—DRUG-NEM : an optimized individualized drug combination strategy for intratumoral heterogeneity using single-cell data— Examples in *HeLa and ALL* single cells

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Abstract

Cancers are comprised of populations of cells with distinct molecular and phenotypic features, a phenomenon termed intratumor heterogeneity. Cells in general within a tumor have biomarkers that can be exposed to drug(s) and the response measured using high-throughput single-cell technologies like CyTOF. Some of the markers denoted here as lineage biomarkers can be used to characterize cell types eg. T or B cells including malignant cell types and the others used to define the functional states of a cell eg. apoptotic or intracellular signaling states producing a complex viewpoint of intratumor heterogeneity. Intratumor heterogeneity poses an important challenge to cancer therapy [1]. Drug resistance mechanisms from targeted single drugs have been identified due to intratumor heterogeneity [2], motivating the need for combination therapy. The increasing number of potential FDA approved targeted drugs and corresponding resistance mechanisms identified due to intratumor heterogeneity warrants a systematic and optimal combination therapy that accounts for intratumor heterogeneity.

We introduce a model framework called DRUG-Nested Effects Models (DRUG-NEM) for analyzing heterogeneous drug response data across a population of single cells to optimize and prioritize(rank) combination therapy particularly for single drugs and in general any perturbation response data. This algorithm combines (1) Population identification, (2) Estimating of desired effects (3) Nested effects modeling and (4) Score-Rank analysis to identify and rank multi-target drug combinations from single drug effects measured at the level of single cells. The main function drugnemmain takes as input either (1) a folder containing all drug response FCS files or (2) A data matrix of pooled drug responses for all proteins(columns) across all cells(rows) or (3) a folder with manually gated cells for drug responses from mass cytometry comprising of n drugs and m target (marker) responses on t cells taken from single or several patient samples and returns several pdf files including both the drug target nested network and the sorted list of all possible drug combinations and their scores with the best drug regimen at the top derived from pre-specified desired effects such as down regulation effects or effects associated with a desired response of a desired marker e.g death marker or from a model with no effects. The algorithm also varies the desired effects, computes the DRUG-NEM score and chooses the model with the desired effects that produce the maximal score.

The specific output of the algorithm is a list of 33 items. Item 2 corresponds to the DRUG-NEM ranking using "Any effect or no prior", item 10 corresponds to the DRUG-NEM ranking using prior "Up regulation effects", item 12 correspond to the DRUG-NEM ranking using prior "Down regulation effects", item 14 corresponds to the DRUG-NEM ranking using prior "No effects" and item 33 corresponds the best ranking after comparing priors "Up effects" and "Down effects". The algorithm also generates several pdf(Tiff) files corresponding to various diagnostic plots including plots of initial and final optimized CCAST decision trees for all cell lineage sub groups, Heatmaps of fold changes for all functional or intracellular markers across all sub populations and DRUG-NEM network and ranking list of all drug combinations with corresponding score.

A patent has been filed for the DRUG-NEM framework by Stanford University.

1 Introduction

This document demonstrates some of the functionality of the drugnem package by providing a detailed description of how to use the functions in the package based on different types of input data. In a nutshell,

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drugnem uses data derived from mass cytometry (MCM) (CyTOF), which is a recently developed high throughput MCM technology for labeling single cells with metal-chelated antibodies that greatly reduce the auto fluorescence effect of individual protein signals, thereby circumventing the need for robust compensation after data generation. MCM has been adapted to generate high throughput drug screening single-cell signaling data using mass-tag cellular barcoding (MCB) [3]. This provides the input data for drugnem although other molecular datasets with a similar input data structure can be adapted as well.

2 Installation

drugnem relies on R (>= 3.4.0) and other R libraries: flowCore, party,nem, RColorBrewer, limma, RBGL, MASS, PerformanceAnalytics, fastcluster, cluster, ggplot2, car,stats4, mvtnorm, grid, modeltools, plottrix, xts, sandwich, and zoo. The current implementation runs on Mac OS X 10.7 and above operating systems. R can be installed in these systems by simply downloading the most recent R-x.y.z.tar.gz file from http://www.r-project.org and following the system specific instructions.To install the package using the devtools package in R simply open an R console or click the Rstudio drugnem.Rproj object in the package folder which automatically opens the drugnem libraries in a new stand alone Rstudio window.

3 Installing and Loading the Library using R

We start by installing the drugnem library.

```
>library("devtools")
>library(roxygen2)
>setwd("path/to/drugnem folder")
install("drugnem")
```

We start by loading the drugnem library. Next we create a working directory (drugnem output path) to save all drugnem output figures and data.

```
>library(drugnem)
>dir.create(path/to/drugnem output)
```

A complete installation of drugnem requires that all dependencies are loaded.

4 Overview of the drugnem algorithm

"drugnem" makes use of four different modeling processes: (1) Population identification, (2) Estimating of desired effects (3) Nested effects modeling and (4) Score-Rank analysis to optimize and prioritize possible combination therapeutic strategies from experimentally derived single-drug responses on single cells using CyTOF. The flowchart of algorithm is shown in Figure2 in the main text [4]. CyTOF produces mass cytometry (MCM) data typically stored in a flow cytometry standard (FCS) file as a data frame with rows representing the cells or events and the columns corresponding to the markers of interest. Each FCS file or data matrix would ideally contain lineage markers used to define baseline subpopulations and intracellular markers including death effector proteins such as caspases or CPARP which allow us to compare both survival and intracellular signaling pre and post drug treatment. Briefly stated, in the absence of manually gated subpopulations, we pool all population of single-cell expression data into a single data matrix for all drugs. CCAST [5] is next used to identify and match homogeneous population of cells using the lineage markers across all treatment conditions to define subpopulations. A set of apoptotic marker(s) is selected from data and then CCAST is applied again using these marker(s) to separate survival from death cells for each subpopulation of cells identified from the previous step. We then pool the mixture of labeled survival cells derived into a new data matrix and then estimate the drug effects for each subpopulation. With respect to the baseline treatment (C), we estimate the probability of a protein to be differentially expressed under each drug using linear models [6] for all cell types. We next build an integrated drug-nested

network using nested effects models [7] from a drug desired effects matrix using the weighttype parameter defining the type of desired phenotype information to use for optimizing treatment effects. Values include: (i) 'T-stat': Regulation using T-stat of intracellular signaling effects (ii) 'FC'(default): Regulation based on Foldchange of intracellular signaling effects, and (iii) 'deathmarker': effects associated with regulation of a death effector protein or any other desired marker. (See main text for description). Finally, we score and rank all drug combinations using posterior weights of the target positions on the derived drug-target network with the best regimens on the top. Drug combinations are scored both under the independence and nested assumption of drug effects.

In the following we focus only on key technical details and implementation steps required for running the algorithm and further demonstrate the usage of the main function of drugnem called "drugnemmain" on various data input types specifically, on CyTOF drug response data derived from an cervical cancer cell line (HeLa) and Acute Lymphoblastic Leukemia (ALL) cells showing the expected results. All data sets can be downloaded from https://stanford.box.com/s/ljb09b7wzwuqmm49ahbsb6qzcqo4ruew

4.1 DRUG-NEM uses 3 different input data format of single-cell mass cytometry data for analysis

Drugnem takes as input either (1) a folder containing all drug response FCS files or (2) A data matrix of pooled drug responses for all proteins(columns) across all cells(rows) or (3) a folder with manually gated cells for drug responses from mass cytometry comprising of n drugs and m target (marker) responses on t cells taken from single or several patient samples. The default is an input file path or directory path name with all FCS files of interest. If the input is a cxm flow cytometry expression matrix of c number of cells and m number of markers, the last 2 columns for the data matrix must be labeled "treatment" and "groups" respectively corresponding to the index combination of drug label and cell cluster for each cell. Alternatively, if the input folder contains manually gated cells for various drug responses, the names of each FCS file must contain the name of the corresponding treatment (basal file must be labeled first in each batch) with their corresponding sub population labels. So if we have 3 drugs including a basal treatment and 5 subpopulations we should have 20 manually gated FCS files in the input folder. Note that an input folder with manual gates must be accompanied by the logical specification of the "manualgates" input parameter as "TRUE".

4.2 DRUG-NEM uses density downsampling for FCS files with hundreds of thousands of cells.

If the total number of cells across all FCS files > 100000, we recommend performing the analysis on a subset of cells from each file. DRUG-NEM makes use of the density downsampling approach introduced by Qiu et al. [8] when using the default input data format (1) corresponding to the folder with drug response FSC files or random down sampling when using manually gated FCS files. The default number of cells to subsample from each file is currently 20000 cells. Note that subsampling of cells is only carried out when accompanied with the specification of the logical input parameter "subanalysis" as TRUE.

4.3 DRUG-NEM requires the specification of a set of key input parameters for population indentification, estimation of drug effects and corresponding DRUGNEM-network.

DRUG-NEM requires (1) the specification of a lower bound for the number of sub populations, (2) Vector set of all marker names of length equal to the length of the columns in each FCS file, (3) set of lineage marker names and column ids for population identification as well as functional or intracellular protein names, (4) Network search approach to determine the best drug network. Default is "search" for exhaustive search for maximum 5 drugs. For large networks we recommend the use of "nem.greedy" (5) Parameter defining the type of data and likelihood for scoring the Nested effect models: Default "CONTmLL" called by the "nem" function in nem package [9] which correspond to the effect probabilities while "CONTmLLBayes" correspond to logodds that each protein is differentially expressed (6) Method to weight the intracellular signaling effect data by setting the weighttype parameter to (i) "FC" for intracellular signaling effects associated

with decrease in fold change, (ii) "T-stat" for intracellular signaling effects using T-statistics , (iii) "death marker" for intracellular signaling changes associated with death markers e.g. cPARP. Other important parameters are (7) optimizenoeffect for optimizing effects based probability odds of "No effect" in targeted subpopulations e.g odds of no intracellular signaling effects associated with non-malignant cells (8) Logical parameter Filter (default is TRUE) to to filter out death or dying cells or not and (9) Logical parameter (Fidelity) to test for fidelity of pre vs post treatment effect on characterization of lineage subpopulations using lineage markers.

4.4 DRUG-NEM requires the user to specify whether to filter our out death cells or not.

Studying intratumor signaling using CyTOF is limited to survival cells. After treatment, we identify pro-survival cells using death effector proteins such as caspaces and cPARP. One possibility to remove these cells is by manual gating. Alternatively, drugnem automatically perform such a process by using an unsupervised approach based on the pre-specified apoptotic effectors. This specification is accompanied with the input logical parameter "filter" to filter out death or dying cells. Default is TRUE.

5 Results

5.1 Integration of perturbed TRAIL pathway single-cell CyTOF drug response data show combination of TRAIL stimulator and Mek inhibitor as indispensible for synergistic killing of HeLa cells.

TRAIL is a death ligand member of the TNF ligand superfamily. It is a potent stimulator of apoptosis, and tumour cells are significantly more sensitive to TRAIL-induced apoptosis than normal cells making it an attractive therapeutic target for the treatment of cancer. Once TRAIL binds to its receptors on the cell surface, it recruits pro-caspace8 and FAS-associated protein with death domain (FADD) to form a death-inducing signalling complex (DISC) that triggers a relay of signals leading to cell death (See Figure2a in the main text). The canonical view of apoptotic signaling down stream of activated caspase 8 leads to the activation of key death effector proteins such as PARP, Caspases 3 and 7. Alternatively, TRAIL has been shown to mediate several signal transduction pathways leading to cell proliferation, cell survival as well as cell death. These pathways include nuclear factor kappa B (NFkB), mitogen activated protein kinases (MAPKs), including extracellular signal regulated kinases (ERKs), JUN N-terminal kinases (JNKs) and p38, phosphoinositide 3-kinase (PI3K) and Akt.

Pathway dependent resistance mechanisms to TRAIL have been elucidated by several studies mainly due to intratumor signaling heterogeneity. Here, we show that given treatment response single-cell data comprising of 4 independent targeted treatment strategies (3 inhibitors and a TRAIL pathway activator), 24 cell death related proteins measured in HeLa-cells, we can integrate these 4 data sets to determine a unique drug regimen required to maximize cell death across all cells. TRAIL reflects a standard therapy which when used to treat the cells will likely result in not all the cells dying and consequently developing resistance to TRAIL. We would like to determine a unique drug combination that will be instrumental in the killing of all or most of the cancer cells. The 3 inhibitors used are: (1) Mek inhibitor (GSK); (2) pP38MAPK inhibitor (SB); and (3) PI3K inhibitor (GDC).

We demonstrate the usage of drugnem main function "drugnemmain" on the various 3 input data formats for the HeLa cell data.

5.2 DRUG-NEM analysis on HeLa cells using drug response input data matrix.

Given a drug response data matrix with rows as cells and columns corresponding to markers including "treatment" and "groups" markers as the last 2 columns, the only parameter that might require changing is the patient label parameter. Running the algorithm under such an input data format is shown below. Such an input guarantees the fastest run of the algorithm since there is no clustering step involved.

```
If you have already preprocessed your  input files into a data matrix such as Supplemenetary file
>load("Heladatamatrix.rdata")
>input=Heladatamatrix
> data(input)
> patient="HeLa" # Sample name. String must be present in the each file name.
>antibody1<-c("Time","Cell_length","BC1","BC2","BC3","BC4","BC5","BC6","pHistoneH3","Normbeads1",
"pBadS136","pRb","pHSP27","pErk","IKBalpha","cPARP","pS6","pAMPK","MCL-1","DNA1","DNA2","File num
>drugs1 <- c("BASAL","TRAIL","GSK","GDC", "SB")

>ylabel="cCaspase3"

>clustmarkers<- c("cCaspase3","cCaspase7","cPARP")

>pProteins1=c("pErk","pMAPK2","pP38","pHSP27","pAMPK","pRb","Ki67","pHistoneH3","pP90RSK","RSK2",
>clustersize <- 2
>clustids=match(clustmarkers,antibody1)

> R1=drugnemmain(patient,file=input,outputfile=getwd(), manualgates=FALSE,
celltype=NULL,drugs=drugs1,drugids=c(1:5),antibody=antibody1,pProteins=
pProteins1,transformlogic=FALSE,asinhp=1,colid=clustids,coln=clustmarkers,
rown=NULL,k=clustersize,param=NULL,respondermarker="cPARP",
infer="search",fcsdes=NULL,ylabel=ylabel,subanalysis=FALSE,
subsamplesize=20000,type="CONTmLL",filter=FALSE,weighttype="FC",Fidelity=FALSE)

R1[[2]] : DRUG-NEM ranking using all "intracellular effects" as desired effects
R1[[4]] : DRUG-NEM ranking assuming effects are associated with an upregulation response
R1[[6]] : DRUG-NEM ranking assuming effects  are associated with a down regulation response
R1[[8]] : DRUG-NEM ranking assuming effects  are associated with a no response
R1[[10]] : DRUG-NEM ranking using only prior "Up regulation effects" after controlling for other
R1[[12]] : DRUG-NEM ranking using only prior "Down regulation effects" after controlling for othe
R1[[14]] : DRUG-NEM ranking using only  prior "No effects" after controlling for other desired ef
R1[[33]]  : DRUG-NEM ranking after comparing prior models "Up regulation" and "Down regulation".
```

5.3 DRUG-NEM analysis on HeLa cells using drug response FCS files (Supplementary Data File 1(Supplementary Data S4)).

```
>input=path/to/input FCS files # FCS Files can be downloaded from online supplementary files
>dir.create(path/to/drugnem output)
>setwd(path/to/drugnem output)
>antibody1<-c("Time","Cell_length","BC1","BC2","BC3","BC4","BC5","BC6","pHistoneH3",
"Normbeads1","pBadSer112","cCaspase3","p4EBP1","RSK2","pP38","cCaspase7","pP90RSK",
"pNFkB","S6","Eubeads1","Ki67","pMAPK2","pBCL-2","Bid","pAkt","pBadS136","pRb","pHSP27",
"pErk","IKBalpha","cPARP","pS6","pAMPK","MCL-1","DNA1","DNA2","File number","beadDist",
"barcode","treatment","treatment","tSNE1","tSNE2") ## column names in each FCS file

>drugs1 <- c("BASAL","TRAIL","GSK","GDC", "SB") # vector of drug names

>ylabel="cCaspase3" # lineage marker for  showing marker cut-offs for CCAST algorithm.
> patient="HeLa" # name of sample
> clustmarkers<- c("cCaspase3","cCaspase7","cPARP") # population identification markers?
> pProteins1=c("pErk","pMAPK2","pP38","pHSP27","pAMPK","pRb","Ki67","pHistoneH3",
"pP90RSK","RSK2","pS6","S6","pAkt","p4EBP1","pNFkB","IKBalpha","pBadSer112",
"pBadS136","Bid","pBCL-2","MCL-1","cPARP") # vector of functional markers
```

```
> clustersize <- 2 # Lower bound of number of clusters
> clustids=match(clustmarkers,antibody1) #  population identification column ids
>respondermarkers="cPARP"
> R2=drugnemmain(patient,file=input,outputfile=getwd(), manualgates=FALSE,
celltype=NULL,drugs=drugs1,drugids=c(1:5),antibody=antibody1,pProteins=pProteins1,
transformlogic=FALSE,asinhp=1,colid=clustids,coln=clustmarkers,rown=NULL,k=clustersize,
param=NULL,respondermarker=respondermarkers,infer="search",fcsdes=NULL, ylabel=ylabel,
subanalysis=FALSE,subsamplesize=20000,type="CONTmLL",filter=FALSE,weighttype="FC",Fidelity=FALSE)
```

Figure 1 summarizes the DRUG-NEM analysis on HeLa cells. Figure 1A shows the decision tree that is used to predict the 2 clusters on all treatment samples. Figure 1B shows the heatmap of the normalized log fold change (FC) with respect to the baseline across all treatments. In POP1, TRAIL and GSK show strong downregulation of intracellular markers. POP2, which corresponds to the most resistant cells, shows signs for more up regulation than down regulation across all the treatment conditions especially under SB. Integrating the downregulated effects across all 2 cell states results in the nested drug network shown in Figure 1C. Interestingly, the network structure in Figure 1C predicts GSK and TRAIL as the most essential drug combination likely to be associated with the most downregulated effects across all cells. The complete distribution of ranking and scores of the drug regimens is also shown in Figure 1D beside the predicted network in Figure 1C .

5.4 DRUG-NEM analysis on HeLa cells using drug response manually gated FCS files.

Given manually gated FCS files as input , all of the input specifications stays the same as before except the "manualgates", "celltype" and "drugs" parameters. These parameters with the corresponding main driver function are specified as follows:

```
> patient="POP" # Sample name. String must be present in the each file name.
> celltype=c("POP1","POP2") # Sub population names must also be present in the each file name in
```

```
> R3<- drugnemmain(patient,file=input,outputfile=getwd(), manualgates=
TRUE,celltype=celltype,drugs=drugs1,drugids=c(1:5),antibody=antibody1,
pProteins=pProteins1,transformlogic=FALSE,asinhp=1,colid=clustids,
coln=clustmarkers,rown=NULL,k=clustersize,param=NULL,
respondermarker="cPARP",infer="search",fcsdes=NULL,ylabel=ylabel,
subanalysis=FALSE,subsamplesize=20000,type="CONTmLL",filter=FALSE,weighttype="FC",Fidelity=FALSE)
```

6 DRUG-NEM analysis on ALL drug response data based on a subset of treatment conditions (Supplementary Data File 3(Supplementary Data S5-S7))

We next demonstrate the use of drugnem on an invitro experimental drug response single cell data on a B-cell Philadelphia chromosome positive Ph+ALL patient data denoted here as UPN7. The single-cell drug response CyTOF data generated from the sample comprise of 21 B-cell related developmental proteins denoted in this study as lineage markers and 18 intracellular protein expression responses (intracellular markers) measured before and after 30 minutes of administering 3 FDA approved drugs: Dasatinib (Bcr-abl inhibitor), Tofacitinib (JAK/STAT inhibitor) and BEZ-235 (PI3k/mTOR inhibitor) denoted as Das, Tof and Bez and all pair-wise combination treatment conditions. In a nutshell we have 7 treatment samples including a baseline sample (Basal). However, we would like to run drugnem algorithm condition on only the single drug samples. Also, we allow for discarding of death cells using the "filter" parameter by setting it to TRUE. Furthemore we will like to test whether the data supports the hypothesis that downregulated intracellular effects correlate with our desired response by setting the parameter weighttype="FC" or "T-stat" and looking only at results from output R4[[33]]. It is also often a good idea to compare DRUG-NEM

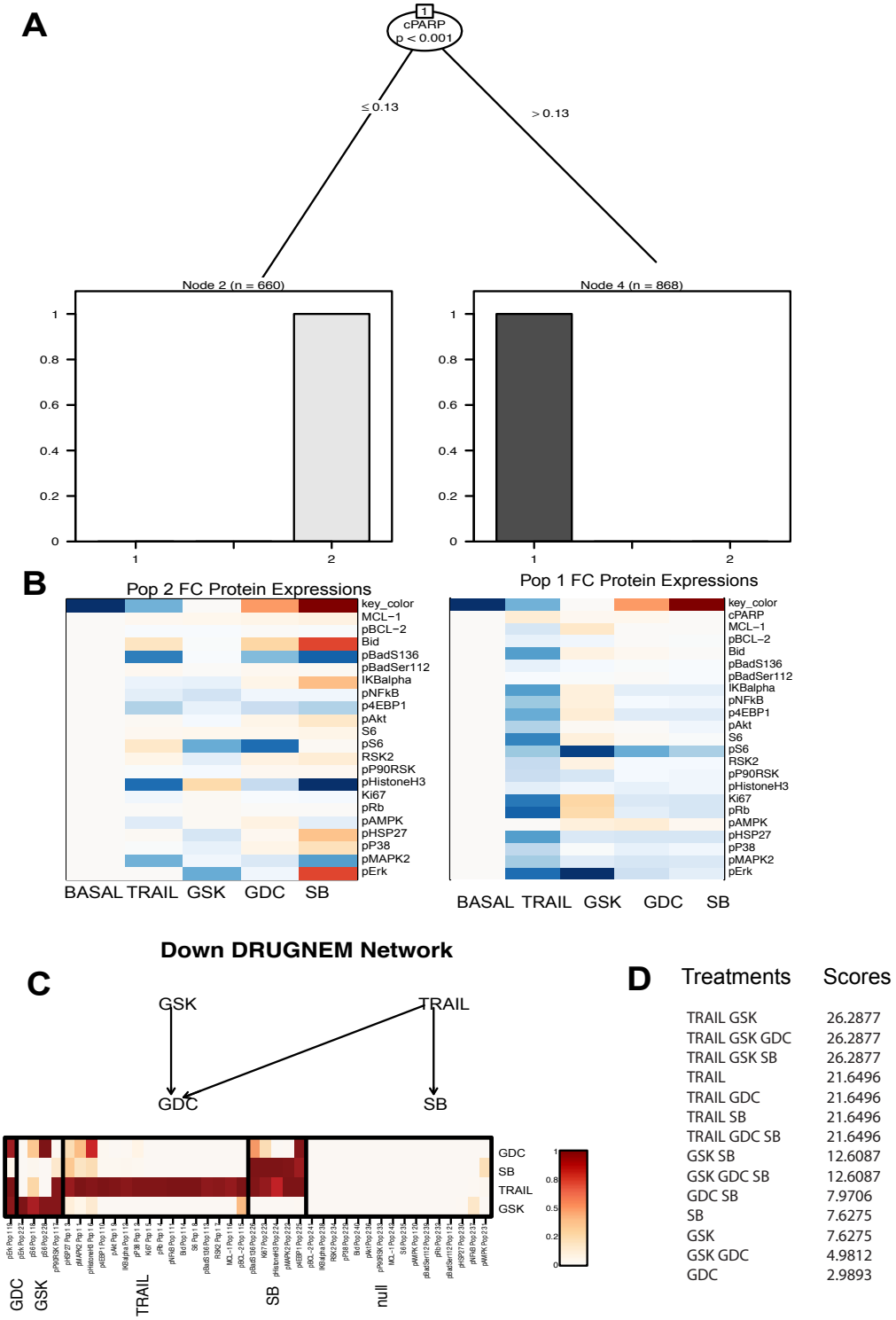

Figure 1: **DRUG-NEM analysis on HeLa cells. A** Decision tree used to predict the 2 clusters on all treatment samples. **B** Heatmap of the normalized log fold change(FC) of functional proteins with respect to the baseline across all treatments. **C** Integrated nested drug network across all cell states portraying the targets of GDC and SB are nested within the targets of TRAIL and GSK. **D** Ranking and scoring of all drug regimens derived from Structure of the network in C

rankings with rankings under independence effects particularly when DRUG-NEM predicts a disjoint nested effects models. Finally, we use "CONTmLLBayes" corresponding to logodds that each protein is differentially expressed instead of "CONTmLL" for probability. measures.

```
>data(input2)
>dir.create(path/to/drugnem output)
>antibody1<-c( "Time" ,"Cell_length","BC1","BC2","BC3","BC4","BC5",
"BC6","CD235_61","CD45","I127","cPARP","Ce140","pPLCg1/2","CD19",
"CD22","4EBP1","tIkaros","CD79b","CD20","CD34","CD179a","pStat5",
"CD123","Ki67","IgMi","Kappa_lambda","pIkaros","CD10","157","CD179b","pAkt","CD24",
"TSLPr","CD127","RAG1","TdT","Pax5","pSyk","CD43","CD38","CD58","CD3","myeloid_FITC","pS6",
"pErk", "HLA-DR", "IgMs","pCreb","DNA1","DNA2","viability","beadDist","barcode")

>drugs1=c("Basal","Bez","Bez+Das","Bez+Tof","Das","Das+Tof","Tof")

>ylabel="HLA-DR"
> patient="UPN7"
> clustmarkers<- c("CD235_61","CD45", "CD19","CD22","CD79b","CD20",
"CD34", "CD179a","CD123","IgMi","Kappa_lambda", "CD10","CD179b",
"CD24","CD127","CD43","CD38","CD58","CD3","HLA-DR","IgMs")

> pProteins1=c("cPARP","pPLCg1/2","4EBP1","tIkaros","pStat5","Ki67","pIkaros","pAkt",
"TSLPr","RAG1","TdT","Pax5","pSyk","pS6","pErk", "pCreb")
> clustersize <- 5
> clustids=match(clustmarkers,antibody1)
>respondermarkers="cPARP"

The ALL input file in a data matrix is stored as Supplemenetary file denoted as ``UPN7datamatrix.
> R4=drugnemmain(patient,file=input,outputfile=getwd(), manualgates=FALSE, celltype=NULL,drugs=dr
pProteins=pProteins1,transformlogic=FALSE,asinhp=1,colid=clustids,coln=clustmarkers,rown=NULL,
k=clustersize,param=NULL,respondermarker="cPARP",infer="search",fcsdes=NULL,ylabel=ylabel,
subanalysis=FALSE,subsamplesize=20000,type="CONTmLLBayes",filter=TRUE,weighttype="FC",Fidelity=FA
```

DRUG-NEM uses CCAST to identify and match 7 cell types across all 3 inhibitors including baseline. Figure 2A shows the decision tree that is used to predict the 7 clusters on all treatment samples. The heat maps in Figure 2B corresponding to each leaf node represent standardized fold changes before (Basal) and after treatment for survival cells. Notice the strong down regulation effect of p4EBP1 by BEZ-235 across almost all subtypes in all 3 samples. BEZ-235 down regulates a lot of targets in subpopulation P7. The nested effect model from integrating all regulated effects across all subpopulations is shown in Figure 2C. The best drug target network corresponds to the model with the targets of Tofacitinib nested within the targets of Das and Bez. The ranking and scores of the drug regimens is shown in Figure 2D beside the predicted network under both nested (left) and additive independence (right) assumptions which are both produced by DRUG-NEM after comparing models optimized for both up and down regulation desired effects. The results for the weighted downregulated intracellular effects is shown in Figure 2E . In this case, the predicted best 2-drug regimen by DRUGNEM based on the single drug data also corresponds to the best prediction under independence of drug effects. Note that under independence score the regimen with the highest score corresponds to all 3 drugs (results not shown).

7 DRUG-NEM analysis on ALL drug response data account for Malignant Vs Non-malignant cells(Supplementary Data S8-S9)

DRUG-NEM can be extended to contextually optimize for different responses in different cell types, opening the possibility for optimizing drug combinations based on desired intracellular responses in malignant cells and possibly a different set of desired effects in non-malignant cells, for example. Here we show an example how DRUG-NEM can be used to optimize drug regimens with the potential to increase cell death in the

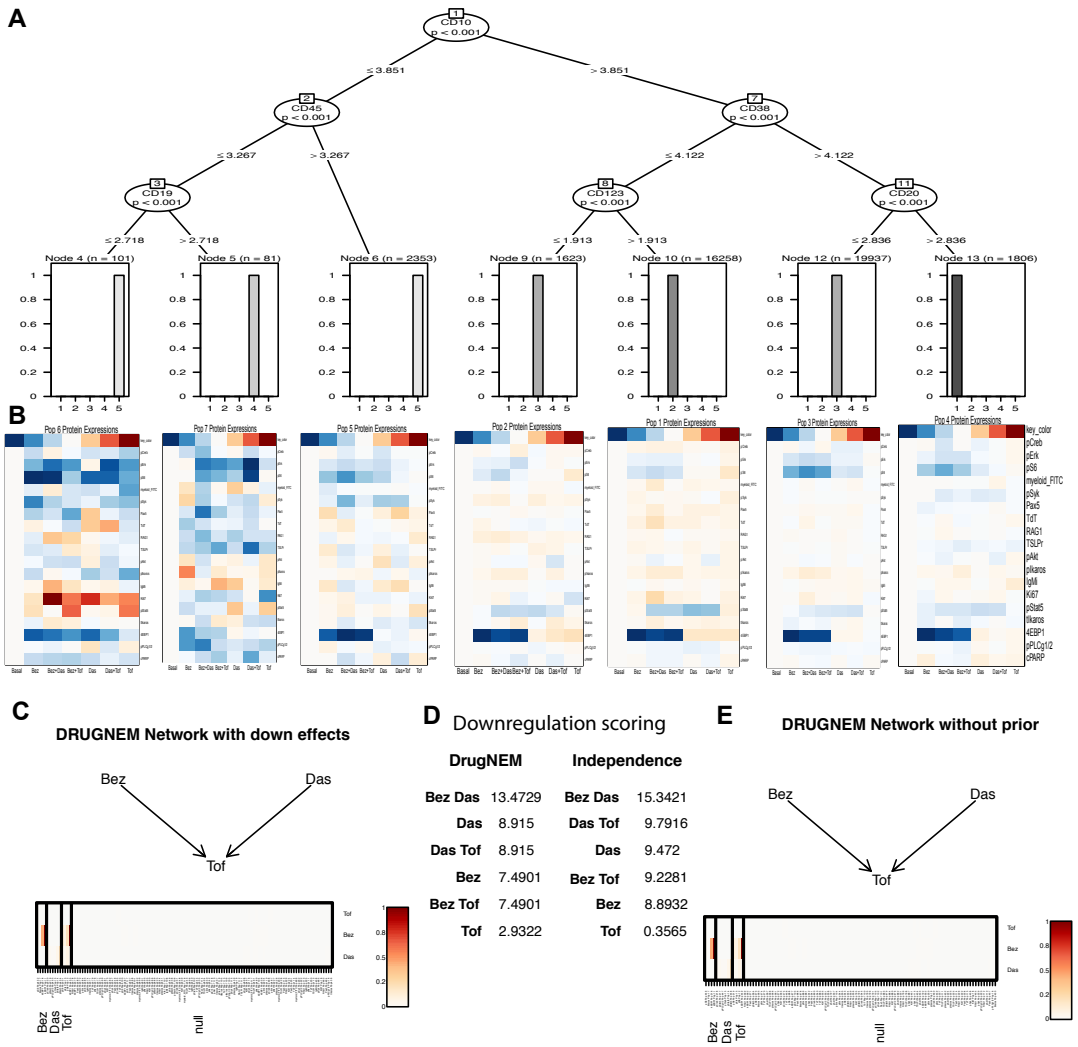

Figure 2: **ALL DRUG-NEM analysis for patient UPN7. A** DRUG-NEM CCAST output for the UPN7 cells derived from all 21 lineage markers showing 7 distinct homogeneous subpopulations at the leaf nodes using only 5 markers: CD38, CD10, CD34, CD123 and CD24. **B** Heat maps corresponding to cPARP lowly expressed cells from all 7 sub types derived in A showing normalized fold changes before (Basal) and after treatment. Notice the strong down regulation effect of 4EBP1 by Bez across all subtypes and the corresponding strong up regulation of effects by Tof. **C** The DRUG-NEM networks derived from integrating only down regulated estimated effects across all subpopulations for all 3 samples showing that the effects of Tof are nested within the effects of Bez and Das. **D** Ranking of all drug combinations under both nested and independence assumptions with their respective scores under model (C) showing the combination of Bez and Das on top across all samples. **E** DRUG-NEM network (top) and heatmap (bottom) from scoring with no prior desired effects showing a similar profile with (C) implying that most of the intracellular effects are actually associated with down regulation.

malignant subpopulation while simultaneously avoiding the non-malignant cells. Since our desired effects are measured in terms of probability, If we assume $log\big(\frac{E_{ij|k}}{1-E_{ij|k}}\big) = B_{ij|k}$ represents the log odds probability of a desired effect associated with intracellular signaling marker M_i under drug S_j in subpopulations k, then with respect to non-malignant sub populations, the $log\big(\frac{1-E_{ij|k}}{E_{ij|k}}\big) = -B_{ij|k}$ represents the probability odds of the complimentary or opposite or no effect. In practice, we use the logical optimizenoeffect parameter to optimize drug regimens with "no effects" as desired effects . For this example we focus on patient UPN7 data. Figure 3A show t-SNE plots of distinct malignant and non-malignant cells with the non-malignant cells significantly much smaller in size. Figure 3B shows the decision trees from CCAST that gives rise to 7 subpopulations of non-malignant cells. Figure 3C-D shows the corresponding DRUGNEM network

and heatmap under optimizenoeffect = TRUE. For this particular analysis, using the DRUG-NEM from the malignant subpopulation for drug combination optimization is optimal because of the equivalent relationship in the non-malignant subgroup. Output list number 14 (R5[[14]]) from running the code below produces the DRUG-NEM ranking when optimized for "No effects".

```
> R5=drugnemmain(patient,file=input,outputfile=getwd(), manualgates=FALSE,
celltype=NULL,drugs=drugs1,drugids=c(1,2,5,7),antibody=antibody1,
pProteins=pProteins1,transformlogic=FALSE,asinhp=1,colid=clustids,coln=clustmarkers,rown=NULL,
k=clustersize,param=NULL,respondermarker="cPARP",infer="search",fcsdes=NULL,ylabel=ylabel,
subanalysis=FALSE,subsamplesize=20000,type="CONTmLLBayes",filter=TRUE,weighttype="FC",optimizenoe
```

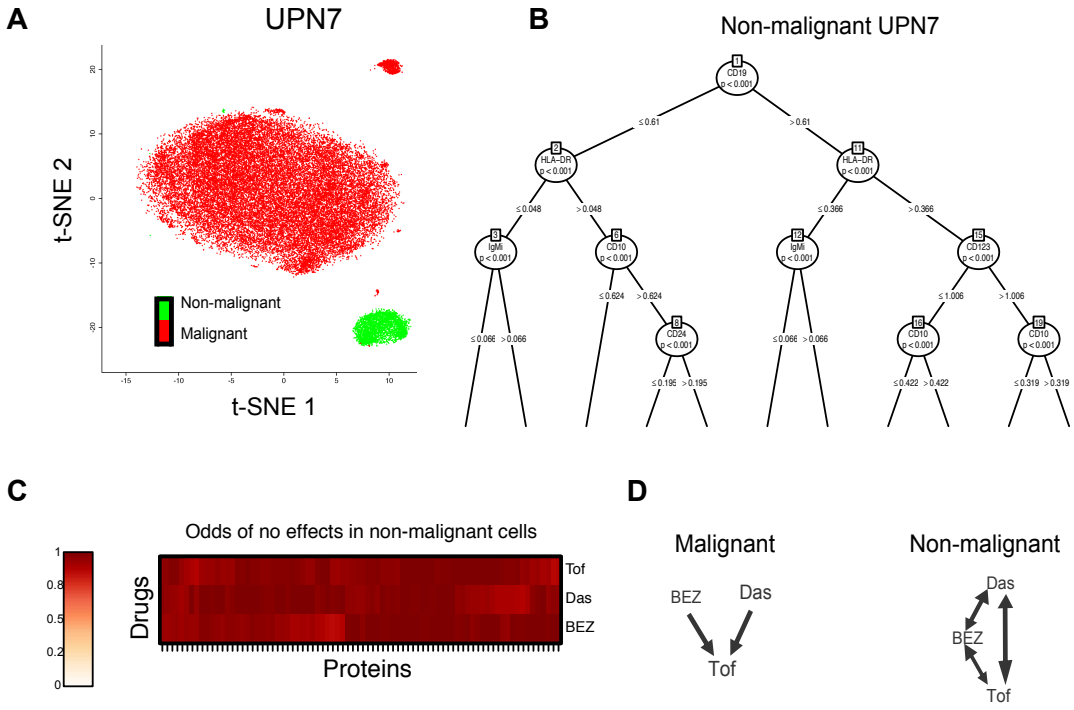

Figure 3: **ALL DRUG-NEM heterogeneous desired effect analysis for patient UPN1. A** t-SNE plot for malignant cells(red) and Non-malignant cells (blue). **B** DRUGNEM network (top) and heatmap (bottom) under "No effect" when we assume a homegeneous desired effect for the non-malignant population of cells

8 Sensitivity analysis of DRUG-NEM on fidelity of pre-treatment versus post-treatment characterization of subpopulations

The above analysis is done based on the assumption that there is no change in the lineage subpopulations before and after treatment. To investigate whether the lineage markers used by the DRUG-NEM algorithm are unaffected by the drugs before CyTOF analysis, we make use of Simes' test [10] by setting the input parameter Fidelity= "TRUE" which tests the global null hypothesis $H_0 = \cap_i H_i = 0$. Focusing particularly on patients UPN7, we generate 21 p-values associated with the difference in expression before and after treatment of lineage markers estimated from limma for each drug across all the 7 subpopulations (Figure 4A). We next estimate the global Simes' adjusted p-value, which uses $\min_i \frac{21 p_i}{i}$ with p_i corresponding to i^{th} smallest p-value in the distribution. The heatmap in Supplementary Figure 4B summarizes the statistical significant changes associated with adjusted p-values based on a 0.05 cut-off for both patients with blue corresponding to no change and a red to a significant change. Clearly subpopulations that show very small effect sizes also tend to be those with the largest number of cells (Supplementary Figure S7A in Supplementary Text S1) providing more power to detect small changes.

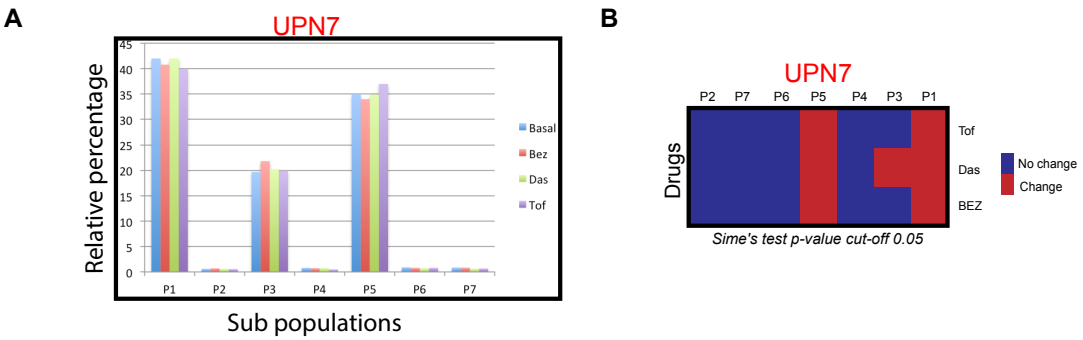

Figure 4: **Fidelity of pre-treatment versus post-treatment characterization of subpopulations for patient UPN7. A** Barplot for 7 linaege subpopulations for UPN7 across all treatment conditions including basal. **B** Heatmaps for UPN7 showing statistical significance based on Simes' global testing of effect of pre-treatment versus post-treatment characterization of the 7 lineage subpopulations shown in Figure 2 for drugs BEZ, Das and Tof. Blue represents no significant change and red represents significant effects, which are also associated with subgroups with the largest sample size.

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