**In silico Drosophila Patient Model Reveals Optimal Combinatorial Therapies for Colorectal Cancer**

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Abstract

In silico models of biomolecular regulation in cancer, annotated with patient-specific gene expression data can aid in the development of novel personalized cancer therapeutics strategies. Drosophila melanogaster is a well-established animal model that is increasingly being employed to evaluate preclinical personalized cancer therapies. Here, we report five Boolean network models of biomolecular regulation in cells lining the Drosophila midgut epithelium and annotate them with patient-specific mutation data to develop an in silico Drosophila Patient Model (DPM). The network models were validated against cell-type-specific RNA-seq gene expression data from the FlyGut-seq database and through three literature-based case studies on colorectal cancer. The results obtained from the study help elucidate cell fate evolution in colorectal tumorigenesis, validate cytotoxicity of nine FDA-approved cancer drugs, and devise optimal personalized drug treatment combinations. The proposed personalized therapeutics approach also helped identify synergistic combinations of chemotherapy (paclitaxel) with targeted therapies (pazopanib, or ruxolitinib) for treating colorectal cancer. In conclusion, this work provides a novel roadmap for decoding colorectal tumorigenesis and in the development of personalized cancer therapeutics through a DPM.

KEYWORDS: Personalized in silico cancer models; Boolean network models; Cancer systems biology; Preclinical in silico drug screening; Combinatorial therapeutics
Introduction

Cancer development is a multistep process that is driven by a heterogeneous combination of somatic mutations at the genetic and epigenetic levels [1,2]. Specific mutations in oncogenes [3] and tumor suppressor genes, [4,5] that result in their activation and inactivation, respectively, manifest themselves at tissue-level in the form of polyps, multi-layering, and metastasis [1,6,7]. These system-level properties resulting from heterogeneous biomolecular aberrations are also acclaimed as “hallmarks of cancer” [1,7]. The heterogeneity amongst individual cancer cells stems from factors such as genomic instability, clonal evolution, and variations in the microenvironment [8,9]. This fosters plasticity in cancer cells which leads to drug resistance – a leading impediment in the treatment of the disease [8–10]. As a result, despite major research initiatives and resultant advancements in decoding the molecular basis of cancer, a comprehensive treatment for the disease still alludes researchers. The limited therapeutic regimens approved by the Food and Drug Administration (FDA) [11–13] exhibit variable efficacies across patients besides a multitude of toxic side effects and, multi-drug resistance [14].

Towards designing efficacious personalized cancer therapeutics, recent advances in high-throughput omics-based approaches complemented by patient-specific gene expression data can provide significant assistance [15,16]. Several online databases and portals provide such freely available datasets including cBioPortal [17], The Cancer Genome Atlas (TCGA) [18], and International Cancer Genome Consortium (ICGC) [19] amongst others [20–22]. However, effective and seamless utilization of such patient-specific genomic data to design personalized cancer therapies is still a fledgling area. Researchers are increasingly employing whole-animal models [23–26] such as mouse, zebrafish, and fruit fly for preclinical in vivo validation of therapeutic hypotheses generated from personalized therapeutics studies. Amongst the animal models, Drosophila melanogaster has become a popular platform for gene manipulation, investigating site-specific changes in the genome, and high-throughput whole-animal screening [15,27]. Importantly, a comparative study of human and fly genome showed that 60% of disease causing genes in humans are conserved in Drosophila [28,29]. Additionally, ease of handling and significantly lower genetic redundancy imparts further advantage to the employment of fly models [5]. As a result, over 50 different data repositories, and tools are now available for hosting data on the fly genome, RNAi screens, and expression data including Flybase [30], FlyGut-seq [31], FlyAtlas [32] databases. Specifically in the case of cancer, several in vivo studies have been designed to elicit novel
therapeutic targets using Drosophila model system [33–37]. One salient example is the validation of indomethacin, which is reported to enhance human Adenomatous Polyposis Coli (hAPC) induced phenotype in Drosophila eye [38] and therefore, employed for treating colorectal cancer (CRC). Vandetanib, another approved targeted therapy that was also validated by using Drosophila system, suppressed Ret activity, and was later approved for medullary thyroid carcinoma (MTC) [33,34].

However, a major shortcoming of using such mono-therapeutic agents for cancer treatment stems from the tumor heterogeneity which results in the selection of resistant cells [39,40] besides acting specifically on singular pathways. To overcome these issues, multiple therapeutic agents acting on multiple pathways in synergy can significantly increase drug efficacy, besides lowering the therapeutic dosage [40]. To evaluate potential high-efficacy synergistic drug combinations, researchers have employed Drosophila model in preclinical studies to elicit optimal drug combinations [36,37]. The Drosophila Lung Cancer Model by Levine et al. [36] helped identify trametinib and fluvastatin as combinatorial drug therapy for lung cancer. Further, an EGFR induced lung tumor model was also designed in Drosophila which assisted in providing an alternative combination of drugs for lung cancer treatment through screening an FDA-approved compound library [37]. However, combinatorial therapies pose unique challenges such as multidrug resistance in chemotherapy [14] and cross drug resistance [41,42] besides the continuing need for higher therapeutic efficacies [43]. Towards tackling these issues, researchers are now ‘personalizing’ live animal platforms for employment in preclinical studies to design efficacious therapeutic regimens. For instance, a comprehensive state-of-the-art in vivo Drosophila Patient Model using a personalized therapeutics approach was described in flies [44]. This particular study involved genetic manipulation of the fly genome to induce mutations specific to KRAS-mutant metastatic colorectal cancer. Combinatorial therapies were then given to the transgenic flies, harbouring mutations that were identified in the patient, to discover high-efficacy synergistic drug combinations.

Here, we propose an in silico counterpart of the in vivo Drosophila Patient Model (DPM) which will facilitate in the modeling and analysis of patient-specified CRC models besides overcoming the challenges of administering combinatorial therapies in animal models [45,46]. We have constructed five biomolecular network models of cells regulating the maintenance of adult Drosophila midgut epithelium lining. These include multipotent intestinal stem cells (ISCs) [47–51], enteroblasts (EBs) [52], enterocytes (ECs) [53–56], enteroendocrine cells (EEs) [57] and visceral muscle (VM) cells [58]. Next, we evaluated each network’s ability to program cell fates in normal conditions as well as under minor perturbations. The networks were then subjected to three types of inputs including
physiological inputs (referred to as “normal”), aberrant inputs such that the fly homeostatic midgut regulation is perturbed (referred to as “stress”), and oncogenic inputs (referred to as “cancer”). The cell fate outcomes under normal and cancer conditions were validated against published literature. The individual output node propensities were also validated against RNA-seq gene expression values taken from FlyGut-seq [31,59] database. Finally, three literature-based case studies were constructed to further validate the proposed in silico DPM. The first case study replicates colorectal tumorigenesis under progressive mutations using Martorell’s CRC model [60]. In the second case study, we employed Markstein et al.’s [61] model to perform therapeutic interventions to validate the cytotoxicity of nine FDA-approved drugs. Finally, in the third case study, we reproduced Bangi’s KRAS-mutant CRC model [44] for evaluating optimal personalized drug treatment combinations by incorporating key patient-specific mutations into our model followed by combinatorial therapeutic screening. Building on these case studies, we devised a novel synergistic combination of paclitaxel (a chemotherapeutic agent) and pazopanib, and ruxolitinib (targeted therapies) for treating ten CRC patients taken from cBioPortal [17,62]. The results obtained from combinatorial chemo- and targeted therapies show up to 100% increase in anti-cancerous cell fates such as apoptosis and a 100% reduction in tumorigenesis promoting cell fates such as hyper-proliferation.

Taken together, we have proposed a computational framework in the form of an in silico DPM to provide personalized CRC therapeutics. This approach can help reduce cancer treatment costs and facilitate in the development of higher efficacy combinatorial therapies for cancer as well as to elucidate novel therapeutic targets.
Results and discussion

Network construction and robustness analysis of regulatory homeostasis in *Drosophila melanogaster* midgut

To investigate the biomolecular signaling regulating homeostasis in *Drosophila melanogaster* midgut, we undertook an extensive literature survey and constructed five cell-type-specific rules-based network models. These models correspond to the five cellular phenotypes lining the *Drosophila* midgut which include: intestinal stem cells (ISCs) [47–51], enteroblasts (EBs) [52], enterocytes (ECs) [53–56], enteroendocrine cells (EEs) [57], and visceral muscle (VM) [58] (Tables S1-5). The scheme of pathways integration for ISC, EB, EC, EE, and VM is provided in Figures S1-5 and the resultant network models consisted of 33 nodes and 51 edges, 30 nodes and 46 edges, 24 nodes and 36 edges, 24 nodes and 36 edges, and 27 nodes and 38 edges, respectively (Figures 1A and B, Figures S6-10).

Next, to evaluate the biological plausibility of the networks, we analyzed each network under normal conditions. Specifically, the biomolecular network of ISC–Apical cells exhibited extrusion, apoptosis, proliferation, and differentiation (or EB fate) with 0.182, 0.179, 0.168, and 0.168 propensities, respectively. EC network exhibited dpp production, and extrusion with corresponding propensities of 0.428, and 0.256. Lastly, for EB and VM cells, extrusion and dpp production were programmed with propensities of 0.335 and 0.450, respectively. Robustness analysis performed by inducing a 10% perturbation in the input stimuli showed the highest variations in ISC’s propensity for apoptosis (SEM=0.0014). Similarly, for EB, EC, and VM, the highest variations in propensity were observed for apoptosis with SEM=0.0027, 0.0034, and 0.0024, respectively (Figure 1C and Figure S11). These results indicate that all five networks are biologically plausible as they exhibited robustness against random perturbations and are hence feasible for employment in onwards analyses [63,64] (Table S6 and Figure S12).
Fig. 1. Regulatory schema of networks for the three cell types present in Drosophila melanogaster midgut. (A) The overall scheme of six conserved pathways involved in the regulation and homeostasis of an adult Drosophila midgut. (B) The mapping between input, processing, and output nodes present in the biomolecular network models of three cell types i.e. ISC, EB, and EC. (C) Cellular fate propensities for ISC–Apical, EBs, ECs, and VM, along with their respective SEMs.
Evaluation and validation of biomolecular network models under normal, stress and colon cancer conditions

To evaluate the proposed networks against published literature and RNA-seq data from FlyGut-seq [31], Deterministic Analysis (DA) was performed [65] under normal, stress, and cancerous conditions (construed as a combination of inputs) (Table S7). Results from our analyses (Figure 2) revealed that in normal conditions, ISC–Apical network programmed extrusion, apoptosis, proliferation, and differentiation (or EB fate) with propensities of 0.183, 0.178, 0.168, and 0.168, respectively (Table S8). Under stress conditions, the propensity for differentiation increased to 0.213, while proliferation, and extrusion reached to 0.211, and 0.213, respectively (see Materials and methods). Lastly, in cancerous conditions, propensities for multi-layering, and apoptosis increased to 0.317, and 0.225, respectively. The results were again validated from the literature which supports elevated apoptosis in ISC’s when under extreme toxic conditions [66,67]. Literature reports also that ISC’s upon encountering extreme stress, exhibit epithelium multi-layering, augmented by overgrowth [68]. Alongside, we also observed a reduction in proliferation, which corroborated with studies showing that tumor cells typically experience limited nutrient availability [69] which also slows down normal ISC cell division rate [70,71] (Figures S13-15).

For network regulation of ISC–Basal cells in physiological conditions (Table S7), the cell fate outcomes included differentiation (or EE fate), apoptosis and delta production, with propensities of 0.341, 0.264, and 0.132, respectively (Table S8). Under stress, Upd production fate increased (from 0.014 to 0.028), and differentiation rate decreased to 0.248. However, delta production remained steady. For cancer conditions, the propensity of differentiation and proliferation decreased to 0.149 and 0.020, respectively, whereas both apoptosis and multi-layering increased to 0.375 and 0.395, respectively. Both of these results, along with the relatively negligible delta expression, are in accordance with previously published reports. Moreover, extreme cellular environments are known to increase apoptosis rate in Enterocytes [66], suggesting that in absence of mutations, normal cells residing in toxic and oncogenic environments can be stressed leading to high apoptosis rates along with an inhibition of cell proliferation (Figures S16-18).

Next, we evaluated cell fate programming of the EB network under normal conditions (Table S7). The results showed extrusion, differentiation (or EC fate) and apoptosis cell fates with propensities of 0.335, 0.197, and 0.152, respectively (Table S8). Alongside, Upd production was also observed with a propensity of 0.130. However, in stress conditions, the propensity for apoptosis and multi-layering increased to 0.253 and 0.136, respectively, whereas, extrusion and differentiation (or
EC fate) decreased to 0.235, and 0.111, respectively. In cancerous conditions, the salient cell fates programmed included multi-layering, apoptosis, and extrusion with propensities of 0.381, 0.291, and 0.161, respectively. Also, differentiation was suppressed to 0.140 due to toxic cellular environments. The trend in cell fate propensities in cancerous conditions also exhibited multi-layering [68] along with low delta production and extrusion (Figures S19-21), which corroborates with published literature which states that delta is a known marker for ISC and in case ISC proliferation, is reduced along with delta production [66]. Extrusion is triggered by over population of cells [72], however, in high stress conditions, cells preferentially inhibit proliferation followed by an enhanced apoptosis thereby limiting extrusion.

Moreover, the EC network was also analyzed for response under normal conditions (Table S7). The emergent cell fates included dpp production, extrusion, and multi-layering with propensities of 0.429, 0.258, and 0.130, respectively (Table S8). Under stress, extrusion rate decreased to 0.101, while apoptosis and dpp production increased to 0.135 and 0.620, respectively. In cancer conditions, however, an increase in propensities of multi-layering (0.378) and apoptosis (0.295) was observed which is in agreement with published studies [66,68] (Figures S22-24).

Fig. 2. Stack bar chart representation of cell fate propensities for intestinal stem cells (ISCs) in apical and basal compartments, enteroblasts (EBs) and enterocytes (ECs) in normal, stress and cancer conditions. (A) ISC–Apical cells adopt nine different cell fates while one remains uncharacterized in three ambient conditions. In normal conditions, the highest propensity was observed for extrusion followed by apoptosis, proliferation, and EB fate, in order. In the case of stress, the highest propensity is that of extrusion, followed by EB fate and proliferation. In cancer, the highest propensity is that of multi-layering, followed by apoptosis and extrusion. (B) ISC–Basal adopts nine different cell fates with the highest propensity being for EE fate in normal conditions, apoptosis in stress conditions while in the case of cancer, multi-layering and apoptosis showed the highest propensity. (C) Seven cellular fates in EB, with the highest propensity for extrusion in normal, apoptosis in stress, and multi-layering in cancer. (D) Five cellular fates in EC, with the highest propensity for dpp production in normal, stress and cancer conditions.
Lastly, a comparison of output node values for ISC–Apical, EB, and EC networks in normal conditions was performed against experimental RNA-seq data from the FlyGut-seq database [31]. Note that due to the paucity of regulatory dynamics in the literature on EE and VM cells, we could not evaluate their networks further. The output node propensities for ISC, EB, and EC were found to be comparable with values from the FlyGut-seq database [31] (Figure 3 and Table S9).
Fig. 3. TISON output nodes propensities (in silico results) validation from FlyGut-seq database (in vivo results). (A) Comparison of nine output nodes propensities in ISC–Apical network: adenomatous polyposis coli (Apc2), cdc42 (Cdc42), head involution defective (hid), suppressor of hairless (Su(H)), prospero (pros), discs large 1 (dlg1), signal-transducer and activator of transcription protein at 92E (Stat92E), rolled (rl) and pangolin (pan). (B) Comparison of eight output nodes propensities in EB network: adenomatous polyposis coli (Apc2), cdc42 (Cdc42), discs large 1 (dlg1), head involution defective (hid), rolled (rl), signal-transducer and activator of transcription protein at 92E (Stat92E), suppressor of hairless (Su(H)), and pangolin (pan). (C) Comparison of seven output nodes propensities in EC network: adenomatous polyposis coli (Apc2), cdc42 (Cdc42), discs large 1 (dlg1), head involution defective (hid), rolled (rl), suppressor of hairless (Su(H)), and pangolin (pan) (Supplementary Table S10).

Case Study 1 – Investigating colorectal tumorigenesis under progressive mutations in Drosophila midgut

To decode the emergent cell fates during initiation and progression of colorectal cancer (CRC) in the adult Drosophila midgut, two salient driver mutations [60] in adenomatous polyposis coli (Apc, in WNT pathway) [73] and Ras (in the EGFR pathway) [74] were incorporated into the ISC–Apical network. These mutations were initially incorporated to act individually and later simultaneously (Figure S25). The emergent cell fates in the control case included apoptosis, proliferation, and differentiation, along with loss of polarity, multi-layering, and extrusion with propensities of 0.180, 0.168, 0.168, 0.00, 0.105 and 0.182, respectively. Upon incorporation of Apc mutation into the ISC–Apical network, a slight decrease in apoptosis and proliferation was observed as their propensities decreased to 0.165 and 0.138, respectively. Differentiation and extrusion also got reduced to 0.138 and 0.148, respectively, while multi-layering increased to 0.349, and loss of polarity remained unaffected. Next, upon introducing Ras mutation, a decrease in apoptosis (0.089) and an increase in proliferation (0.186) was observed, which indicated cellular overgrowth. Furthermore, in line with Martorell et al. [60], differentiation remained unchanged while the loss of polarity and extrusion increased to 0.089 and 0.255, respectively.

On the other hand, the concurrent incorporation of Apc and Ras mutations resulted in hyper-proliferation and overgrowth as apoptosis decreased to 0.066 and proliferation increased to 0.203. Differentiation rate was observed to be 0.138 and loss of polarity, multi-layering and extrusion increased to 0.066, 0.203, and 0.203, respectively. Hence, with concurrent mutations in Apc and Ras, the emergent cell fates started exhibiting the hallmarks of cancer including abnormal proliferation and loss of differentiation, etc [75]. These results were also coherent with both the experimental findings reported by Martorell et al. [60] (Figure 4 and Table S11) and differential gene expression data [76] (Table S12).
Fig. 4. Cell fate outcomes after the introduction of progressive CRC mutations and their validation against Martorell et al.’s Drosophila CRC model. A high rate of extrusion and loss of polarity was observed in Apc-Ras as well as Ras clones. Alongside, an increased proliferation rate with a decreased apoptosis and differentiation is also highlighted by Martorell et al. in their in vivo model.

Case Study 2 – Therapeutic evaluation of CRC in Drosophila midgut using targets from the literature

Introduction of gain-of-function Raf-specific driver mutations in our ISC–Apical network enabled the replication of Markstein et al.’s [61] therapeutic screen towards a comparative cytotoxicity evaluation of nine FDA-approved drugs. In their gain-of-function Raf tumor model, Markstein and colleagues had classified FDA approved drugs into class I and II drugs. According to the study class I drugs induced CRC reversal in mutated cells without effecting the wild type cells, whereas class II drugs besides reversing CRC in mutated cells, also induced CRC in wild type cells (Table S13). The result of our network analysis of the control case exhibited proliferation and apoptosis with propensities of 0.167 and 0.179, respectively. However, after the induction of Raf mutations, proliferation (0.187) rate increased along with a decrease in apoptosis (0.088). Treatment of a Raf-mutated network using class I drugs led to a decrease in proliferation (0.108) and an increase in apoptosis (0.167). No effect was observed on proliferation, which remained steady at 0.168 whereas a slight decrease was observed in apoptosis (0.167) for the wild type in comparison with the control. This confirmed the action of class I drugs which act to significantly reduce cancerous fates in CRC without having a major impact on wild type cells.

Alternatively, in the case of class II drugs, the wild type also exhibited hyper-proliferation after therapy with its propensity reaching up to 0.240 and apoptosis decreasing to 0.068. Importantly, for the CRC network, drug action continued to show cancer reversal with the propensity of proliferation around 0.108 and apoptosis at 0.132. These results suggest that class II drugs are indeed associated
with drug cytotoxicity as they revert cancer phenotype but at the same time induce malignancy in normal cells under therapy. This again confirms Markstein et al.’s study which hypothesized that the extracellular environment in animal models is crucial in drug delivery and cytotoxicity (Figure 5 and Table S14).

![Fig. 5. Evaluating cell fates under therapeutic screens taken from Markstein et al.’s Drosophila model.](image)

(A) The effect of class I drugs on cell proliferation in wild type and CRC networks, (B) The effect of class II drugs on apoptosis in wild type and CRC networks, (C) The effect of class I drugs on apoptosis in wild type and CRC mutated networks, (D) The effect of class II drugs on apoptosis in wild type and mutated network.
Case Study 3 – Employing the *in silico* Drosophila Patient Model for personalized therapeutics

Towards developing a *Drosophila-based* platform for employment in orchestrating patient-centric cancer therapeutics, we adopted Bangi *et al*.’s [44] *in vivo* Drosophila Patient Model (DPM). The *in vivo* model was first translated into an *in silico* DPM which incorporated patient-specific mutations from Bangi *et al*.’s study. These mutations included eight tumor suppressors: Apc, Tp53, Fbxw7, Tgfr2, Smarca4, Fat4, Mapk14, and Cdh1, along with one oncogenic mutation in Kras (Table S15). After inducing these patient-specific mutations into the ISC–Apical network, we administered the combinatorial therapy of trametinib and zoledronate. Our results showed that in control (i.e. healthy cells), the cell fate propensities for proliferation and apoptosis came out to be 0.167 and 0.182, respectively. Upon induction of mutations, proliferation increased to 0.250 and apoptosis decreased to 0.000, respectively. Next, with the administration of trametinib, an inhibitor of MEK kinase (mitogen-activated protein kinase kinase), used to treat patients with Kras mutation [44], the propensities for proliferation and apoptosis reverted to 0, however, upon augmentation of therapy with the addition of zoledronate in combination with trametinib, a decrease in proliferation to 0.168 and an increase in apoptosis to 0.131 was observed. These results exhibited cancer reversal on the administration of the drug combination and corroborate with Bangi *et al*.’s findings (*Figure 6*).

**Figure 6.** Cell fate propensities obtained from the *in vivo* Drosophila Patient Model using Bangi *et al*.’s study.
Identification and evaluation of personalized therapeutics for CRC patients using *in silico* DPM

Towards developing personalized combinatorial therapies for treating colorectal cancer patients, we coupled our *in silico* DPM with patient-specific gene expression data from cBioPortal [17]. Patient-specific potential druggable targets were identified and their oncogenic cell fate propensities were obtained using DA pipeline. Each node was then queried in the PanDrugs database [77] to find out the drugs that targeted them directly or indirectly (Table S16). The results from this exercise elicited paclitaxel [78] and several other targeted therapies including pazopanib, and ruxolitinib depending on patient-specific mutations (Table S17). Follow up literature review showed that these drugs and their combinations are currently being used in several studies and clinical trials [79–82]. Specifically, the combination of paclitaxel and ruxolitinib was evaluated in 2018 to treat human ovarian cancer [79], while the paclitaxel-pazopanib combination was evaluated for treating metastatic melanoma [80] and is in clinical trials for Non-Small Cell Lung Cancer (NSCLC) [81] as well as angiosarcoma [82].

To test the efficacy of these drug combinations in CRC patients, we administered these therapies using the proposed *in silico* DPMs to ten patients with colorectal adenocarcinoma obtained from cBioPortal [17]. To implement the simultaneous action of chemotherapy wherein the drug introduces widespread inhibition of mitosis by stabilizing polymerized microtubules and not allowing them to function during cell division for that, we surveyed the existing literature and constructed a microtubule network (Table S18) with 23 nodes and 28 edges (Figure S26). This network was then integrated into our existing ISC-Apical network to study the behaviour of microtubule stabilization-induced cell fates in chemotherapy (Table S19). The resultant integrated network consistent of 39 nodes and 64 edges (Figure S27). Our results from combinatorial chemo- and targeted therapy using the integrated network showed up to a 100% increase in apoptosis cell fate and a 100% decrease in proliferation rate (Table S20). With administration of targeted therapy only, our results showed up to a 600% increase in apoptosis cell fate and a 100% decrease in proliferation rate (*Figure 7* and Table S21).
Fig. 7. Comparison of oncogenic cell fate propensities obtained from chemotherapy + targeted therapy results versus targeted therapy results. (A) Chemotherapy + targeted therapy for ten colorectal cancer patients for personalized screening. Patient ID and mutation data were extracted from cBioPortal and cell fates for apoptosis and proliferation were plotted to observe before and after therapy results. (B) Targeted therapy for five colorectal cancer patients for personalized screening. Patient ID and mutation data were extracted from cBioPortal and cell fates for apoptosis and proliferation were plotted to observe before and after therapy results.
Conclusion

Taken together, in our study we present a computational framework using a literature-derived in silico Drosophila Patient Model (DPM) for treating colorectal cancer (CRC). We carried out an extensive literature survey to construct five biomolecular network models (intestinal stem cells (ISCs) [47–51], enteroblasts (EBs) [52], enterocytes (ECs) [53–56], enteroendocrine cells (EEs) [57], and visceral muscle (VM) [58]) regulating the maintenance of the epithelium in Drosophila midgut. The networks were analyzed in normal conditions for their robustness against minor perturbations followed by an evaluation in normal, stress, and cancer conditions. The network model was further validated against RNA-seq datasets from FlyGut-seq database as well as three literature-based case studies. Therapeutic screening using the proposed in silico DPM helped personalize treatment for individual patients taken from cBioPortal (Table S17). Outcomes from the in silico screening of ten patients highlight the need for a detailed evaluation of paclitaxel, a chemotherapeutic agent, and targeted therapy synergy to treat CRC patients. To the best of our knowledge, the proposed model is the first of its kind to model fly gut homeostasis and tumorigenesis using the five cells lining the midgut epithelium.

The proposed model can be deployed by wet-lab biologists in preclinical settings to evaluate potential drug targets before their in vivo evaluation. The flexibility offered by this model can also facilitate the incorporation of patient-specific gene expression data towards directly evaluating potential drugs. It will be interesting to employ the proposed model by investigating fly embryo formation and development by incorporating developmental genes. The in silico DPM further stands to strengthen the fly community by providing a tool for value addition in the development of novel therapeutic strategies using personalized therapeutics approaches.
Materials and methods

Data collection and Boolean modeling of five cell-type-specific networks in Drosophila midgut

To construct the biomolecular network models involved in cellular regulation of Drosophila midgut, a comprehensive review of the existing literature and databases was undertaken. The databases employed included the Kyoto Encyclopedia of Genes and Genomes (KEGG) [83], Drosophila Interactions Database (DroID) [84], and data repositories such as FlyGut-seq [31], and Flybase [85]. Alongside, network models of Drosophila by Giot et al. [86], Formstecher et al. [87], and Toku et al. were used to construct five rule-based Boolean biomolecular networks of the conserved signaling pathways in intestinal stem cells (ISCs) [47–51], enteroblasts (EBs) [52], enterocytes (ECs) [53–56], enteroendocrine cells (EEs) [57], and visceral muscle (VM) [58]. Six major pathways involved in maintaining the overall homeostatic nature of the fly midgut were selected from the available literature. These included Notch [88], BMP [88], EGFR [89], WNT [90], JAK-STAT [90,91], and Robo [92] pathways for each cell type lining the midgut. The network steady states were used to program cell fate outcomes such as cellular differentiation, proliferation, apoptosis, EC fate determination, etc. Boolean equations [93] were used to model the regulation of each node in the biomolecular network. TISON, an in-house theatre for in silico systems oncology (https://tison.lums.edu.pk) was used to translate Boolean rules into network models (see Supplementary Data).

Robustness analysis

To validate the biological plausibility of the proposed networks, robustness analysis was performed. Physiological conditions were maintained during this process and the input node values were taken from the FlyGut- seq database [31,59]. The normal node states for ISC, EB, EC, and VM were perturbed by ±10%. Bootstrapping was employed on 10,000 network states. The means and standard deviations of the emergent cell fates were then calculated and standard error of means (SEM) was
plotted for each cell fate to determine the biological plausibility of the scale-free networks [94] (see Supplementary Data 1).

**Deterministic analysis**

The Boolean network models were analyzed using Deterministic Analysis (DA) [65,95] performed in TISON, an in-house web-based multi-scale modeling platform for *in silico* systems oncology. The DA pipeline was derived from ATLANTIS [96]. DA was used to identify ‘cell fate attractors’ – the most probable biological states of a cell and compute their propensities. TISON’s DA pipeline requires three different input files including (i) network file, (ii) fixed node states file, and (iii) cell fate classification file. The network file contained the Boolean rules for rules-based biomolecular networks. The fixed node states file contained fixed values for generating environmental conditions such as normal, stress, or cancer conditions. The cell fate classification file was used to map network states onto the biological cell fates in the light of particular cell fate markers [96] (Table S22). For DA, bootstrapping was employed on 10,000 network states. TISON’s *Therapeutics Editor* (TE) was used to undertake therapeutic evaluation on the network using the DA pipeline, with mutation and drug data integrated. Fixed node states for normal conditions were obtained from FlyGut-*seq* database while for cancer conditions, literature was surveyed to find out if the pathway is up or downregulated. For stress, abnormal values were abstracted by perturbing the stimuli in normal conditions (see Supplementary Data 2).

**Output node validation against Flygut-*seq* database**

To validate the output node propensities of ISC-Apical, EB, and EC networks with FlyGut-*seq* database, we exported the RNA-seq, rpkm values, from the database. The dataset was then used to extract the relevant genes present in our networks (ISC, EB, and EC) using their biological names. Expression data across the five regions of the midgut (i.e. R1, R2, R3, and R5) [97] was normalized for each gene in specific cells. The normalized values were taken as normal input fixed node states for onwards
analyses. The normalized values were then compared with the output node propensities from DA that was performed in normal cell fate conditions in TISON (see Supplementary Data 2).

**Cell fate data collection for case studies and their validation**

To validate and exemplify our network models, we used three literature-based case studies on colorectal tumorigenesis in *Drosophila melanogaster*. For case study 1, data including cell fates under Apc and Ras single and simultaneous mutations were obtained from Martorell *et al.*’s model [60]. The differential gene expression screens and data were also obtained from Martorell *et al.* [76] (see Supplementary Data 3). TISON’s TE was used to implement the mutations in our network using TE’s horizontal therapy pipeline. For case study 2, therapeutic screens including the existing list of FDA-approved drugs for targeting ISC in *Drosophila* were adapted from Markstein *et al.*’s [61] study. Existing databases on drugs and drug-gene interactions such as PharmacoDB [98], PanDrugs [77], and DGIdb [99], etc [100,101] were then used to identify nodes in our ISC–Apical network, which were targets of the drugs mentioned in Markstein *et al.*’s study. TE was employed to deliver drug data into the CRC mutated network using TE’s vertical therapy pipeline. Different fixed node states and cell fate classification files were employed to perform network evaluations in wild type and CRC to mimic different cellular environments in normal and cancerous conditions (see Supplementary Data 4). For case study 3, patient-specific mutations, along with combination therapy drug candidates were taken from Bangi *et al.*’s [44] study. Drug databases were used to identify nodes in the ISC–Apical network which were targets of drugs mentioned in Bangi *et al.*’s study. Drugs which did not have direct targets in the network were implemented indirectly using literature-based mechanisms (see Supplementary Data 5).

**Development of an in silico Drosophila Patient Model (DPM) and its validation**

Towards devising a novel drug combination for the treatment of colorectal tumorigenesis, we performed an exhaustive evaluation of each node in our ISC–
Apical network using TISON’s TE therapy panel. For that, we started with the sensitivity analysis of both tumor suppressor genes and oncogenes involved in CRC using data from existing databases and literature [60,76,98,100,101] against patient-specific mutations taken from cBioPortal [17]. The therapeutic screening was performed by upregulating the tumor suppressors and downregulating the oncogenes (Table S23), to evaluate potential drug combination targets using PanDrugs [77] database, a platform that prioritizes direct and indirect targeting of genomic mutations (see Supplementary Data 6).

**Combination of chemotherapy and targeted therapy to treat CRC patients**

To induce the effect of chemotherapy we carried an extensive survey of the existing literature and constructed a microtubule network. The network consisted of 23 nodes and 28 edges. Next, this network was integrated with ISC-Apical network via up- and downstream signaling interaction. The resultant integrated network contained 39 nodes and 64 interactions. This integrated network was then utilized for chemotherapeutic screening. The combinatorial personalized therapy was used to treat the CRC patients, in a vertical therapy scheme through targeting specific nodes in our ISC-Apical network in light of patient mutations. DA pipeline was used to carry out the therapeutic evaluation (see Supplementary Data 6).
Authors’ contributions

SUC designed and supervised the study. MNG carried out the literature review, construction of the model, and undertook the analyses. MNG and RNB designed the personalized treatment pipeline, SUC, MNG, RNB, ZN, and HK drafted the manuscript. OSS helped construct Boolean networks. RH critically reviewed the model development and performed validations, MT and AF assisted in the study design and manuscript development. All authors read and approved the final manuscript.

Competing interests

The authors have declared no competing interests.

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Figure legends

Figure 1  Regulatory schema of networks for the three cell types present in
Drosophila melanogaster midgut

A. The overall scheme of six conserved pathways involved in the regulation and
homeostasis of an adult Drosophila midgut. B. The mapping between input,
processing, and output nodes present in the biomolecular network models of three cell
types i.e. ISC, EB, and EC. C. Cellular fate propensities for ISC-Apical, EBs, ECs,
and VM, along with their respective SEMs.

Figure 2  Stack bar chart representation of cell fate propensities for intestinal
stem cells (ISCs) in apical and basal compartments, enteroblasts (EBs) and
enterocytes (ECs) in normal, stress and cancer conditions

A. ISC-Apical cells adopt nine different cell fates while one remains uncharacterized
in three ambient conditions. In normal conditions, the highest propensity was
observed for extrusion followed by apoptosis, proliferation, and EB fate, in order. In
the case of stress, the highest propensity is that of extrusion, followed by EB fate and
proliferation. In cancer, the highest propensity is that of multi-layering, followed by
apoptosis and extrusion. B. ISC-Basal adopts nine different cell fates with the highest
propensity being for EE fate in normal conditions, apoptosis in stress conditions while
in the case of cancer, multi-layering and apoptosis showed the highest propensity. C.
Seven cellular fates in EB, with the highest propensity for extrusion in normal,
apoptosis in stress, and multi-layering in cancer. D. Five cellular fates in EC, with the
highest propensity for dpp production in normal, stress and cancer conditions.

Figure 3  TISON output nodes propensities (in silico results) validation from
FlyGut-seq database (in vivo results)

A. Comparison of nine output nodes propensities in ISC-Apical network:
adenomatous polyposis coli (Apc2), cdc42 (Cdc42), head involution defective (hid),
suppressor of hairless (Su(H)), prospero (pros), discs large 1 (dlg1), signal-transducer
and activator of transcription protein at 92E (Stat92E), rolled (rl) and pangolin (pan).
Comparison of eight output nodes propensities in EB network: adenomatous polyposis coli (Apc2), cdc42 (Cdc42), discs large 1 (dlg1), head involution defective (hid), rolled (rl), signal-transducer and activator of transcription protein at 92E (Stat92E), suppressor of hairless (Su(H)), and pangolin (pan). C. Comparison of seven output nodes propensities in EC network: adenomatous polyposis coli (Apc2), cdc42 (Cdc42), discs large 1 (dlg1), head involution defective (hid), rolled (rl), suppressor of hairless (Su(H)), and pangolin (pan) (Table S10).

Figure 4  Cell fate outcomes after the introduction of progressive CRC mutations and their validation against Martorell et al.’s Drosophila CRC model
A high rate of extrusion and loss of polarity was observed in Apc-Ras as well as Ras clones. Alongside, an increased proliferation rate with a decreased apoptosis and differentiation is also highlighted by Martorell et al. in their in vivo model.

Figure 5  Evaluating cell fates under therapeutic screens taken from Markstein et al.’s Drosophila model
A. The effect of class I drugs on cell proliferation in wild type and CRC networks, B. The effect of class II drugs on apoptosis in wild type and CRC networks, C. The effect of class I drugs on apoptosis in wild type and CRC mutated networks, D. The effect of class II drugs on apoptosis in wild type and mutated networks.

Figure 6  Cell fate propensities obtained from the in vivo Drosophila Patient Model using Bangi et al.’s study

Figure 7  Comparison of oncogenic cell fate propensities obtained from chemotherapy + targeted therapy results versus targeted therapy results
A. Chemotherapy + targeted therapy for ten colorectal cancer patient for personalized screening. Patient ID and mutation data were extracted from cBioPortal and cell fates for apoptosis and proliferation were plotted to observe before and after therapy results, B. Targeted therapy for five colorectal cancer patients for personalized screening. Patient ID and mutation data were extracted from cBioPortal and cell fates
for apoptosis and proliferation were plotted to observe before and after therapy results.

Supplementary material

**Supplementary Figure 1** Schematic representation of regulation in Intestinal Stem Cells

Intestinal Stem Cell (ISC) in both apical and basal compartments employ six major signaling pathways including EGFR, WNT, JAK/STAT, BMP, NOTCH and Robo to maintain homeostasis and regeneration in the midgut. The inputs (green boxes) to these pathways are EGFs, Wg, Upds, Dpp, Delta and Slit, respectively. Each input is mapped to the output through an intermediate layer of nodes. The outputs (orange boxes) include Rolled, Cdc42, Hid, Dlg, Apc-Arm, TCF-LEF, STAT92E, Su(H), and Pros. The output layer is used to program cell fates which includes Extrusion, Apoptosis, Polarity, Division, Multilayering, Delta and Upd Production, EB Fate and EE Fates.

**Supplementary Figure 2** Schematic representation of regulation in Enteroblast

Enteroblasts employ five major signaling pathways including EGFR, WNT, JAK/STAT, BMP, and NOTCH. The inputs (green boxes) to these pathways are EGFs, Wg, Upds, Dpp, and Delta, respectively. Each input is mapped to the output through an intermediate layer of nodes. The outputs (orange boxes) include Cdc42, Hid, Dlg, Apc-Arm, STAT92E, and Su(H). The output layer is used to program cell fates which include Extrusion, Apoptosis, Polarity, Multilayering, Delta and Upd Production and EC Fate.

**Supplementary Figure 3** Schematic representation of regulation in Enterocyte

Enterocytes employ four major signaling pathways including EGFR, WNT, BMP, and NOTCH. The inputs (green boxes) to these pathways are EGFs, Wg, Dpp, and Delta, respectively. Each input is mapped to the output through an intermediate layer of nodes. The outputs (orange boxes) include Cdc42, Hid, Dlg, Apc-Arm, and Su(H).
The output layer is used to program cell fates which include Extrusion, Apoptosis, Polarity, Multilayering, Dpp and Upd Production.

**Supplementary Figure 4  Schematic representation of regulation in Enteroendocrine**

Enteroendocrine cells employ four major signaling pathways including EGFR, WNT, BMP, and NOTCH. The input (green boxes) to these pathways are EGFs, Wg, Dpp, and Delta, respectively. Each input is mapped to the output through an intermediate layer of nodes. The outputs (orange boxes) include Cdc42, Hid, Dlg, Apc-Arm, and Su(H). The output layer is used to program cell fates which include Extrusion, Apoptosis, Polarity, Multilayering, and Upd Production.

**Supplementary Figure 5  Schematic representation of regulation in Visceral Muscle cells**

Visceral Muscle cells employ five major signaling pathways including EGFR, WNT, JAK/STAT, BMP, and NOTCH. The inputs (green boxes) to these pathways are EGFs, Wg, Upds, Dpp, and Delta, respectively. Each input is mapped on to outputs through an intermediate layer of nodes. The outputs (orange boxes) include Cdc42, Hid, Dlg, Apc-Arm, STAT92E, and Su(H). The output layer is used to program cell fates which include Apoptosis, Wnt target genes, Delta, Upd and Dpp Production.

**Supplementary Figure 6  TISON implementation of biomolecular pathways involved in regulating intestinal stem cells (apical and basal)**

The network contains 32 nodes and 50 edges with 6 input, 9 output and 17 processing nodes.

**Supplementary Figure 7  TISON implementation of biomolecular pathways involved in regulating Enteroblast**

The network contains 29 nodes and 45 edges with 5 input, 6 output and 18 processing nodes.

**Supplementary Figure 8  TISON implementation of biomolecular pathways involved in regulating Enterocyte**
The network contains 23 nodes and 35 edges with 4 input, 6 output and 13 processing nodes.

Supplementary Figure 9  TISON implementation of biomolecular pathways involved in regulating Enteroendocrine

The network contains 23 nodes and 35 edges with 4 input, 6 output and 13 processing nodes.

Supplementary Figure 10  TISON implementation of biomolecular pathways involved in regulating Visceral Muscle

The network contains 26 nodes and 37 edges with 5 input, 5 output and 16 processing nodes.

Supplementary Figure 11  Standard Error of Means (SEM) for ISC, EB, EC and VM

The SEM with highest for Apoptosis in ISC (0.00128), Upd production for EB (0.00287), Dpp Production for EC (0.0026) and WNT target gene fate for VM (0.0039).

Supplementary Figure 12  Circos plot of biomolecular regulatory networks in ISC (purple), EB (blue), EC (orange), EE (red) and VM (green) cells

The plots shows the interaction relationship between 5 cell types, 12 cell fates and their respective pathways.

Supplementary Figure 13  Intestinal Stem Cell – Apical, in Normal Condition

Supplementary Figure 14  Intestinal Stem Cell – Apical, in Stress Condition

Supplementary Figure 15  Intestinal Stem Cell – Apical, in Cancer Condition

Supplementary Figure 16  Intestinal Stem Cell – Basal, in Normal Condition

Supplementary Figure 17  Intestinal Stem Cell – Basal, in Stress Condition

Supplementary Figure 18  Intestinal Stem Cell – Basal, in Cancer Condition

Supplementary Figure 19  Enteroblast, in Normal Condition

Supplementary Figure 20  Enteroblast, in Stress Condition

Supplementary Figure 21  Enteroblast, in Cancer Condition
Supplementary Figure 22  Enterocyte, in Normal Condition

Supplementary Figure 23  Enterocyte, in Stress Condition

Supplementary Figure 24  Enterocyte, in Cancer Condition

Supplementary Figure 25  Schematic of homeostasis, differentiation and tumorigenesis in normal and diseased midgut

A. In normal midgut, basal ISCs maintain stemness or differentiate into EE while apical ISCs get converted into EB. EBs can then differentiate into ECs under certain conditions; however, they mostly remain dormant in homeostatic conditions. B. In diseased midgut, depending on the mutation type, the gut can either form adenocarcinoma or carcinoma. APC mutation can lead to development of an adenocarcinoma in the gut with a further Ras mutation can result in carcinoma.

Supplementary Figure 26  Schematic Representation of Regulation in Mitochondria

The inputs (green boxes) to these pathways are GF, Upds, Slit and Wg, respectively. Each input is mapped to the output through an intermediate layer of nodes. The outputs (orange boxes) include Stathmin, CLASP, CDK, and Apc-Arm. The output layer is used to program cell fates which includes Destabilize and Stabilize microtubule, corresponding to Proliferation and Apoptosis cell fates, respectively.

Supplementary Figure 27  Schematic Representation of Regulation an Integrated Network of Intestinal Stem Cells and Microtubule

The inputs (green boxes) to these pathways are GF, EGFs, Wg, Upds, Dpp, Delta, and Slit, respectively. Each input is mapped to the output through an intermediate layer of nodes. The outputs (orange boxes) include Rolled, Cdc42, Hid, Dlg, Apc-Arm, TCF-LEF, STAT92E, Su(H), Stathmin, CLASP, CDK and Pros. The output layer is used to program cell fates which includes Extrusion, Apoptosis, Polarity, Division, Multilayering, Delta and Upd Production, EB Fate and EE Fates.
Supplementary Table 1  Detailed node interaction rules and experimental evidences supporting different interactions and logical functions for Intestinal Stem Cells (ISC) model

Supplementary Table 2  Detailed node interaction rules and experimental evidences supporting different interactions and logical functions for Enteroblast (EB) model

Supplementary Table 3  Detailed node interaction rules and experimental evidences supporting different interactions and logical functions for Enterocyte (EC) model

Supplementary Table 4  Detailed node interaction rules and experimental evidences supporting different interactions and logical functions for Enteroendocrine (EE) model

Supplementary Table 5  Detailed node interaction rules and experimental evidences supporting different interactions and logical functions for Visceral Muscle (VM) cells model

Supplementary Table 6  Robustness analysis cell fates and corresponding SEMs for ISC, EB, EC and VM

Supplementary Table 7  Fixed node input states in normal, stress and cancer for ISC-Apical, ISC-Basal, EB and EC network models and their literature validation

Supplementary Table 8  Cell fate propensities of Intestinal Stem Cells (ISC) in Apical and Basal compartments; Enteroblast (EB) and Enterocytes (EC) in Normal, Stress and Cancer conditions along with literature validations

Supplementary Table 9  A comparison of model and experimental output node propensities

Supplementary Table 10  Tabulation of network nodes, gene IDs, annotation symbols, gene symbols, and FlyBase genes
Supplementary Table 11  Martorell et al.'s predictions: experiment versus model

Supplementary Table 12  Differential gene expression comparison between prediction and the model

Supplementary Table 13  Results of class I and class II drugs from Markstein et al.'s therapeutics screens

Supplementary Table 14  Cell fate propensities for proliferation and apoptosis in class I and class II drugs

Supplementary Table 15  Details of the Bangi et al.'s case study: mutations, therapy, and induction of therapy in the in silico DPM

Supplementary Table 16  Oncogenic cell fate propensities of potential target nodes

Supplementary Table 17  Personalized therapeutic combinations for individual patients

Supplementary Table 18  Detailed node interaction rules and experimental evidences supporting different interactions and logical functions Microtubule model (MT)

Supplementary Table 19  Detailed node interaction rules and experimental evidences supporting different interactions and logical functions for Integrated (ISC+MT) model

Supplementary Table 20  Results from chemotherapy + targeted therapy of colorectal cancer patients

Supplementary Table 21  Results from targeted therapy of colorectal cancer patients

Supplementary Table 22  Mapping of cell fate classification logic

Supplementary Table 23  Details of tumor suppressors and oncogenes in ISC-Apical network
Supplementary URL link

The supplementary data of manuscript titled “In silico Drosophila Patient Model Reveals Optimal Combinatorial Therapies for Colorectal Cancer” including input files, output files and analysis results are available at GitHub on this URL:

https://github.com/BIRL/DrosophilaPatientModel