITX2	3758	35967	33879	27313	16543
EP300-FZD2-PITX2	3771	12315	30396	16799	22718	CSNK1D-FOXN1-PITX2	3778	6280	4678	21772	36345
FZD1-GSK3A-PITX2	3798	30685	3123	6034	15265	CCND3-PITX2-SFRP4	3846	53365	17972	862	38266
DVL1-EP300-PITX2	3886	37126	11319	53194	36735	DAAM1-GSK3A-PITX2	3913	55523	36819	2364	4273
DKK1-PITX2-SFRP4	3919	36216	54630	36550	651	GSK3B-PITX2-SFRP4	3969	51591	11242	44587	45162
FBXW2-JUN-PITX2	4023	15973	52731	42180	49503	FRZB-FZD2-PITX2	4081	5620	20625	16286	21997
DIXDC1-PITX2-PPP2CA	4123	13781	1911	14325	45522	DAAM1-LRP6-PITX2	4134	55453	49218	56874	3254
FOSL1-FOXN1-PITX2	4149	3704	3591	20411	19698	CTNNB1-FOXN1-PITX2	4154	3460	4095	19749	19696
PITX2-WNT1-WNT2B	4158	13925	1462	19718	12422	CCND3-PITX2-SENP2	4176	37072	17505	1404	25804
FZD5-PITX2-WNT2B	4182	39042	16284	52876	11251	FZD7-GSK3A-PITX2	4302	20752	736	4869	12197
AXIN1-EP300-PITX2	4304	11293	12887	53659	13073	FRAT1-GSK3A-PITX2	4363	56251	2247	18588	44926
CSNK1G1-FZD2-PITX2	4407	7002	19144	19885	20203	FOSL1-FZD2-PITX2	4478	4516	12555	16266	17423
FBXW11-PITX2-SFRP4	4547	50563	47219	11287	28318	CSNK1G1-PITX2-SENP2	4579	38292	32335	49857	17717
PITX2-PYGO1-TLE2	4588	2003	47249	8274	54767	CTBP2-FOXN1-PITX2	4611	37603	5195	14491	24302
DVL1-FOXN1-PITX2	4624	42047	4932	11016	38406	CTBP1-JUN-PITX2	4720	2611	40385	42676	12432
PITX2-WNT1-WNT4	4724	908	3272	33901	10244	DIXDC1-PITX2-RHOU	4739	31170	5147	8935	19768
FZD6-PITX2-TCF7	4754	25933	18851	11368	22426	DKK1-GSK3A-PITX2	4759	23623	33694	50522	6502
FZD6-PITX2-SENP2	4827	47413	31625	7317	23700	APC-PITX2-SLC9A3R1	4832	13233	25730	31544	21292
FBXW11-PITX2-WNT2B	4991	55437	27760	53601	30191	DIXDC1-PITX2-TCF7	5005	35931	12459	6681	32860
CSNK1D-PITX2-SFRP4	5096	37446	11233	10582	41257	FZD5-CCND3-PITX2	5106	9271	1305	14097	20152
FZD6-PITX2-WNT2	5113	30267	46069	12925	22168	AES-FOXN1-PITX2	5216	17207	8200	21874	15128
CSNK1G1-PITX2-RHOU	5258	16813	32518	47070	15599	FZD7-NKD1-PITX2	5310	12439	25127	40994	7486
FGF4-JUN-PITX2	5343	2634	32672	53764	32133	CCND1-NLK-PITX2	5399	39293	9478	4030	37469
FZD6-PITX2-SFRP1	5615	37130	25262	13890	9233	CXXC4-FGF4-PITX2	5669	13032	45784	50894	54142
AXIN1-FOXN1-PITX2	5682	1318	3400	19758	15375	FOSL1-PITX2-SFRP4	5766	29791	9666	5926	21290
FOSL1-NKD1-PITX2	5819	8607	19299	34454	29628	DKK1-PITX2-WNT2B	5967	20722	56765	57114	736
FZD5-PITX2-RHOU	5980	46930	21039	1256	11863	GSK3B-PITX2-SENP2	6007	41103	11874	39352	36153
FZD6-PITX2-TCF7L1	6019	6483	34081	5505	18404	CTBP1-FOXN1-PITX2	6078	7952	6550	21251	24027
FBXW2-GSK3A-PITX2	6113	23145	5408	17937	19874	EP300-GSK3B-PITX2	6131	11194	7972	26156	25577
CTBP1-FGF4-PITX2	6132	3149	40895	31072	24686	FRAT1-FZD2-PITX2	6168	56131	24624	14249	36492
AES-FZD2-PITX2	6222	26972	33042	43053	11722	FBXW11-GSK3A-PITX2	6249	47414	27591	23081	13112
FZD8-JUN-PITX2	6263	42057	38010	54749	2980	CXXC4-FZD2-PITX2	6270	7825	20641	14923	45220
PITX2-SFRP4-TLE2	6342	43420	38012	10662	35267	DIXDC1-FZD2-PITX2	6448	5815	17969	18298	39281

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Table 2. Rankings of PITX2-X-X. A list of approximately first 125 combinations with rankings below 10,000 out of 57,155. SA - HSIC; Kernel - rbf

RANKING @ t_i USING HSIC - RBF

3rd order comb.	t_1	t_3	t_6	t_{12}	t_{24}	3rd order comb.	t_1	t_3	t_6	t_{12}	t_{24}
APC-PITX2-SFRP4	6639	22831	16274	5172	51942	APC-PITX2-SENP2	2251	31790	24604	4114	39754
APC-PITX2-TCF7	5809	43852	29659	2608	54162	PITX2-PORCN-TLE2	13743	33091	10461	36155	54286
PITX2-PORCN-WNT4	9247	2492	36647	13332	52478	PITX2-PORCN-SENP2	9499	29209	4986	29421	35908
DVL2-JUN-PITX2	22623	16521	37051	52719	15020	FZD5-PITX2-SENP2	10435	42695	24116	3954	33353
APC-PITX2-PPP2CA	4042	33446	8698	18579	52910	APC-PITX2-RHOU	4699	39874	24475	1820	49384
PITX2-PORCN-WNT5A	11335	32674	6548	15458	54809	PITX2-PORCN-FBXW4	2782	19946	19676	35972	50951
DIXDC1-PITX2-SENP2	3537	43865	15972	13329	19669	CTNNBIP1-JUN-PITX2	55688	6273	51543	54725	26305
CCND1-FGF4-PITX2	55688	6273	51543	54725	26305	PITX2-PORCN-TCF7L1	13222	48332	9570	21227	51753
DIXDC1-PITX2-SFRP4	9005	33898	35036	1595	29334	PITX2-PORCN-WNT2B	6802	25276	16938	13140	54357
APC-PITX2-WNT4	6441	34135	28508	16023	43494	FRAT1-JUN-PITX2	28349	55075	50998	56838	16856
PITX2-PORCN-TLE1	13872	39019	23792	7146	28106	DKK1-JUN-PITX2	24208	3989	4050	56945	15274
AES-AXIN1-PITX2	37489	47814	10383	53313	22724	PITX2-PORCN-PPP2R1A	11355	50277	26063	28070	55628
PITX2-PORCN-RHOU	11795	30595	6426	8297	54722	CCND1-JUN-PITX2	44312	43519	25962	49672	2955
FOSL1-JUN-PITX2	42099	14135	52254	57017	24935	FZD6-PITX2-WNT2B	8135	7300	14987	7418	3456
FZD5-PITX2-SFRP4	8368	50438	38825	1203	52857	PITX2-PORCN-SFRP4	7652	26224	19994	9630	41059
CCND1-CTBP1-PITX2	7652	26224	19994	9630	41059	FSHB-FZD2-PITX2	11301	38653	1373	55358	36056
PITX2-WNT3-WNT5A	50071	36976	3198	23809	43096	CCND2-LRP6-PITX2	20931	44333	44558	56656	20738
PITX2-PORCN-SFRP1	20244	35446	37738	24843	55456	AES-EP300-PITX2	46927	53837	1549	50756	32506
FZD6-PITX2-SFRP4	7135	52408	16969	864	34132	PITX2-WNT3-WNT3A	36927	41437	49172	9526	14468
DAAM1-FOXN1-PITX2	1685	41262	42245	51064	684	CSNK2A1-JUN-PITX2	25321	4747	44298	56844	22732
FBXW11-LRP6-PITX2	29615	47349	39438	51084	16601	CCND1-CTNNBIP1-PITX2	17845	54257	2633	44741	19765
CSNK2A1-FOXN1-PITX2	1130	13168	44037	50356	32422	FOXN1-GSK3A-PITX2	35488	50787	34525	56639	52918
AXIN1-FZD6-PITX2	33737	44557	47717	57090	46710	FRZB-GSK3A-PITX2	5619	18480	55073	54146	46967
PITX2-WNT1-WNT5A	29674	16693	38632	17220	34621	BTRC-GSK3A-PITX2	2406	44730	43962	54217	10925
PITX2-SFRP4-WNT2B	13474	47608	14111	19231	2624	DAAM1-JUN-PITX2	35874	49308	2846	56449	4164
CXXC4-JUN-PITX2	5093	2871	54929	54382	25342	APC-PITX2-WNT3	18467	40797	31945	31204	47530
PITX2-PORCN-PPP2CA	9603	23189	295	19554	39138	DIXDC1-PITX2-WNT2B	12649	21037	9654	4112	8993
FBXW11-FOXN1-PITX2	661	3042	46901	46989	62	CSNK1D-FGF4-PITX2	21808	22200	7826	55044	47261
LRP5-PITX2-SFRP4	1850	34385	2623	13213	34281	DIXDC1-PITX2-WNT4	11062	47027	42885	4269	14148
FOSL1-GSK3A-PITX2	22118	26574	53921	57069	41599	FBXW11-JUN-PITX2	17945	27019	14279	54975	2329
CCND3-PITX2-WNT3A	14471	39233	2731	800	1147	CSNK2A1-MYC-PITX2	21651	30519	49254	52766	16814
EP300-FZD2-PITX2	21066	1261	6278	52068	50788	CSNK1D-FOXN1-PITX2	11143	1695	49037	55776	7145
FZD1-GSK3A-PITX2	6732	30000	53910	56520	34560	CCND3-PITX2-SFRP4	4452	50957	21986	3532	9719
DVL1-EP300-PITX2	56689	28520	27891	57038	12113	DAAM1-GSK3A-PITX2	1726	56240	42341	56628	12934
DKK1-PITX2-SFRP4	9818	37503	3735	2305	35936	GSK3B-PITX2-SFRP4	5309	55194	34830	18305	53111
FBXW2-JUN-PITX2	42532	23223	17767	54368	980	FRZB-FZD2-PITX2	22096	10208	42171	51487	54482
DIXDC1-PITX2-PPP2CA	5166	50281	13595	5982	18374	DAAM1-LRP6-PITX2	25969	54325	26609	56867	3176
FOSL1-FOXN1-PITX2	10188	3461	55891	51582	2726	CTNNB1-FOXN1-PITX2	7149	4991	55880	53565	3414
PITX2-WNT1-WNT2B	26078	12206	23538	5721	30071	CCND3-PITX2-SENP2	10221	40135	22112	6819	7030
FZD5-PITX2-WNT2B	22980	38933	9787	4035	21144	FZD7-GSK3A-PITX2	3127	14890	47575	57093	5868
AXIN1-EP300-PITX2	56749	23129	25963	57120	24869	FRAT1-GSK3A-PITX2	4495	57021	56892	56898	45139
CSNK1G1-FZD2-PITX2	45412	7430	10137	54602	39686	FOSL1-FZD2-PITX2	12121	784	39252	52428	46106
FBXW11-PITX2-SFRP4	8480	50287	2608	4536	27519	CSNK1G1-PITX2-SENP2	8978	31266	11421	7149	23975
PITX2-PYGO1-TLE2	1782	35079	27236	39307	56772	CTBP2-FOXN1-PITX2	18240	37720	54077	51182	7949
DVL1-FOXN1-PITX2	7131	54107	45985	52139	6298	CTBP1-JUN-PITX2	33380	1220	50964	56252	25801
PITX2-WNT1-WNT4	32926	5300	37141	2973	19943	DIXDC1-PITX2-RHOU	6079	35454	22214	14529	24323
FZD6-PITX2-TCF7	5172	16188	13820	2800	28962	DKK1-GSK3A-PITX2	605	17271	39751	55202	27215
FZD6-PITX2-SENP2	3363	52204	8127	5114	16199	APC-PITX2-SLC9A3R1	6853	5291	25242	18176	16828
FBXW11-PITX2-WNT2B	30130	56623	1327	7485	2572	DIXDC1-PITX2-TCF7	4373	37581	13658	8585	32638
CSNK1D-PITX2-SFRP4	4175	45358	33459	4847	37993	FZD5-CCND3-PITX2	40000	10437	46397	54792	20187
FZD6-PITX2-WNT2	10709	28286	1099	25993	43215	AES-FOXN1-PITX2	3577	24818	43679	53324	2571
CSNK1G1-PITX2-RHOU	11965	12955	21369	13669	45243	FZD7-NKD1-PITX2	25856	8298	48445	57028	4035
FGF4-JUN-PITX2	24971	2275	32263	54277	17002	CCND1-NLK-PITX2	30491	29337	45690	39544	1454
FZD6-PITX2-SFRP1	18690	52116	9763	7836	11500	CXXC4-FGF4-PITX2	3350	14873	39377	55805	47209
AXIN1-FOXN1-PITX2	17522	6988	53149	55878	9496	FOSL1-PITX2-SFRP4	7348	5890	31972	1744	49355
FOSL1-NKD1-PITX2	29467	27288	56470	57104	13679	DKK1-PITX2-WNT2B	17111	13223	40502	378	15000
FZD5-PITX2-RHOU	12500	51905	7871	1962	44656	GSK3B-PITX2-SENP2	915	34496	33084	22723	32507
FZD6-PITX2-TCF7L1	4289	24535	11228	6141	16611	CTBP1-FOXN1-PITX2	8239	9962	54422	50573	10742
FBXW2-GSK3A-PITX2	13991	18085	38156	55688	24970	EP300-GSK3B-PITX2	22813	7384	28301	49131	16578
CTBP1-FGF4-PITX2	24027	10442	12158	56632	50733	FRAT1-FZD2-PITX2	19475	56951	30336	56309	51026
AES-FZD2-PITX2	56089	10520	10321	45652	34757	FBXW11-GSK3A-PITX2	6618	46116	51128	49797	28514
FZD8-JUN-PITX2	40592	40343	33795	57114	7221	CXXC4-FZD2-PITX2	28941	8301	39688	54135	51166
PITX2-SFRP4-TLE2	23954	41848	2647	36476	20017	DIXDC1-FZD2-PITX2	15533	7397	34069	54461	17799

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Table 3. Rankings of PITX2-X-X. A list of approximately first 125 combinations with rankings below 10,000 out of 57,155. SA - SOBOL; Implementation - 2002

	RANKING @ t_i USING SOBOL - 2002										
3rd order comb.	t_1	t_3	t_6	t_{12}	t_{24}	3rd order comb.	t_1	t_3	t_6	t_{12}	t_{24}
APC-PITX2-SFRP4	17003	19660	7752	22464	43680	APC-PITX2-SENP2	16777	21369	5264	21757	43696
APC-PITX2-TCF7	19670	34554	8057	26773	38941	PITX2-PORCN-TLE2	14738	34228	17868	7003	24689
PITX2-PORCN-WNT4	38340	23219	45866	44679	30684	PITX2-PORCN-SENP2	49351	13297	36355	31422	8016
DVL2-JUN-PITX2	23671	1038	12434	27999	54466	FZD5-PITX2-SENP2	521	47250	5679	3657	50275
APC-PITX2-PPP2CA	24251	10201	10127	26604	27627	APC-PITX2-RHOU	39285	21142	46215	32608	9407
PITX2-PORCN-WNT5A	4366	18564	14041	19978	22471	PITX2-PORCN-FBXW4	8808	13705	22864	22147	31748
DIXDC1-PITX2-SENP2	55978	6920	46363	55209	16814	CTNNBIP1-JUN-PITX2	11329	1937	10201	6114	40219
CCND1-FGF4-PITX2	43513	2928	44305	52341	16554	PITX2-PORCN-TCF7L1	7523	29695	12381	16214	18408
DIXDC1-PITX2-SFRP4	46369	10535	43650	52909	13347	PITX2-PORCN-WNT2B	9536	5794	12824	18961	17911
APC-PITX2-WNT4	24477	25329	3199	26367	36930	FRAT1-JUN-PITX2	19630	54721	9834	10848	22067
PITX2-PORCN-TLE1	49615	27631	44758	40897	38885	DKK1-JUN-PITX2	2939	11664	23494	19389	44598
AES-AXIN1-PITX2	47687	38614	55070	30813	9874	PITX2-PORCN-PPP2R1A	10644	13926	20214	8562	45301
PITX2-PORCN-RHOU	7818	43749	20769	25705	49200	CCND1-JUN-PITX2	17592	41812	11511	4995	53682
FOSL1-JUN-PITX2	29544	47341	44123	52018	7644	FZD6-PITX2-WNT2B	42547	47083	32974	29428	5335
FZD5-PITX2-SFRP4	2340	11183	13226	1835	33599	PITX2-PORCN-SFRP4	52035	24948	36616	49778	2115
CCND1-CTBP1-PITX2	47450	37599	38364	47623	15808	FSHB-FZD2-PITX2	21425	53181	12066	7803	49923
PITX2-WNT3-WNT5A	3631	37403	19670	4320	50237	CCND2-LRP6-PITX2	45303	45054	55240	46174	9660
PITX2-PORCN-SFRP1	5118	32289	20516	7388	55055	AES-EP300-PITX2	27429	13782	22774	24073	54041
FZD6-PITX2-SFRP4	2191	34024	23773	8678	52481	PITX2-WNT3-WNT3A	1072	29636	4611	11649	49643
DAAM1-FOXN1-PITX2	23402	15789	15327	5498	42400	CSNK2A1-JUN-PITX2	4226	47140	759	5831	41428
FBXW11-LRP6-PITX2	19674	55920	14183	7236	52984	CCND1-CTNNBIP1-PITX2	23479	13649	20332	26184	43389
CSNK2A1-FOXN1-PITX2	484	10646	14993	25101	56105	FOXN1-GSK3A-PITX2	55917	8017	43971	38630	16813
AXIN1-FZD6-PITX2	45899	20191	43656	43529	4707	FRZB-GSK3A-PITX2	42536	49066	41808	51663	3945
PITX2-WNT1-WNT5A	34	29476	27919	8439	25717	BTRC-GSK3A-PITX2	53128	50908	30242	52659	1281
PITX2-SFRP4-WNT2B	7927	31067	16874	24744	41200	DAAM1-JUN-PITX2	24371	14681	11567	17251	47118
CXXC4-JUN-PITX2	7081	3972	8713	3640	32183	APC-PITX2-WNT3	14234	12446	706	24449	41729
PITX2-PORCN-PPP2CA	48624	10701	36145	29228	15037	DIXDC1-PITX2-WNT2B	3174	55687	15258	13757	29932
FBXW11-FOXN1-PITX2	13141	28980	22463	8005	34303	CSNK1D-FGF4-PITX2	45761	16034	33072	48233	21295
LRP5-PITX2-SFRP4	315	27436	8203	2763	45221	DIXDC1-PITX2-WNT4	44341	8459	33406	35610	21988
FOSL1-GSK3A-PITX2	3336	36071	6692	8964	29002	FBXW11-JUN-PITX2	27953	2910	15908	22	55248
CCND3-PITX2-WNT3A	34394	29164	30661	48295	16862	CSNK2A1-MYC-PITX2	9129	7731	935	12301	36666
EP300-FZD2-PITX2	24012	15242	9973	25729	44122	CSNK1D-FOXN1-PITX2	18384	31000	27911	15993	34137
FZD1-GSK3A-PITX2	49460	16018	43507	46060	9217	CCND3-PITX2-SFRP4	22350	19459	24189	16717	42599
DVL1-EP300-PITX2	548	36053	11918	25848	49131	DAAM1-GSK3A-PITX2	40753	11421	43484	37867	5004
DKK1-PITX2-SFRP4	694	29442	14079	22614	54046	GSK3B-PITX2-SFRP4	14898	11432	9059	22640	47560
FBXW2-JUN-PITX2	12579	4618	17745	364	46971	FRZB-FZD2-PITX2	35229	41345	36872	51138	49950
DIXDC1-PITX2-PPP2CA	53041	37364	46121	33605	10550	DAAM1-LRP6-PITX2	16446	14829	15814	16673	38836
FOSL1-FOXN1-PITX2	54512	5376	32845	52595	9349	CTNNB1-FOXN1-PITX2	29063	19206	49013	56402	27258
PITX2-WNT1-WNT2B	3768	50433	24960	15608	43963	CCND3-PITX2-SENP2	22323	19475	21774	24829	24809
FZD5-PITX2-WNT2B	45840	6879	31831	55937	11210	FZD7-GSK3A-PITX2	8237	8741	24973	13187	10820
AXIN1-EP300-PITX2	1837	53367	4684	5496	23397	FRAT1-GSK3A-PITX2	44421	52054	29767	47337	1646
CSNK1G1-FZD2-PITX2	4443	9218	19882	21920	41937	FOSL1-FZD2-PITX2	18628	35229	27305	27474	41987
FBXW11-PITX2-SFRP4	22007	13276	18287	6972	23461	CSNK1G1-PITX2-SENP2	42929	46890	54375	42726	15165
PITX2-PYGO1-TLE2	19850	10379	18180	6093	40940	CTBP2-FOXN1-PITX2	12690	32085	14231	16779	36765
DVL1-FOXN1-PITX2	13716	11451	12180	2594	46581	CTBP1-JUN-PITX2	24353	53693	13796	3746	36415
PITX2-WNT1-WNT4	55244	8137	33549	51863	7332	DIXDC1-PITX2-RHOU	1183	50113	10813	1973	40351
FZD6-PITX2-TCF7	58	54592	26110	20224	55626	DKK1-GSK3A-PITX2	51992	863	44516	56355	32287
FZD6-PITX2-SENP2	432	817	17937	22896	56866	APC-PITX2-SLC9A3R1	17574	24386	9813	27607	44761
FBXW11-PITX2-WNT2B	29169	24449	32207	29680	32687	DIXDC1-PITX2-TCF7	53422	53152	36175	42927	22338
CSNK1D-PITX2-SFRP4	26609	44292	24260	17256	33381	FZD5-CCND3-PITX2	19393	32161	13355	11683	5330
FZD6-PITX2-WNT2	14637	10126	24195	27724	51796	AES-FOXN1-PITX2	17914	21798	703	10325	20162
CSNK1G1-PITX2-RHOU	14243	10287	2792	14397	41970	FZD7-NKD1-PITX2	1376	4501	11215	16178	40845
FGF4-JUN-PITX2	18457	36152	1440	2969	44768	CCND1-NLK-PITX2	48102	11112	41884	52866	812
FZD6-PITX2-SFRP1	56728	56346	39190	34205	286	CXXC4-FGF4-PITX2	38294	56427	50581	38505	7533
AXIN1-FOXN1-PITX2	4350	36135	20767	6550	49005	FOSL1-PITX2-SFRP4	48140	54056	50405	43960	31198
FOSL1-NKD1-PITX2	9221	29087	12816	22289	29068	DKK1-PITX2-WNT2B	54626	45844	39615	30595	28144
FZD5-PITX2-RHOU	55488	4203	52989	44916	13289	GSK3B-PITX2-SENP2	16336	29784	11616	16048	44166
FZD6-PITX2-TCF7L1	57098	2574	31025	36904	1546	CTBP1-FOXN1-PITX2	8046	12489	6826	22448	30443
FBXW2-GSK3A-PITX2	36714	12730	37356	49810	4808	EP300-GSK3B-PITX2	26038	11762	5959	1368	52250
CTBP1-FGF4-PITX2	41855	4408	56680	38243	15293	FRAT1-FZD2-PITX2	55832	24730	44050	40886	5856
AES-FZD2-PITX2	29806	34809	44098	30337	5206	FBXW11-GSK3A-PITX2	35583	52800	48903	44758	21694
FZD8-JUN-PITX2	14227	28361	22535	6380	50233	CXXC4-FZD2-PITX2	49278	49869	33082	55290	10056
PITX2-SFRP4-TLE2	16042	46434	18733	25251	47254	DIXDC1-FZD2-PITX2	7897	43672	18332	7315	28673

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Table 4. Rankings of PITX2-X-X. A list of approximately first 125 combinations with rankings below 10,000 out of 57,155. SA - SOBOL; Implementation - martinez

	RANKING @ t_i USING SOBOL - MARTINEZ										
3rd order comb.	t_1	t_3	t_6	t_{12}	t_{24}	3rd order comb.	t_1	t_3	t_6	t_{12}	t_{24}
APC-PITX2-SFRP4	21135	9002	6211	25291	18551	APC-PITX2-SENP2	8228	7160	4560	17437	34258
APC-PITX2-TCF7	10603	6873	14988	17051	4307	PITX2-PORCN-TLE2	34575	49797	39649	34525	4049
PITX2-PORCN-WNT4	9395	23474	32883	37297	24846	PITX2-PORCN-SENP2	20617	22563	38695	45455	12377
DVL2-JUN-PITX2	36525	53951	49133	9931	6910	FZD5-PITX2-SENP2	41803	45522	15594	45235	8865
APC-PITX2-PPP2CA	13736	5292	47096	11939	9629	APC-PITX2-RHOU	51464	18486	41767	41508	48864
PITX2-PORCN-WNT5A	23589	48950	34402	37236	3755	PITX2-PORCN-FBXW4	6203	42677	48707	17993	5734
DIXDC1-PITX2-SENP2	22480	35744	10230	30579	35447	CTNNBIP1-JUN-PITX2	22154	22207	5596	2608	22098
CCND1-FGF4-PITX2	50568	42194	37514	30676	15317	PITX2-PORCN-TCF7L1	4744	47264	23302	15463	5925
DIXDC1-PITX2-SFRP4	6126	5499	2542	31975	46995	PITX2-PORCN-WNT2B	32916	28214	19614	42831	17840
APC-PITX2-WNT4	33809	3550	4717	20161	40028	FRAT1-JUN-PITX2	12383	53362	24248	28478	2424
PITX2-PORCN-TLE1	26366	14552	49163	2938	25505	DKK1-JUN-PITX2	12302	18067	6655	22598	48385
AES-AXIN1-PITX2	12302	18067	6655	22598	48385	PITX2-PORCN-PPP2R1A	28551	48685	56979	48784	335
PITX2-PORCN-RHOU	5900	36986	56261	27068	40618	CCND1-JUN-PITX2	26952	23887	45413	42573	55139
FOSL1-JUN-PITX2	6068	2098	52232	48612	54047	FZD6-PITX2-WNT2B	10663	27608	9003	33499	56550
FZD5-PITX2-SFRP4	20840	47471	15797	20287	564	PITX2-PORCN-SFRP4	34090	15340	54473	55067	54878
CCND1-CTBP1-PITX2	30618	10940	32193	972	31794	FSHB-FZD2-PITX2	1274	54630	28998	48951	32890
PITX2-WNT3-WNT5A	11058	51240	29498	43821	8108	CCND2-LRP6-PITX2	33683	15677	29830	6781	45787
PITX2-PORCN-SFRP1	3773	36533	56407	54176	6456	AES-EP300-PITX2	1201	15547	40455	15398	55710
FZD6-PITX2-SFRP4	37787	24023	8434	15004	34203	PITX2-WNT3-WNT3A	27307	12715	32115	33802	9431
DAAM1-FOXN1-PITX2	1465	52252	45599	49025	5462	CSNK2A1-JUN-PITX2	27303	36268	42893	48996	26416
FBXW11-LRP6-PITX2	41653	34792	56343	41510	9933	CCND1-CTNNBIP1-PITX2	5144	23911	28169	30083	48576
CSNK2A1-FOXN1-PITX2	42869	56525	55768	53229	9861	FOXN1-GSK3A-PITX2	8151	3885	48407	38343	35200
AXIN1-FZD6-PITX2	30708	20336	36283	2992	46685	FRZB-GSK3A-PITX2	38546	36100	12520	26239	9128
PITX2-WNT1-WNT5A	34426	18511	22931	30346	5013	BTRC-GSK3A-PITX2	47166	48343	22973	655	24369
PITX2-SFRP4-WNT2B	927	39222	24017	42692	21210	DAAM1-JUN-PITX2	1061	37083	38686	12471	9610
CXXC4-JUN-PITX2	40461	38937	29744	13401	491	APC-PITX2-WNT3	4548	9482	3841	24293	17208
PITX2-PORCN-PPP2CA	13665	21071	35213	8665	8832	DIXDC1-PITX2-WNT2B	1930	455	20150	24837	6597
FBXW11-FOXN1-PITX2	21045	14442	3872	11989	28492	CSNK1D-FGF4-PITX2	44579	43462	767	11821	46293
LRP5-PITX2-SFRP4	44579	43462	767	11821	46293	DIXDC1-PITX2-WNT4	7785	53066	8449	29987	33965
FOSL1-GSK3A-PITX2	41751	14274	10983	10196	4550	FBXW11-JUN-PITX2	17329	8127	25753	12601	5194
CCND3-PITX2-WNT3A	17329	8127	25753	12601	5194	CSNK2A1-MYC-PITX2	17421	12288	5142	54094	13707
EP300-FZD2-PITX2	42426	55258	55923	31463	6536	CSNK1D-FOXN1-PITX2	39725	19950	32730	49412	6271
FZD1-GSK3A-PITX2	48467	33105	24061	31062	11460	CCND3-PITX2-SFRP4	4204	22026	19937	33723	37285
DVL1-EP300-PITX2	3179	18813	1618	47057	47210	DAAM1-GSK3A-PITX2	55926	34919	2198	26476	34092
DKK1-PITX2-SFRP4	41420	54154	2348	32631	39954	GSK3B-PITX2-SFRP4	33905	8750	32946	40959	35507
FBXW2-JUN-PITX2	7565	3918	56772	31902	36091	FRZB-FZD2-PITX2	34589	22932	3335	4626	9832
DIXDC1-PITX2-PPP2CA	13451	49710	9306	15472	45623	DAAM1-LRP6-PITX2	4226	51422	34496	17910	19674
FOSL1-FOXN1-PITX2	34136	42061	42013	21630	10002	CTNNB1-FOXN1-PITX2	30090	3174	40964	41038	24723
PITX2-WNT1-WNT2B	30090	3174	40964	41038	24723	CCND3-PITX2-SENP2	26238	35315	30434	4445	31508
FZD5-PITX2-WNT2B	54327	51773	28527	10450	30322	FZD7-GSK3A-PITX2	53257	11920	36889	50223	7187
AXIN1-EP300-PITX2	35819	48198	7128	5864	15595	FRAT1-GSK3A-PITX2	54984	44532	31462	10423	18225
CSNK1G1-FZD2-PITX2	27767	17670	44181	29136	6588	FOSL1-FZD2-PITX2	46849	14143	26285	25720	2468
FBXW11-PITX2-SFRP4	24062	50479	55339	34232	43276	CSNK1G1-PITX2-SENP2	21368	35661	31239	903	35353
PITX2-PYGO1-TLE2	4242	25999	8726	47004	18848	CTBP2-FOXN1-PITX2	31599	17908	37379	3256	50472
DVL1-FOXN1-PITX2	5966	14588	26787	49885	38378	CTBP1-JUN-PITX2	52350	15661	6369	48385	43960
PITX2-WNT1-WNT4	29539	42190	49683	54991	25868	DIXDC1-PITX2-RHOU	53274	4427	27249	13322	6203
FZD6-PITX2-TCF7	34797	1642	14315	5723	2841	DKK1-GSK3A-PITX2	19149	27520	14397	22460	50424
FZD6-PITX2-SENP2	3638	21706	10102	1780	5953	APC-PITX2-SLC9A3R1	17135	12041	37141	24875	35496
FBXW11-PITX2-WNT2B	21655	37846	5599	10411	15406	DIXDC1-PITX2-TCF7	14448	13847	2584	29387	42594
CSNK1D-PITX2-SFRP4	783	12794	45331	28854	43563	FZD5-CCND3-PITX2	2967	9267	43490	56564	1817
FZD6-PITX2-WNT2	18122	28288	4331	4664	2986	AES-FOXN1-PITX2	35956	4067	8624	18700	37957
CSNK1G1-PITX2-RHOU	3677	41232	10163	26187	14032	FZD7-NKD1-PITX2	21282	14799	25761	53461	20283
FGF4-JUN-PITX2	47903	44221	6764	16153	53529	CCND1-NLK-PITX2	34312	8877	197	29441	23686
FZD6-PITX2-SFRP1	47783	46454	12281	1010	33862	CXXC4-FGF4-PITX2	15949	22104	53797	53381	41155
AXIN1-FOXN1-PITX2	41205	52384	56750	25249	1165	FOSL1-PITX2-SFRP4	9026	16248	48877	1791	54481
FOSL1-NKD1-PITX2	50255	20028	7545	39345	9274	DKK1-PITX2-WNT2B	31169	20699	272	25542	32964
FZD5-PITX2-RHOU	38238	30956	45448	44396	35805	GSK3B-PITX2-SENP2	53063	6524	48104	47493	2780
FZD6-PITX2-TCF7L1	23995	53280	5248	34635	32343	CTBP1-FOXN1-PITX2	40602	56308	50160	12837	53360
FBXW2-GSK3A-PITX2	30236	5683	49721	53618	29062	EP300-GSK3B-PITX2	32327	39363	15231	36079	50640
CTBP1-FGF4-PITX2	22020	31745	53245	9706	10584	FRAT1-FZD2-PITX2	38027	8675	30814	7454	12758
AES-FZD2-PITX2	28125	7101	38481	20396	36144	FBXW11-GSK3A-PITX2	55105	985	398	46541	28183
FZD8-JUN-PITX2	51716	20227	40146	38514	47991	CXXC4-FZD2-PITX2	54393	44977	17036	3028	55722
PITX2-SFRP4-TLE2	810	13707	22911	51972	37332	DIXDC1-FZD2-PITX2	616	9921	12975	22276	34068

6.3.1. Examining the behaviour of WNT-PITX2-X combinations

Kioussi et al. [19] report that PITX2 is rapidly induced by the WNT/DVL/β-catenin pathway and is required for effective cell-type-specific proliferation by directly activating specific growth-regulating genes. Regulated exchange of HDAC1/β-catenin converts PITX2 from repressor to activator (analogous to control of TCF/LEF1) which then serves as a competence factor required for the tempo-

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rally ordered and growth factor-dependent recruitment of a series of specific coactivator complexes that prove necessary for CCND2 gene induction. Since the WNT pathway is strongly involved in ovarian development and cancer, Basu and Roy [20] focused on the possible association between PITX2 and WNT pathway in ovarian carcinoma cells and found that PITX2 interacts and regulates WNT2/5A/9A/6/2B genes of the canonical, noncanonical, or other pathways in the human ovarian adenocarcinoma cell SKOV-3. Chromatin immunoprecipitation and promoter-reporter assays further indicated the significant association of PITX2 with WNT2 and WNT5A promoters. Looking at the tables above, one finds the following combinations for member of WNT family along with PITX2, to be prominent at 3rd order level - PITX2-PORCN-WNT4, PITX2-PORCN-WNT5A, APC-PITX2-WNT4, PITX2-WNT3-WNT5A, PITX2-WNT1-WNT5A, PITX2-SFRP4-WNT2B, CCND3-PITX2-WNT3A, PITX2-WNT1-WNT2B, FZD5-PITX2-WNT2B, PITX2-WNT1-WNT4, FBXW11-PITX2-WNT2B, FZD6-PITX2-WNT2, PITX2-PORCN-WNT2B, FZD6-PITX2-WNT2B, PITX2-WNT3-WNT3A, APC-PITX2-WNT3, DIXDC1-PITX2-WNT2B, DIXDC1-PITX2-WNT4 and DKK1-PITX2-WNT2B. All these combinations indicate the existence of a possible synergy when they take a higher rank in the list of combinations.

6.3.2. Examining the Behaviour of FGF / CCND -PITX2-X Combinations

PITX2 is mutated in the haploinsufficient Rieger Syndrome type 1 that includes dental, ocular and abdominal wall anomalies as cardinal features. Analysis of the craniofacial phenotype of PITX2-null mice has revealed that PITX2 was both a positive regulator of fibroblast growth factor 8 (FGF8) and a repressor of BMP4-signaling. Liu et al. [21] show by analysis that PITX2 allelic combinations that encode varying levels of PITX2 showed that repression of BMP signaling requires high PITX2 while maintenance of FGF8 signaling requires only low PITX2.

Cleft palate is a common congenital birth defects. Transforming growth factor β (TGFβ) signaling regulates craniofacial development, and loss of TGFβ receptor type II in cranial neural crest cells leads to craniofacial malformations, including cleft palate in mice (Tgfbr2$^{fl/fl}$;WNT1-Cre mice). Iwata et al. [22] indicate that a TGFβ-FGF9-PITX2 signaling cascade regulates cranial neural crest cell proliferation during palate formation. They found that FGF9 and PITX2 expressions were significantly down-regulated in the palate of Tgfbr2$^{fl/fl}$;WNT1-Cre mice, and FGF9 and PITX2 loss of function mutations resulted in cleft palate in mice. PITX2 expression was found to be down-regulated by siRNA knockdown of FGF9, suggesting that FGF9 is upstream of PITX2. Further, exogenous FGF9 restores expression of CCND1 and CCND3 in a PITX2-dependent manner and rescues the cell proliferation defect in the palatal mesenchyme of Tgfbr2$^{fl/fl}$; WNT1-Cre mice.

Looking at the tables above, one finds the following combinations for member of FGF family along with PITX2, to be prominent at 3rd order level - CCND1-FGF4-PITX2, FGF4-JUN-PITX2, CTBP1-FGF4-PITX2, CSNK1D-FGF4-PITX2 and CXXC4-FGF4-PITX2. Also, looking at the tables above, one finds the following combinations for member of CCND family along with PITX2, to be prominent at 3rd order level - CCND1-FGF4-PITX2, CCND1-CTBP1-PITX2, CCND3-PITX2-WNT3A, CCND1-JUN-PITX2, CCND2-LRP6-PITX2, CCND1-CTNNBIP1-PITX2, CCND3-PITX2-SFRP4, CCND3-PITX2-SENP2, FZD5-CCND3-PITX2 and CCND1-NLK-PITX2. All these combinations indicate the existence of a possible synergy when they take a higher rank in the list of combinations.

6.3.3. Examining the behaviour of FSHB-PITX2-X combinations

PITX regulate the activity of pituitary hormone-encoding genes and Lamba et al. [23] examined mechanisms through which the family of PITX proteins control murine follicle stimulating hormone β-subunit (FSHB) transcription. They found that both PITX-1/2C regulated murine and human Fshb/FSHB transcription through a conserved cis-element in the proximal promoter. Looking at the tables above, one finds the following combinations FSHB along with PITX2, to be prominent at 3rd order level - FSHB-FZD2-PITX2. All these combinations indicate the existence of a possible synergy when they take a higher rank in the list of combinations.

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6.3.4. Examining the behaviour of FOX-PITX2-X combinations

Axenfeld–Rieger ocular dysgenesis is associated with mutations of the human PITX2 and forkhead box C1 (FOXC1) genes. Berry et al. [24] identified a functional link between FOXC1 and PITX2 which underpins the similar Axenfeld–Rieger phenotype caused by mutations of these genes. They show that FOXC1 and PITX2A physically interact, and this interaction requires crucial functional domains on both proteins: the C-terminal activation domain of FOXC1 and the homeodomain of PITX2. Immunofluorescence further showed FOXC1 and PITX2A to be colocalized within a common nuclear subcompartment. Looking at the tables above, one finds the following combinations for members of FOX family along with PITX2, to be prominent at 3rd order level - DAAM1-FOXN1-PITX2, CSNK2A1-FOXN1-PITX2, FBXW11-FOXN1-PITX2, FOSL1-FOXN1-PITX2, DVL1-FOXN1-PITX2, AXIN1-FOXN1-PITX2, FOXN1-GSK3A-PITX2, CSNK1D-FOXN1-PITX2, CTNNB1-FOXN1-PITX2, CTBP2-FOXN1-PITX2, AES-FOXN1-PITX2 and CTBP1-FOXN1-PITX2. All these combinations indicate the existence of a possible synergy when they take a higher rank in the list of combinations.

6.3.5. Examining the behaviour of JUN-PITX2-X combinations

Briata et al. [25] report that PITX2 mRNA displays a rapid turnover rate and that activation of the Wnt/β-catenin pathway stabilizes PITX2 mRNA as well as other unstable mRNAs,including c-JUN and CCND-1/2 encoded pathway. There might not be an interaction between JUN and PITX2, but they might be synergistically getting stabilized due to the WNT pathway. Looking at the tables above, one finds the following combinations for JUN along with PITX2, to be prominent at 3rd order level - DVL2-JUN-PITX2, FOSL1-JUN-PITX2, CXXC4-JUN-PITX2, FBXW2-JUN-PITX2, FGF4-JUN-PITX2, FZD8-JUN-PITX2, CTNNBIP1-JUN-PITX2, FRAT1-JUN-PITX2, DKK1-JUN-PITX2, CCND1-JUN-PITX2, CSNK2A1-JUN-PITX2, DAAM1-JUN-PITX2, FBXW11-JUN-PITX2 and CTBP1-JUN-PITX2. All these combinations indicate the existence of a possible synergy when they take a higher rank in the list of combinations.

6.3.6. Examining the behaviour of EP300-PITX2-X combinations

Malformations of the septum, outflow tract and aortic arch are the most common congenital cardiovascular defects and occur in mice lacking CITED2, a transcriptional coactivator of TFAP2. Bamforth et al. [26] show that CITED2 and TFAP2 were detected at the PITX2C promoter in embryonic hearts, and they activate PITX2C transcription in transient transfection assays. They propose an abnormal NODAL-PITX2C pathway presents a mechanism for the cardiovascular malformations observed in CITED2$^{-/-}$ mice, and that such malformations may be the sole manifestation of a laterality defect. Further, EP300 and CREBBP interact with high affinity with CITED2. CITED2 also physically interacts with and coactivates TFAP2 (transcription factor AP2) and LIM-domain containing transcription factors by linking them to EP300 and CREBBP (Bamforth et al. [27]). Looking at the tables above, one finds the following combinations for EP300 along with PITX2, to be prominent at 3rd order level - EP300-FZD2-PITX2, DVL1-EP300-PITX2, AXIN1-EP300-PITX2, AES-EP300-PITX2 and EP300-GSK3B-PITX2. All these combinations indicate the existence of a possible synergy when they take a higher rank in the list of combinations.

7. Conclusion

This manuscript studies the time behaviour of 3rd order combinations of PITX2 in WNT3A stimulated HEK 293 cells. Based on the extablished 2nd order combinations of the PITX2, 3rd order combinations emerge using the machine learning based search engine. These 3rd order combinations might be of interest for further wet lab investigations.

Supplementary Materials: The following files (ending with .txt and can be opened in R or in simple text processing program) with these names are made available with this manuscript. For every WNT family member, (1) **-3-odr-TP-ranking-linear.txt**, (2) **-3-odr-TP-ranking-rbf.txt**, (3) **-3-odr-TP-ranking-2002.txt**, and (4) **-3-odr-TP-**

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ranking-martinez.txt, contain rankings for 3rd order combinations across each time point for, HSIC (linear kernel), HSIC (rbf kernel), SOBOL (2002 implementation) and SOBOL (martinez implementation), respectively.

Author Contributions: SS conceived and designed the experiments; wrote the code; performed the experiments; analyzed the data; wrote the manuscript.

Data Availability Statement: Code for time series data available at CERN based Zenodo on https://zenodo.org/records/14637456.

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