Axitinib exposure triggers endothelial cells senescence through ROS accumulation and ATM activation

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Supplementary Materials

1. RNA-Seq reads quality control, trimming and mapping

Read quality was examined using FastQC (v0.11.7). After adapter sequence removal, read ends were trimmed if base quality scores were lower than Q20, using Trimmomatic (Bolger *et al*., 2014). All FastQC tests were passed, with the exception of Per-base Sequence Content, Sequence Duplication Levels and Kmer Content, which is common in RNA-seq experiments. After trimming, reads with the total length shorter than 25 bases, with average quality less than Q20, or with the fraction of undetermined bases >2% were discarded. The resulting cleaned reads were mapped to the GRCh38 human genome assembly using Bowtie v2.3.4.1 (Langmead and Salzberg, 2012) and TopHat v2.1.1 (Kim *et al*., 2013), using default parameters. The total number of reads per replicate, the reads surviving trimming and the mapped reads are reported in Table 1. The read alignment rate ranges from a minimum of 98.2% to a maximum of 98.6%.

	Total reads	Reads after trimming	Mapped reads
HUVEC rep1	25,052,056	24,993,794	24,606,227
HUVEC rep2	23,951,111	23,882,880	23,459,693
HUVEC rep3	25,299,157	25,241,364	24,826,760
DOXO rep1	24,057,993	23,999,785	23,621,719
DOXO rep2	25,356,344	25,298,268	24,909,066
DOXO rep3	21,769,915	21,719,496	21,377,982
AXI rep1	22,514,354	22,460,874	22,120,794
AXI rep2	26,541,942	26,477,103	26,097,757
AXI rep3	19,125,654	19,069,705	18,752,745

Table 1. Breakdown of the total number of reads, reads after trimming and mapped reads for each replicate. HUVEC: Human Umbilical Vein Endothelial Cells; DOXO: HUVEC cells after Doxorubicin (5 μM) one-hour pulse; AXI: HUVEC cells after Axitinib (25 μM) one-hour pulse.

2. Expression estimates and differential expression analysis

From the mapped reads, transcripts were reconstructed and their expression levels were measured using Cufflinks v2.1.1 (Trapnell *et al*., 2012) provided with human gene structures in GTF format, downloaded from Ensembl (release 91); expression was reported as Fragments Per Kilobase of transcript per Million mapped reads (FPKM). Differential expression was tested using the CuffDiff module of Cufflinks, comparing HUVEC cells normal expression with both drug treatments (i.e. Doxorubicin (5 μM) one-hour pulse and Axitinib (25 μM) one-hour pulse). Genes were considered differentially expressed by setting a significance threshold for FDR-corrected p-values at 0.05. The total number of differentially expressed genes for each comparison is reported in Figure 8 in the main text.

The output of CuffDiff was analyzed with cummerbund (Trapnell *et al*., 2012), to perform quality controls and global statistics on the differential analysis tests. Figure 1 reports the count vs. dispersion plot, showing that, even though dispersion is spread, it remains in an acceptable range. Similarly, Figure 2 reports the squared coefficient of variation, a normalized measure of cross-replicate variability, showing a standard behavior.

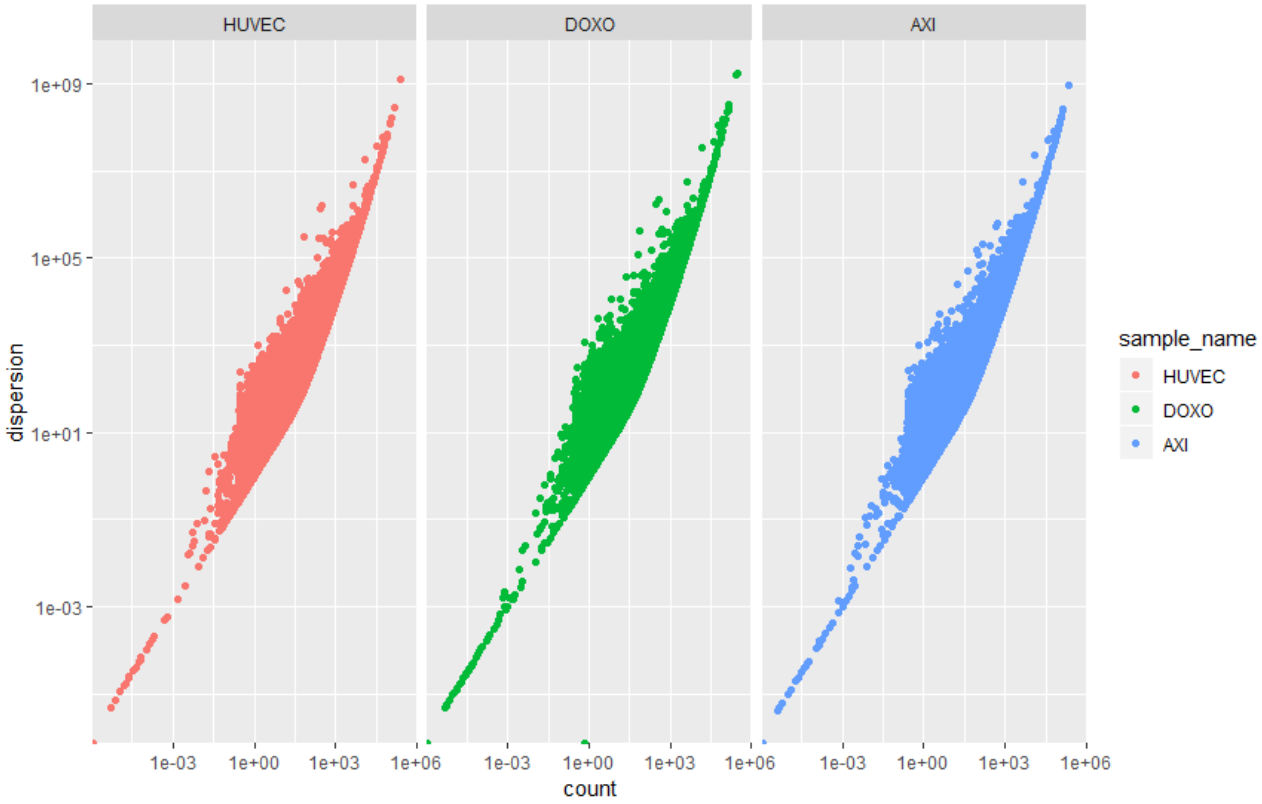

Figure 1. Dispersion vs. count plot for the three RNA-seq samples.

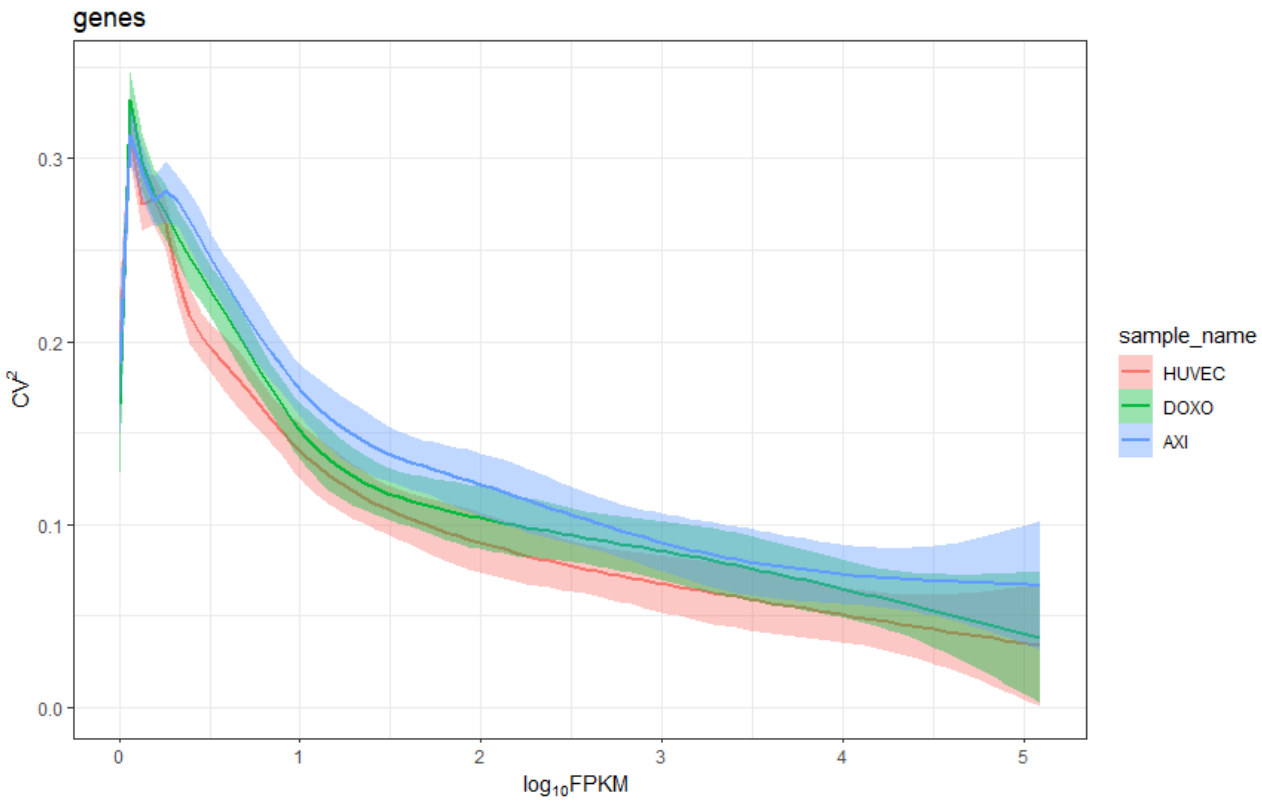

Figure 2. Squared coefficient of variation (CV^2) vs log_{10}FPKM plot.

The distributions of expression values in FPKM units are reported in Figure 3 as density curves and Figure 4 as boxplots. The overall trend is a general similarity, is range and shape, among the three analyzed conditions

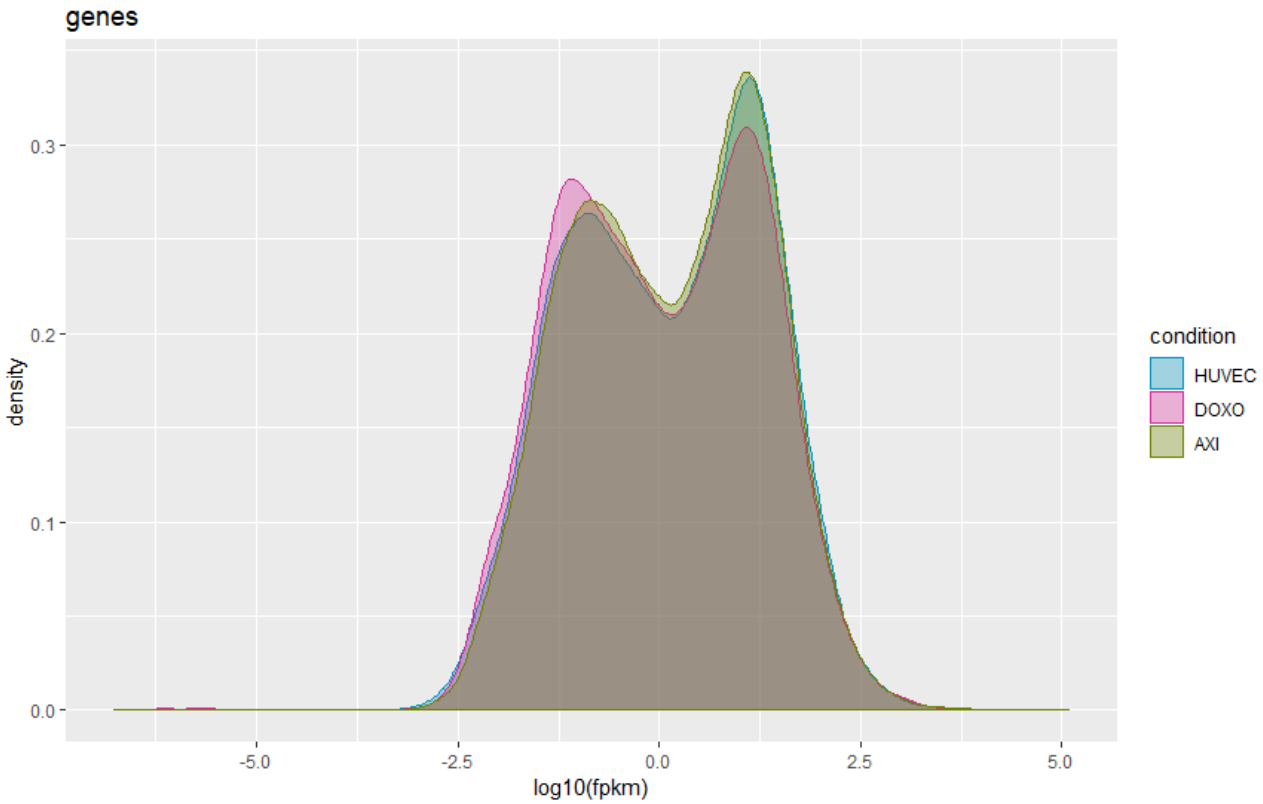

Figure 3. Density plot of the expression score distributions.

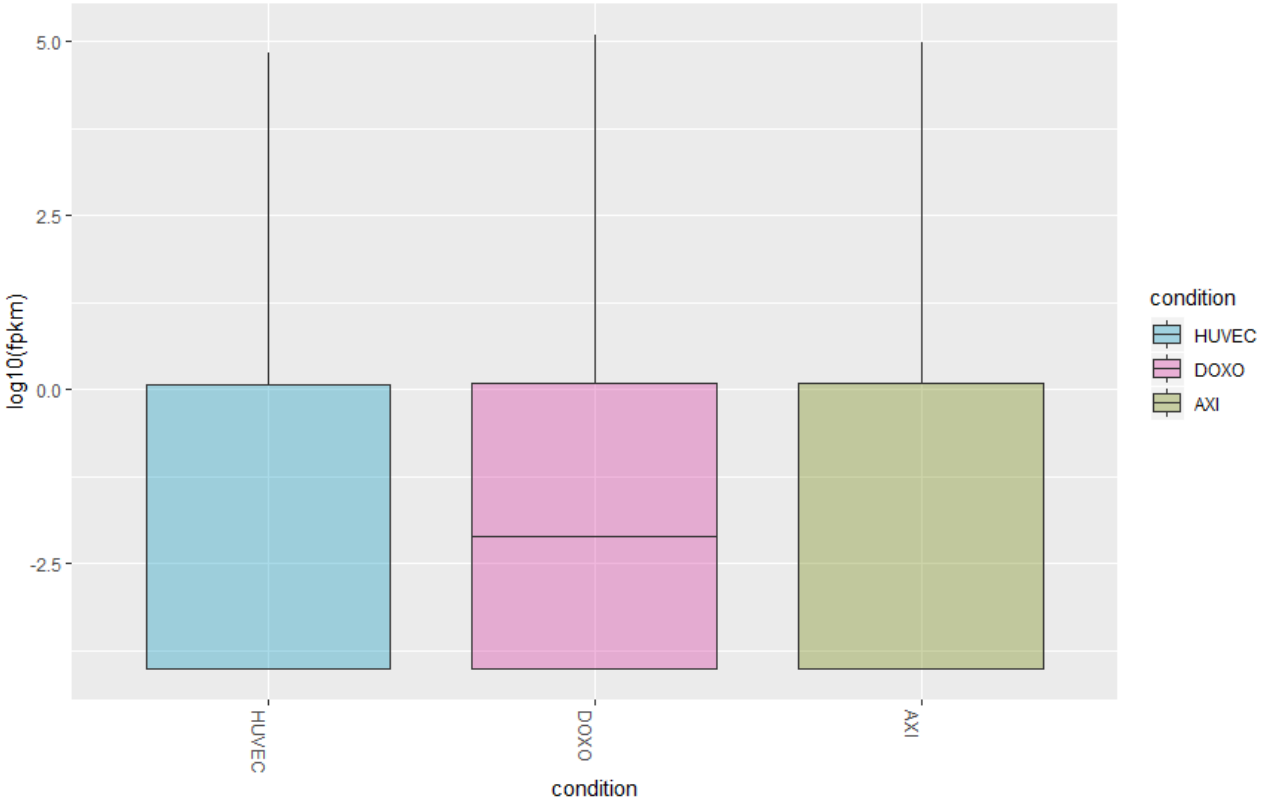

Figure 4. Boxplot of the expression score distributions.

A more precise breakdown is reported as a scatterplot matrix, in which each FPKM estimates are compared for each gene in all pairs of conditions (Figure 5).

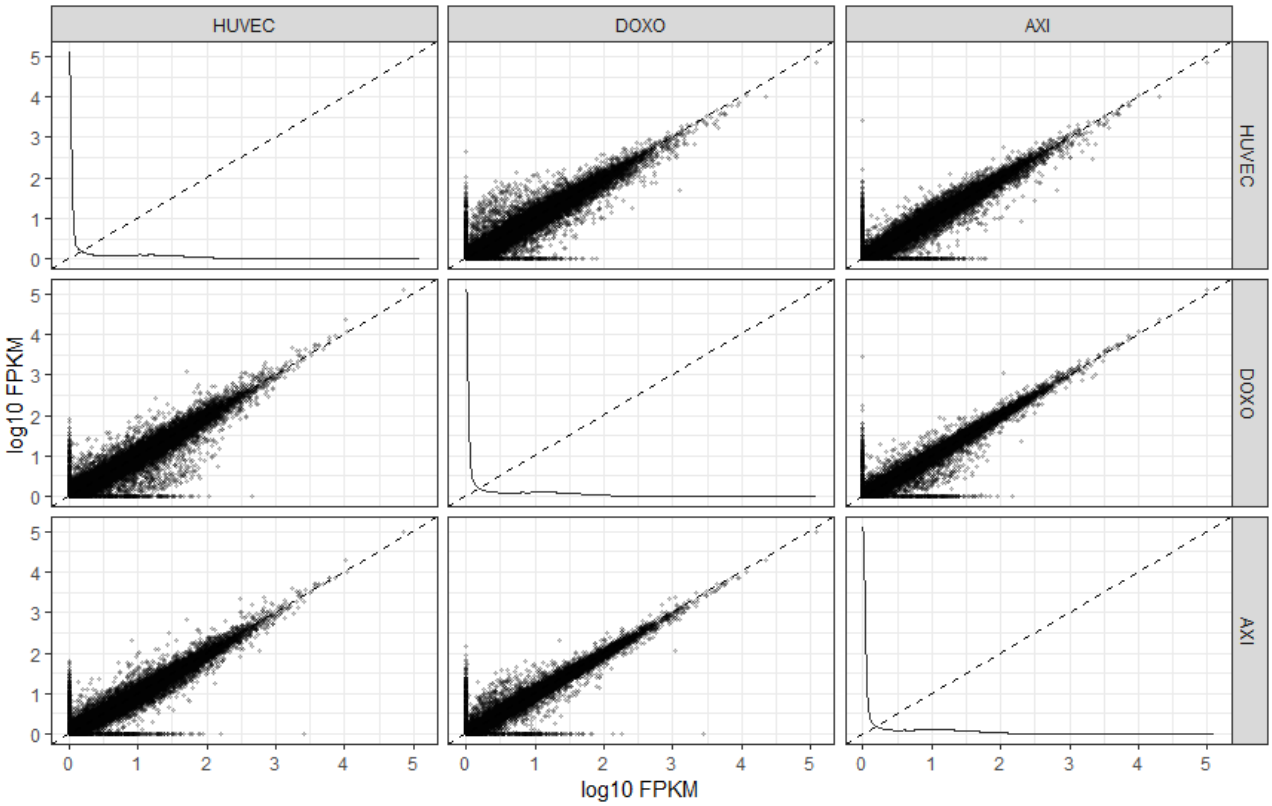

Figure 5. FPKM scatterplot matrix of scores for all possible pairs of conditions.

MA-plots, reporting fold-change vs. signal intensity also show no evident abnormalities, with a smaller correlation at low expression levels that increases towards higher expression values (Figure 6 and 7).

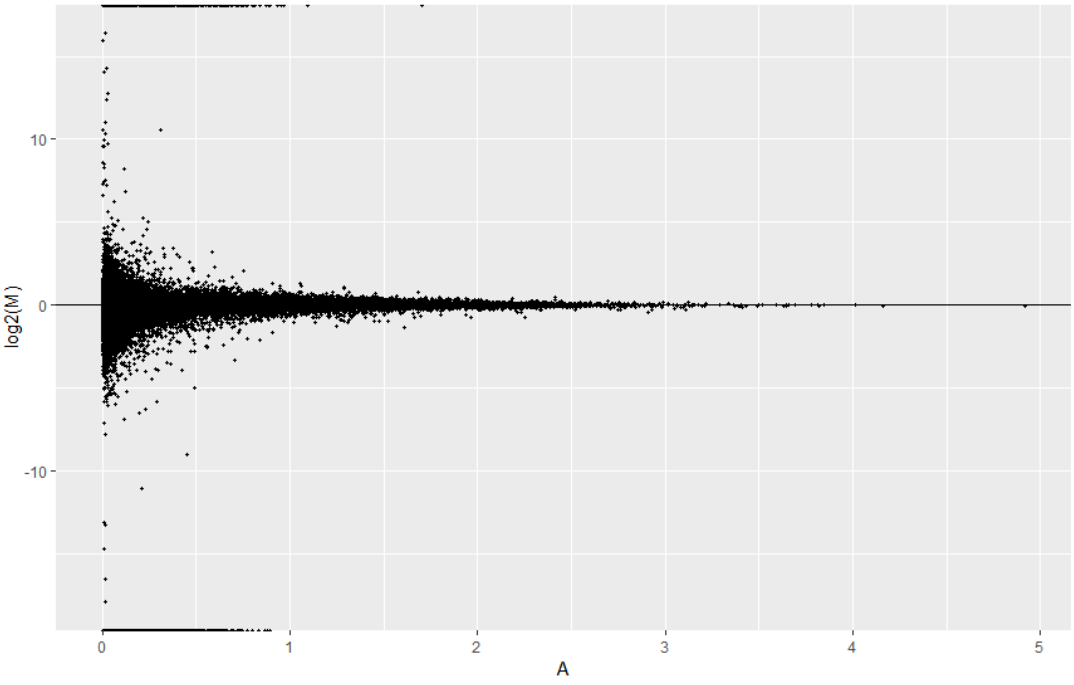

Figure 6. MA-plot for the HUVEC vs. DOXI comparison.

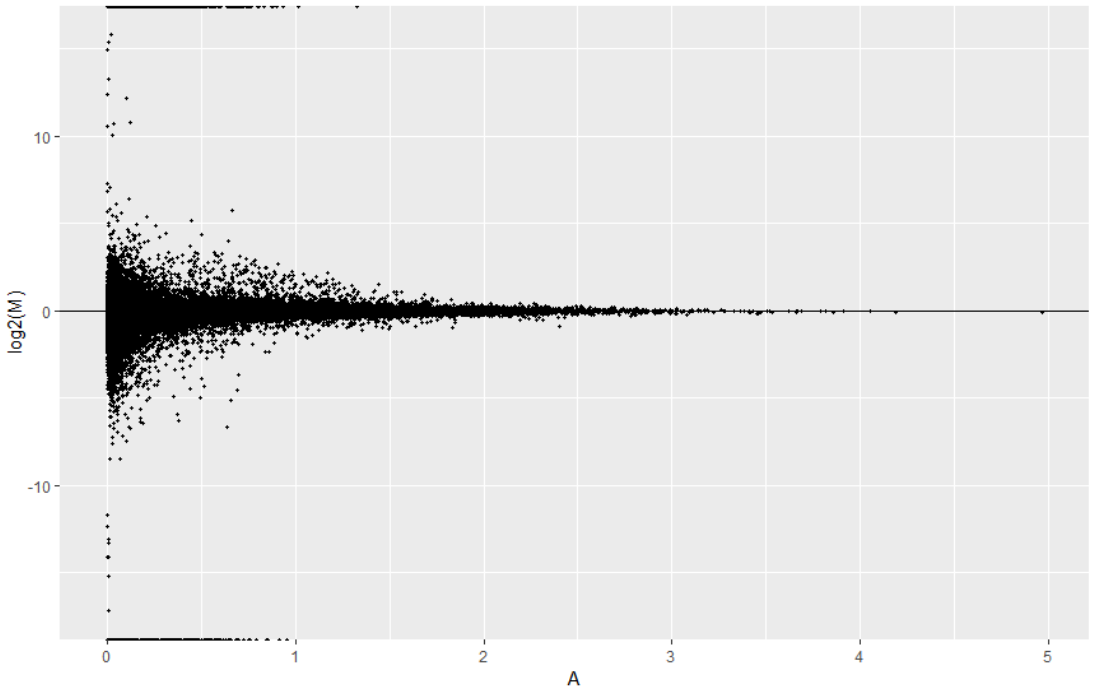

Figure 7. MA-plot for the HUVEC vs. AXI comparison.

Volcano plots, showing the relation between fold-change and statistical significance, show a relevant number of genes having relatively limited fold-change that was deemed significant (Figure 8). This behavior can be considered consistent with the overall similar expression distributions, the degree of dispersion and the fairly high sequencing coverage.

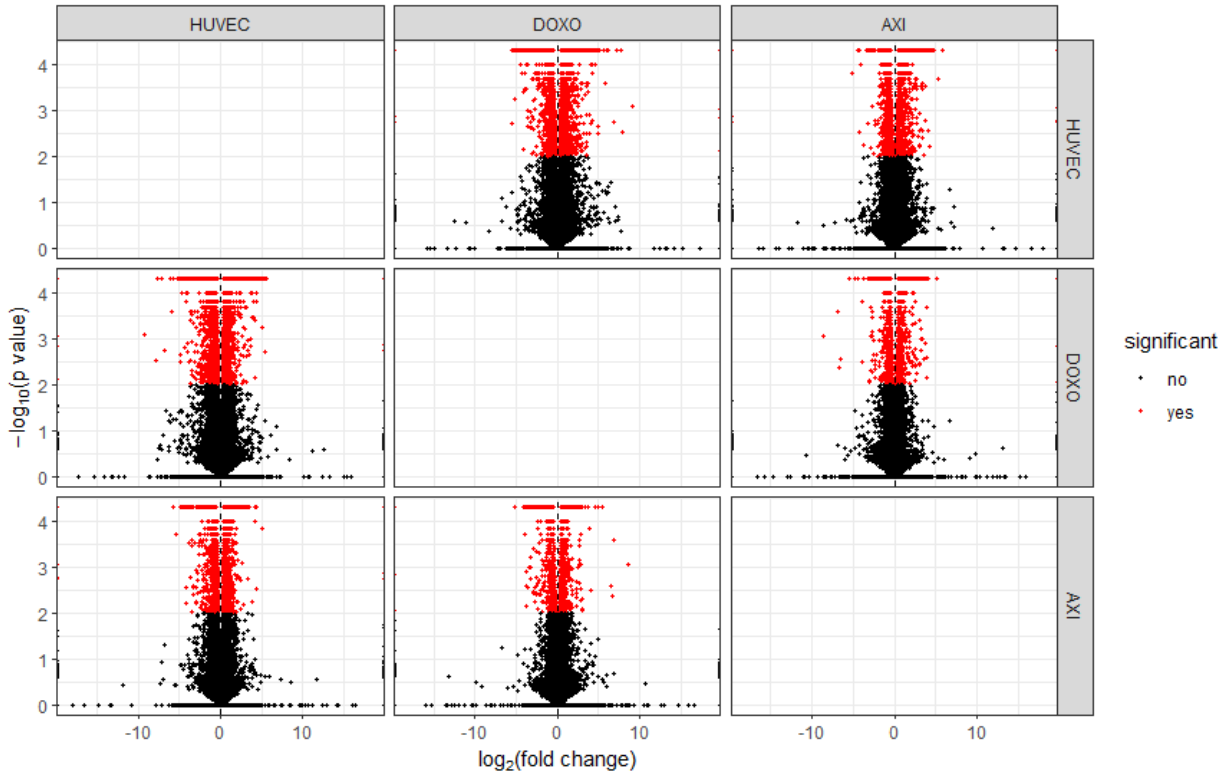

Figure 8. Matrix of volcano plots (p-value vs. fold-change) for all pairs of comparisons.

We also assessed the concordance of the three biological replicates that were produced for each condition. Figure 9 shows a dendrogram computed on the Jensen-Shannon distance of the gene expression profile of each replicate. Replicates for the same condition are grouped together, and the two treatments (AXI and DOXO) are closer to each other than to untreated cells (HUVEC)

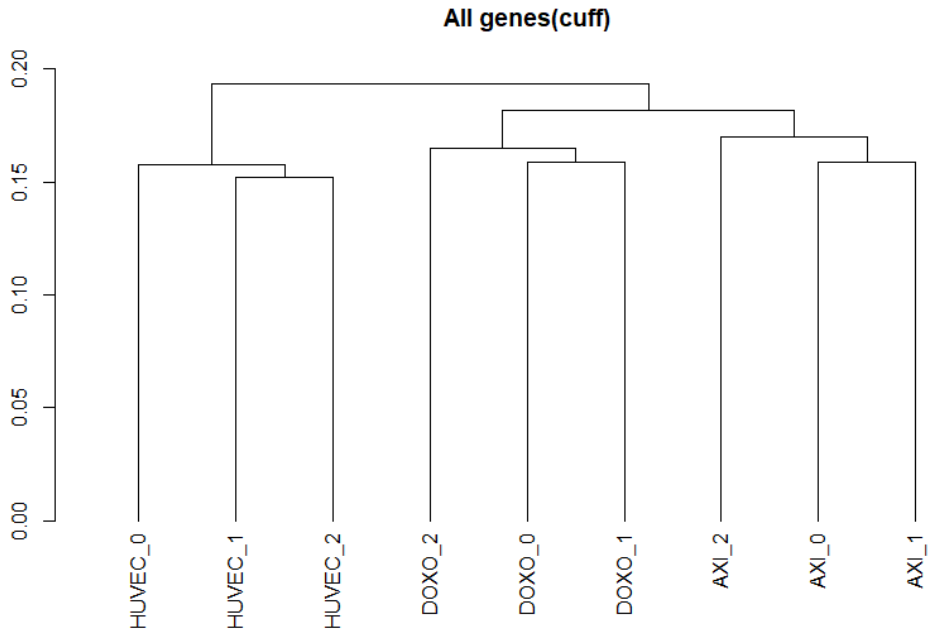

Figure 9. Matrix of volcano plots (p-value vs. fold-change) for all pairs of comparisons.

Dimensionality reductions also confirm the biological replicates concordance, by means of both a multi-dimensional scaling (Figure 10) and principal components analysis (Figure 11).

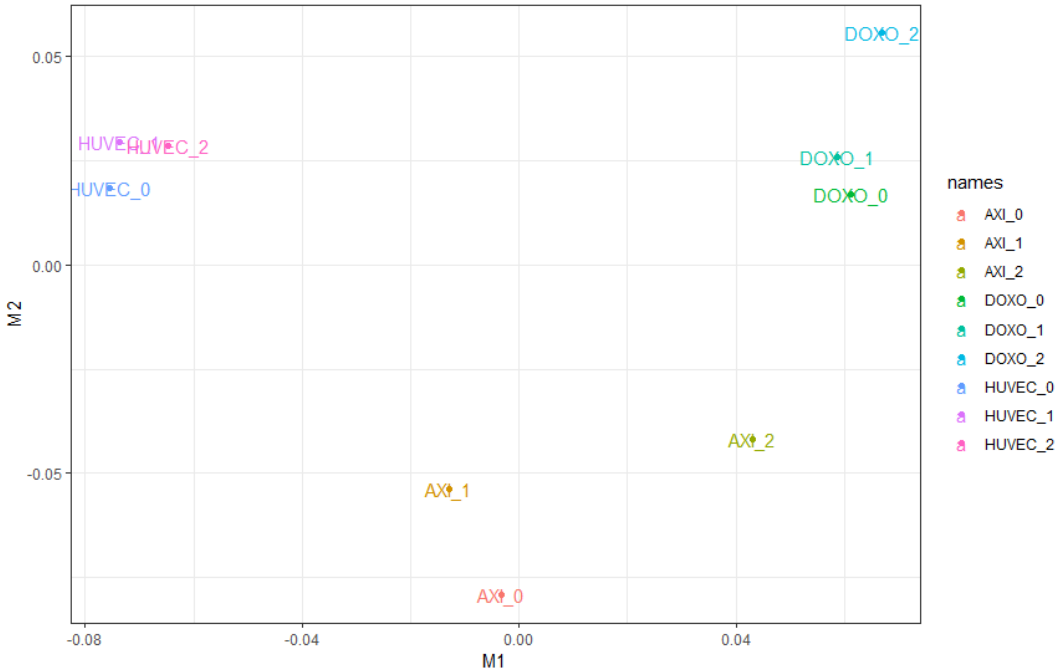

Figure 10. MDS (multi-dimensional scaling) of gene-level expression estimate profiles of individual replicates.

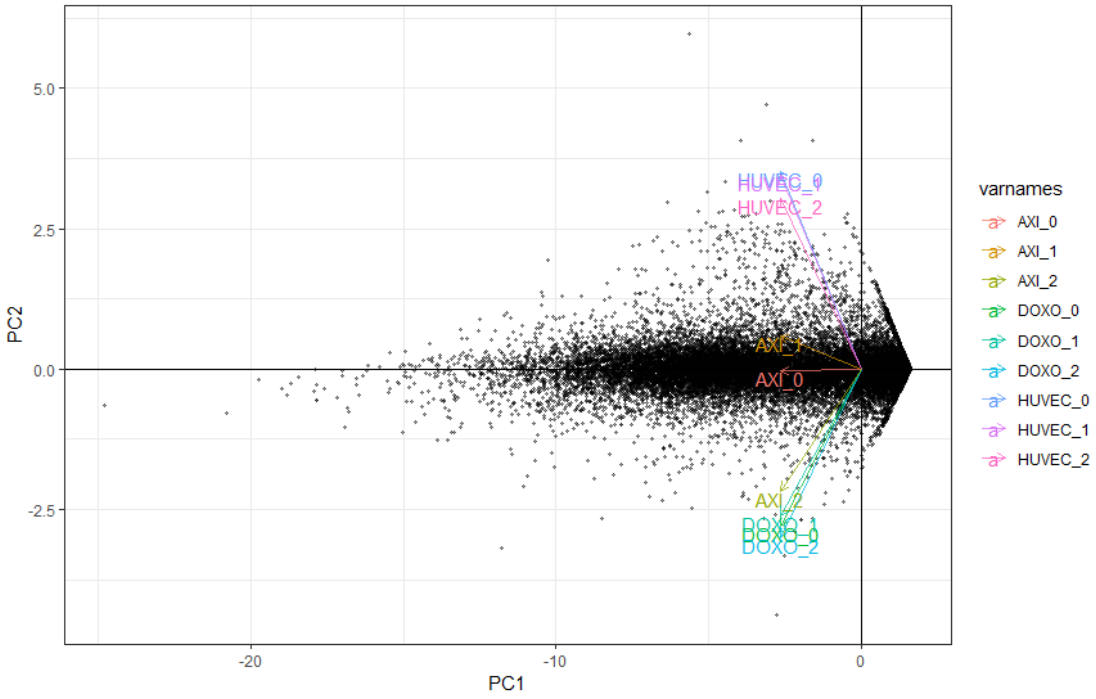

Figure 11. PCA (principal components analysis) of gene-level expression estimate profiles of individual replicates.

Overall, all controls do not evidence any abnormalitie that might introduce biases in the identification of differentially expressed genes in the compared condition pairs.

3. Functional Interpretation

3.1 Differentially expressed genes Gene Ontology terms enrichment

Functional enrichment analysis was performed using the DAVID web server (Huang *et al*., 2009). Functional enrichment analysis of all Axitinib-induced differentially expressed genes (DEGs) revealed significant enrichment (at significance threshold 0.05) of 235 Gene Ontology (GO) terms belonging to the Biological Process domain, 66 GO terms belonging to the Cellular Component (CC) domain, and 65 belonging to the Molecular Function (MF) domain. For the Doxorubicin-treated cells, we detected significant enrichment of 626 GO BP terms, 141 GO CC terms and 138 GO MF terms. The top 10 most significantly enriched GO BP terms are reported in Tables 2 and 3.

Term	Description	Fold Enrichment	P-value
GO:0007059	chromosome segregation	2.65454728835	1.05E-09
GO:0007062	sister chromatid cohesion	3.94626895991	1.41E-09
GO:0000819	sister chromatid segregation	3.06037184646	2.11E-09
GO:0098813	nuclear chromosome segregation	2.77539794983	2.15E-09
GO:0000278	mitotic cell cycle	1.83205025289	6.09E-09
GO:1903047	mitotic cell cycle process	1.87112511161	6.77E-09
GO:0022402	cell cycle process	1.67259081745	7.44E-09
GO:0000280	nuclear division	2.10777644987	9.19E-09
GO:0007049	cell cycle	1.57756855506	1.57E-08
GO:0048285	organelle fission	2.03678397931	2.2E-08

Table 2: Top-ten most significantly enriched GO BP terms in differentially expressed genes after Axitinib treatment

Term	Description	Fold Enrichment	P-value
GO:1903047	mitotic cell cycle process	2.71406256367	1.99118E-47
GO:0000278	mitotic cell cycle	2.61504529161	8.99339E-47
GO:0022402	cell cycle process	2.29198191628	9.4994E-46
GO:0007049	cell cycle	2.1230372506	3.76591E-45
GO:0007059	chromosome segregation	3.45613654238	3.87713E-32
GO:0000819	sister chromatid segregation	4.05376958964	4.54944E-31
GO:0007067	mitotic nuclear division	3.03775708525	1.8261E-30
GO:0098813	nuclear chromosome segregation	3.58108251926	5.33154E-30
GO:0000280	nuclear division	2.68501896899	6.22502E-30
GO:0048285	organelle fission	2.57154355964	2.26491E-28

Table 3: Top-ten most significantly enriched GO BP terms in differentially expressed genes after Doxorubicin treatment

Enriched GO terms were categorized and mapped to higher-level, representative Go terms using CateGOrizer (Na *et al.*, 2014), for facilitating the representation and interpretation of functional enrichments. The representative GO BP terms associated to Axitinib-induced differentially expressed genes are reported in Table 4 and as a pie chart in Figure 12, while the representative terms for Doxorubicin-treated cells are in Figure 13 and Table 5.

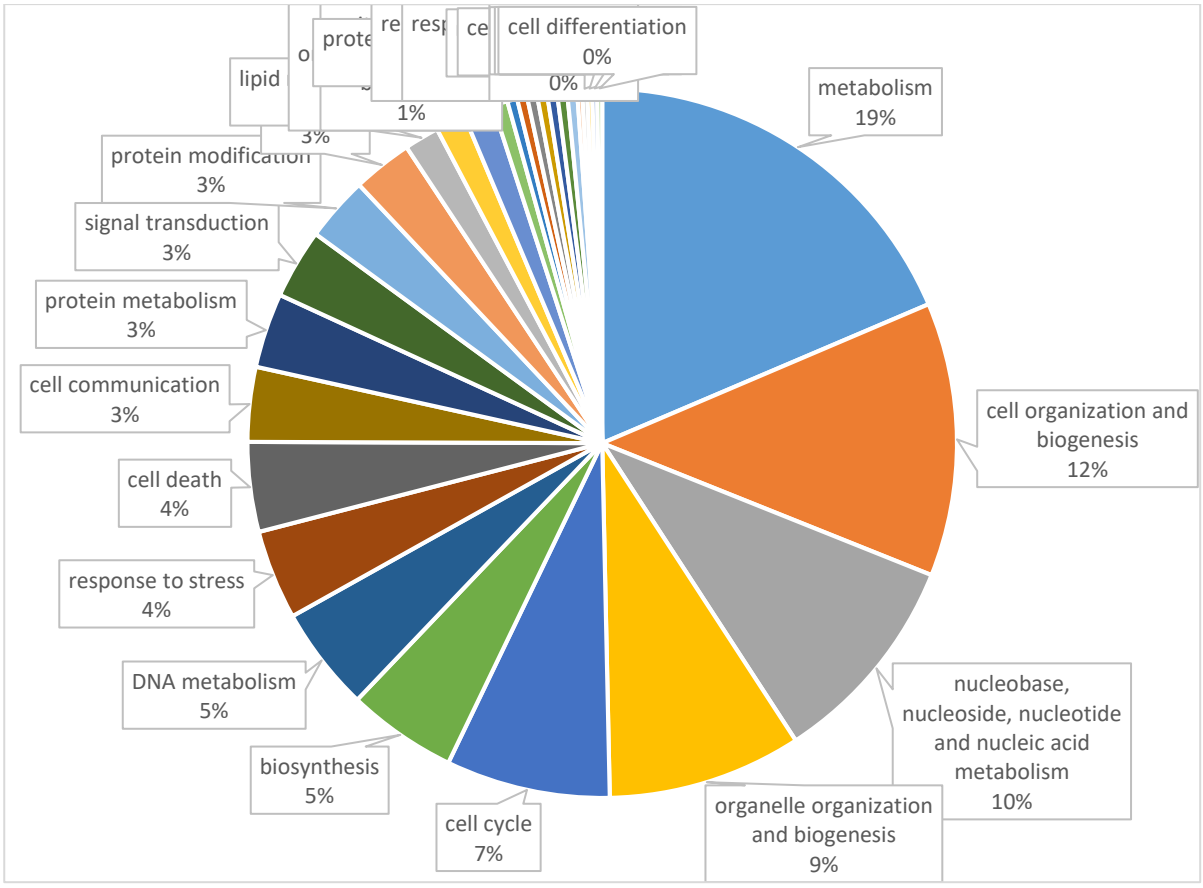

Figure 12: Representative GO terms for the domain Biological Process for differentially expressed genes in cells treated with Axitinib

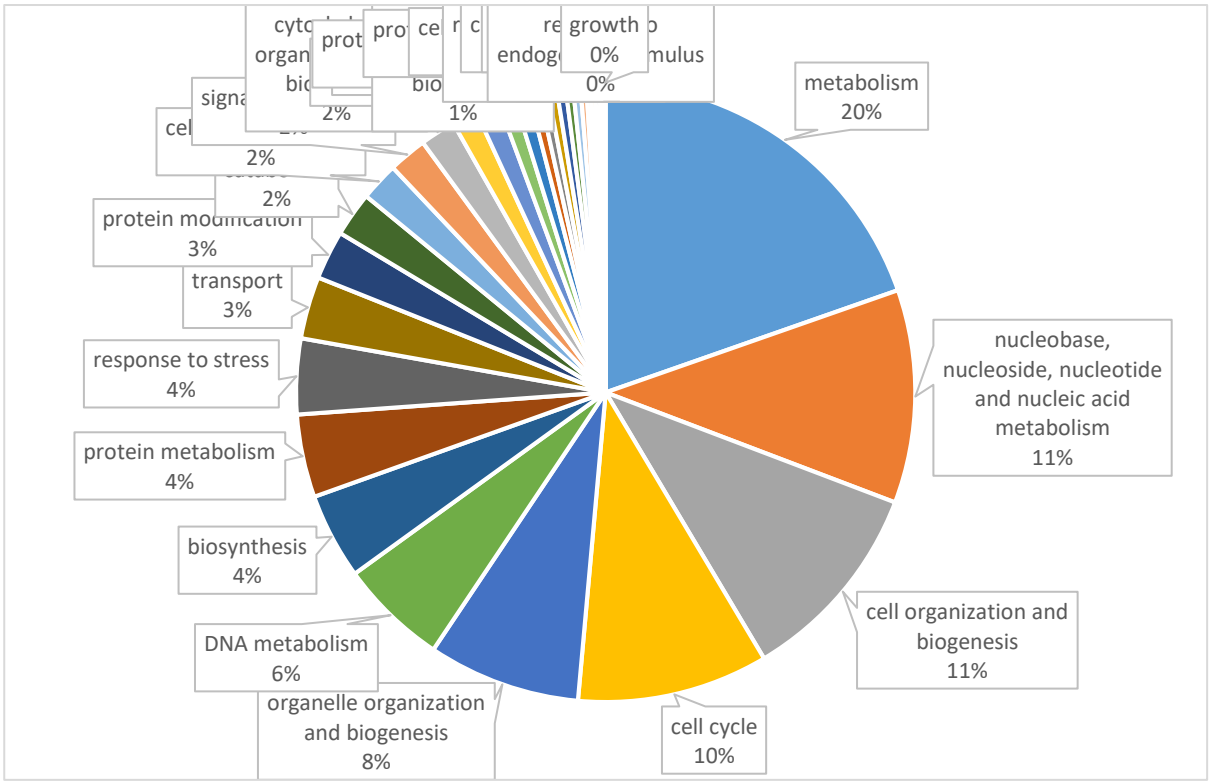

Figure 13: Representative GO terms for the domain Biological Process for differentially expressed genes in cells treated with Doxorubicin

GO Class ID	Definitions	Counts	Fractions
GO:0008152	metabolism	82	18.59%
GO:0016043	cell organization and biogenesis	55	12.47%
GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	43	9.75%
GO:0006996	organelle organization and biogenesis	39	8.84%
GO:0007049	cell cycle	33	7.48%
GO:0009058	biosynthesis	22	4.99%
GO:0006259	DNA metabolism	21	4.76%
GO:0006950	response to stress	18	4.08%
GO:0008219	cell death	18	4.08%
GO:0007154	cell communication	15	3.40%
GO:0019538	protein metabolism	15	3.40%
GO:0007165	signal transduction	14	3.17%
GO:0006464	protein modification	13	2.95%
GO:0006810	transport	12	2.72%
GO:0006629	lipid metabolism	7	1.59%
GO:0000003	reproduction	6	1.36%
GO:0007010	cytoskeleton organization and biogenesis	6	1.36%
GO:0007005	mitochondrion organization and biogenesis	3	0.68%
GO:0006412	protein biosynthesis	2	0.45%
GO:0007275	development	2	0.45%
GO:0009056	catabolism	2	0.45%
GO:0009605	response to external stimulus	2	0.45%
GO:0009628	response to abiotic stimulus	2	0.45%
GO:0009653	morphogenesis	2	0.45%
GO:0019725	cell homeostasis	2	0.45%
GO:0005975	carbohydrate metabolism	1	0.23%
GO:0006811	ion transport	1	0.23%
GO:0008283	cell proliferation	1	0.23%
GO:0015031	protein transport	1	0.23%
GO:0030154	cell differentiation	1	0.23%

Table 4: Representative GO terms for the domain Biological Process for differentially expressed genes in cells treated with Axitinib

GO Class ID	Definitions	Counts	Fractions
GO:0008152	metabolism	254	19.64%
GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	144	11.14%
GO:0016043	cell organization and biogenesis	138	10.67%
GO:0007049	cell cycle	129	9.98%
GO:0006996	organelle organization and biogenesis	103	7.97%
GO:0006259	DNA metabolism	73	5.65%
GO:0009058	biosynthesis	58	4.49%
GO:0019538	protein metabolism	56	4.33%
GO:0006950	response to stress	51	3.94%
GO:0006810	transport	42	3.25%
GO:0006464	protein modification	33	2.55%
GO:0009056	catabolism	30	2.32%
GO:0007154	cell communication	26	2.01%
GO:0007165	signal transduction	26	2.01%
GO:0007010	cytoskeleton organization and biogenesis	24	1.86%
GO:0000003	reproduction	17	1.31%
GO:0007275	development	16	1.24%
GO:0015031	protein transport	11	0.85%
GO:0008219	cell death	10	0.77%
GO:0007005	mitochondrion organization and biogenesis	7	0.54%
GO:0006412	protein biosynthesis	6	0.46%
GO:0007267	cell-cell signaling	6	0.46%
GO:0016032	viral life cycle	6	0.46%
GO:0006811	ion transport	5	0.39%
GO:0009628	response to abiotic stimulus	5	0.39%
GO:0030154	cell differentiation	4	0.31%
GO:0006629	lipid metabolism	3	0.23%
GO:0008283	cell proliferation	3	0.23%
GO:0009790	embryonic development	3	0.23%
GO:0009653	morphogenesis	2	0.15%
GO:0009719	response to endogenous stimulus	1	0.08%
GO:0040007	growth	1	0.08%

Table 5: Representative GO terms for the domain Biological Process for differentially expressed genes in cells treated with Doxorubicin

As an alternative way to facilitate the interpretation of functional enrichment results, we used REViGO (Supek *et al*., 2011), which provides a clustering of semantically similar Gene Ontology terms, and summarized the functional enrichment results by removing redundant terms and computing parameters related to dispensability (how much a term is redundant) and uniqueness (i.e. how much a GO term differs from all other terms) for each term. The Figures 14 and 15 report the results of the REViGO analysis for the significantly enriched GO BP terms in cells treated with Axitinib and Doxorubicin, respectively.

Figure 14: Semantic similarity clustering of significantly enriched GO BP terms in differentially expressed genes in cells treated with Axitinib. Each circle represents a distinct GO term. The color scale reports its uniqueness, i.e. how much a term is representative. The size of each circle is proportional to the number of occurrences of each term in the differentially expressed gene list. Representative members for each cluster, whose description is reported, were selected manually.

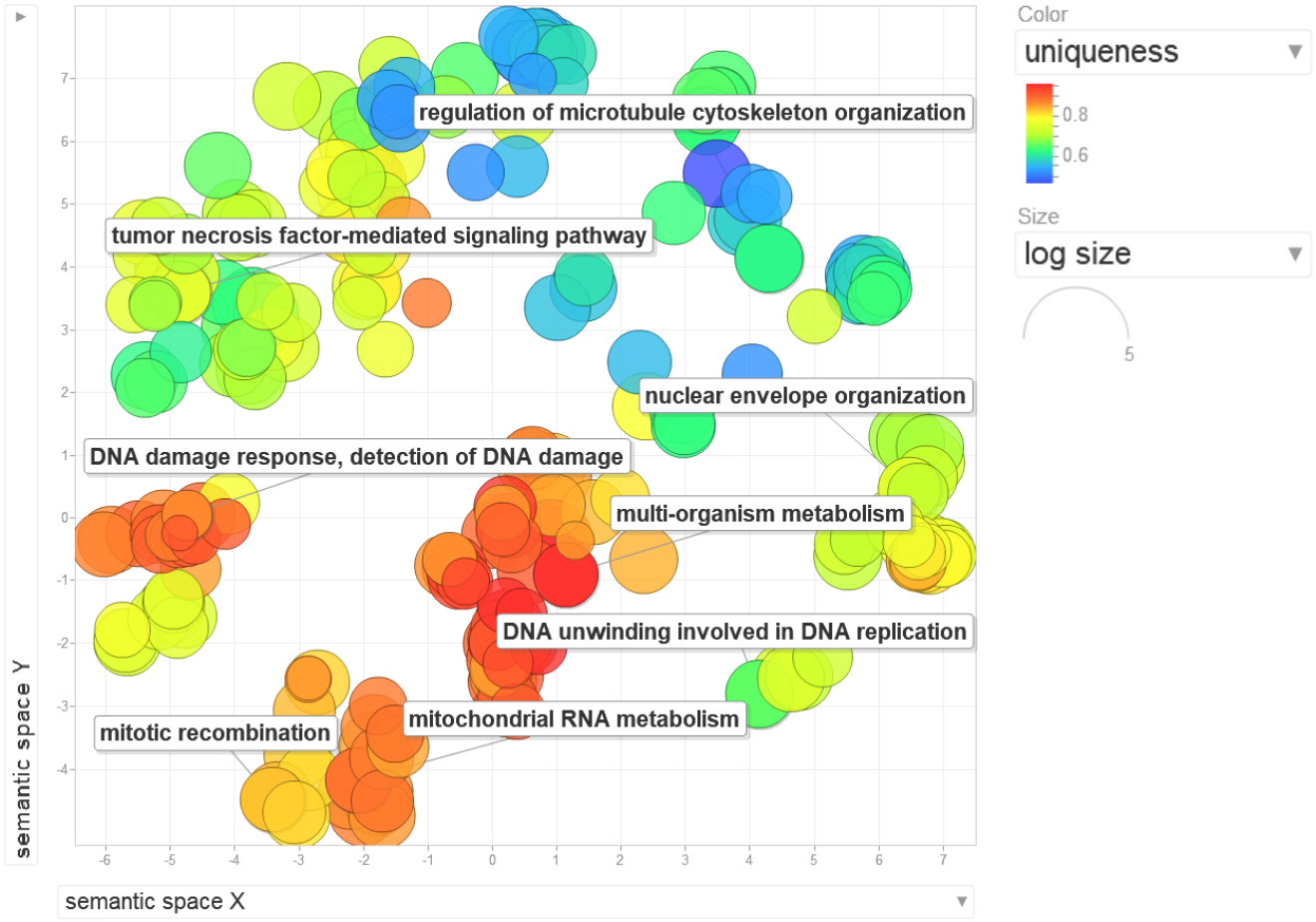

Figure 15: Semantic similarity clustering of significantly enriched GO BP terms in differentially expressed genes in cells treated with Doxorubicin. Each circle represents a distinct GO term. The color scale reports its uniqueness, i.e. how much a term is representative. The size of each circle is proportional to the number of occurrences of each term in the differentially expressed gene list. Representative members for each cluster, whose description is reported, were selected manually.

From these analyses, it seems that the two treatments led to differential expression of genes having in general similar functional characteristics, as summarized by Gene Ontology terms. While the exact functional terms and their significance and proportion may vary, still the same general categories seem affected.

3.2 Differentially expressed genes pathway enrichment

We tested enrichment in genes participating in specific pathways, retrieved from the KEGG and BioCarta databases. For the Axitinib-treated cells, 15 pathways resulted significantly enriched (significance threshold 0.05), of which 5 from the BioCarta database and 10 from KEGG. The full list of significantly enriched pathways is reported in Table 6. As an example, Figure 16 reports the Cell Cycle pathway, as described by KEGG, with highlighted the genes differentially expressed after Axitinib treatment, showing that the majority of the pathway' genes are affected. Most of the significant pathways recapitulate what was already highlighted with Gene Ontology terms enrichment analysis.

Source	Identifier and Description	Fold Enrichment	p-value
KEGG_PATHWAY	hsa04110:Cell cycle	2.87246425008	5.2E-05
BIOCARTA	h_smPathway:Spliceosomal Assembly	6.10902255639	0.00672
KEGG_PATHWAY	hsa04142:Lysosome	2.20776177899	0.00744
KEGG_PATHWAY	hsa04114:Oocyte meiosis	2.28743024686	0.00759
KEGG_PATHWAY	hsa03030:DNA replication	3.46291523482	0.01384
KEGG_PATHWAY	hsa03013:RNA transport	1.76021937185	0.03103
KEGG_PATHWAY	hsa03040:Spliceosome	1.874660879	0.0348
KEGG_PATHWAY	hsa03430:Mismatch repair	3.87158225011	0.03681
KEGG_PATHWAY	hsa00790:Folate biosynthesis	5.088365243	0.04001
BIOCARTA	h_plateletAppPathway:Platelet Amyloid Precursor Protein Pathway	4.56140350877	0.05138
BIOCARTA	h_ctcfPathway:CTCF: First Multivalent Nuclear Factor	3.42105263158	0.05213
KEGG_PATHWAY	hsa03015:mRNA surveillance pathway	1.957063555	0.06751
KEGG_PATHWAY	hsa03460:Fanconi anemia pathway	2.35216883875	0.07418
BIOCARTA	h_mta3Pathway:Downregulated of MTA-3 in ER-negative Breast Tumors	3.80116959064	0.08137
BIOCARTA	h_arfPathway:Tumor Suppressor Arf Inhibits Ribosomal Biogenesis	3.80116959064	0.08137

Table 6: Pathways enriched in differentially expressed genes in cells treated with Axitinib

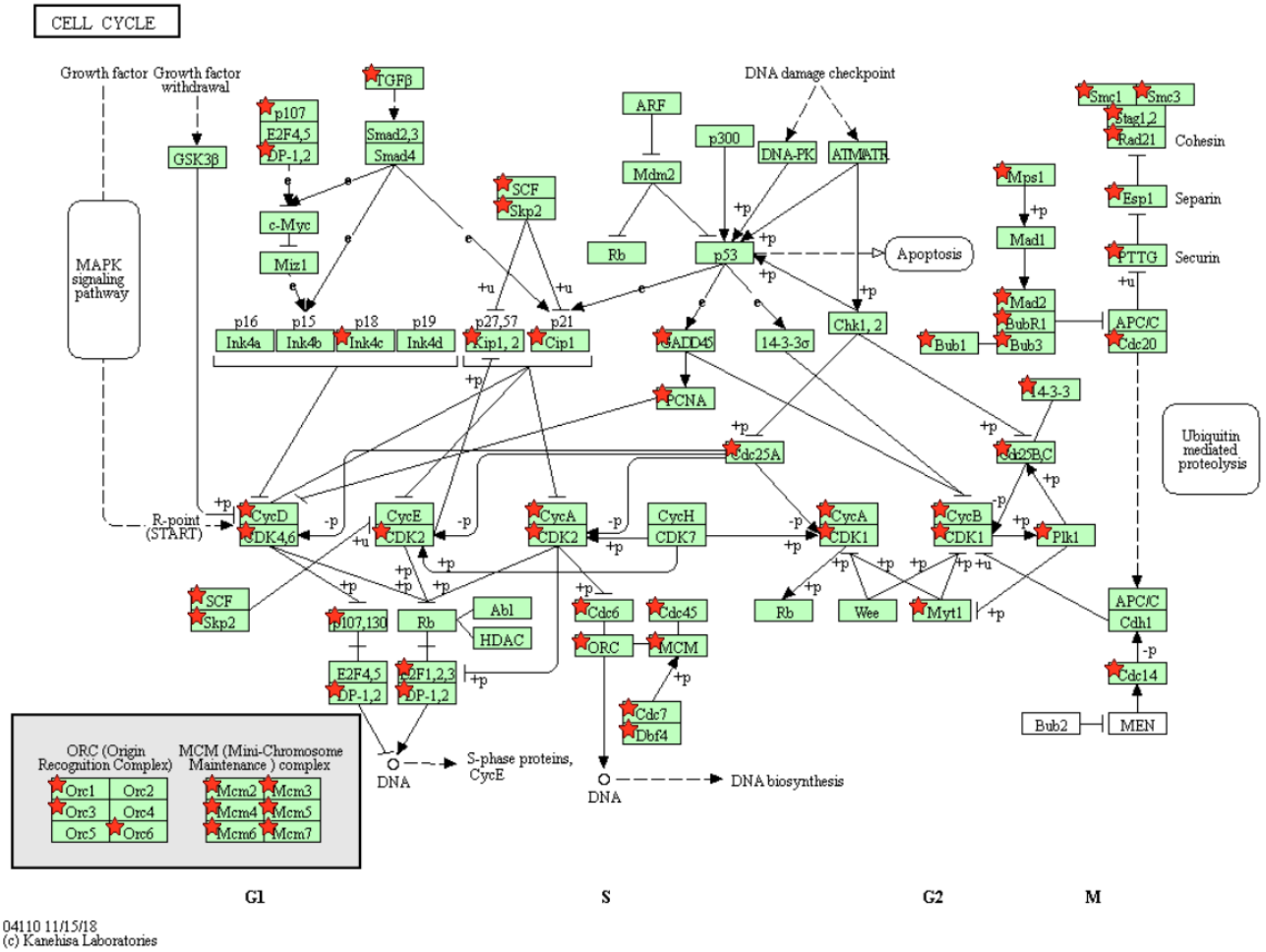

Figure 16: The KEGG Cell Cycle pathway. Differentially expressed genes in Axitinib-treated cells are highlighted by a red star.

For the Doxorubicin-treated cells, 31 pathways resulted significantly enriched (significance threshold 0.05), of which 10 from the BioCarta database and 21 from KEGG. The full list of significantly enriched pathways is reported in Table 7. Also in this case, most of the significant pathways recapitulate what was already highlighted with Gene Ontology terms enrichment analysis, and most of the identified pathways are shared by the two treatments.

Source	Identifier and Description	Fold Enrichment	p-value
KEGG_PATHWAY	hsa04110:Cell cycle	3.32928004405	2E-12
KEGG_PATHWAY	hsa03460:Fanconi anemia pathway	4.45100512628	3.3E-10
KEGG_PATHWAY	hsa03030:DNA replication	5.18768768769	1.7E-09
KEGG_PATHWAY	hsa03008:Ribosome biogenesis in eukaryotes	3.38941482317	3E-09
KEGG_PATHWAY	hsa00240:Pyrimidine metabolism	2.74086333297	9E-07
KEGG_PATHWAY	hsa03013:RNA transport	2.28588441563	9.2E-07
KEGG_PATHWAY	hsa03040:Spliceosome	2.29104054589	1.9E-05
KEGG_PATHWAY	hsa03430:Mismatch repair	4.70097099388	3.7E-05
BIOCARTA	h_mcmPathway:CDK Regulation of DNA Replication	4.69653179191	0.00023
KEGG_PATHWAY	hsa00230:Purine metabolism	1.89884262253	0.00034
KEGG_PATHWAY	hsa03440:Homologous recombination	3.72835630549	0.00036
KEGG_PATHWAY	hsa04114:Oocyte meiosis	2.16425019902	0.00049
KEGG_PATHWAY	hsa03050:Proteasome	2.90411224622	0.00108
KEGG_PATHWAY	hsa03410:Base excision repair	3.27643432907	0.00116
KEGG_PATHWAY	hsa05169:Epstein-Barr virus infection	1.75892790297	0.00138
BIOCARTA	h_smPathway:Spliceosomal Assembly	4.69653179191	0.00188
BIOCARTA	h_cellcyclePathway:Cyclins and Cell Cycle Regulation	3.38150289017	0.00298
BIOCARTA	h_ranMSPathway:Role of Ran in mitotic spindle regulation	5.12348922754	0.00341
BIOCARTA	h_atrbrcaPathway:Role of BRCA1, BRCA2 and ATR in Cancer Susce	3.41565948502	0.00566
KEGG_PATHWAY	hsa04914:Progesterone-mediated oocyte maturation	2.0336488939	0.00587
BIOCARTA	h_g2Pathway:Cell Cycle: G2/M Checkpoint	3.00578034682	0.01212
KEGG_PATHWAY	hsa03020:RNA polymerase	2.76449146515	0.01259
KEGG_PATHWAY	hsa03015:mRNA surveillance pathway	1.83624341519	0.01988
KEGG_PATHWAY	hsa01100:Metabolic pathways	1.16062616706	0.02698
BIOCARTA	h_g1Pathway:Cell Cycle: G1/S Check Point	2.50481695568	0.03259
KEGG_PATHWAY	hsa04141:Protein processing in endoplasmic reticulum	1.51220045957	0.03428
KEGG_PATHWAY	hsa04115:p53 signaling pathway	1.90717819155	0.03561
KEGG_PATHWAY	hsa03450:Non-homologous end-joining	3.78050114892	0.03581
BIOCARTA	h_rbPathway:RB Tumor Suppressor/Checkpoint Signaling in respo	3.61271676301	0.04036
BIOCARTA	h_p27Pathway:Regulation of p27 Phosphorylation during Cell Cyc	3.61271676301	0.04036
BIOCARTA	h_bard1Pathway:BRCA1-dependent Ub-ligase activity	4.69653179191	0.04372
KEGG_PATHWAY	hsa00790:Folate biosynthesis	3.51046535257	0.04621

Table 7: Pathways enriched in differentially expressed genes in cells treated with Doxorubicin.

Figure 17 reports the Cell Cycle pathway, as described by KEGG, with highlighted the genes differentially expressed after Doxorubicin treatment, showing, similarly to what observed after Axinitib treatment, that the majority of the pathway' genes are affected. While the list of differentially expressed genes in the pathway is slightly different for the two treatments, most genes are in common.

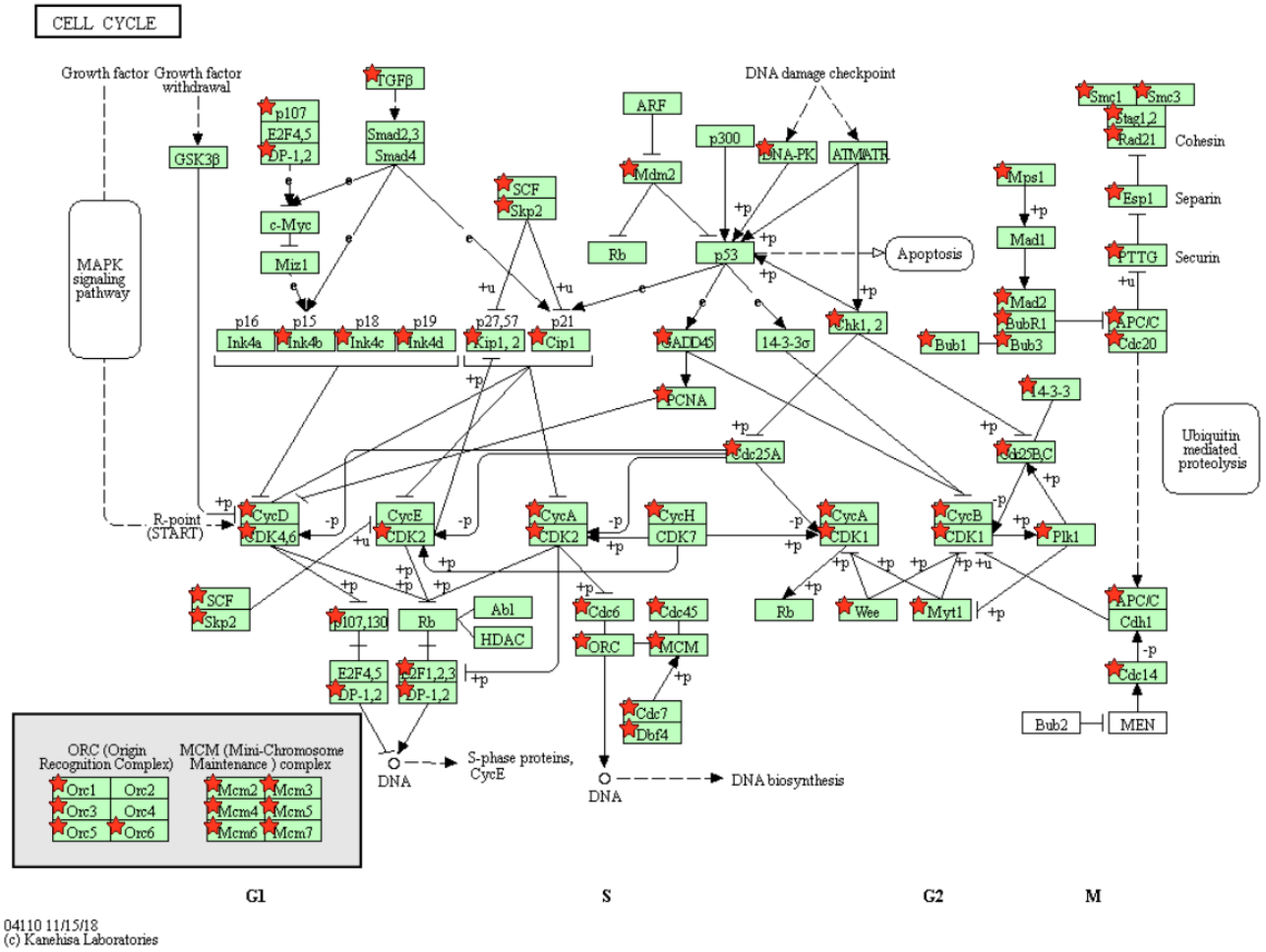

Figure 17: The KEGG Cell Cycle pathway. Differentially expressed genes in Doxorubicin-treated cells are highlighted by a red star.

As observed for Gene Ontology enrichment analysis, pathway analysis did not reveal marked differences in the differentially expressed genes after the two treatments.

3.3 Differentially expressed genes transcription factor enrichment

Then, we investigated whether differentially expressed genes are under the control of different transcription factors (TF), by using a mapping of TF binding site consensus sequences from the UCSC Genome Browser. A total of 70 TF binding sites are significantly over-represented in promoters of differentially expressed genes after Axitinib treatment, while 45 are significantly over-represented in promoters of differentially expressed genes after Doxorubicin treatment (the top-ten most significantly enriched are reported in Table 8 and Table 9, respectively), at significance threshold 0.05. 39 transcription factors are shared by the two treatments, while 6 (CP2, NKX25, EVI1, AP2, ELK1, OCT1) resulted specifically enriched after Doxorubicin treatment, and 31 (NKX22, SOX5, NFAT, STAT5B, HSF1, STAT3, EGR2, RREB1, CART1, SEF1, BRACH, RFX1, ARNT, HFH3, FOXJ2, PAX4, HLF, TGIF, XBP1, HNF3B, AP4, TAL1ALPHAE47, HAND1E47, FREAC4, PPARG, HNF4, MYCMAX, CMYB, BACH2, RORA1, EN1) are specific of the Axitinib treatment. These specific TFs might highlight a different response to the two treatments, nevertheless only 3 of the 31 Axitinib-specific TFs are also differentially expressed in Axitinib-treated cells (STAT3, XBP1, PPARG), and none of the 8 Doxorubicin-specific TFs are also differentially expressed in Doxorubicin-treated cells.

Transcription Factor	Fold Enrichment	P-value
CETS1P54	1.24771007567	4.59E-05
IRF7	1.1504322023	9.7E-05
HMX1	1.14232641558	0.000195
HOX13	1.12865440672	0.000698
SRF	1.07954150979	0.000713
CDPCR1	1.14220904298	0.001019
MYCMAX	1.08543767221	0.001627
CDP	1.08983790325	0.001691
GATA1	1.0576011687	0.002047
STAT3	1.10857619165	0.002673

Table 8: Enriched transcription factors binding sites in differentially expressed genes in cells treated with Axitinib.

Transcription Factor	Fold Enrichment	P-value
E2F	1.12378602801	1.33E-07
NFY	1.12215909207	6.92E-06
NRF2	1.17110204285	1.95E-05
HMX1	1.12018325815	2.97E-05
CETS1P54	1.18023020262	6.6E-05
NMYC	1.13185806602	0.000546
CEBP	1.05381490639	0.000585
GATA1	1.04797427026	0.000719
MEF2	1.05466786883	0.00087
USF	1.06969449611	0.001566

Table 9: Enriched transcription factors binding sites in differentially expressed genes in cells treated with Doxorubicin.

3.4 Comparison of differentially expressed genes between Axitinib and Doxorubicin treatments

Overall, it was difficult to highlight a different response to the treatment with the two drug, that seem to affect a similar set of genes having similar functional characteristics, belonging to the same pathways and that are under the control of a similar set of transcription factors. To facilitate the analysis, we treated separately genes that are differentially expressed in cells treated with one drug but not with the other, and genes that are differentially expressed in both treatments. In cells treated with Axitinib, 3,221 genes resulted differentially expressed with respect to control cells (at p-value < 0.05). In Doxorubicin treated cells, the number of differentially expressed genes is 4,447. Of these two sets of differentially expressed genes, 2,601 are shared by both treatments (from now on “common DEGs”), while 620 are Axitinib-specific (“AXI DEGs”) and 1,846 are Doxorubicin-specific (“DOXO DEGs”).

Functional enrichment of the AXI DEGs highlighted significant enrichment (at significance threshold 0.05) of 54 Gene Ontology (GO) terms belonging to the Biological Process domain, 20 GO terms belonging to the Cellular Component (CC) domain, and 10 belonging to the Molecular Function (MF) domain. As before, significantly enriched GO BP terms were categorized to a representative selection of high-level terms, clustered by semantic similarity and reported in Table 10, Figure 18 and Figure 19.

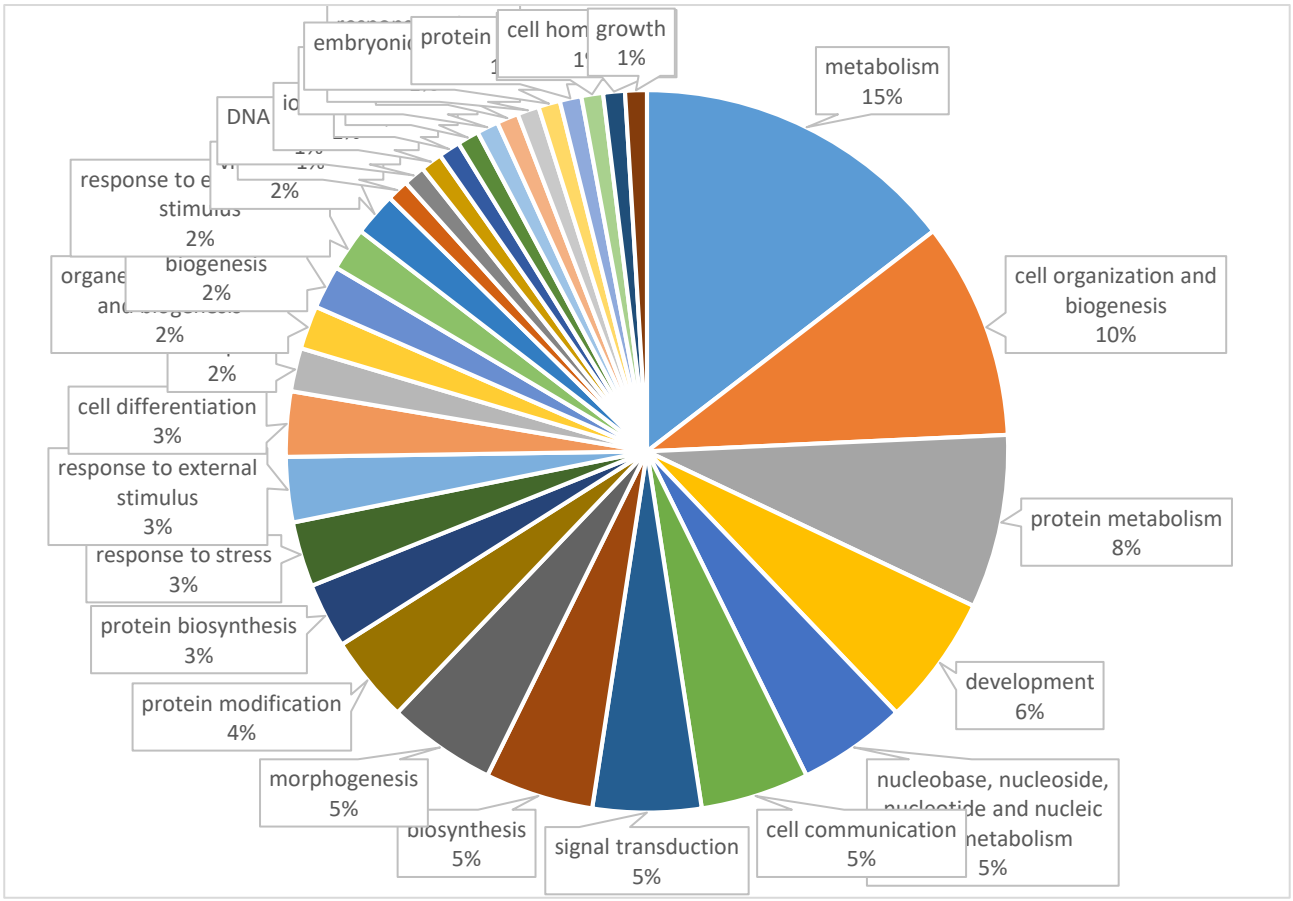

Figure 18: Representative GO terms for the domain Biological Process for differentially expressed genes in cells treated with Axitinib, that are not differentially expressed in cells treated with Doxorubicin.

GO Class ID	Definitions	Counts	Fractions
GO:0008152	metabolism	15	14.56%
GO:0016043	cell organization and biogenesis	10	9.71%
GO:0019538	protein metabolism	8	7.77%
GO:0007275	development	6	5.83%
GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	5	4.85%
GO:0007154	cell communication	5	4.85%
GO:0007165	signal transduction	5	4.85%
GO:0009058	biosynthesis	5	4.85%
GO:0009653	morphogenesis	5	4.85%
GO:0006464	protein modification	4	3.88%
GO:0006412	protein biosynthesis	3	2.91%
GO:0006950	response to stress	3	2.91%
GO:0009605	response to external stimulus	3	2.91%
GO:0030154	cell differentiation	3	2.91%
GO:0006810	transport	2	1.94%
GO:0006996	organelle organization and biogenesis	2	1.94%
GO:0007010	cytoskeleton organization and biogenesis	2	1.94%
GO:0009719	response to endogenous stimulus	2	1.94%
GO:0016032	viral life cycle	2	1.94%
GO:0000003	reproduction	1	0.97%
GO:0006259	DNA metabolism	1	0.97%
GO:0006811	ion transport	1	0.97%
GO:0007049	cell cycle	1	0.97%
GO:0008219	cell death	1	0.97%
GO:0008283	cell proliferation	1	0.97%
GO:0009056	catabolism	1	0.97%
GO:0009607	response to biotic stimulus	1	0.97%
GO:0009790	embryonic development	1	0.97%
GO:0015031	protein transport	1	0.97%
GO:0016049	cell growth	1	0.97%
GO:0019725	cell homeostasis	1	0.97%
GO:0040007	growth	1	0.97%

Table 10: Representative GO terms for the domain Biological Process for differentially expressed genes in cells treated with Axitinib, that are also not differentially expressed in cells treated with Doxorubicin.

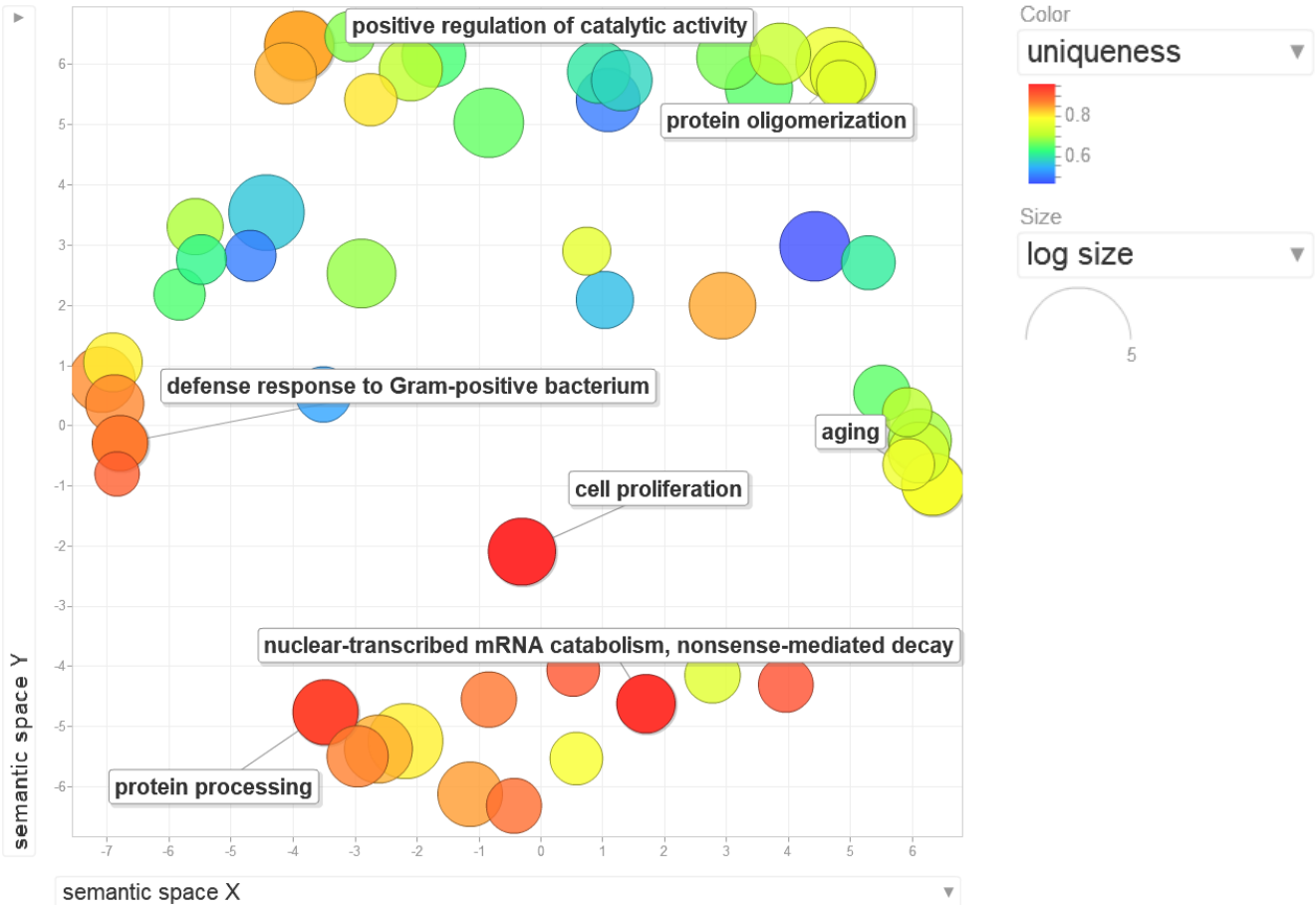

Figure 19: Semantic similarity clustering of significantly enriched GO BP terms in differentially expressed genes in cells treated with Axitinib, that are also not differentially expressed in cells treated with Doxorubicin. Each circle represents a distinct GO term. The color scale reports its uniqueness, i.e. how much a term is representative. The size of each circle is proportional to the number of occurrences of each term in the differentially expressed gene list. Representative members for each cluster, whose description is reported, were selected manually.

Functional enrichment of the DOXO DEGs highlighted significant enrichment (at significance threshold 0.05) of 82 Gene Ontology (GO) terms belonging to the Biological Process domain, 48 GO terms belonging to the Cellular Component (CC) domain, and 30 belonging to the Molecular Function (MF) domain. Significantly enriched GO BP terms were categorized to a representative selection of high-level terms, clustered by semantic similarity and reported in Table 11, Figure 20 and Figure 21.

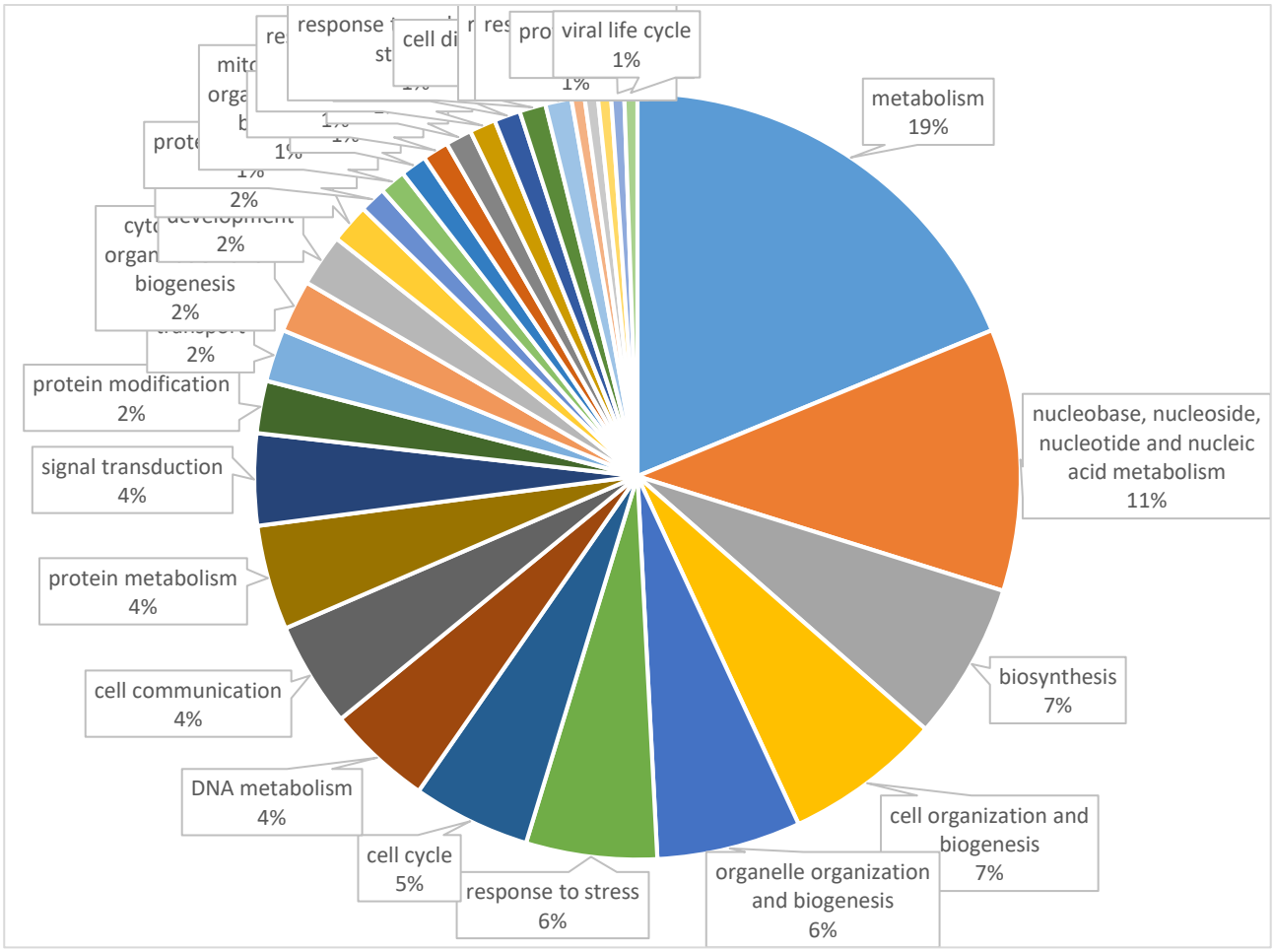

Figure 20: Representative GO terms for the domain Biological Process for differentially expressed genes in cells treated with Doxorubicin, that are not differentially expressed in cells treated with Axitinib.

GO Class ID	Definitions	Counts	Fractions
GO:0008152	metabolism	34	18.78%
GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	20	11.05%
GO:0009058	biosynthesis	12	6.63%
GO:0016043	cell organization and biogenesis	12	6.63%
GO:0006996	organelle organization and biogenesis	11	6.08%
GO:0006950	response to stress	10	5.52%
GO:0007049	cell cycle	9	4.97%
GO:0006259	DNA metabolism	8	4.42%
GO:0007154	cell communication	8	4.42%
GO:0019538	protein metabolism	8	4.42%
GO:0007165	signal transduction	7	3.87%
GO:0006464	protein modification	4	2.21%
GO:0006810	transport	4	2.21%
GO:0007010	cytoskeleton organization and biogenesis	4	2.21%
GO:0007275	development	4	2.21%
GO:0006629	lipid metabolism	3	1.66%
GO:0006412	protein biosynthesis	2	1.10%
GO:0007005	mitochondrion organization and biogenesis	2	1.10%
GO:0008219	cell death	2	1.10%
GO:0008283	cell proliferation	2	1.10%
GO:0009056	catabolism	2	1.10%
GO:0009605	response to external stimulus	2	1.10%
GO:0009653	morphogenesis	2	1.10%
GO:0009719	response to endogenous stimulus	2	1.10%
GO:0030154	cell differentiation	2	1.10%
GO:0000003	reproduction	1	0.55%
GO:0009607	response to biotic stimulus	1	0.55%
GO:0009628	response to abiotic stimulus	1	0.55%
GO:0015031	protein transport	1	0.55%
GO:0016032	viral life cycle	1	0.55%

Table 11: Representative GO terms for the domain Biological Process for differentially expressed genes in cells treated with Doxorubicin, that are also not differentially expressed in cells treated with Axitinib.

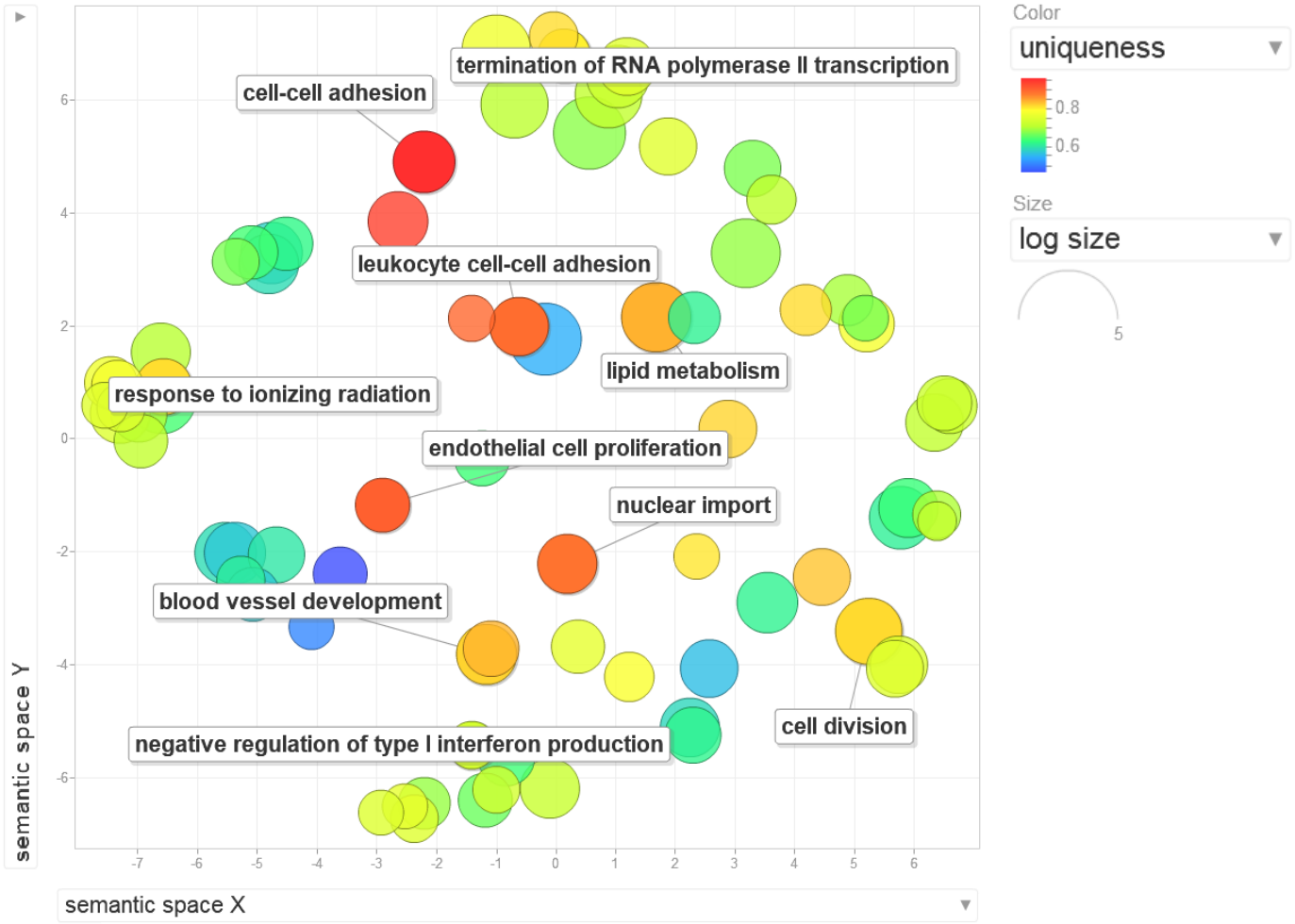

Figure 21: Semantic similarity clustering of significantly enriched GO BP terms in differentially expressed genes in cells treated with Doxorubicin, that are also not differentially expressed in cells treated with Axitinib. Each circle represents a distinct GO term. The color scale reports its uniqueness, i.e. how much a term is representative. The size of each circle is proportional to the number of occurrences of each term in the differentially expressed gene list. Representative members for each cluster, whose description is reported, were selected manually.

Finally, we analyzed the functional enrichment of genes differentially expressed after treatment with both Axitinib and Doxorubicin. The analysis of these common DEGs highlighted significant enrichment (at significance threshold 0.05) of 238 Gene Ontology (GO) terms belonging to the Biological Process domain, 99 GO terms belonging to the Cellular Component (CC) domain, and 78 belonging to the Molecular Function (MF) domain. Significantly enriched GO BP terms were categorized to a representative selection of high-level terms, clustered by semantic similarity and reported in Table 12, Figure 22 and Figure 23.

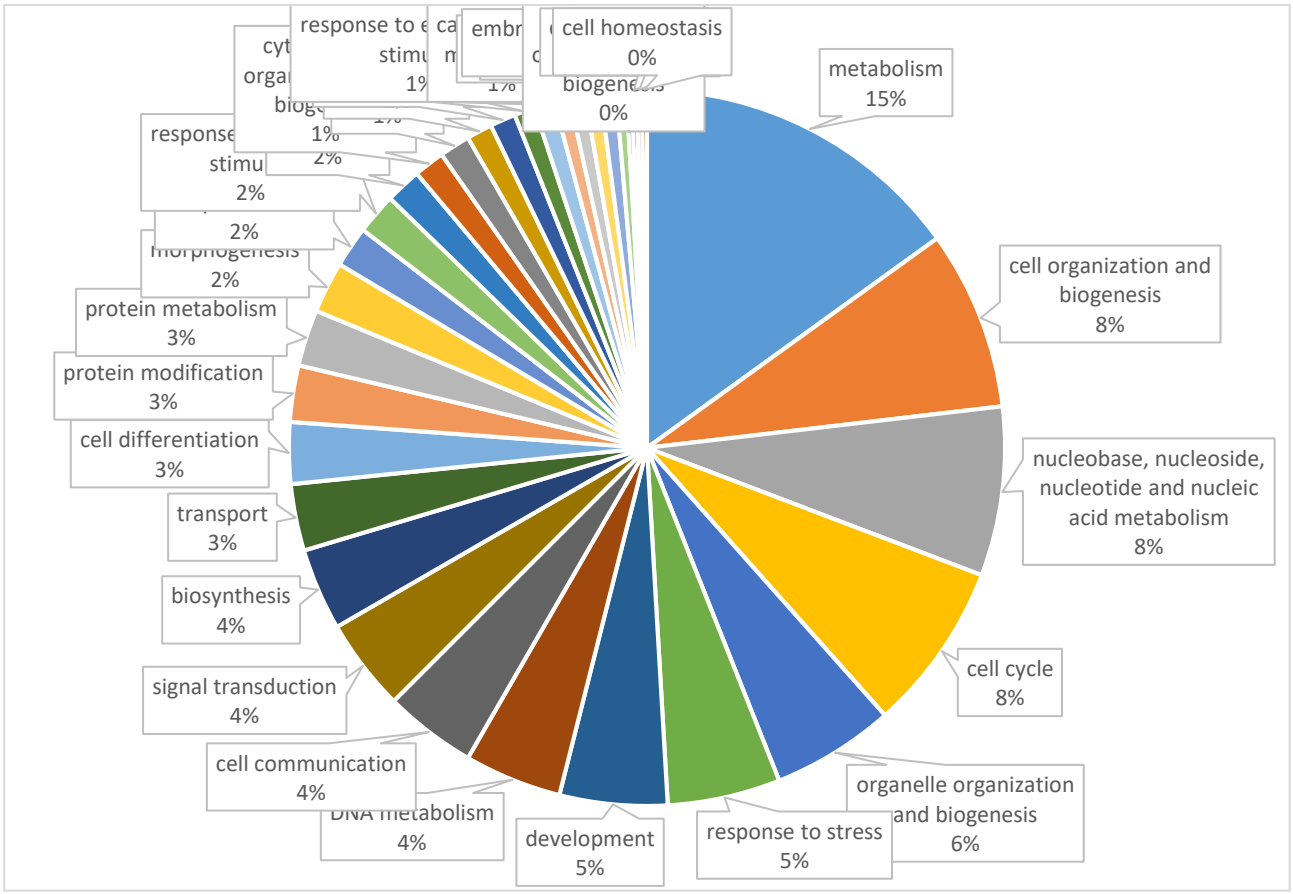

Figure 22: Representative GO terms for the domain Biological Process for genes differentially expressed both in cells treated with Doxorubicin and in cells treated with Axitinib.

GO Class ID	Definitions	Counts	Fractions
GO:0008152	metabolism	65	15.05%
GO:0016043	cell organization and biogenesis	35	8.10%
GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	33	7.64%
GO:0007049	cell cycle	33	7.64%
GO:0006996	organelle organization and biogenesis	24	5.56%
GO:0006950	response to stress	22	5.09%
GO:0007275	development	21	4.86%
GO:0006259	DNA metabolism	19	4.40%
GO:0007154	cell communication	18	4.17%
GO:0007165	signal transduction	18	4.17%
GO:0009058	biosynthesis	16	3.70%
GO:0006810	transport	13	3.01%
GO:0030154	cell differentiation	12	2.78%
GO:0006464	protein modification	11	2.55%
GO:0019538	protein metabolism	11	2.55%
GO:0009653	morphogenesis	10	2.31%
GO:0008283	cell proliferation	8	1.85%
GO:0009605	response to external stimulus	8	1.85%
GO:0009056	catabolism	7	1.62%
GO:0007010	cytoskeleton organization and biogenesis	6	1.39%
GO:0008219	cell death	6	1.39%
GO:0000003	reproduction	5	1.16%
GO:0006629	lipid metabolism	5	1.16%
GO:0009628	response to abiotic stimulus	4	0.93%
GO:0009719	response to endogenous stimulus	4	0.93%
GO:0005975	carbohydrate metabolism	3	0.69%
GO:0006811	ion transport	3	0.69%
GO:0016032	viral life cycle	3	0.69%
GO:0040007	growth	3	0.69%
GO:0009790	embryonic development	2	0.46%
GO:0007005	mitochondrion organization and biogenesis	1	0.23%
GO:0008037	cell recognition	1	0.23%
GO:0016049	cell growth	1	0.23%
GO:0019725	cell homeostasis	1	0.23%

Table 12: Representative GO terms for the domain Biological Process for genes differentially expressed both in cells treated with Doxorubicin and in cells treated with Axitinib.

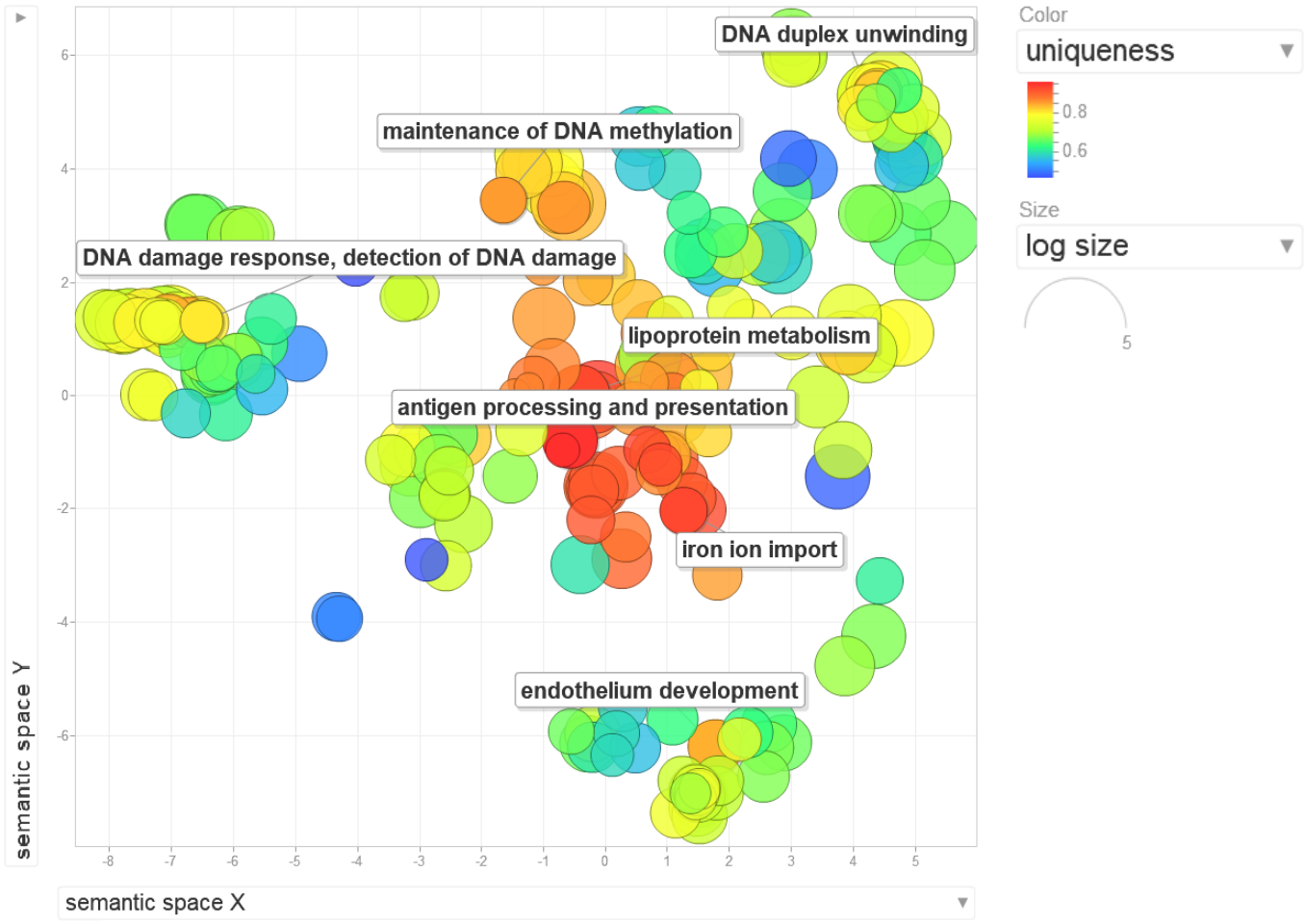

Figure 23: Semantic similarity clustering of significantly enriched GO BP terms in genes that are differentially expressed both in cells treated with Doxorubicin and in cells treated with Axitinib. Each circle represents a distinct GO term. The color scale reports its uniqueness, i.e. how much a term is representative. The size of each circle is proportional to the number of occurrences of each term in the differentially expressed gene list. Representative members for each cluster, whose description is reported, were selected manually.

From these analyses, there appear to be only slight differences in specific enriched terms or groups of terms in the AXI, DOXO and Common DEGs.

Pathway enrichment test revealed 4 KEGG pathways enriched in AXI DEGs (Table 13), 23 KEGG pathways and 6 BioCarta Pathways enriched in DOXO DEGs (Table 14), and 29 KEGG pathways and 8 BioCarta pathways enriched in the Common DEGs (Table 15).

Category	Identifier and Dexcription	PValue
KEGG_PATHWAY	hsa03010:Ribosome	7.88E-04
KEGG_PATHWAY	hsa05132:Salmonella infection	0.005647
KEGG_PATHWAY	hsa05131:Shigellosis	0.006132
KEGG_PATHWAY	hsa05130:Pathogenic Escherichia coli infection	0.042932

Table 13: Pathways enriched in genes differentially expressed in cells treated with Axitinib but not cells treated with Doxorubicin.

Category	Identifier and Description	PValue
KEGG_PATHWAY	hsa00062:Fatty acid elongation	0.003248
KEGG_PATHWAY	hsa03460:Fanconi anemia pathway	0.003427
KEGG_PATHWAY	hsa04510:Focal adhesion	0.005686
KEGG_PATHWAY	hsa04110:Cell cycle	0.006007
KEGG_PATHWAY	hsa03015:mRNA surveillance pathway	0.007123
KEGG_PATHWAY	hsa03013:RNA transport	0.007296
KEGG_PATHWAY	hsa03440:Homologous recombination	0.007899
KEGG_PATHWAY	hsa01040:Biosynthesis of unsaturated fatty acids	0.009224
KEGG_PATHWAY	hsa05222:Small cell lung cancer	0.009226
KEGG_PATHWAY	hsa03008:Ribosome biogenesis in eukaryotes	0.011281
KEGG_PATHWAY	hsa01100:Metabolic pathways	0.013013
KEGG_PATHWAY	hsa03420:Nucleotide excision repair	0.013323
KEGG_PATHWAY	hsa05200:Pathways in cancer	0.014185
BIOCARTA	h_atrbrcaPathway:Role of BRCA1, BRCA2 and ATR in Cancer Susceptibility	0.015332
BIOCARTA	h_cellcyclePathway:Cyclins and Cell Cycle Regulation	0.028496
BIOCARTA	h_il10Pathway:IL-10 Anti-inflammatory Signaling Pathway	0.030585
KEGG_PATHWAY	hsa00020:Citrate cycle (TCA cycle)	0.033208
BIOCARTA	h_bard1Pathway:BRCA1-dependent Ub-ligase activity	0.035012
KEGG_PATHWAY	hsa05223:Non-small cell lung cancer	0.038388
KEGG_PATHWAY	hsa05214:Glioma	0.039138
KEGG_PATHWAY	hsa05205:Proteoglycans in cancer	0.039487
BIOCARTA	h_egfPathway:EGF Signaling Pathway	0.040497
KEGG_PATHWAY	hsa01212:Fatty acid metabolism	0.04096
KEGG_PATHWAY	hsa00520:Amino sugar and nucleotide sugar metabolism	0.04096
KEGG_PATHWAY	hsa05169:Epstein-Barr virus infection	0.042417
KEGG_PATHWAY	hsa01200:Carbon metabolism	0.044206
BIOCARTA	h_pdgfPathway:PDGF Signaling Pathway	0.047539
KEGG_PATHWAY	hsa00564:Glycerophospholipid metabolism	0.047579
KEGG_PATHWAY	hsa04068:FoxO signaling pathway	0.048738

Table 14: Pathways enriched in genes differentially expressed in cells treated with Doxorubicin but not cells treated with Axitinib.

Category	Term	PValue
KEGG_PATHWAY	hsa04110:Cell cycle	8.83E-12
KEGG_PATHWAY	hsa04142:Lysosome	2.14E-10
KEGG_PATHWAY	hsa03030:DNA replication	1.03E-07
BIOCARTA	h_mcmPathway:CDK Regulation of DNA Replication	1.00E-05
KEGG_PATHWAY	hsa01130:Biosynthesis of antibiotics	1.62E-04
KEGG_PATHWAY	hsa04115:p53 signaling pathway	2.51E-04
KEGG_PATHWAY	hsa00531:Glycosaminoglycan degradation	3.02E-04
KEGG_PATHWAY	hsa03040:Spliceosome	3.93E-04
KEGG_PATHWAY	hsa00100:Steroid biosynthesis	4.86E-04
KEGG_PATHWAY	hsa04512:ECM-receptor interaction	6.30E-04
KEGG_PATHWAY	hsa00511:Other glycan degradation	0.001116
KEGG_PATHWAY	hsa03430:Mismatch repair	0.001636
BIOCARTA	h_cellcyclePathway:Cyclins and Cell Cycle Regulation	0.002248
BIOCARTA	h_smPathway:Spliceosomal Assembly	0.00233
KEGG_PATHWAY	hsa00532:Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan s	0.002522
KEGG_PATHWAY	hsa01100:Metabolic pathways	0.004664
KEGG_PATHWAY	hsa04514:Cell adhesion molecules (CAMs)	0.005067
KEGG_PATHWAY	hsa04145:Phagosome	0.006212
KEGG_PATHWAY	hsa00230:Purine metabolism	0.007142
BIOCARTA	h_g2Pathway:Cell Cycle: G2/M Checkpoint	0.008424
KEGG_PATHWAY	hsa05166:HTLV-I infection	0.00844
KEGG_PATHWAY	hsa04114:Oocyte meiosis	0.008569
BIOCARTA	h_ranMSPathway:Role of Ran in mitotic spindle regulation	0.016719
BIOCARTA	h_granulocytesPathway:Adhesion and Diapedesis of Granulocytes	0.01774
KEGG_PATHWAY	hsa05144:Malaria	0.02203
KEGG_PATHWAY	hsa03410:Base excision repair	0.023006
KEGG_PATHWAY	hsa05222:Small cell lung cancer	0.025651
KEGG_PATHWAY	hsa00030:Pentose phosphate pathway	0.029337
BIOCARTA	h_g1Pathway:Cell Cycle: G1/S Check Point	0.029442
BIOCARTA	h_p27Pathway:Regulation of p27 Phosphorylation during Cell Cycle Progress	0.035919
KEGG_PATHWAY	hsa01200:Carbon metabolism	0.035932
KEGG_PATHWAY	hsa00604:Glycosphingolipid biosynthesis - ganglio series	0.037501
KEGG_PATHWAY	hsa00240:Pyrimidine metabolism	0.037601
KEGG_PATHWAY	hsa03460:Fanconi anemia pathway	0.039041
KEGG_PATHWAY	hsa03018:RNA degradation	0.039943
KEGG_PATHWAY	hsa04350:TGF-beta signaling pathway	0.043761
KEGG_PATHWAY	hsa01230:Biosynthesis of amino acids	0.04481

Table 15: Pathways enriched in genes differentially expressed both in cells treated with Doxorubicin and in cells treated with Axitinib.

Some differences among the three lists of DEGs can be observed. For example, the most significantly enriched pathway for the DOXO DEGs (Fatty Acids Elongation) is not enriched for the AXI and Common DEGs, while the Lysosome pathway, the second most significantly enriched pathway for the Common DEGs is not enriched in neither of the treatment-specific DEGs.

We then analyzed whether the identified DEGs are known to be interacting partners, using the STRING database (Szklarczyk et al., 2019). Among the AXI DEGs, there are 398 known interactions, selected as those considered as high confidence (STRING interaction score > 0.7), which is significantly more (p-value 8.76e-09) than what would be expected for a random set of proteins of similar size, drawn from the genome. Such an enrichment indicates that the proteins are at least partially biologically connected, as a group. Other parameters, such as the clustering coefficient (0.329) and the average node degree (1.74) supports the observation of a highly connected network. The protein-protein interaction (PPI) network for the AXI DEGs is shown in Figure 24.

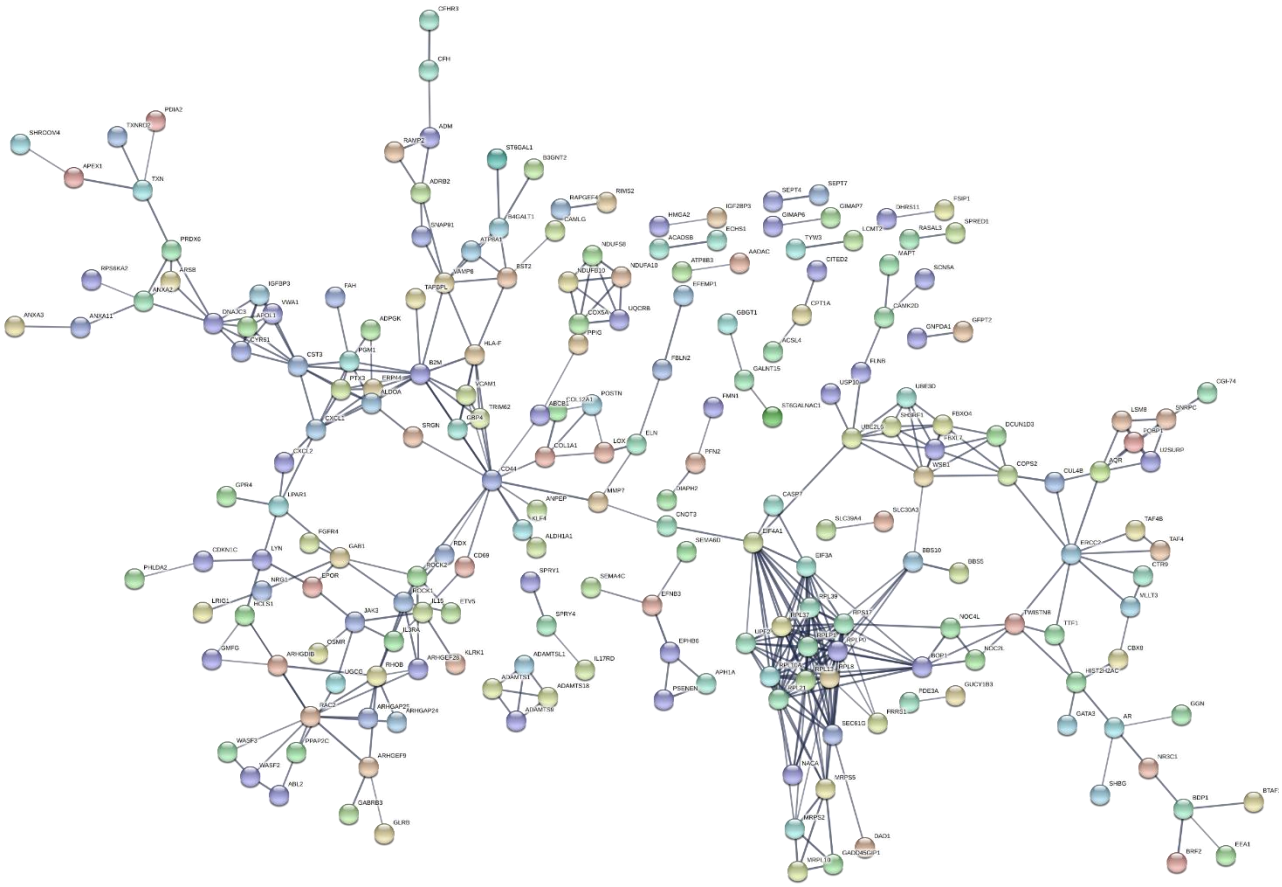

Figure 24: Protein-protein interaction network among the AXI DEGs, as reconstructed by STRING.

The DOXO DEGs form an even more highly interconnected network, having a number of high confidence interactions significantly higher than expected (4569 interactions, p-value 8.66e-15), clustering coefficient 0.431 and average node degree 5.99 (Figure 25).

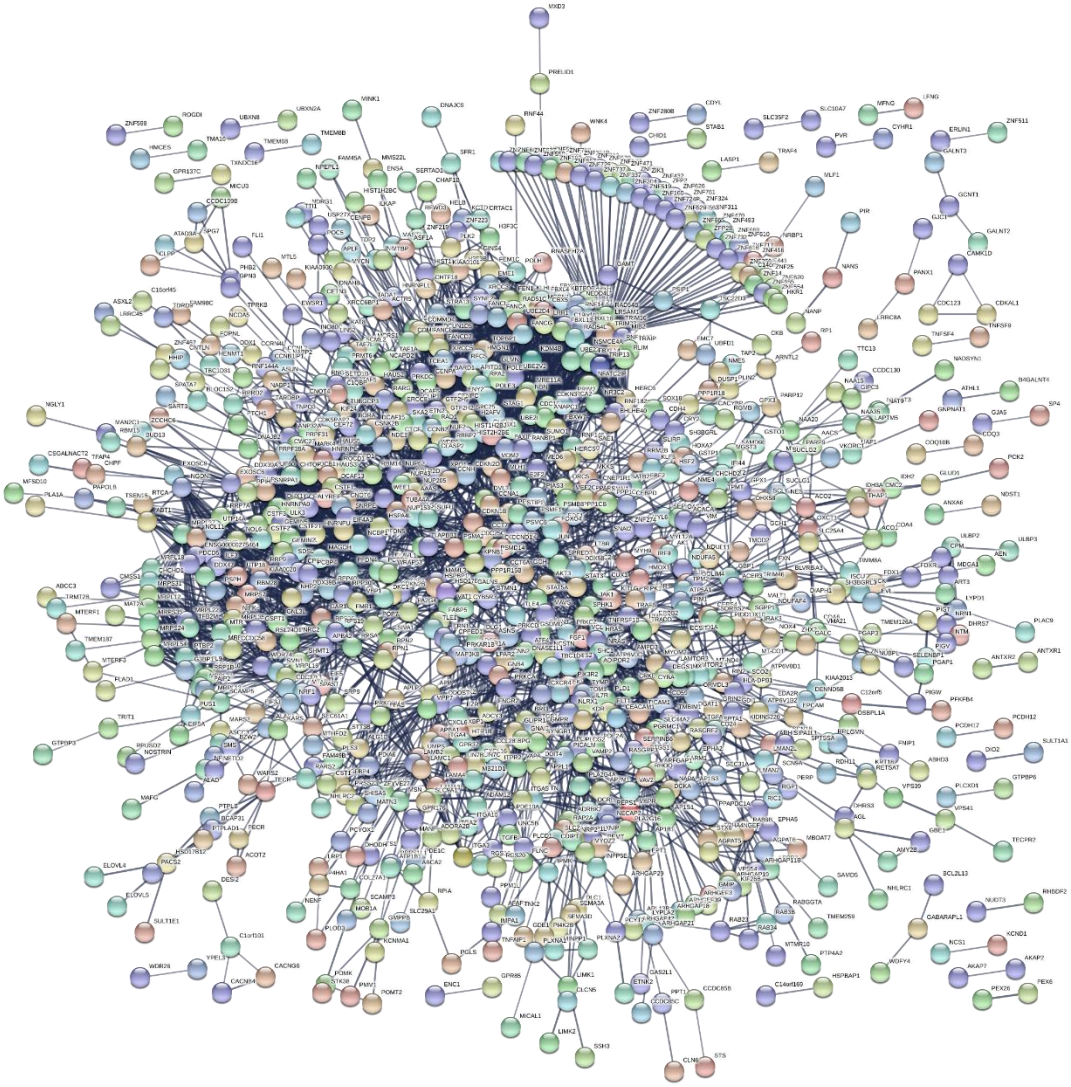

Figure 25: Protein-protein interaction network among the DOXO DEGs, as reconstructed by STRING.

The Common DEGs also form a very highly interconnected PPI network, having a number of high confidence interactions significantly higher than expected (9855 interactions, p-value <1.0e-16), clustering coefficient 0.437 and average node degree 11.7 (Figure 26).

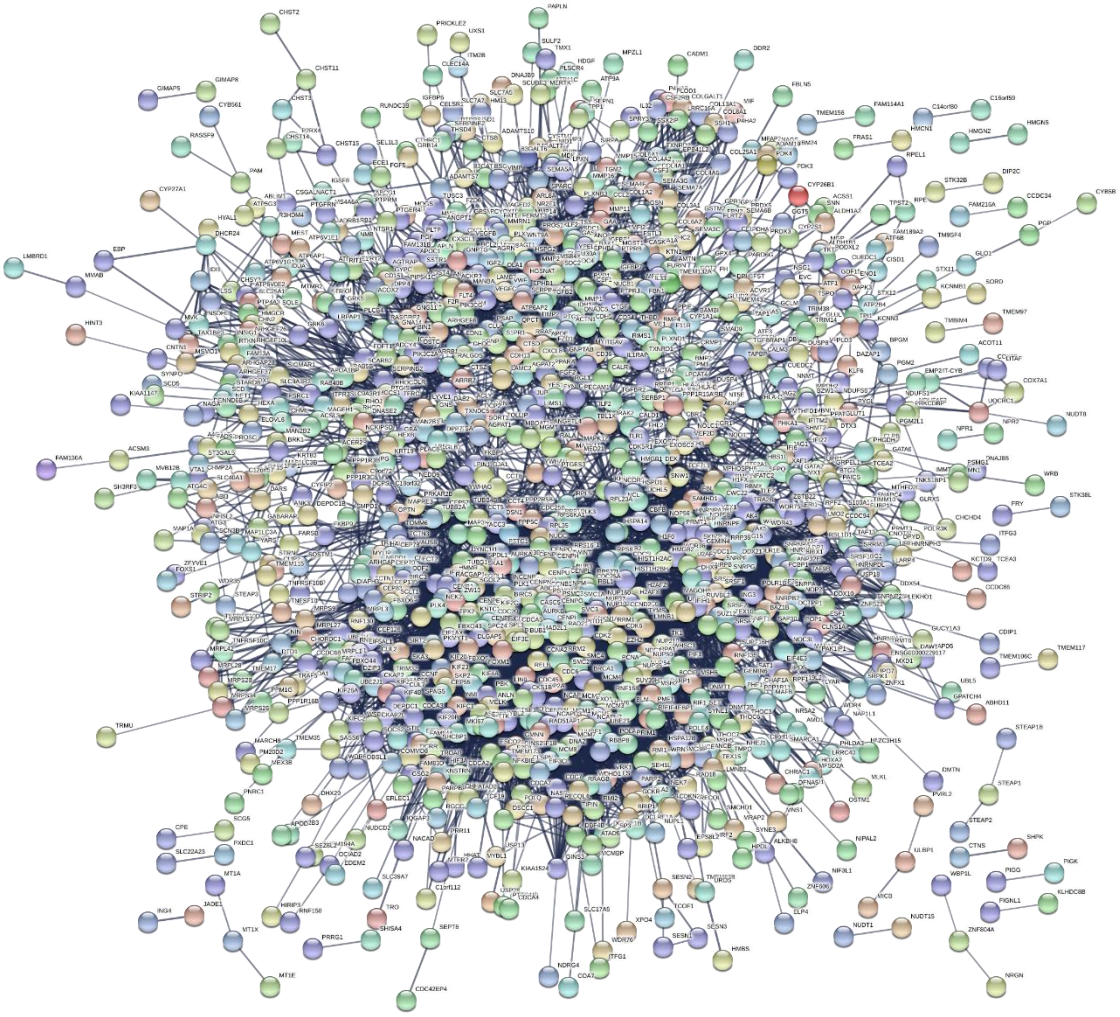

Figure 26: Protein-protein interaction network among the Common DEGs, as reconstructed by STRING.

The Gene Ontology enrichment of the proteins in the PPI network highlight some differences in the three DEG groups. For example, the AXI DEGs PPI genes are enriched in terms associated to cell motility and migration (Table 16), which are not significantly enriched in neither the DOXO or the Common DEGs.

#pathway ID	pathway description	observed gene count	false discovery rate
GO.0051270	regulation of cellular component movement	50	0.00011
GO.0022603	regulation of anatomical structure morphogenesis	50	0.00058
GO.0030334	regulation of cell migration	42	0.00058
GO.0032502	developmental process	177	0.00058
GO.0040012	regulation of locomotion	46	0.00058
GO.1901564	organonitrogen compound metabolic process	172	0.00058
GO.2000026	regulation of multicellular organismal development	78	0.00058
GO.2000145	regulation of cell motility	44	0.00058
GO.0048583	regulation of response to stimulus	134	0.00059
GO.0050793	regulation of developmental process	93	0.00059
GO.0051271	negative regulation of cellular component movement	22	0.00059
GO.2000146	negative regulation of cell motility	21	0.00059
GO.0040013	negative regulation of locomotion	22	0.00098
GO.0051239	regulation of multicellular organismal process	102	0.0011
GO.0048856	anatomical structure development	163	0.0013
GO.0009966	regulation of signal transduction	108	0.0015
GO.0030336	negative regulation of cell migration	19	0.0021
GO.0030155	regulation of cell adhesion	34	0.0023
GO.0048519	negative regulation of biological process	158	0.0023
GO.0051128	regulation of cellular component organization	86	0.0028
GO.0023051	regulation of signaling	115	0.0031
GO.0019538	protein metabolic process	137	0.0035
GO.0010646	regulation of cell communication	113	0.0049
GO.0044267	cellular protein metabolic process	120	0.0057
GO.0048523	negative regulation of cellular process	142	0.0065
GO.0051093	negative regulation of developmental process	42	0.0068
GO.0035239	tube morphogenesis	32	0.0069
GO.0030198	extracellular matrix organization	20	0.0075
GO.0016043	cellular component organization	159	0.0092
GO.0045765	regulation of angiogenesis	19	0.0092

Table 16: GO BP enrichment in the PPI network formed by the AXI DEGs. Significance threshold 0.01.

4. References

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