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ADAPTIVE WNT SIGNALING GOVERNS DIAPAUSE-LIKE PERSISTENCE IN TRIPLE-NEGATIVE BREAST CANCER

MECHANISTIC INSIGHTS INTO CHEMOTHERAPY-INDUCED DRUG-TOLERANT STATES

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيْمِ

In the name of Allah, the Most Compassionate, the Most Merciful.

وَأَن لَّيْسَ لِلْإِنسَٰنِ إِلَّا مَا سَعَىٰ (٣٩) وَأَنَّ سَعْيَهُۥ سَوْفَ يُرَىٰ (٤٠)

(سورة النجم، ٣٩-٤٠)

And that man shall have nothing but what he strives for. And that his striving will soon be seen.

وَقُلْ رَبِّ زِدْنِي عِلْمًا

(سورة طه، ١١٤)

And say: "My Lord, increase my knowledge."

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Summary

Breast cancer is the most common and most deadly neoplastic disease among women worldwide. Triple-negative breast cancer is a molecular subtype of breast cancer characterized by the lack of expression of hormone and growth factor receptors. Clinically, this subtype presents highly aggressive features, poor prognosis, and high relapse rates. The lack of hormone and growth factor receptors renders targeted therapies, which are highly efficient in battling other subtypes of breast cancer, obsolete.

Despite the striking advancements in the therapeutic landscape of triple-negative breast cancer, cytotoxic chemotherapy remains the primary standard of care treatment. In response to neoadjuvant chemotherapy, triple-negative breast cancer patients achieve pathologic complete response at significantly higher rates compared to non-triple-negative patients. However, despite the better response rates, tumors belonging to the triple-negative subtype paradoxically present higher rates of relapse and recurrence. Relapse is a direct consequence of treatment failure, often hinging on drug resistance; which can either be intrinsic or acquired – or both.

In recent years, several key studies have highlighted that in treatment failure and residual disease in triple-negative breast cancer cells is driven by adaptative transcriptional rewiring-based mechanisms rather than the mere selection of pre-existing resistance-mediating genotypes. Drug-tolerant persisters describe a subpopulation of cancer cells that acquire a reversible drug-refractory phenotype in response to therapeutic pressure. Since their first identification in 2010 in Non-small cell lung cancer, drug-tolerant persister cells have since been described in a wide range of tumor types in response to various therapeutic modalities and have now become a focal research point in the field of cancer resistance. However, we still lack a fundamental understanding of the mechanisms driving the entry of cancer cells into a persister state and, therefore, lack actionable targets to prevent chemotherapy adaptation and acquisition of resistance.

In this thesis, we sought to investigate the early molecular mechanisms, namely the signaling cascades, that are involved in driving entry into and/or exit from a drug-tolerant persister state in triple-negative breast cancers in response to chemotherapy – using *in vitro*, 3D *ex vivo*, and *in vivo* models.

Transcriptomic profiling of in-house-generated early drug-persister cells revealed significant enrichment of the canonical Wnt/β-catenin signaling pathway. Similarly, chemotherapeutic treatment of triple-negative breast cancer *in vitro* cell lines, *in vivo* cell line-derived xenograft models, *ex vivo* patient-derived organoid models, and patient tumors validated transcriptional activation of the canonical Wnt/β-catenin signaling pathway.

Upon early-persister (and chemotherapy naïve) cells, only Wnt-active (Wnt^{High}) persister cells exhibit a true transcriptional diapause-like cell-phenotype and function as *bona fide* early diapause-like drug-tolerant persisters, highlighting that Wnt transcriptional activity serves a distinct transcriptional and functional biomarker for that state. Moreover, in chemotherapy naïve conditions, Wnt-activation promoted a paused proliferative state and enhanced chemoresistance, recapitulating major key features of a diapause-like persister cell phenotype.

Chemotherapy-mediated Wnt-activation guiding entry into a diapause-like persister cell phenotype results namely from a *de novo* action rather than solely representing a manifestation of an inherently chemotherapy-resistant subpopulation selected under treatment pressure. Moreover, this activation displayed a transient and plastic nature, exhibiting a reversible capacity upon drug withdrawal.

Mechanistically, chemotherapy-induced Wnt-activation (and its loss upon drug withdrawal) hinged on the expression and secretion of Wnt-ligands, -enhancers, and -ligand secretion machinery.

Notably, inhibition of Wnt-ligand secretion is able to disrupt chemotherapy-mediated Wnt-activation and the subsequent induction of a diapause-like persister state – sensitizing *in vitro* cell lines, *in vivo* cell line-derived xenograft models, and *ex vivo* patient-derived organoid models to chemotherapy.

Altogether, our findings indicate that Wnt-transcriptional activity contributes to the induction of a diapause-like persister state in triple-negative breast cancer cells, providing a pivotal route of chemotherapeutic resistance and offering new insights into the identification and exploitation of this diapause drug-tolerant persister population.

Samenvatting

Borstkanker is wereldwijd de meest voorkomende en dodelijkste neoplastische ziekte bij vrouwen. Triple-negatieve borstkanker is een moleculair subtype van borstkanker waarbij hormoon- en groeifactorreceptoren ontbreken. Dit subtype vertoont klinisch gezien zeer agressieve kenmerken, slechte prognoses en hoge percentages van herval. Waar bij de bestrijding van andere subtypes van borstkanker gerichte therapieën zeer effectief zijn, is dit, door het ontbreken van hormoon- en groeifactorreceptoren, niet het geval bij dit subtype.

Ondanks de aanzienlijke vooruitgang in de therapeutische behandeling van triple-negatieve borstkanker, blijft cytotoxische chemotherapie de primaire standaardbehandeling. Ten opzichte van niet-triple-negatieve borstkankerpatiënten, vertonen patiënten met triple-negatieve borstkanker significant hogere percentages pathologische complete respons als reactie op neoadjuvante chemotherapie. Ondanks deze hogere responspercentages vertonen tumoren van het triple-negatieve subtype paradoxaal genoeg hogere percentages van herval. Herval is een direct gevolg van het falen van de behandeling, vaak te wijten aan resistentie tegen geneesmiddelen, die intrinsiek en/of verworven kan zijn.

In de afgelopen jaren hebben verschillende belangrijke studies aangetoond dat het falen van behandeling en restziekte bij triple-negatieve borstkankercellen voornamelijk worden veroorzaakt door adaptieve, transcriptie-herprogrammeringsmechanismen, en niet louter door de selectie van reeds aanwezige, resistentie-verlenende genotypen. *Drug-tolerant persisters* vormen een subpopulatie kankercellen die, als reactie op therapeutische druk, een omkeerbaar geneesmiddel-refractair fenotype verwerven. In 2010 werden *drug-tolerant persister*-cellen voor het eerst geïdentificeerd bij niet-kleincellige longkanker. Sindsdien worden ze beschreven in een breed scala aan tumortypen, als reactie op uiteenlopende therapeutische modaliteiten, en vormen ze inmiddels een centraal onderzoeksgebied binnen het domein van kankerresistentie. Een fundamenteel begrip van de mechanismen die de overgang van kankercellen naar een *persister*-toestand aansturen, en bijgevolg aangrijpingspunten voor het voorkomen van chemotherapie-adaptatie en het verwerven van resistentie, ontbreekt echter nog steeds.

In deze thesis werd onderzocht welke vroege moleculaire mechanismen, met name de signaalcascades, betrokken zijn bij de overgang naar en/of het verlaten van een *drug-tolerant persister*-toestand in triple-negatieve borstkankers als reactie op chemotherapie. Daarbij werd gebruik gemaakt van *in vitro*-,3D-*ex vivo*- en *in vivo*-modellen.

Transcriptomische profilering van in eigen gegenereerde vroege *drug-persister*-cellen toonde een significante verrijking van de canonieke Wnt/β-catenine-signaalroute. Evenzo bevestigde chemotherapeutische behandeling van triple-negatieve borstkanker de transcriptionele activatie van de canonieke Wnt/β-catenine-signaalroute in *in vitro*-cellijnen, *in vivo* uit cellijnen afgeleide xenograftmodellen, *ex vivo* patiënt-afgeleide orgaanmodellen en patiënttumoren.

Bij vroege *persister*- (en chemotherapienaïeve) cellen vertonen uitsluitend Wnt-actieve (Wnt^{High}) *persister*-cellen een werkelijk diapauze-achtig transcriptioneel cel-fenotype en functioneren zij als *bona fide* vroege diapauze-achtige *drug-tolerant persisters*. Dit geeft aan dat Wnt-transcriptionele activiteit een onderscheidende transcriptionele en functionele biomarker vormt voor die toestand. Bovendien bevorderde Wnt-activatie onder chemotherapienaïeve omstandigheden een gepauzeerde proliferatieve staat en verhoogde het de chemoresistentie, waarmee essentiële kenmerken van het diapauze-achtige *persister*-celphenotype werden gereproduceerd.

Chemotherapie-gemedieerde Wnt-activatie, die leidt tot een diapauze-achtig *persister*-celfenotype, is het resultaat van een *de novo*-werking en vormt niet louter de manifestatie van een reeds aanwezige chemoresistente subpopulatie die onder therapeutische druk is geselecteerd. Bovendien vertoonde deze activatie een tijdelijk en plastisch karakter, met een omkeerbaar vermogen na het stopzetten van de behandeling. Mechanistisch gezien hing de door chemotherapie geïnduceerde Wnt-activatie (en het verlies ervan na staken van de behandeling) af van de expressie en secretie van Wnt-liganden, -versterkers en het -ligandsecretie-mechanisme.

Opmerkelijk is dat remming van Wnt-ligandsecretie de door chemotherapie geïnduceerde Wnt-activatie en de daaropvolgende inductie van een diapauze-achtige *persister*-toestand kan verstoren, waardoor *in vitro*-cellijnen, *in vivo* uit cellijnen afgeleide xenograftmodellen en *ex vivo* patiënt-afgeleide orgaanmodellen gevoeliger worden voor chemotherapie.

Samengenomen wijzen onze bevindingen erop dat Wnt-transcriptionele activiteit bijdraagt aan de inductie van een diapauze-achtige *persister*-toestand in triple-negatieve borstkankercellen, hetgeen een cruciale route vormt naar chemotherapeutische resistentie en nieuwe inzichten biedt voor de identificatie en het benutten van deze diapauze-*drug-tolerant persister*-populatie.

List of Abbreviations

5-FU	5-Fluorouracil
ABC	ATP-binding cassette
ADC	antibody-drug conjugate
ALDH	aldehyde dehydrogenase
AML	acute myeloid leukemia
APC	adenomatosus poluposis coli
AR	androgen receptor
ATR	ataxia telangiectasia and Rad3 related-protein kinase
AXIN	axis inhibition protein
BC	breast cancer
BCO	breast cancer organoid
BCSC	breast cancer stem cell
BIO	6-Bromoindirubin-3'-oxime
CAF	cancer associated fibroblast
CAR	carboplatin
CDK	cyclin-dependent kinase
CDX	cell line derived xenograft
CHIR	CHIR99021
CM	conditioned media
CPS	combined positive score
CR	complete response
CRC	colorectal cancer
CSC	cancer stem cell
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DAPI	4’,6-diamidino-2- phenylindole
Dato-DXd	datopotamab deruxtecan
DCIS	ductal carcinoma in situ
DDR	DNA damage response
DDR	DNA damage repair
DEG	differentially expressed gene
DKK	dickkopf-related protein
DOC	docetaxel
DTP	drug-tolerant persister
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FZD	frizzled
GSEA	gene set enrichment analysis
GSK3β	glycogen synthase kinase β
HDAC	histone deacetylase
HDI	human development index
HER2	human epidermal growth factor receptor 2
HIF	hypoxia-inducible factor
HK	housekeeping gene

HR	homologous recombination
HRD	homologous recombination deficiency
IC50	half-maximal inhibitory concentration
ICI	immune checkpoint inhibitor
IDC	invasive ductal carcinoma
ILC	invasive lobular carcinoma
ITH	intratumor heterogeneity
IVIS	in vivo live imaging system
IWP-2	Inhibitor of Wnt Production
KDM5A	lysine-specific demethylase 5A
LAG-3	lymphocyte-activation gene 3
LCIS	lobular Carcinoma in situ
LGR	Leu-rich repeat-containing G-protein coupled receptor
LRP	low-density lipoprotein receptor-related protein
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MaSC	mammary gland stem cell
MDR1	multi-drug resistance 1
MDSC	myeloid-derived suppressor cell
miRNA	microRNA
MMRD	mismatch repair deficiency
mTOR	mammalian target of rapamycin
NAC	neoadjuvant chemotherapy
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cell
NSCLC	non-small cell lung cancer
ORR	objective response rate
OS	overall survival
PARP	poly-ADP ribose polymerase
pCR	pathologic complete response
PD	progressive disease
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PDO	patient derived organoid
PFS	progression free survival
PI3K	phosphatidylinositol 4,5-biphosphate 3-kinase
PORCN	porcupine O-acyltransferase
PR	partial response
PR	progesterone receptor
PTEN	phosphatase and tensin homolog
RAS	rat sarcoma
RNA-seq	RNA-sequencing
ROR	receptor tyrosine kinase-like orphan receptor
ROS	reactive oxygen species
RSPO	R-spondin
RT-qPCR	real-time quantitative polymerase chain reaction
RYK	receptor-like tyrosine kinase
scRNA-seq	single-cell RNA-sequencing
SD	stable disease
SFRP	secreted frizzled related protein
SG	sacituzumab govitecan
shRNA	short hairpin RNA
siRNA	small interfering RNA

TAM	tumor-associated macrophage
TCF/LEF	T cell factor/lymphoid enhancer factor
TGF-β	transforming growth factor β
TIL	tumor-infiltrating lymphocyte
TIME	tumor immune microenvironment
TK	tyrosine kinase
TMB	tumor mutational burden
TME	tumor microenvironment
TNBC	triple negative breast cancer
TNF	tumor necrosis factor
Treg	regulatory T cell
Trop-2	trophoblast cell surface antigen-2
TSG	tumor suppressor gene
UNT	untreated
VEGF	vascular endothelial growth factor
VEH	vehicle
WIF	wnt inhibitory factor 1
WLS	wntless

Chapter 1: Introduction

This chapter contains sections and illustrations which are part of a review published in May 2022 in the journal Frontiers in Cell and Developmental Biology under the title: ***"Wnt Signaling in the Breast: From Development to Disease"****.*

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1. Chapter 1: Introduction

1.1. Cancer

1.1.1. Historical Overview

Cancer has affected humans and animals alike throughout recorded and pre-recorded history, with some of the earliest credible evidence of cancer in mammals comprising tumor masses found in fossilized dinosaur and human bones from pre-historic times (1). Though not yet labeled as "cancer", the earliest written records believed to describe human cancer are found in ancient Egyptian manuscripts (e.g., the Edwin Smith and George Ebers papyri, ~ 3000 BC), describing pharmacological, surgical, and magical treatments (1–4). Of the 49 cases of injuries described in the Edwin Smith Papyrus, case number 45 is believed to contain the first reference of breast cancer, referring to tumors or ulcers of the anterior chest (1,5). The ancient Egyptians attempted to treat tumors via cauterization of open wounds, use of salts, and an arsenic-based paste (referred to as the "Egyptian Ointment" until the 19th century) (3,6). In distant and later civilizations, Sumerians, Indians, Chinese, Hebrews, and Persians employed herbal treatments (e.g., fruit juices, teas, and figs) but resorted to chemical-based agents (e.g., iron, sulfur, copper, and mercury) in advanced cases. Many of these formulations and remedies remained in use for over 3000 years (3,7).

Thousands of years later, in ancient Greece, Hippocrates (460-370 BC), considered as the Father of Medicine, used the ancient Greek word *karkinos* (καρκίνος) meaning crab (later Latinized as *cancer*) to describe malignant tumors (1–4). Medical writers (7th century AD), Stephanus of Alexandria and Paul of Aegina, hypothesized that naming the disease after the crab was on based on **i)** its character; whereby the disease, similar to the crab, can be very aggressive and obstinate and **ii)** its appearance; whereby the veins surrounding the area in which the cancer has developed resemble the legs of a crab (8,9). Another Greek physician, Galen (130-200 AD), used the Greek word for swelling – *oncos*, pl. *onkoi* (όγκος) to describe abnormal incidents of swelling that happen to human bodies, causing discomfort (10). It is likely that misdiagnoses of swelling were frequent considering the difficulty of differentiating benign and malignant tumors. Nonetheless, despite the crab analogy of Hippocrates still being used to describe malignant tumors to this day, Galen's term provided the basis for naming cancer specialists – oncologists.

Throughout the Middle Ages and Renaissance period, cancer was attributed to humoral imbalances (11). Anatomical discoveries by Andreas Vesalius and pathological insights by Giovanni Morgagni and Rudolf Virchow established cancer as a disease of organs and later of cells – marking the beginning of modern pathology (11). These advances set the stage for the 20th century discoveries of carcinogens, oncogenes, and cancer therapeutics, laying the foundation for where we stand today.

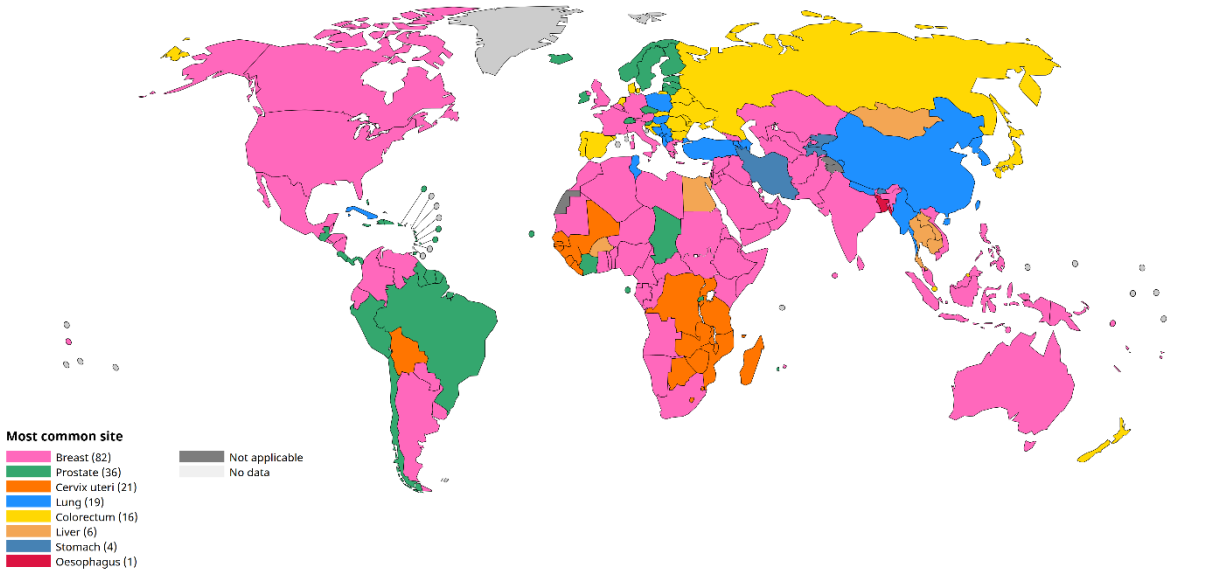
Cancer is both a biological phenomenon and a group of diseases. As a phenomenon, it describes abnormal cell growth due to a dysregulated balance between proliferation and cell death, leading to cells capable of invading surrounding tissues and metastasizing to distance organs – causing significant morbidity, and, if untreated, death of the host (12). Clinically, cancer refers to a collection of diseases in multicellular organisms characterized by the disruption of homeostatic control, favoring uncontrolled tumor growth.

1.1.2. Overview in Numbers

Cancer is a major global health challenge. In 2022, nearly 20 million new cases and 9.7 million cancer-related deaths were reported globally, a 90% increase compared to the early 2000s, 216% increase compared to the 1980s, and a 375% increase compared to the 1950s (13,14). Projections estimate 35 million cases and 16 million deaths by 2050 – estimating increases (vs. 2022) of 77% for new cases and 65% for cancer-related deaths. The rapidly growing global cancer burden is driven by varying factors including aging populations, lifestyle changes, and exposure to risk factors (often associated with region and socioeconomic background).

Notably, 1 in 5 people will develop cancer in their lifetime; approximately 1 in 9 men and 1 in 12 women die from the disease. According to the Global Cancer Observatory[I], lung (2.5 million new cases, 12.4% | 1.8 million deaths, 18.7%), breast (female – 2.3 million new cases, 11.6% | 670,000 deaths, 6.9%), colorectal (1.9 million cases, 9.6% | 900,000 deaths, 9.3%), prostate (1.5 million cases, 7.3% | 400,000 deaths, 5.5%),stomach (970,000 new cases, 4.9% | 660,000 deaths, 6.8%), and liver (870,000 new cases, 4.4% | 760,000 deaths, 7.8%) cancers comprise nearly two-thirds of all new diagnoses and deaths (Fig. 1.1) (15). It's noteworthy to mention that while some cancers, such as lung and liver cancer, exhibit both high incidence and disproportionately higher mortality rates, others, like prostate and breast cancer, show high incidence but significantly lower mortality (15).

Estimates by the Union for International Cancer Control revealed striking disparities in the cancer burden according to indices of human development. Countries that rank low (<0.55) on the Human Development Index (HDI) are at a much higher risk of "bearing the brunt of the global cancer burden" due to limited healthcare access and late-stage diagnoses (16).



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Cancer TODAY | IARC
https://gco.iarc.who.int/today
Data version: Globocan 2022 (version 1.1) - 08.02.2024
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International Agency for Research on Cancer
World Health Organization

Fig. 1.1: Global Incidence of Cancer, World Health Organization, GLOBOCAN 2025 – Inside parentheses: the most common site per country – absolute numbers, incidence, both sexes, in 2022 (excl. Non-Melanoma Skin Cancer).

[I] Statistics collected for 2022 and data covers 185 countries and 36 cancers.

1.1.3. Classifications of Cancer

Cancers are traditionally classified based on their anatomical site and the cell or tissue of origin. The latter classifies cancers into the follow groups (Table 1.1 – overview of prominent cancer types).

Cancer Type	Tissue or Cell of Origin	Examples
Carcinoma	**Cancers of epithelial origin**, arising from the internal or external linings of the body	**Adenocarcinoma** (organs or glands): breast, lung, colon, etc. | **Squamous cell carcinoma** (squamous epithelium): cervix, skin, etc.
Sarcoma	**Cancers of mesenchymal origin**, arising from connective and supportive tissues	Osteosarcoma (bone), Rhabdomyosarcoma (skeletal), etc.
Leukemia	**Cancers of the hematopoietic system**, originating in blood-forming tissues of the bone marrow	Acute lymphoblastic leukemia, chronic myeloid leukemia, etc.
Lymphoma	**Cancers of the lymphatic system**, developing in lymph nodes or lymphatic organs	Hodgkin lymphoma, non-Hodgkin lymphoma, etc.
Myeloma	**Cancers of plasma cell origin**, arising from plasma cells in the bone marrow	Multiple myeloma
Brain and Spinal Cord Cancers	**Cancers of the central nervous system**, originating in the brain or spinal cord	Glioblastoma, Astrocytoma, etc.

Table 1.1: Classification of cancers according to tissue or cellular origin.

The evolution of cancer classification has been marked by a shift from general and simplistic categorization based on cell origin (as indicated in Table 1.1), to the incorporation of more detailed diagnostic tools such as histological grading, staging systems, molecular and genomic subtyping, and immunophenotyping classifications (Table 1.2). As our understanding of cancer biology deepens, classification systems continue to evolve, guiding treatment development and improving overall patient outcomes. These advances highlight the ongoing transformation of cancer classification from traditional, broad categories to dynamic, multidimensional systems that reflect the complexity of the disease (17–21).

Classification method	Focus	Key Features	Example(s)
Histological grading	Morphological assessment of tumor cells relative to their normal (healthy) tissue counterparts	Grading based on cell size, shape, mitotic rate, and differentiation. Low-grade tumors resemble normal cells; high-grade tumors are more atypical and proliferative	Gleason score (prostate cancer) and Nottingham grade (breast cancer)
Staging systems	Anatomical extent of tumor spread	Assesses (primary) tumor size (T), regional lymph node involvement (N), and distant metastasis (M)	TNM staging system and FIGO
Molecular & genomic subtyping	Genetic and transcriptomic profiling	Identification of driver mutations, gene expression profiles, and chromosomal alterations	HER2 status (breast cancer), MSI status (colorectal cancer), and Oncotype DX (breast cancer)
Immunophenotypic classification	Immune landscape and tumor–immune interactions	Assesses immune markers (e.g., PD-L1 expression) – predicting response to immune checkpoint inhibitors.	PD-L1 expression

Table 1.2: Dynamic and multidimensional systems of cancer classification.

1.2. Hallmarks of Cancer

The conceptual framework of cancer biology has been shaped by the notion of "hallmarks" – a set of distinct biological capabilities that define the transformation of normal cells into malignant ones. Initially introduced by Douglas Hanahan and Robert Weinberg in 2000, and expanded and refined in subsequent updates, these hallmarks capture the essential biological processes that underpin tumorigenesis and progression (Fig. 1.2) (22–24).

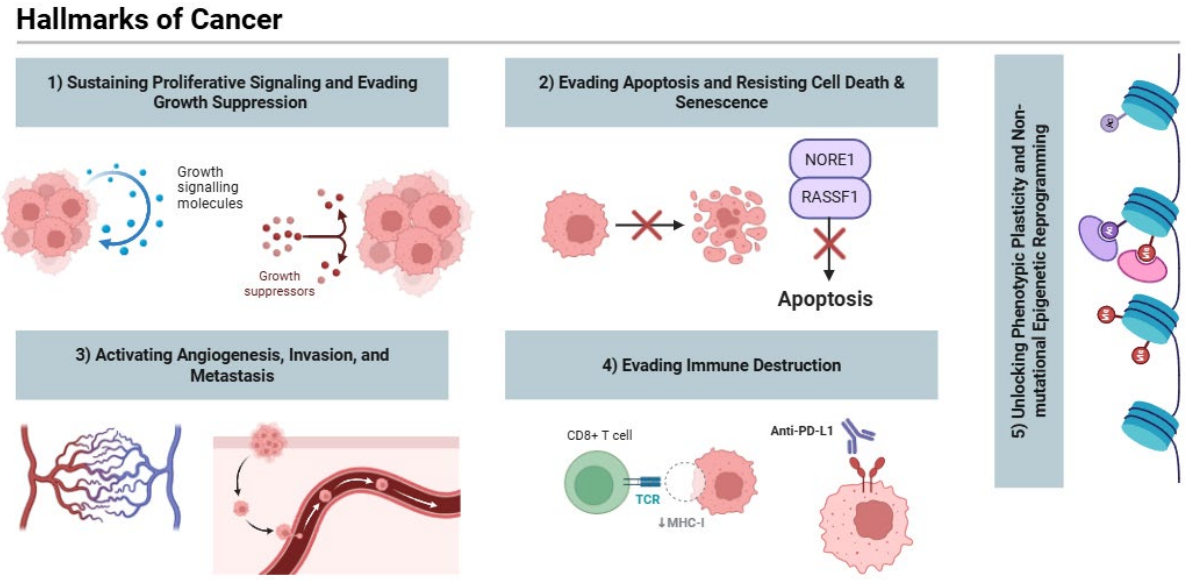


Fig. 1.2: Hallmarks of Cancer.

1.2.1. Sustaining Proliferative Signaling and Evading Growth Suppression

Uncontrolled proliferation is a foundational trait of cancer cells. Unlike normal cells, which rely on tightly regulated "growth-promoting" (also referred to as pro-proliferation) signals, cancer cells deregulate such signals, essentially regulating their own proliferative destiny by bypassing dependence on external growth factors through multiple strategies. These include autocrine production of growth factors such as PDGF and TGF-α, overexpression of receptor tyrosine kinases (TKs) like Human Epidermal Growth Factor Receptor 2 (HER2) and Epidermal Growth Factor Receptor (EGFR), and paracrine interactions within the tumor microenvironment (TME) (25–27). Additionally, mutations in downstream effectors (e.g., RAS/RAF/MAPK and PI3K/AKT/mTOR) enable ligand-independent signaling, while loss of negative regulators like phosphatase and tensin homolog (PTEN) further enhances proliferative signaling (28–30). Lastly, key advancements in delineating the complexities of the TME highlighted key systemic factors, including immune and metabolic influences, as crucial contributors to sustained proliferative signaling (31,32).

Concomitantly, cancer cells evade growth suppression by inactivating tumor suppressor genes (TSGs) such as *TP53* and *RB1*, which normally function to regulate DNA damage responses and cell-cycle checkpoints (33,34). Loss-of-function mutations in TSGs *TP53* (present in 40-50% of all cancers) and *RB1* are some of the earliest described dysregulated TSGs – impairing cancer cells' ability to respond to DNA damage and lead to subsequent uncontrolled cell proliferation (*TP53*) or loss of control in cell cycle progression (G1 phase – *RB1*) (33). Beyond mutations, epigenetic silencing also contributes to TSG inactivation (35,36). Furthermore, recent research has also highlighted the critical role of TME in aiding tumors in evading anti-proliferative signals, namely via inflammation-signals and immune-suppression (37,38).

1.2.2. Evading Apoptosis and Resisting Cell Death & Senescence

The ability of cancer cells to expand in number does not only hinge on the rate of proliferation and cell growth but is also severely reliant on the rate of cell attrition. Apoptosis, one of the most prominent forms of programmed cell death, is commonly evaded via *TP53* loss, overexpression of anti-apoptotic proteins (e.g., Bcl-2 and Bcl-xL), and suppression of pro-apoptotic factors (e.g., Bax, Bim, Puma) (39–41). Autophagy, another form of programmed cell death, also represents an important cell-physiologic response induced in certain states of cellular stress, namely nutrient deficiency (42). Studies have highlighted some crosstalk between apoptosis and autophagy and suggested that induction of the latter can also serve as a barrier to tumorigenesis, which cancer cells must also circumvent during tumor development (43–45). Similarly, other modes of cell death, including necrosis and senescence, have been shown to be suppressed or bypassed in cancer cells, further driving cancer cell survival and tumor progression (46,47).

Notably, these processes may paradoxically contribute to tumor progression: apoptotic bodies can provide mitogenic signals (48), autophagy can supply pro-growth metabolic substrates under stress (49), necrosis can create a pro-inflammatory microenvironment (50), and senescent cells can secrete growth-promoting factors (51). These dual roles highlight the complexity of targeting cell death pathways as anticancer treatment strategies and emphasize the need for further research to unravel the nuanced impacts of inducing such programs.

1.2.3. Activating Angiogenesis, Invasion, and Metastasis

Like normal tissues, tumors require sustenance in the form of nutrients and oxygen as well as an ability to evacuate metabolic waste, relying on the process of angiogenesis (52). In cancer, angiogenesis is sustained by persistent expression of Vascular Endothelial Growth Factor-A (VEGF-A), VEGF signaling via three receptor TKs (VEGFR-1-3), and members of the fibroblast growth factor (FGF) family, often in response to hypoxia via hypoxia-inducible factor (HIF) activation (53–56). Unlike physiological angiogenesis, which is transitory and tightly regulated, tumors permanently activate the “angiogenetic switch”, keeping the process chronically active (23).

In advanced stages, primary tumors initiate local invasion into adjacent tissues and may subsequently disseminate to distant sites, where they can establish secondary growths or new colonies. These distant settlements of cancer cells – metastases – are responsible for around 90% of cancer-related deaths (57). Loss of E-cadherin, a key cell-to-cell adhesion molecule, was one of the earliest studied contributors to potentiating invasion and metastasis (58). Later research highlighted the developmental regulatory program “epithelial-to-mesenchymal transition” (EMT) as one of the key regulators involved in promoting invasion and metastasis (59). Moreover, the TME actively contributes to metastasis through stromal remodeling and paracrine signaling, highlighting that the metastatic phenotype does not arise in a strictly cell-autonomous manner (60,61). Cellular plasticity in the invasive and metastatic phenotype has also been highlighted as a key characteristic as evidenced by the inverse of the EMT program, namely the mesenchymal-to-epithelial transition (MET) program, utilized by disseminated tumor cells (DTCs) (62,63). The current landscape of metastatic oncology is extended to include contributions to tumor dormancy, whereby DTCs remain quiescent but capable of reactivation to drive recurrence, as well as critical contributions by the immune system (64,65).

1.2.4. Evading Immune Destruction

Absent from the original hallmarks, immune evasion is now central to cancer biology. The concept of cancer immunoediting – comprising elimination, equilibrium, and escape – describes the evolutionary

interaction between tumor cells and the immune system (66,67). The final phase, the escape, is what has been acknowledged as a hallmark in the 2011 iteration as evading immune destruction. Key mechanisms of immune evasion include the suppression of cytotoxic T lymphocytes, the recruitment of inflammatory immunosuppressive cells (e.g., regulatory T cells; Tregs and myeloid-derived suppressor cells; MDSCs) and the disabling of the immune system by secretion of immunosuppressive cytokines (e.g., TGF-β and IL-10) (68–71). Additionally, in some cases, tumors also upregulate immune checkpoint molecules such as the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death-ligand 1 (PD-L1), which essentially act as "brakes" to prevent excessive immune reactions, thereby suppressing effective anti-tumor immune responses and facilitating immune escape.

1.2.5. Unlocking Phenotypic Plasticity and Non-mutational Epigenetic Reprogramming

Cancer cell plasticity refers to the dynamic ability to transition between phenotypic states such as EMT, lineage switching, or dedifferentiation into stem-like states, without fixed genetic alterations. (72,73). Instead, they often result from dynamic transcriptional programs or environmental cues, enabling cancer cells to adapt to stress, resist therapy, and drive invasion and metastasis. Factors that promote cellular plasticity during development and wound healing often overlap with those that generate phenotypic plasticity in cancer. These include developmental signaling cascades/pathways (e.g., TGF-β, Wnt/β-catenin, and Notch), transcription factors (e.g., SNAIL, SLUG, ZEB1/2, and TWIST), and cues from the TME (hypoxic conditions activating HIFs) (72,74). In parallel, non-mutational epigenetic reprogramming allows cancer cells to modify gene expression via DNA methylation, histone modifications, and chromatin remodeling – independently of mutations (75). While originally described in embryonic development, differentiation, and organogenesis (76,77), growing bodies of evidence support the notion that analogous epigenetic alterations can allow cancer cells to drive proliferation, advance metastasis, and evade therapeutic pressure (75,78).

1.3. The Mammary Gland

The mammary gland, originating from the Latin word *mamma*, is the defining organ of mammals, functioning as a hormonally regulated secretory gland specialized for lactation. Anatomically, it is composed of a complex branched tubuloalveolar ductal system that produces, transports, and delivers milk through the papilla (nipple) (79). Structurally, the gland comprises epithelial and stromal compartments that interact dynamically throughout development and adulthood. The epithelium forms a bi-layered structure: an inner luminal layer that lines the ducts and alveoli, and an outer basal layer of myoepithelial cells that form the outer layer of the mature mammary ducts as well as harbor stem and progenitor cells with the capacity to form both the luminal and myoepithelial layers. Surrounding this is a diverse stroma containing adipocytes (mammary fat pad), fibroblasts, vasculature, immune cells, and extracellular matrix (ECM) components, including the basement membrane, which separates the epithelium from the stroma and regulates polarity and signaling (79,80). In the normal duct-lobular unit – the primary site of origin for most breast cancers (BCs) – epithelial cells are supported by a specialized hormone-responsive stroma composed of fibroblasts, immune cells, adipocytes, and ECM. This microenvironment actively responds to endocrine cues, modulating epithelial proliferation, differentiation, and ductal remodeling (81). Alterations in stromal-epithelial interactions, including aberrant hormonal signaling, may contribute to early carcinogenic events and influence the phenotype of the emerging tumor.

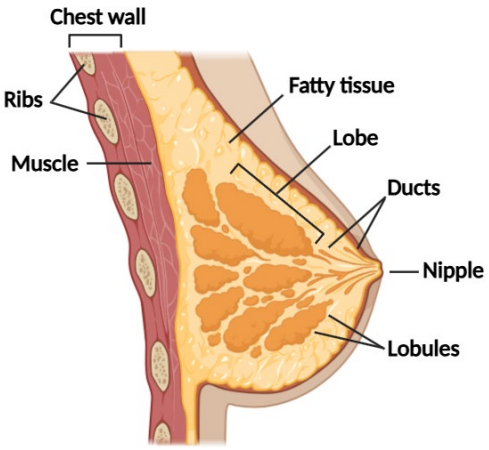


Fig. 1.3: Anatomy of the female breast. *This figure was adapted from a pre-existing figure on Biorender.*

Due to the limited and/or lack of availability and access to human prenatal samples, and despite major differences between species, much of what we know about the molecular journey of mammary gland development mainly stems from studies in murine models (82). In mice, embryonic signaling events establish a ductal network within the fat pad. In humans, a similar process occurs during early gestation, resulting in a rudimentary ductal tree at birth. The gland remains largely quiescent until puberty, yet the rudimentary mammary structure continues to grow isometrically with the rest of the body (79).

In adult females, the mammary gland is uniquely dynamic, undergoing cyclical remodeling in response to hormonal cues during the menstrual cycle, pregnancy, lactation, and involution (79,80,82). This dynamic remodeling capacity and plasticity – driven by epithelial progenitor activity and regulated by developmental pathways such as Wnt/β-catenin signaling – underlies the tissue's regenerative capacity but also its vulnerability to transformation. The stem/progenitor-rich basal layer, along with hormone- and growth factor-responsive luminal cells, represents a potential origin site for breast tumorigenesis. Understanding this cellular hierarchy is therefore fundamental for contextualizing mechanisms of phenotypic plasticity and its implications in Wnt-driven adaption and subsequent therapy resistance described in later chapters of this thesis.

1.4. Breast Cancer

1.4.1. Overview

BC is the most frequently diagnosed malignancy among women worldwide and the leading cause of cancer-related death in women, with over 2.3 million new cases and more than 685,000 deaths reported annually (Fig. 1.4). The global incidence of BC has been on a steady rise, with an annual increase of 6.2% – a trend that is likely to continue. While the global cancer burden in women is rising across countries of all income levels, the incidence of BC is significantly higher in countries with a high HDI (e.g., North America and New Zealand) compared to countries with a low(er) HDI (e.g., Middle Africa and Eastern Asia). This disparity is due to increased life expectancy, urbanization, lifestyle-related risk factors, the availability and use of screening technologies such as mammography, and overall diagnostic capacity (83). Notably, Belgium has the highest age-standardized BC incidence rate worldwide, likely due to the combination of lifestyle-related risk factors and a strong national screening and recording system. The Belgian National Cancer Registry (BNCR) projects an estimate of 12,125 new cases in 2025, a 16% increase compared to 2014 (84). However, the BNCR does not expect a significant increase in age-standardized incidence rates, attributing the increase in cases to the advancing aging population.

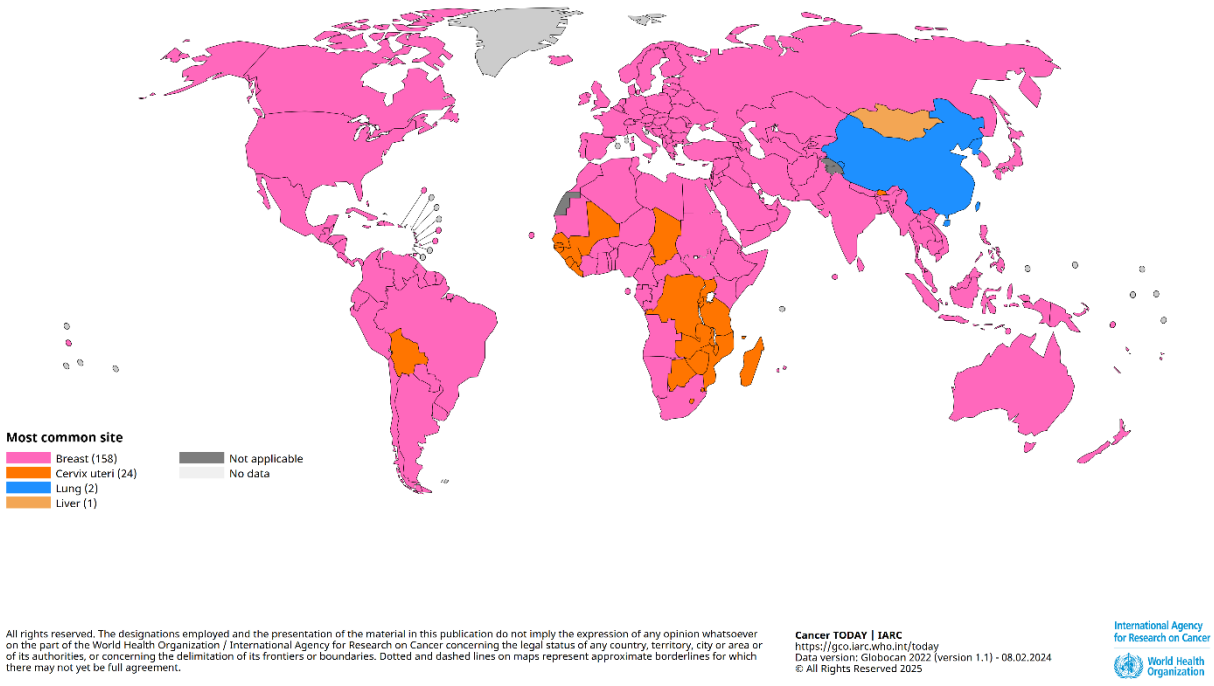


Fig. 1.4: Global Incidence of Cancer, World Health Organization, GLOBOCAN 2025 – Inside parentheses: the number of countries in which that cancer type ranks first for incidence in females, in 2022 (excl. Non-Melanoma Skin Cancer).

In contrast to rising incidence rates, BC mortality has been declining in high-HDI countries, largely due to increased public awareness, the implementation of organized screening programs enabling earlier detection, broad access to healthcare services, advances in therapeutic strategies, and improvements in supportive care. However, this decline is not uniformly observed worldwide and remains limited to settings where such interventions are accessible and effectively implemented.

1.4.2. Risk Factors

Only a fraction of breast cancers are hereditary, with only 7-10% of cases being caused by germline mutations that increase the cumulative risk of developing BC (85). Two of the most frequently studied tumor suppressor genes, *BRCA1* (17q21) and *BRCA2* (13q13), which encode proteins involved in DNA repair, follow an autosomal-dominant inheritance pattern. Mutations in *BRCA1* and *BRCA2* are associated with an average cumulative risk of developing BC by age 80 of 72% and 69%, respectively (86).

Approximately 80% of BC patients are over 50 years old, and more than 40% are older than 65 years, underscoring age as a significant risk factor (83). Numerous studies highlighted a strong relationship between exposure to endogenous hormones – particularly estrogen and progesterone – and increased BC risk (87). Therefore, hormonal imbalance caused by specific events and their duration, such as first menstruation, pregnancy, lactation and breastfeeding, and menopause are crucial for the potential induction of breast carcinogenesis (88). Early first full-term pregnancy (around the early 20s) and higher parity are associated with reduced risk (88). In addition to genetic factors, various non-genetic risk factors conducive to the development of BC have been identified. These include physical inactivity, obesity, alcohol and tobacco use, poor dietary habits, and use of hormonal contraceptives (88). Notably, an estimated one-third of BC cases could be prevented through healthier lifestyle choices (89).

1.4.3. Initiation of Breast Cancer

The mechanisms underlying the initiation of BC remain incompletely understood. It is widely believed that the progressive accumulation of somatic mutations in epithelial or basal cells of the mammary gland – driven by defects in DNA damage repair (DDR) pathways and/or environmental exposures – can lead to malignant transformation. This process promotes clonal evolution, allowing mutated cells to acquire a selective growth advantage and initiate tumor development (85,90).

An alternative hypothesis proposes that adult mammary gland stem cells (MaSCs) may serve as the cell of origin for BC (90). These hormone/steroid-responsive cells regulate proliferation, tissue remodeling, and ductal branching of the mammary gland, particularly during pregnancy and lactation. As self-renewing, multipotent progenitors of both luminal and basal cell lineages, MaSCs are also thought to contribute to the intratumor heterogeneity (ITH) observed in BC – a topic discussed in later sections.

1.4.4. Classification of Breast Cancer

BC represents a heterogeneous group of diseases with distinct biological, pathological, and clinical characteristics. This diversity influences disease presentation, diagnostic interpretation, therapeutic strategies, and patient outcomes. While breast tissue contains multiple cell types, over 99% of BCs originate from the epithelial compartment and are thus classified as carcinomas; while mesenchymal tumors such as sarcomas account for less than 1% of cases (91).

1.4.4.1. Histological Subtypes

Historically, breast carcinomas are classified based on both the degree of invasiveness – reflecting how far tumor cells have spread beyond their site of origin – and their architectural pattern, which describes the structural organization and growth characteristics of the tumor tissue. Tumors are broadly divided into three major groups: non-invasive (*in situ* – pre-invasive), invasive, and metastatic disease (90).

i. *In situ* (Non-invasive)

The two major *in situ* lesions are ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS). DCIS is characterized by neoplastic cells confined within the ducts, often distorting the ductal architecture, but without stromal invasion. While non-invasive, DCIS has malignant potential and may progress to invasive disease if not adequately and timely treated. LCIS originates in the lobules of the milk-producing glands of the breast and is regarded both as a risk factor and as a non-obligate precursor lesion for invasive BC rather than a direct *in situ* carcinoma. Although historically grouped with DCIS, LCIS is now recognized as a marker of increased BC risk, with a relatively low progression rate if untreated (92). A long-term follow-up study by McDivitt and colleagues reported cumulative risks of subsequent invasive BC after LCIS diagnosis at 5% after 5 years, 15% after 10 years, 27% after 15 years, 35% after 20 years, and over 50% after 23 years (93). LCIS itself encompasses distinct histologic variants, including classic, pleomorphic, and florid forms. Classic LCIS is typically indolent and associated with a bilateral increase in BC risk, whereas pleomorphic LCIS exhibits more aggressive cytologic features, a higher proliferative index, and a stronger association with invasive carcinoma. At the molecular level, LCIS frequently shows loss of E-cadherin and *CDH1* mutations, features shared with invasive lobular carcinoma, supporting their biological link (92).

ii. Invasive

Invasive carcinomas infiltrate the surrounding stromal tissue and carry metastatic potential. The most prevalent subtype is invasive ductal carcinoma (IDC), accounting for ~80% of invasive BCs. Invasive lobular carcinoma (ILC) is the second most common subtype (~10-15%), and exhibits distinct growth patterns, molecular alterations (e.g., loss of E-cadherin), and treatment responses. While IDC and ILC

share overlapping features, their histological architecture, genomic alterations, and clinical behavior differ, necessitating tailored management strategies. Overall, 90-95% of BC cases are invasive at diagnosis.

iii. Metastatic

Metastatic disease, also referred to as stage IV disease, represents the most advanced form of BC and is defined by the spread of malignant cells beyond the breast and regional lymph nodes to distant organs. Common sites of distant metastasis include the lungs, liver, bones, and brain. Patients may be diagnosed with *de novo* metastatic breast cancer, where metastases are present at initial diagnosis, or they may experience distant recurrence months or years after completing initial treatment for early-stage disease. While the risk of recurrence or metastasis varies among individuals, the use of established prognostic tools and biomarkers such as tumor grade, hormone receptor (HR) status, HER2 status, and genomic assays are used to estimate recurrence risk and guide clinical decision-making.

Despite therapeutic advances, metastatic BC remains largely incurable, and long-term survival is limited. It's estimated that approximately 30% of patients initially diagnosed with early-stage BC will eventually develop metastatic disease, where prognosis and survival rates are poor.

1.4.4.2. Molecular (intrinsic) subtypes

While histological classification remains foundational in BC diagnosis, it offers limited insight into biological behavior, therapeutic response, and prognosis – presenting the need for updated and improved frameworks of classifications based on better understanding of the disease. The advent of high-throughput genomic technologies, particularly next-generation sequencing technologies, has transformed our understanding of BC heterogeneity, further classifying subtypes according to relevant genotypic and gene expression patterns.

Landmark studies by Perou, Sørlie, and colleagues in the early 2000s, laid the groundwork for a new chapter of BC subtyping, defining the first iteration of the intrinsic molecular subtypes and stratifying breast tumors into four primary subtypes – Luminal A, Luminal B, HER2-enriched, and Basal-like – using hierarchical clustering of mRNA expression data from tumor samples (18). Collectively, these works caused a paradigm shift in classifying and subtyping breast carcinomas, underscoring the complexity of the disease and highlighting that tumors of similar histology could have vastly different molecular characteristics with significant impacts on clinical outcomes (Fig. 1.5). A fifth, Normal-like subtype was later proposed. Initially thought to represent breast tissue contamination during early sequencing experiments, it was maintained in subsequent classification with controversies around its clinical significance (94–96).

In clinical settings, BC diagnosis typically begins with a clinical breast examination to assess for palpable masses, skin changes, nipple abnormalities, or axillary lymphadenopathy (97). While imaging and histopathology provide definitive diagnosis, clinical breast examinations remain a cornerstone of initial assessment, especially in low-resource settings, and can guide further diagnostic workup. Imaging modalities such as mammography or ultrasound are then used, followed by a core needle biopsy of suspicious lesions. Histopathological evaluation of biopsy tissue includes immunohistochemical (IHC) staining for estrogen receptor (ER), progesterone receptor (PR), HER2, and the proliferation marker Ki-67, which serve as surrogate markers to assign tumors to clinically meaningful intrinsic subtypes.

To translate the molecular taxonomy of BC into routine clinical care, IHC-based surrogate classifications have been developed. These definitions categorize tumors into Luminal A, Luminal B, HER2-enriched,

and triple-negative breast cancer (TNBC) based on the combined status of ER, PR, HER2, and Ki-67. This classification guides key therapeutic decisions, including the use of endocrine therapy, HER2-targeted therapy, and cytotoxic chemotherapy. While gene expression-based subtyping offers a deeper understanding of tumor biology, its use remains largely restricted to specific clinical contexts. For instance, multigene assays such as Oncotype DX, MammaPrint, and Prosigna (PAM50) are primarily applied in early-stage, HR-positive, HER2-negative tumors to assess the risk of recurrence and inform the need for adjuvant chemotherapy (98). These assays integrate molecular features beyond what IHC can capture, including proliferation signatures and transcriptomic risk scores. In parallel, more advanced and cutting-edge techniques including single-cell RNA sequencing (scRNA-seq), spatial transcriptomics, and epigenetic profiling, are uncovering additional complexity in subtype architecture, especially in relation to the tumor immune microenvironment (TIME).

i. Luminal Breast Cancer

Approximately 70-75% of BCs express ER and/or PR and are classified as luminal tumors, reflecting gene expression profiles akin to luminal epithelial cells (99). The luminal A subtype accounts for approximately 40% of all BCs, characterized by ER and/or PR expression, HER2 absence, and a low proliferative index (low Ki-67). Luminal A tumors are frequently low-grade and slow growing, and are associated with favorable prognosis (90). Luminal B tumors comprise 15-20% of BCs, characterized by ER and/or PR expression, either HER2 expression or absence, and a high proliferative index (high Ki-67). This subtype presents a more aggressive phenotype, highlighted by higher histological grades, fast-growing tumors, and worse prognosis (90,99). In the majority of cases of luminal cancers, endocrine therapy is utilized, with aromatase inhibitors used for postmenopausal patients or tamoxifen for premenopausal patients (100,101). Chemotherapy is rarely required and only used in cases of higher tumor burden or resistance to endocrine therapy. In advanced and metastatic cases, cyclin-dependent kinase 4/6 (CDK4/6) inhibitors are also used alongside endocrine therapy (102).

ER-low tumors, defined as having ER expression between 1-10% by IHC, represent a unique clinical and biological category. While traditionally grouped under luminal tumors and often treated with endocrine therapy, mounting evidence indicates that ER-low tumors differ significantly from classic ER-positive tumors (103). Molecular profiling studies have revealed that many ER-low tumors exhibit basal-like gene expression patterns and share characteristics with TNBC, including poor responsiveness to endocrine therapy and a more aggressive clinical course (103). Consequently, treatment paradigms for ER-low tumors remain controversial, with some guidelines suggesting that chemotherapy may be more beneficial than hormone therapy in these cases. The ASCO/CAP guidelines now recommend explicit reporting of ER-low status, though its optimal management remains an area of active investigation (104).

ii. HER2-enriched Breast Cancer

HER2 is a member of the EGFR family of receptor TKs (105). Its activation leads to downstream induction of several pro-proliferative and pro-survival signaling cascades such as the mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and the phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K) (90,106). Between 15-20% of BCs are classified as HER2-enriched by gene expression profiling, a subtype characterized by high expression of the HER2 gene and other genes associated with the HER2 pathway and/or HER2 amplicon located on the 17q12 chromosome alongside frequent absence of ER and PR expression (99). HER2-enriched tumors are clinically more aggressive than luminal tumors, characterized by hyperproliferation, high histological grade, and presenting *TP53* mutations in more than 40% of cases (107,108). Clinically, HER2-positive BC is diagnosed by IHC and/or

in situ hybridization and this group is more heterogeneous: while a subset mirrors the HER2-enriched intrinsic subtype, a substantial proportion are HR-positive ($HER2^+/HR^+$), displaying distinct biology, prognosis, and therapeutic responses compared to HER2-enriched/HR-negative disease (99). Endocrine therapy is not indicated for HER2-enriched/HR-negative tumors, but it remains an important component of treatment for $HER2^+/HR^+$ cases. Across HER2-positive disease, targeted therapies aimed at the HER2 protein (i.e., anti-HER2 therapy), are the backbone of treatment, typically combined with different regimens of chemotherapy (109). Emerging research is currently exploring the benefits of immunotherapy, namely the use of immune checkpoint inhibitors (ICIs) in combination with HER2-targeted therapies.

A growing subset of tumors, those with HER2 IHC score 1+ or 2+ but without gene amplification, are now categorized as HER2-low (110). Although these tumors do not qualify for conventional HER2-targeted therapy, they have demonstrated responsiveness to novel antibody-drug conjugates (ADCs), particularly in HR-positive cases. The HER2-low subtype is not yet an established intrinsic subtype but is emerging as a clinically actionable category.

iii. Basal-like and Triple-Negative Breast Cancer

The final major subtype in routine classification is TNBC, defined by the absence of ER, PR, and HER2 expression by IHC. TNBC accounts for approximately 15-20% of BC cases and is more frequently diagnoses in younger women, particularly those of African or Hispanic ancestry (90). Clinically, it is considered the most aggressive subtype, characterized by rapid progression, high histological grade, and limited therapeutic options. While TNBC frequently overlaps with the basal-like intrinsic subtype, the two are not synonymous; not all TNBCs are basal-like and not all basal-like tumors are triple-negative. Bertucci et al. reported that 71% of TNBCs are basal-like, while 77% of basal-like tumors are triple-negative (111). Furthermore, since TNBC is a diagnosis of exclusion rather than by presence of any unifying features, it remains a heterogeneous disease entity that presents a formidable challenge to developing effective treatment (112). Given its poor prognosis and the absence of targetable receptors, TNBC is the primary focus of this thesis, in which we explore mechanisms of therapeutic resistance and investigate potential vulnerabilities using *in vitro*, *ex vivo*, and *in vivo* models.

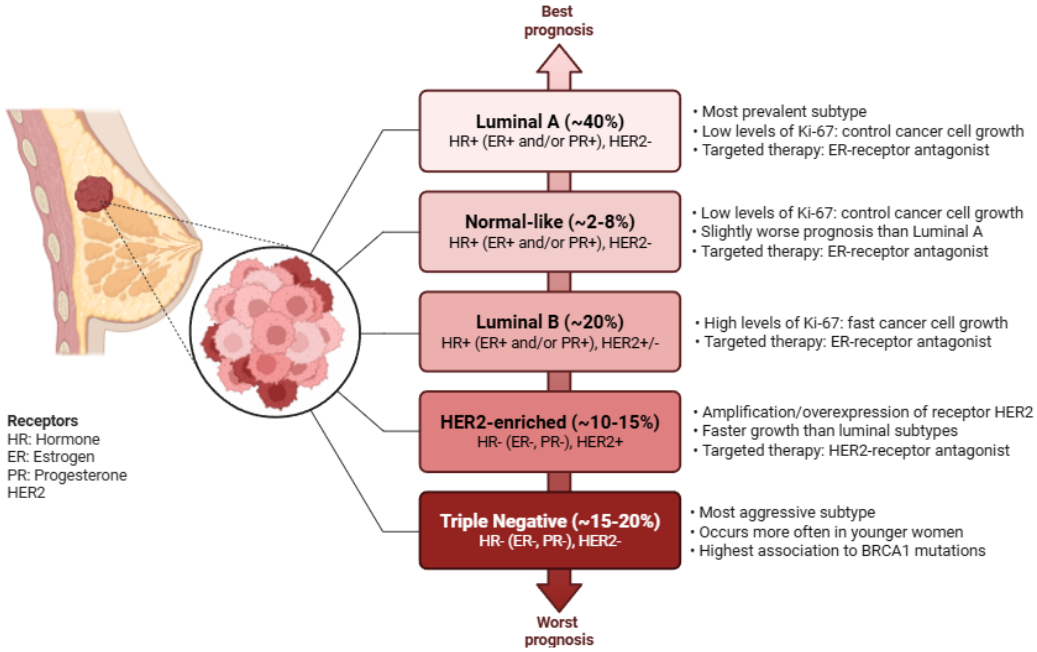


Fig. 1.5: Intrinsic (molecular) subtypes of breast cancer. *This figure was adapted from a pre-existing figure on Biorender.*

1.5. Triple Negative Breast Cancer

TNBC is not a biologically uniform entity, and significant efforts have been made to dissect its molecular heterogeneity. A pivotal contribution in this area was made by Lehmann et al., who in 2011, performed gene expression analysis on 587 TNBC cases across 21 BC datasets and identified six transcriptionally distinct subtypes: Basal-like 1 and 2 (BL1 and BL2), Immunomodulatory (IM), Mesenchymal (M), Mesenchymal stem-like (MSL), and Luminal androgen receptor (LAR) (113). The BL1 and BL2 subtypes are enriched in genes related to proliferation and DDR, though BL1 is more tightly associated with cell cycle regulation (e.g., G1/S checkpoint, replication machinery), while BL2 shows enrichment in growth factor signaling pathways, including the epidermal growth factor (EGF) pathway, the nerve growth factor pathway, and the Wnt/β-catenin pathway (113). The IM subtype is characterized by high expression of genes involved in immune cell signaling (e.g., T helper 1/2 pathway, natural killer-cell signaling, and B cell receptor signaling), cytokine signaling (e.g., IL-7 and IL-12 signaling), and core immune signal transduction pathways (e.g., Nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) pathway and tumor necrosis factor (TNF) pathway) (113). Both the M and MSL subtypes share features of enhanced cell motility, ECM receptor interaction, cell differentiation pathways (e.g., Wnt/β-catenin pathway and transforming growth factor β (TGF-β) signaling), and EMT (113). The MSL subtype, however, shows lower proliferative activity and higher expression of stem cell-related genes (e.g., *ABCA5*, *ALDHA1*, and *BMP2*), suggesting a more quiescent, stem-like phenotype. The LAR subtype is unique in expressing androgen-receptor (AR)-related transcriptional programs despite lacking ER expression. It is enriched for genes associated with steroid synthesis, androgen metabolism, and other hormonally regulated pathways (113).

In 2016, the Lehmann group refined their model, collapsing the classification into four tumor-intrinsic subtypes: BL1, BL2, M, and LAR, after excluding the IM and MSL groups, which were likely confounded by immune and stromal cell infiltration rather than tumor-intrinsic features (114). This refinement underscores the importance of distinguishing tumor-cell-specific features from the surrounding microenvironment when defining molecular subtypes.

Functionally, these subtypes demonstrate distinct therapeutic vulnerabilities. In pre-clinical models, BL1 cell lines showed sensitivity to DNA-damaging agents such as cisplatin, consistent with their DDR enrichment. LAR models responded to AR antagonists (e.g., enzalutamide), while the MSL/M subtypes showed vulnerability to PI3K/mTOR inhibitors. These findings were further validated in clinical samples, where BL1 tumors achieved a pathologic complete response (pCR) rate of ~41% following neoadjuvant chemotherapy (NAC), highlighting the efficacy of standard chemotherapy and possible synergy with DNA-damaging agents (114). In contrast, pCR rates were lower in BL2 (~18%) and LAR (~29%) tumors, emphasizing the limited efficacy of standard cytotoxic therapy in these subtypes and the need for alternative strategies. Moreover, a study by Masuda and colleagues revealed vast differences among TNBC subtypes in response to chemotherapy, where BL1 tumors achieved pCR in 52% of cases, while BL2, AR, and M tumors achieved markedly lower pCR rates (0%, 10%, and 23%, respectively) (115). These findings underscore the clinical relevance of molecular stratification in TNBC and offer a framework for personalized treatment selection. Nevertheless, these subtypes have not yet been integrated into routine clinical practice due to the absence of standardized diagnostic assays, limited prospective validation, and technical barriers in implementing transcriptomic profiling (116).

An alternate molecular taxonomy was proposed by Burstein et al., who identified four TNBC subtypes: LAR, mesenchymal, basal-like immune suppressed (BLIS), and basal-like immune activated (BLIA) – using integrated RNA and DNA profiling of 198 TNBC tumors. These groups differ not only in gene expression but also in clinical outcome: BLIA tumors show the best prognosis, whereas BLIS tumors have the worst disease-free and disease-specific survival rates. Importantly, this classification proposed subtype-specific therapeutic targets: LAR tumors – expressing AR and MUC1; mesenchymal tumors – overexpressing growth factor receptors such as PDGFRA and CD117; BLIS tumors – harboring immunosuppressive proteins like VTCN1; and BLIA tumors – enriched in STAT pathway components and cytokine signaling (117). These findings nominate potential vulnerabilities for tailored therapeutic strategies. While Lehmann's approach used unsupervised clustering across large TNBC cohorts to define tumor-intrinsic subtypes, Burstein's classification placed a greater emphasis on the immune context and clinical outcome, integrating prognosis into subtype stratification and linking molecular features directly into actionable targets. While there is partial overlap between the two classification frameworks, their methodological differences highlight the ongoing challenge of harmonizing molecular taxonomies for clinical application.

Beyond the Lehmann and Burstein classifications, several newer TNBC subtyping frameworks have emerged, leveraging multi-dimensional molecular profiling to enhance clinical relevance. The Fudan University (FUSCC) classification integrated mRNA and long non-coding RNA expression to define four subtypes: IM, LAR, mesenchymal, and BLIS – with an IHC-based surrogate enabling practical clinical application (118). Jézéquel et al. applied fuzzy clustering to transcriptomic data to identify three prognostically distinct clusters (C1, C2, and C3). The C1 cluster included tumors with a molecular apocrine phenotype that showed a better prognosis, and C2 and C3 were enriched in basal-like properties. C2 tumors displayed biological aggressiveness and an immune-suppressive phenotype, whereas C3 outlines the adaptive immune response and immune checkpoint upregulation (119). More recent proteomic, phosphoproteomic, and spatial transcriptomic approaches further dissect the TIME and signaling pathway dependencies, revealing subtype-specific vulnerabilities (120). Emerging integrative classifiers that combine genomic, transcriptomic, proteomic, and clinicopathologic data promise finer patient stratification and improved therapeutic matching, marking a shift toward multi-omics-driven TNBC taxonomy.

1.5.1. Genomic Characteristics and Therapeutic Implications

At the genomic level, TNBCs are marked by a high somatic mutational burden, with an average of ~60 mutations per tumor (approximately 1.68 mutations per Mb of coding DNA). The most frequent genetic alteration is *TP53* mutation, found in 60-70% of TNBCs, followed by *PIK3CA* mutations in ~10% of cases. Most other mutations occur at low (<5%) to very low (<1%) frequencies (121,122). A defining hallmark of TNBC tumors is their impaired capacity for DDR often related to impairment in the homologous recombination (HR) pathway. Although the majority of TNBCs are sporadic and lack germline *BRCA1* mutations (<5%), many display phenotypic similarities to BRCA-mutated tumors – a concept known as BRCAness (123). Additionally, there is a strong link between the BRCA1 pathway and TNBCs, whereby 75% of women carrying germline mutations of this gene develop TNBC. As *BRCA1* is critically involved in mediating the repair of double-strand DNA breaks via HR (124), drugs blocking single-strand DNA repair mechanisms could be selectively lethal to tumor cells lacking functional *BRCA1* (*BRCA*-mutant tumors and BRCAness tumors) (125).

1.5.2. Clinical Outcomes and Prognosis

TNBC is associated with a poorer prognosis compared to other BC subtypes, driven by both biological aggressiveness (e.g., high proliferation and genomic instability) and limited treatment options. On average, the median overall survival (mOS) for patients with TNBC is 4.2 years, compared to 6 years for non-TNBC cases. TNBC is also characterized by higher recurrence rates (1 in 3 patients vs. 1 in 5 in non-TNBC cases), with a shorter median time to recurrence (2.6 vs. 5 years) (126). Survival outcomes are particularly poor in the metastatic setting: while regional-stage TNBC has a 5-year survival rate of ~65%, this drops to ~11% in patients with distant metastases, highlighting the urgent need for improved treatment strategies (126).

1.6. Treatment Landscape for TNBC

Due to the lack of hormone and/or growth factor receptor expression, TNBC patients do not benefit from established endocrine or HER2-targeted therapies, putting them at a significant disadvantage from a therapeutic and clinical point of view. Surgical intervention, radiotherapy, and systemic cytotoxic chemotherapy have long been considered the foundational pillars in the management of TNBC, particularly in early-stage disease. However, given the aggressive nature of the disease and the lack of targeted therapies available, conventional treatments are often insufficient, especially in the metastatic setting. Recent years have seen a rapid expansion of therapeutic research, leading to the development of novel approaches including immunotherapy, ADCs, Poly-ADP ribose polymerase (PARP) inhibitors, and therapies targeting specific signaling pathways (Fig. 1.6).

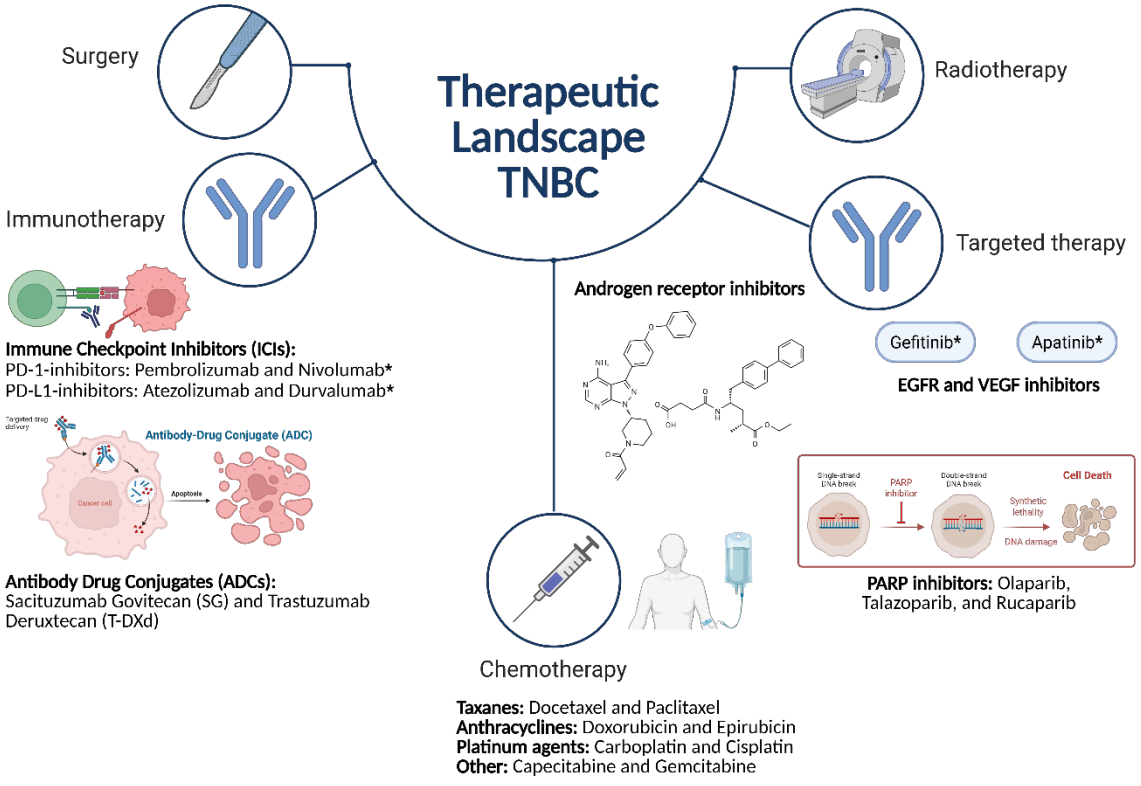


Fig. 1.6: Schematic overview of therapeutic landscape for TNBC – Drugs marked with an * (Gefitinib, Apatinib Nivolumab, and Durvalumab) are still being actively explored in clinical trials and their roles in TNBC treatment are still under investigation and have not yet established consistent efficacy.

1.6.1. Surgery and Radiotherapy

Surgical resection remains a cornerstone in the management of early-stage TNBC, with the choice between a lumpectomy (breast-conserving surgery) and a mastectomy (removal of the whole breast) determined by tumor size, grade, lymph node status, genetic predisposition, and patient preference (127). In most cases of breast-conserving surgery, adjuvant radiotherapy is administered to reduce the risk of locoregional recurrence, particularly in patients with high-risk features such as lymph node involvement, large tumor size, or close/positive surgical margins (128,129).

Radiotherapy in TNBC has demonstrated a benefit in reducing local recurrence, but its impact on distant metastases and overall survival (OS) remains modest. Reported recurrence rates after radiotherapy range from 7% to 12.6% within 5 years, underscoring the aggressive nature of the disease and the potential for the development of radiotherapy resistance (130). Importantly, TNBC patients who recur after radiotherapy often experience rapid progression with limited post-recurrence survival, highlighting the need for combination approaches.

To enhance antitumor effects, emerging clinical trials are exploring the combination of radiotherapy with immunotherapeutic agents, particularly ICIs. This strategy, sometimes referred to as radioimmunotherapy, is based on the rationale that radiation-induced immunogenic cell death may enhance tumor visibility to the immune system and synergize with immune checkpoint blockade (130). These approaches are currently under clinical investigation but are not yet standard of care.

1.6.2. Chemotherapy

Systemic chemotherapy remains the primary therapeutic modality for both early-stage and metastatic TNBC, largely due to the absence of HR or HER2 amplification, which precludes the use of endocrine or HER2-directed therapies. In the early-stage setting, chemotherapy is often administered in the neoadjuvant setting (prior to surgery) to shrink tumors and potentially enable breast-conserving surgeries. Standard neoadjuvant regimens typically include anthracyclines (e.g., doxorubicin and epirubicin), cyclophosphamide, and taxanes (e.g., docetaxel and paclitaxel), either sequentially or in combination (130). Adjuvant (post-operative) chemotherapy is considered in selected cases, particularly when neoadjuvant treatment is not feasible or if there is evidence of residual disease following surgery.

TNBC is characterized by a high proliferative index and frequent defects in DNA repair pathways, making it generally more responsive (sensitive) to cytotoxic chemotherapy compared to HR-positive subtypes (130). Among patients with early-stage TNBC, those who achieve pCR – defined as the absence of invasive cancer in the breast and axillary lymph nodes at the time of surgery – have significantly improved long-term outcomes. pCR is therefore considered a strong surrogate endpoint for relapse-free survival (RFS) and OS, particularly in TNBC. A large pooled analysis by Cortazar et al. involving over 11,000 patients across multiple clinical trials demonstrated that pCR is most associated with favorable long-term outcomes in TNBC and HER2-positive subtypes, with patients achieving pCR showing significantly reduced risk of recurrence and death (131).

Commonly used anthracycline-taxane-based neoadjuvant regimens yield pCR rates ranging from 30%-40% in TNBC. Achieving pCR in this setting is associated with RFS rates of up to 89% at 5 years, compared to 62% in those with residual disease (132,133). Despite these encouraging responses, a substantial proportion of patients with TNBC relapse, particularly those who do not achieve pCR.

Among non-pCR patients, recurrence typically occurs within the first 3 years after diagnosis and is associated with worse post-relapse survival compared to other BC subtypes.

In recent years, platinum-based chemotherapeutic agents, such as cisplatin and carboplatin, have been investigated in TNBC, owing to their ability to induce DNA cross-linking and double-strand breaks – mechanisms that exploit the frequent DNA repair deficiencies in this disease. TNBC tumors with germline or somatic mutations in *BRCA1* or *BRCA2* genes, or those with a BRCAness phenotype (i.e., exhibiting HR deficiency), may be particularly sensitive to platinum compounds. A meta-analysis of 24 studies comprising over 1,700 patients with TNBC reported pCR rates of up to 48% with platinum-containing regimens, compared to 30-40% with standard (non-platinum) agents (134). Importantly, the pCR benefit was even more pronounced in patients with *BRCA1* mutations, with pCR rates exceeding 60% (135,136).

Beyond the early setting, chemotherapy remains the backbone of treatment in metastatic TNBC. First-line therapy typically includes single-agent or combination regimens using taxanes, anthracyclines, or platinum agents, with the choice guided by prior exposure, toxicity profile, and disease burden (137,138). While some TNBCs are initially chemotherapy-sensitive, the development of resistance remains a significant challenge. Responses to chemotherapy in the metastatic setting are often transient, and disease tends to progress rapidly, especially in patients with poor prognostic factors such as visceral metastases or short disease-free intervals.

Overall, although a subset of patients with TNBC are responsive to chemotherapy – particularly those achieving pCR – the lack of durable benefit and the high risk of recurrence in non-responders underscore the urgent need for predictive biomarkers and more effective targeted strategies. In the next sections, we address recent advances in TNBC treatment, including the integration of immunotherapy, PARP inhibitors, and ADCs into clinical practice.

1.6.3. Immunotherapy

TNBC is considered more immunogenic than other BC subtypes due to the frequent presence of tumor-infiltrating lymphocytes (TILs) in its microenvironment, making it a promising candidate for immunotherapy strategies (139). In oncology, immunotherapy refers to a specialized approach that utilizes the body's own immune system to fight cancer cells; different approaches include stimulating or boosting the system and/or modifying it to recognize and eliminate cancer cells more effectively (140).

1.6.3.1. Immune Checkpoint Inhibitors

At the core of cancer immunotherapy are T cells, which play a central role in the recognition and elimination of tumor cells. However, their activity is tightly regulated through a set of inhibitory pathways known as "immune checkpoints". These molecular safeguards prevent excessive immune activation and autoimmunity. In 1995, Jim Allison identified CTLA-4 as the first negative immune checkpoint protein that inhibits T cell activity. Subsequent discoveries revealed additional checkpoint proteins, including PD-1 and its ligand PD-L1, lymphocyte-activation gene 3 (LAG-3), and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) (141).

Among these, the PD-1/PD-L1 axis has become the most extensively studied and clinically exploited in BC. PD-1 is expressed on immune cells including T cells, B cells, natural killer cells, and dendritic cells, while PD-L1 is expressed on both immune and tumor cells as well as normal tissue (38). In the TME, interaction between PD-1 and PD-L1 suppresses T cell effector functions, cytokine secretion,

proliferation, and cytotoxic activity of lymphocytes – thereby enabling tumors to avoid immune-mediated destruction (38). ICIs are monoclonal antibodies (mAbs) that block these inhibitory interactions, effectively "releasing the brakes" on the immune system and restoring antitumor immunity. The application of ICIs in TNBC has primarily focused on targeting the PD-1/PD-L1 pathway, either alone or in combination with chemotherapy.

i. PD-(L)1 Inhibitors – Monotherapy

ICIs such as pembrolizumab, a PD-1 inhibitor, have demonstrated limited efficacy as monotherapy in advanced/metastatic TNBC (140), particularly in heavily pre-treated patients, as assessed by objective response rate (ORR) in early-phase trials (NCT01848834 and NCT02447003) (142–144). However, clinical benefits appears more likely in tumors with high PD-L1 expression, suggesting that biomarker selection is crucial for identifying potential responders. Despite this, pembrolizumab has not shown a significant survival advantage over chemotherapy in unselected patient populations, evaluated by OS (NCT02555657) (145).

In early-stage TNBC, ongoing trials are exploring the role of PD-(L)1 inhibitors as adjuvant therapy for patients with high risk features, such as residual disease after NAC. Trials evaluating pembrolizumab and avelumab (a PD-L1 inhibitor) in this setting aim to improve long-term survival outcomes (NCT02954874 and NCT02926196) (146,147). Results of both of these trials are eagerly anticipated and could provide additional options for the treatment of early-stage, high-risk TNBC patients.

ii. PD-(L)1 Inhibitors – Combination with Chemotherapy

In addition to their cytotoxic effect, several chemotherapeutic agents exhibit immunomodulatory properties. These include enhancing antigen presentation via stimulating the recruitment and maturation of antigen-presenting cells, stimulating the maturation of dendritic cells, promoting the activation and infiltration of effector T cells, and reducing immunosuppressive populations such as Tregs and MDSCs (148–150). Such changes render the TME more permissive to immune-mediated tumor elimination. These observations provide a strong rationale for combining ICIs with chemotherapy to enhance the efficacy of immunotherapy and facilitate synergistic antitumor activity.

In advanced TNBC, the addition of pembrolizumab to chemotherapy improved progression-free survival (PFS) compared to chemotherapy alone, particularly with high PD-1 expression (CPS ≥ 10[II]). This benefit was also reflected in OS within the PD-L1-positive subgroups, leading to the regulatory (Food and Drug Administration – FDA) approval of pembrolizumab in combination chemotherapy as first-line treatment for PD-L1-positive advanced TNBC (NCT02819518) (151,152). Similarly, the combination of atezolizumab (a PD-L1 inhibitor) with chemotherapy showed significant PFS and OS improvements in PD-L1-positive patients (NCT02425891) (153,154). On the basis of these findings, atezolizumab was granted accelerated approval by the FDA to be used in combination with nab-paclitaxel as a first-line treatment for late-stage TNBC patients. However, these results were not replicated when atezolizumab was paired with a different chemotherapy backbone, regardless of PD-L1 expression status, indicating that the choice and sequence of agents may impact clinical outcomes (NCT03125902) (155).

[II] Combined Positive Score (CPS) is a method for assessing the expression of PD-L1 calculated by determining the percentage of PD-L1 stained cells (including tumor, lymphocytes, and macrophages) relative to the total number of viable tumor cells. The CPS is used as a indicative prognostic marker assessing likeliness to respond/benefit to/from ICI-therapy.

An alternative strategy under investigation involves short-term induction with low-dose chemotherapy prior to immune checkpoint blockade. Preliminary findings from trials suggest that brief pre-treatment with agents such as doxorubicin or cisplatin can enhance the responsiveness to ICIs (nivolumab – PD-1 inhibitor) in metastatic TNBC (NCT02499367) (156). Analysis of patient samples suggested that short-term chemotherapy induction can transiently reshape the TIME toward a more inflamed state. This induction approach is currently being evaluated in ongoing studies.

In early-stage TNBC, which tends to exhibit a more immunogenic microenvironment with higher TIL levels and PD-L1 expression (157,158), the addition of ICIs to NAC has demonstrated particularly promising results. Pembrolizumab combined with taxane- and anthracycline-based NAC significantly improved rates of pCR, seen across both PD-L1-positive and -negative subgroups, supporting broad clinical applicability (NCT01042379, NCT02622074, and NCT03036488) (159–161). These findings led to the approval of pembrolizumab in combination with NAC for high-risk early-stage TNBC, followed by adjuvant pembrolizumab monotherapy. Importantly, recent follow-up data confirmed that the early improvements in pCR translate into longer event-free survival with pembrolizumab – benefits observed regardless of PD-L1 expression – reinforcing both the prognostic value of pCR and the long-term efficacy of the preoperative pembrolizumab (162). Ongoing studies (NCT03639948) are now evaluating whether comparable pCR and survival outcomes can be achieved with pembrolizumab when anthracyclines are omitted from the NAC regimen in early-stage TNBC.

Other studies evaluating PD-L1 inhibitors have produced and reported more heterogenous outcomes. For instance, durvalumab (a PD-L1 inhibitor) added to NAC yielded modest improvements in pCR, but long-term follow-up revealed improved invasive disease-free survival and OS, suggesting delayed clinical benefit not fully captured by pCR alone (NCT02685059) (163,164). In contrast, atezolizumab failed to improve pCR when given in combination with taxane- and platinum-based chemotherapy in one clinical trial (NCT02620280), though another trial (NCT03197935) demonstrated significant pCR benefit when atezolizumab was combined with a standard chemotherapy regimen including preoperative anthracyclines (165,166). The discrepancies between these studies highlight the potential importance of both the sequence and selection of chemotherapy – particularly the timing of anthracycline delivery – in determining immunotherapy efficacy in early-stage high-risk TNBC.

Taken together, these results underscore that combining ICIs with chemotherapy represents a major therapeutic advance in both early-stage and metastatic TNBC. However, optimizing chemotherapy partners, identifying predictive biomarkers, and understanding the immunological mechanisms underlying response variability remain critical areas of ongoing investigation.

1.6.3.2. Antibody-Drug Conjugates

ADCs represent an emerging class of targeted cancer therapeutics that combine the specificity of mAbs with the cytotoxic potency of chemotherapeutic payloads (167). These agents are composed of three components: a mAb targeting a tumor-associated antigen, a cytotoxic drug (payload), and a chemical linker connecting the former two components. Upon binding to tumor cells, the ADC is internalized, releasing the payload and inducing cell death (167). While various payload types exist, most approved ADCs rely on antimitotic agents, microtubule inhibitors, antitumor antibiotics, and DNA damaging agents) (167).

Sacituzumab govitecan (SG) is the most prominent ADC in TNBC, which targets trophoblast cell surface antigent-2 (Trop-2) and delivers SN-38, and active metabolite of irinotecan (a topoisomerase I inhibitor)

(168). Trop-2 is highly expressed in TNBC, with expression levels reported as high as 93% in TNBC (vs. 50% in ER-positive and 74% in HER2-positive BCs), making it an attractive target (169,170). As monotherapy, SG showed significant efficacy in patients with various metastatic solid tumors who had received at least 1 prior line of therapy (NCT01631552), leading its "breakthrough therapy" FDA approval (171). In another pivotal trial (NCT02574455), SG significantly improved PFS and OS compared to standard chemotherapy in heavily pre-treated metastatic TNBC, regardless of Trop-2 expression (172). Ongoing trials are assessing the role of SG in earlier disease- stages and in PD-L1-stratified populations (NCT05382299 and NCT05382286). Datopotamab Deruxtecan (Dato-DXd) is another Trop-2 directed ADC using DXd (a topoisomerase I inhibitor) as a toxic payload (168). Early-phase studies showed promising activity and tolerability in metastatic TNBC (NCT03401385) (173). Ongoing clinical trials (NCT05374512) are currently evaluating its efficacy as first-line treatment compared to chemotherapy for patients with metastatic TNBC.

In early-stage, non-metastatic TNBC, the role of ADCs is still being investigated in a number of ongoing clinical trials. SG is under investigation in both the neoadjuvant (NCT04230109) and adjuvant settings (NCT04595565, NCT05633654, and NCT04434040), often in patients with residual disease after chemotherapy, with our without ICIs (174). Dato-DXd is also being evaluated in early-stage TNBC, alone or in combination with immunotherapy (NCT05629585 and NCT01042379). Several trials (NCT04644068 and NCT03742102) are further exploring ADC combinations with PARP inhibitors, particularly in *BRCA*-mutated or DNA repair-deficient tumors. Overall, ADCs represent a promising therapeutic option in TNBC. Ongoing efforts aim to optimize their integration into treatment regimens, enhance efficacy, and address resistance mechanisms.

1.6.4. Targeted Therapies for TNBC

Alongside standard of care chemotherapy and the evolving immunotherapy, the search of more targeted therapeutic strategies aimed at specific molecules, signaling pathways, and deficiencies/weaknesses in TNBC is underway.

1.6.4.1. PARP Inhibitors

The PARP family plays a critical role in the repair of single-stranded DNA breaks (175). Inhibiting PARP leads to blocking and trapping PARP1 and 2 isoforms, resulting in the accumulation of DNA damage and synthetic lethality in tumors with homologous recombination deficiency (HRD), particularly those harboring *BRCA1/2* mutations (176,177).

Two PARP inhibitors, Olaparib and Talazoparib are approved for germline *BRCA1/2*-mutated, HER2-negative metastatic BC (178,179). In clinical trials (NCT02000622 and NCT01945775), both agents showed significant benefits in ORR and PFS compared to chemotherapy, though OS benefits were modest (178,179). Notably, platinum agents were not included in the comparator arms of these trials, limiting direct comparisons between platinum-based chemotherapy and PARP inhibition. Ongoing efforts aim to expand PARP inhibitor use beyond germline *BRCA*-mutated TNBC. Trials evaluating PARP inhibitors (NCT0240134, NCT03981796, NCT03344965, and NCT03367689) with somatic *BRCA* mutations or HRD due to other mechanisms (e.g., *ATM*, *CHEK2*, or *PALB2* mutations) have shown limited but noteworthy benefits (180–183). In early-stage TNBC, PARP inhibitors have been tested in the neoadjuvant setting and are under evaluation as maintenance therapies. Results so far have shown comparable pCR rates to platinum-based chemotherapy (NCT02789332) but remain inconclusive (184). Early-phase studies have also begun investigating PARP inhibitors in combination with immunotherapy (NCT03834519, NCT04191135, and NCT03167619) or other DDR inhibitors (NCT03330847), such as ataxia telangiectasia and Rad3-related protein kinase (ATR) and WEE1 inhibitors (185,186).

1.6.4.2. Anti-androgen Therapy

AR is a steroid hormone receptor involved in cell proliferation, differentiation, apoptosis, or angiogenesis – depending on concurrent signaling pathways and tissue types. Although AR is not routinely assessed in TNBC, its expression has been linked to reduced chemosensitivity (187,188).

Early-phase clinical trials (NCT02353988) of AR antagonists such as bicalutamide and enzalutamide have shown modest clinical benefit in AR-positive TNBC, with disease stabilization and prolonged PFS in a subgroup of patients (189,190). Preclinical data suggest that combining AR blockade with other agents – such as PARP inhibitors or CDK4/6 inhibitors – may enhance efficacy via DNA damage and cell cycle pathways (191,192). These findings have promoted clinical trials testing such combinations in metastatic and early-stage settings. Given the limited efficacy of AR inhibitors as monotherapy, future research is focused on patient selection through AR scoring or genomic profiling and exploring combinatorial regimens that may enhance antitumor responses.

1.6.4.3. EGFR and VEGF Inhibition

EGFR is overexpressed in up to 50% of basal-like TNBC (27-57%) and has been linked with larger tumor size, poor differentiation, and poor clinical outcomes (193). Moreover, cancer cells can express up to 20x more EGFR than normal cells, highlighting EGFR as a potential therapeutic target (194). However, clinical trials using EGFR inhibitors – such as small-molecule TK inhibitors gefitinib, erlotinib, and cetuximab – have yielded disappointing results, with low response rates and no improvement in survival outcomes (195–197). Challenges include poor drug delivery, intrinsic resistance mechanisms, and heterogeneity of EGFR expression within tumors. Ongoing studies are exploring alternative approaches for effective drug delivery, such as the use of immunoliposomes or small interfering RNA (siRNA)-loaded nanocarriers (130).

Similarly, VEGF-driven angiogenesis contributes to tumor growth and poor prognosis in TNBC (198,199). VEGF inhibition has been explored primarily through bevacizumab, an anti-VEGF mAb which is FDA-approved for use in several cancers. Several clinical trials (NCT00028990, NCT01200212, NCT00262067, and NCT00528567) combining bevacizumab with conventional chemotherapy have reported improvements in PFS but demonstrated no OS benefits (200,201). Consequently, its indication in BC was withdrawn by regulatory authorities (202). More recently, the VEGFR TK inhibitor apatinib has shown promise in both early-stage and metastatic TNBC, with improved outcomes when combined with chemotherapy or immunotherapy in early-phase, exploratory studies (NCT03243838, NCT01176669, and NCT03359018) – warranting further investigation (203–206).

1.6.4.4. Epigenetic Modifications – Histone deacetylase Inhibitors

Epigenetic dysregulation, including histone modifications and DNA methylation, plays a role in TNBC pathogenesis and treatment resistance (207). Histone deacetylases (HDACs) are commonly overexpressed in TNBC and contribute to gene silencing, impaired DNA repair, and immune evasion (208). HDAC inhibitors (e.g., entinostat and vorinostat) can restore gene expression patterns by altering chromatin structure, promote apoptosis, and induce a HRD-like phenotype, sensitizing tumors to other treatment modalities such as PARP inhibitors or immunotherapy (209,210). Preclinical studies have demonstrated synergistic effects between HDAC inhibitors and various therapeutic agents. Clinical data, however, remain limited and inconclusive (207,211). Other combinatorial treatment approaches have shown favorable efficacy in metastatic BC (HDAC inhibitors and letrozole) or are yet to be explored (HDAC inhibitors plus PARP inhibitors or ionizing radiation) (212,213).

1.7. Treatment Challenges and Drug Resistance in TNBC

Despite advances in TNBC treatment – including chemotherapy, immunotherapy, ADCs, and targeted agents – clinical outcomes remain suboptimal due to a high rate of intrinsic and acquired resistance. Even patients who initially respond to therapy often relapse with more aggressive, treatment-refractory disease. Resistance is shaped by diverse molecular, cellular, and microenvironmental mechanisms that vary across TNBC subtypes and dynamically evolve during treatment. Understanding these complexities is crucial to improving long-term clinical outcomes. This section outlines key mechanisms of resistance – particularly to chemotherapy – as well as resistance linked to other treatment modalities.

Resistance is typically classified as intrinsic (primary or pre-existing) or acquired (secondary or emerging during treatment) (214). Intrinsic resistance involves baseline tumor features that allow cancer cells to survive therapy, while acquired resistance develops following initial treatment response, often through adaptive genetic or epigenetic changes.

1.7.1. Chemotherapy Resistance in TNBC

As previously emphasized, chemotherapy remains central to TNBC treatment. While many patients initially show high chemotherapy sensitivity, achieving pCR in up to 30-40% of cases, long-term outcomes remain disappointing. TNBC patients paradoxically have worse OS than those with other subtypes, even though they respond well to NAC – a phenomenon known as the "triple-negative paradox" (215). Those who fail achieve pCR face especially poor prognosis, and even among initial responders, relapse rates remain high within the first 3 years. This paradox underscores an urgent need to better understand and overcome resistance mechanisms in TNBC.

1.7.1.1. Drug Efflux and Metabolism-mediated Chemoresistance

One of the best-characterized contributors to chemotherapy resistance in TNBC is the activity of ATP-binding cassette (ABC) transporters, which use ATP to export a wide range of anticancer drugs across cellular membranes (216,217). Among these, *ABCC1* (MRP1), *ABCG2* (BCRP), and *ABCC11* (MRP8) are overexpressed in TNBC and contribute to resistance to agents such as taxanes, anthracyclines, doxorubicin, methotrexate and 5-fluorouracil (5-FU) (216,218–220). The overlapping substrate specificity leads to broad multidrug resistance (221). Strategies to overcome this include inhibiting transporter function or downregulating their expression. Non-steroidal anti-inflammatory drugs have been shown to sensitize ABCC1-overexpressing TNBC cells to chemotherapy and demonstrated preliminary clinical efficacy in combination with epirubicin (222,223). TK inhibitors can also block ABCG2 at high concentrations but can act as transport substrates at lower doses – an important factor often overlooked in trial design (224).

Gene-silencing techniques using siRNA and microRNA (miRNA) technology have effectively downregulated *ABCC1* and *ABCG2* in resistant TNBC cell lines, restoring drug sensitivity (225–228). While RNAi- and miRNA-based approaches offer high specificity, they often face challenges in delivery and stability *in vivo*. In addition, TNBC cells can upregulate enzymes involved in xenobiotic metabolism, such as cytochrome P450 isoforms (e.g., CYP3A4), which enzymatically inactivate chemotherapy agents (e.g., taxanes), lowering their intracellular concentration and hindering their therapeutic efficiency (229,230). Furthermore, elevated levels of reactive oxygen species (ROS) and glutathione in TNBC enhance the detoxification of chemotherapeutic agents (231). Glutathione, through its conjugation reactions catalyzed by glutathione-S-transferases, provides a protective mechanism against cytotoxic agents by accelerating their elimination (229,231).

1.7.1.2. Enhanced DNA Repair

In response to therapy-induced DNA damage, cancer cells activate DNA repair pathways to promote survival and treatment failure (229). One such mechanism involves O^6-methylguanine-DNA methyltransferase (MGMT), a key enzyme which removes alkyl groups from DNA and confers resistance to alkylating agents. Promoter methylation of MGMT correlates with improved response to alkylating agents and may serve as a predictive biomarker in TNBC (232,233). Mismatch repair deficiency (MMRD), present in approximately 6% of TNBCs, allows DNA replication with mismatched bases, leading to resistance to antimetabolites and alkylating agents (234–237). However, MMRD is also associated with higher tumor mutational burden, increased neoantigen production, and upregulated PD-L1 expression – rendering MMRD-TNBCs more susceptible to ICIs (238). Interestingly, since 2017, MMRD has been used as a biomarker for selecting patients with colorectal cancer suitable for pembrolizumab treatment (229). HRD, as discussed previously in 1.6.4.1, increases sensitivity to DNA-damaging agents (239). However, resistance can develop through compensatory DDR pathways. Notably, the ATR-checkpoint kinase 1 cascade can mitigate replication stress and promote resistance in HR-deficient cells (240). Preclinical studies have shown that ATR inhibitors (e.g., AZD6738) sensitize both HR-deficient and proficient TNBC to radiotherapy (241), and these agents are currently under investigation in clinical trials (NCT03330847) in combination with PARP inhibitors.

1.7.1.3. Evasion of Apoptosis

Evasion of apoptosis is a hallmark of cancer and a key driver of chemotherapy resistance in TNBC (22–24). Overexpression of pro-survival and anti-apoptotic proteins such as Bcl-2 and Mcl-1 is associated with poor prognosis in TNBC, and *MCL1* has been identified as one of the most frequently altered genes in chemotherapy-resistant residual TNBC tumors (242–244). Preclinical studies showed that inhibiting Bcl-2 (e.g., ABT-199) can re-sensitize resistant TNBC cells to doxorubicin (245).

Therapeutic strategies to restore apoptotic sensitivity include BH3 mimetics, which mimic pro-apoptotic BH3-only proteins (members of the Bcl-2 family) and are under evaluation in early-phase clinical trials (NCT03878524, NCT03900884, and NCT03584009) in advanced BC (246). Second-mitochondrial-derived activator of caspases (SMAC) is a pro-apoptotic protein which acts as an antagonist of the family of inhibitors of apoptosis (247). SMAC mimetics (e.g., Debio 1143 and LCL161) have shown pre-clinical or early clinical efficacy in TNBC (NCT03270176, NCT01078649, NCT02022098, and NCT01930292) (248). Lastly, elevated autophagy markers (e.g., *LC3B*) correlate with poor prognosis in TNBC. Inhibiting autophagy using chloroquine has been shown to enhance chemotherapy efficacy in preclinical models of TNBC and is being evaluated in clinical trials for advanced BC (NCT03774472 and NCT04316169) (249).

1.7.1.4. Tumor Microenvironment

Cancerous tissues are complex and dynamic, comprised of a plethora of different cell types besides tumor cells. Stromal cells, immune cells, and blood and lymphatic vascular networks collectively comprise the TME, which is increasingly recognized for its crucial role in mediating therapeutic resistance (250).

Cancer-associated fibroblasts (CAFs) are key drivers of chemotherapy resistance in TNBC through matrix remodeling, promotion of metastasis, and suppression of immune responses (229). CAF-secreted factors like C-X-C motif chemokine 12 (CXCL12) and insulin-like growth factor 1, activate pro-survival signaling pathways (e.g., PI3K signaling), promoting TNBC cell migration toward metastatic niches (e.g., the bone marrow). TNBC cells, in turn, secrete Hedgehog ligands to induce CAF-mediated stromal remodeling, enhancing cell-stemness (251). CAFs also promote immunosuppression by recruiting Tregs via CXCL12 signaling (252). Tumor-associated macrophages (TAMs) comprise over half

the immune infiltrate in TNBC and often adopt an M2-like – anti-inflammatory, tumor-promoting phenotype – in response to TNBC-secreted cytokines (253). TAMs contribute to resistance by enhancing PD-L1 expression through ROS-mediated NF-κB activation, thus facilitating immune evasion (254).

Driven by the rapid proliferation of cancer cells and poor vasculature, the TME tends to be a hypoxic environment. In TNBC, hypoxia has been correlated with poor prognosis and disease progression, driving certain drug-resistance phenotypes such as dormancy, redox adaptation, and stemness (250). Upregulation of HIF1α and its stabilizer prolyl 4-hydroxylase subunit alpha 1 is prominent in TNBC and mediates key features of hypoxia-driven resistance (255).

Extracellular vesicles, particularly exosomes, further amplify and promote resistance by shuttling oncogenic proteins and miRNAs between tumor and stromal cells (256). TNBC-derived exosomes enhance invasion and metastasis, in part through integrin subunit β4 delivery and HIF-RAB22A signaling (257,258). miRNAs such as miR-10b, miR-105, miR-122, miR-940, and miR-155 have been implicated in therapy resistance, with miR-155 showing promise as a predictive biomarker for platinum-based NAC response (259–263).

1.7.2. Cancer Stem Cells

Cancer stem cells (CSCs) represent a distinct tumor cell subpopulation endowed with self-renewal and tumor-initiating capacity, contributing to ITH, recurrence, and metastasis. Their intrinsic resistance to conventional therapies positions them as central players in treatment failure and disease progression (264).

The concept of CSCs originated with Lapidot et al.'s discovery in 1994, demonstrating that a minority subpopulation in acute myeloid leukemia (AML) could propagate disease in immunodeficient mice (265). This seminal work laid the groundwork for CSC research across solid tumors, including the brain, colon, liver, breast, and more (266–274). In this hierarchical model of tumor evolution, CSCs occupy the apex, capable of self-renewal and asymmetric division, seeding genetically distinct lineages that drive ITH (Fig. 1.7) (275,276). Moreover, CSCs bring an additional level of complexity to the concept of ITH as subpopulations of differentiated cancer cells with different genetic features existing within a tumor may very well originate from discrete pools of CSCs (277). Whether CSCs represent a stable phenotype or a dynamic, reversible state remains a subject of active debate (278).

In BC, CSCs (BCSCs) were first identified in 2003 by Al-Hajj and colleagues, where their work demonstrated that as few as 100 $CD44^{High/+}/CD24^{Low/-}$ cells could initiate tumor growth in mice (273). Subsequent studies identified activity of aldehyde dehydrogenases (ALDHs) – a family of detoxifying enzymes also prevalent in MaSCs – as another biomarker of BCSCs, with $ALDH^{High}$ cells correlating with enhanced tumor initiation, metastatic relapse, and poor prognosis (279–281). Studies have shown that TNBC tumors are especially enriched in CSCs, relative to other BC subtypes (282,283). Notably, CSCs are enriched in tumors after exposure to chemotherapy, with residual tumors (post-NAC) displaying a higher proportion of CSCs (284,285).

Multiple mechanisms underline CSC-mediated chemotherapeutic resistance. CSCs typically reside in a slow-cycling, quiescent state, rendering them less susceptible to chemotherapies targeting rapidly dividing cells (286,287). Moreover, CSCs also express high levels of ABC transporter, particularly *ABCG2* and *ABCB1*, rendering them resistant to a broad spectrum of cytotoxic agents (288). Hypoxia and therapy-induced stress further increase CSC marker expression and drug transporter activity, reinforcing their resilience (289).

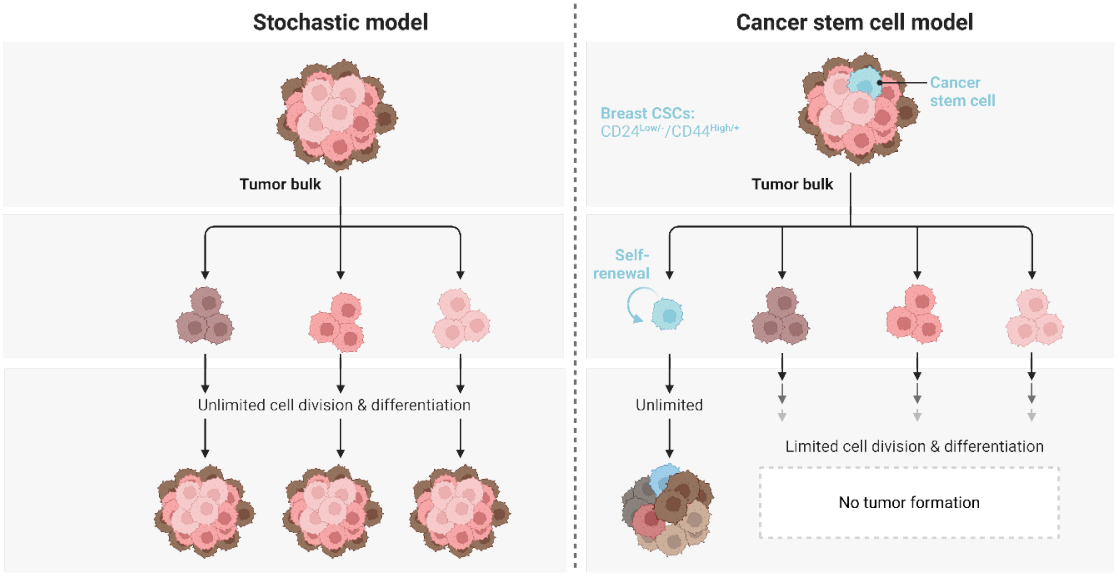


Fig. 1.7: Schematic overview of the Cancer Stem Cell model. *This figure was adapted from a pre-existing figure on Biorender.*

Therapeutic strategies to eliminate CSCs are currently being explored. ABCG2 inhibition with compounds such as YHO-13351 have shown promise in sensitizing CSCs to chemotherapy *in vitro* and *in vivo* (290). Another approach relies on targeting CSC surface agents. Innovative drug delivery methods such as hyaluronic acid-coated nanoparticles targeting CD44 have shown efficacy in delivering chemotherapy selectively to CSCs while sparing normal tissue (291). Various other CD44-targeting nanocarriers are currently being investigated as delivery vehicles for different therapeutic agents, including cytotoxic drugs and siRNAs (292).

Similar to embryonic and adult stem cells, the maintenance and regulation of CSCs is tightly regulated by key developmental signaling cascades, namely transforming growth factor-beta (TGF-β), Notch, Wnt/β-catenin, and Hedgehog signaling pathways (221). In the context of various cancers, including TNBC, aberrant activation of these pathways supports CSC maintenance, reinforces tumor heterogeneity, and drives treatment failure. Each of these signaling axes orchestrates complex transcriptional programs that sustain stemness, promote EMT, and facilitate the dynamic activation of CSCs under therapeutic pressure. Given their central role in maintaining the CSC population and, by extension, their role in mediating chemoresistance, these pathways have emerged as compelling targets for novel therapeutic strategies, although clinical translation remains challenging due to toxicity and pathway complexity.

In TNBC, aberrant activation of TGF-β signaling promotes BCSC enrichment, enhances EMT, and contributes to tumor recurrence and immune evasion (293,294). Inhibition of TGF-β receptors has shown promising results in preclinical TNBC models, preventing tumor establishment post-chemotherapy (295). Similarly, Notch signaling – especially involving Notch-1, -3, and -4 receptors – is frequently upregulated in TNBC and associated with enhanced proliferation, invasion, and stem-like traits (296–298). However, its role may be context-dependent, with some evidence suggesting Notch-3 may exert tumor-suppressive effects in certain settings (299). Therapeutic targeting of Notch is under clinical investigation using γ-secretase inhibitors (e.g., AL101 – a novel GSI), as well as mAbs (e.g., tarextumab – a first-in-class anti-Notch 2/3 antibody), both demonstrating early-phase efficacy (300–

303). The Hedgehog pathway also plays a critical role in sustaining the CSC population in TNBC, largely through glioma-associated factor 1/2 (GLI1 and GLI2) transcriptional activation (304–306). Targeting Hedgehog signaling is mainly focused on inhibition of Smoothened (SMO), a transmembrane protein involved in the pathway's activation. While SMO inhibitors have shown limited success in clinical trials (NCT02027376, NCT01071564, NCT01576666, and NCT01757327), likely due to SMO-independent pathway activation (307), direct inhibition of GLI1/2 with compounds such as GANT61 has yielded encouraging preclinical results, reducing CSC frequency and enhancing sensitivity to chemotherapy (308,309). Lastly and most notably, Wnt/β-catenin signaling plays a key role in regulating CSC properties in TNBC, including self-renewal, tumor initiation, and resistance to therapy (310–313). Aberrant activation of Wnt/β-catenin signaling has been linked to poor clinical outcomes, enhanced metastatic potential, and expansion of the CSC pool. As Wnt/β-catenin signaling is a central focus of this thesis, a detailed discussion of the pathway, its role in TNBC resistance, and associated therapeutic strategies is provided in later sections.

1.7.3. Resistance to Immunotherapy and PARP Inhibitors

While immunotherapy has shown great promise in TNBC, resistance remains a major challenge. Many patients exhibit primary resistance or relapse after an initial response (314). Key mechanisms included impaired antigen presentation, notably through MHC-I downregulation, which limits cytotoxic T cell recognition despite high tumor mutational burden (315–318). The TME further contributes to resistance throughout its immunosuppressive composition, with elevated levels of Tregs, MDSCs, and M2-type TAMs – all of which suppress effective T cell responses (319). Chronic antigen exposure and hypoxic conditions can lead to T cell exhaustion, characterized by diminished cytolytic activity and cytokine production (319). Resistance is exacerbated by upregulation of compensatory immune checkpoints such as TIM-3 and LAG-3, often induced after PD-1/PD-L1 blockade, which inhibit T cell function and limit therapeutic efficacy (320–322). Additionally, aberrant oncogenic pathways, including RAS/MAPK signaling, PTEN loss, and Wnt/β-catenin activation, further suppress immune infiltration and promote immune evasion (323–329). Collectively, these interconnected mechanisms underscore the need for robust biomarkers to guide patient selection and rational combination strategies to enhance immunotherapy responsiveness in TNBC.

Resistance to PARP inhibitors arises in multiple molecular mechanisms that undermine their therapeutic efficacy. One key route is the overexpression of drug-efflux transporters such as multi-drug resistance 1 (MDR1), which actively pump PARP inhibitors out of tumor cells, reducing intracellular drug concentration and cytotoxicity (330,331). Additionally, mutations in the PARP1 protein can impair its ability to bind DNA or disrupt its enzymatic activity, while loss of PARG – the enzyme responsible for degrading PAR chains – can interfere with PARP1 recycling and hinder the DNA repair process (332–334). Crucially, resistance also emerges through the restoration of HR repair, the very pathway PARP inhibitors are designed to exploit. This can occur via secondary mutations that restore the function of *BRCA1/2*, the expression of partially functional isoforms of these genes, or loss of key HR repressors such as 53BP1 and the Shieldin complex, which re-enable DNA end resection (335–341). Together, these alterations enable tumor cells to bypass synthetic lethality. To address these challenges, several combination strategies are being investigated, including co-administration of PARP inhibitors with chemotherapy, ICIs, and cell cycle checkpoint inhibitors, aiming to prevent or overcome resistance and extend the clinical benefit of PARP-targeted therapy in TNBC.

1.8. Phenotypic Plasticity and Non-genetic Resistance – Offering a New Perspective on Reversible Adaptive Resistance

Despite the rapid evolution of therapeutic strategies for TNBC—from conventional chemotherapy and radiotherapy to ICIs, TK inhibitors, ADCs, and DNA repair inhibitors—clinical outcomes remain hampered by the near-universal development of resistance. This resistance is rarely attributable to a single, fixed molecular defect; rather, it emerges from a complex interplay of ITH, plasticity, and adaptive rewiring in response to therapeutic stress (Fig. 1.8) (342,343). The ability of TNBC cells to evade treatment involves not only the selection of pre-existing resistant clones, but also widespread transcriptional and epigenetic reprogramming, phenotypic switching, and reshaping of the TME. These dynamic adaptations enable cancer cells to transiently enter drug-tolerant states that are neither terminally differentiated nor fully proliferative, but rather primed for survival under therapeutic pressure. Such reversible states of drug tolerance—marked by altered chromatin landscapes, shifts in lineage identity, and stress response activation—represent a crucial intermediate step before the development of stable resistance. Understanding these non-genetic, reversible processes is therefore essential, as they offer a window of therapeutic vulnerability that may precede irreversible treatment failure. In the next section, we examine the mechanisms of transcriptional reprogramming in TNBC and introduce the concept of drug-tolerant persister cells (DTPs/DTP cells), which pose one of the most significant challenges to long-term treatment success.

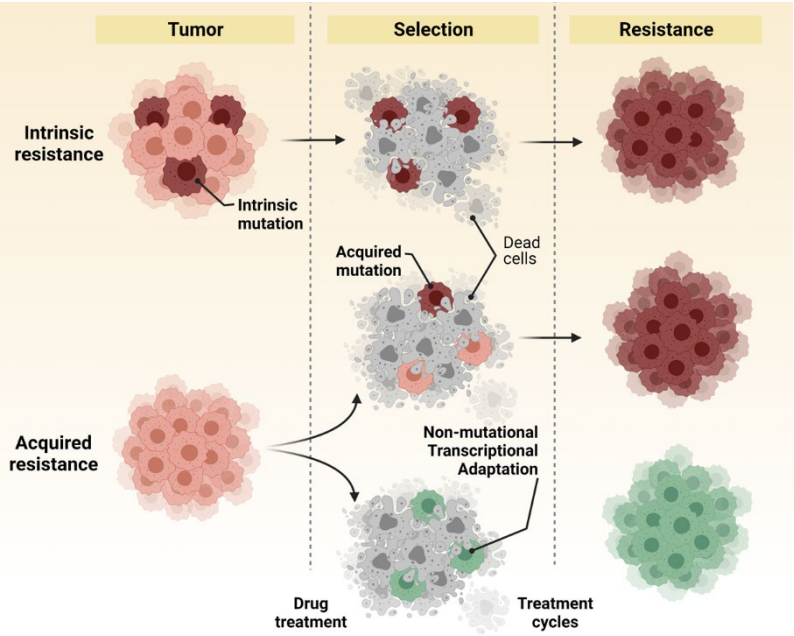


Fig. 1.8: Schematic overview of Intrinsic and Acquired therapeutic resistance. *This figure was adapted from a pre-existing figure in the dissertation by Willy Antoni Abreu De Oliveira under the title "The Role Of Wnt Signaling In Acquired Chemotherapy Resistance In Triple Negative Breast Cancer".*

1.9. Drug Tolerant Persister Cells

Described as "… invincible in peace, invisible in war", DTP cells, also referred to as persister cells or just persisters, comprise a rare subpopulation of cancer cells capable of surviving otherwise lethal drug exposures by entering a transient, non-mutational state of tolerance (344,345). First described by Sharma et al. in non-small cell lung cancer (NSCLC), DTPs evade drug-induced cytotoxicity without

harboring resistance mutations, later re-entering the cell cycle when treatment pressure is lifted (346). This phenotype has since been identified across numerous cancers, including BC, and underlines residual disease and tumor relapse following chemotherapy and other targeted agents (214,344). Although often viewed as a novel concept in oncology, DTPs have origins in microbiology. The term "persister" dates back to the 1940s, when the newly developed antibiotic penicillin was found to eliminate most (99%) bacteria (pneumococci, hemolytic streptococci, and staphylococci) while sparing a small subset (1%) that survived without genetic resistance (347). These so-called "persister" cells were dormant but viable, resuming growth once antibiotic levels were decreased (Fig. 1.9) (348–350).

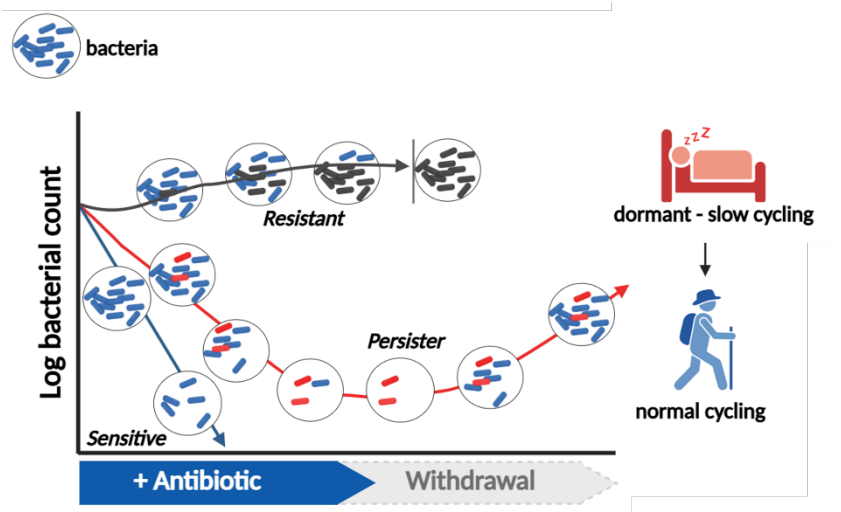


Fig. 1.9: Schematic overview of bacterial persister cells. *This figure was adapted from a pre-existing figure in the review titled "Emerging Insights into Targeted Therapy-Tolerant Persister Cells in Cancer" by Cabanos, H. and Hata, A. (https://doi.org/10.3390/cancers13112666).*

Almost 70 years after the discovery of bacterial persisters, the concept was reintroduced into oncology by Sharma et al., who in 2010 identified a small subpopulation of NSCLC cells that survived EGFR-targeted therapy by entering a reversible DTP state (346). These cancer DTPs mirrored bacterial persisters: they endured high-dose drug exposure without harboring resistance mutations and resumed proliferation upon drug withdrawal. Mechanistically, this phenotype was mediated by non-genetic adaptations, including activation of insulin-like growth factor 1 receptor signaling and chromatin remodeling through lysine-specific demethylase 5A (KDM5A). This work challenged the dominant paradigm that drug resistance arises exclusively through genetic selection, highlighting instead a dynamic, plastic survival program (Fig. 1.10). Since then, DTPs have been documented across numerous malignancies – including melanoma, colorectal cancer (CRC), pancreatic cancer, glioblastoma, AML, and BC – following exposure to chemotherapy, targeted agents, or immunotherapy (343,345,359,360,351–358).

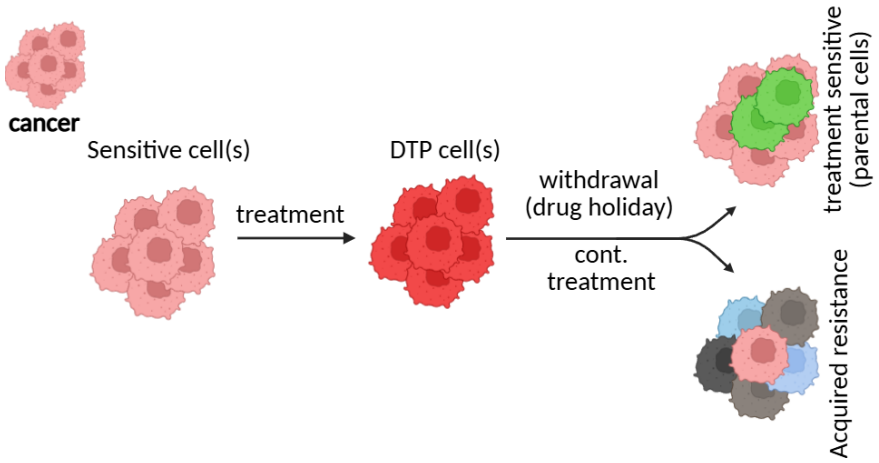


Fig. 1.10: Schematic overview of cancer Drug-Tolerant Persister cells.

1.9.1. Defining Cancer DTPs

Despite increasing recognition of DTPs in oncology, no universal criteria currently define or identify them. However, several hallmark features recur across studies: (i) a non-genetically driven phenotype, (ii) a reversible state of quiescence or dormancy, (iii) the capacity to survive high-dose therapy that eliminates the majority of tumor cells, and (iv) transient entry into and exit from this state (361–363). While other traits – such as metabolic rewiring, altered cell identity, a senescence-associated secretory phenotype, and microenvironmental interactions – have been reported, we focus here on the four characteristics most consistently observed and relevant to our work (Fig. 1.11).

1.9.1.1. Non-genetic Driven Phenotype

Unlike classical models of resistance based on clonal selection and stable mutations, DTPs arise through reversible, non-genetic adaptations (346,364). In response to treatment, sensitive cells may enter a drug-tolerant state characterized by transcriptional, epigenetic, and metabolic reprogramming – without acquiring resistance mutations (361–363). DTPs retain the capacity to revert to a drug-sensitive phenotype upon withdrawal of therapy, distinguishing them from permanently resistant clones. This plastic state often involves chromatin remodeling, altered histone modifications, and dynamic changes in transcription factor activity. For example, increased expression of histone demethylases KDM5A or epigenetic repressors such as HDACs facilitate the establishment of a repressive chromatin environment that supports cell survival under stress (344,365,366). Importantly, the DTP phenotype is not tied to a single oncogenic driver or signaling pathway but rather reflects a flexible survival program shaped by both therapeutic pressure and environmental cues (361–363). However, while initially reversible and non-mutational, this tolerance may serve as a precursor to stable resistance, providing a reservoir from which permanent resistance can emerge via subsequent acquisition of mutations or stable genetic alterations (367,368).

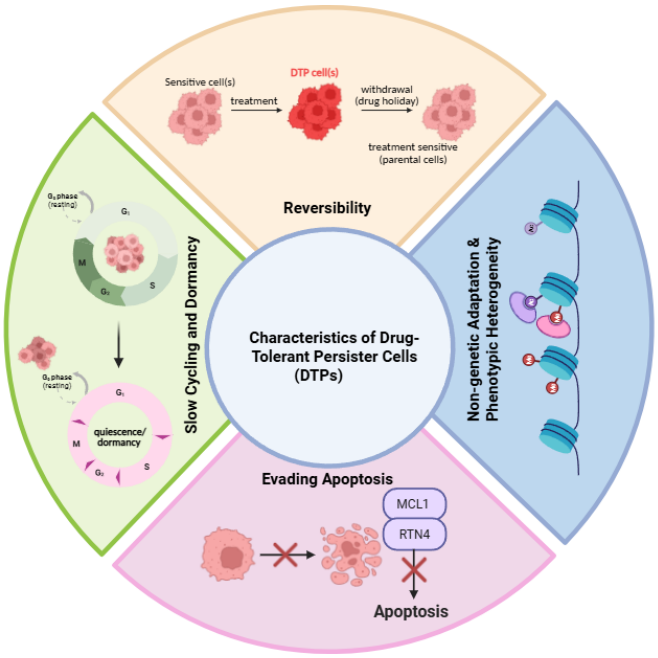


Fig. 1.11: Schematic overview of a key characteristics cancer DTP cells.

1.9.1.2. Slow Cycling and Dormancy

A central hallmark of DTP cells is their entry into a quiescent or slow-cycling state, characterized by diminished proliferation and cell cycle arrest, typically in the G0/G1 phase (346,358,366). Unlike "classic" dormant tumor cells, which may exist prior to therapy, DTP cells enter a transient and reversible slow-cycling state in response to treatment-induced stress, reflecting an induced rather intrinsic form of dormancy (361,362). In CRC for example, a subset of 5-FU treated cells persisted in the G0/G1 phase and remained viable for an extended treatment period, later re-entering the cell cycle and resuming proliferation upon drug removal (369). Similarly, Osimertinib-resistant NSCLC cells showed downregulation of cell cycle gene expression, which reverted upon drug removal (370). This state is maintained by both cell-intrinsic and -extrinsic factors. Intrinsically, histone-modifying enzymes like KDM6A alter chromatin states to suppress proliferation-associated genes (371). Extrinsically, interactions with the TME – such as secretion of secreted Frizzled related protein 1 (SFRP1) by fibroblasts or recruitment of macrophages – support dormancy induction (372,373). Notably, although most DTPs remain dormant under therapy, a subset (approximately 20%) eventually reinitiates proliferation despite continued drug exposure. These "drug-tolerant extended persisters" can proliferate indefinitely in the presence of the drug, serving as an opportunity for the acquisition of resistance-inducing mutations or epigenetic adaptations that can contribute to therapeutic failure and relapse (346).

1.9.1.3. Reversibility

Reversibility is a defining feature that distinguishes DTPs from genetically resistant clones. Once the therapeutic agent is withdrawn (i.e., a drug holiday period), DTPs often re-enter the cell cycle again and regain drug sensitivity. This was first demonstrated in the seminal work by Sharma et al. in NSCLC cells treated with EGFR inhibitors, which resumed proliferation and drug sensitivity after treatment withdrawal (346). Similar reversible phenotypes have been reported in various tumor types, including melanoma, ovarian, colon, lung, and BCs (374–376). A recent exhaustive study by Chang et al. demonstrated that HER2-positive BC tumors exposed to HER2-TK inhibitors entered a reversible DTP state, regaining proliferative capacity upon halting treatment and later displaying the capacity to re-enter the DTP state upon re-treatment (377). Mechanistically, this reversibility is underpinned by epigenetic plasticity, driven by histone modifications, chromatin accessibility, and dynamic shifts in DNA methylation profiles in response to drug exposure and removal (346,370,378,379). The reversible nature of DTPs may also be supported by a senescent-like state (380,381). Studies have shown that eliminating senescent cells after chemotherapy can prevent or delay cancer recurrence and spread to distant tissues, suggesting that their targeted removal could be a therapeutic avenue through which induction of a DTP phenotype could be prevented (381).

1.9.1.4. Surviving Cell Death & Evading Apoptosis

By definition, DTPs are defined by their capacity to evade cell death and persist under drug concentrations lethal to most tumor cells. Unlike survival models reliant on mutations, DTP cells utilize transient, non-genetic, adaptative mechanisms that suppress apoptosis and other forms of cell death. For instance, treatment of EGFR-mutant NSCLC cells with gefitinib led to the upregulation of the mTOR-mediated anti-apoptotic protein MCL1 (382). Similarly, DTPs exhibit decreased reliance on oncogenic signaling and increased sensitivity to inhibitors of pro-survival proteins like Bcl-2 and Bcl-xL, revealing therapeutic vulnerabilities (383–385). Some DTPs undergo "sublethal" or "failed" apoptosis, which activates stress responses without triggering immediate cell death. In this state, incomplete mitochondrial permeabilization and limited caspase activation promote adaptive transcriptional programs through mediators such as eIF2α and ATF4, sustaining a quiescent, resistant phenotype (386–388).

Interestingly, DTP cells are not universally resistant to all cell death modalities. Several studies have suggested that DTP cells are selectively vulnerable to ferroptosis, a non-apoptotic form of iron-dependent cell death driven by lipid peroxidation (389,390). Hiroto et al. showed that osimertinib-resistant cancer cells were significantly more sensitive to ferroptosis inducers like RSL3 (391). Similarly, GPX4 inhibition or KDM5A suppression, both regulators of lipid metabolism and redox homeostasis, increased DTP cells' susceptibility to ferroptosis (390,392). These insights suggest that while DTP cells can evade apoptosis, they may become dependent on alternative metabolic states that can be exploited therapeutically. Beyond escaping cell-autonomous death, DTPs may also leverage immune-evasive strategies. CD70 expression, recently identified in osimertinib-resistant NSCLC cells, confers resistance to $CD8^+$ T cell-mediated cytotoxicity via PI3K signaling (393,394). These $CD70^+$ DTP cells can therefore be targeted using anti-CD70 ADCs or CD70-directed CAR-T cells (394). Similarly, DTP-like cells in anti-PD-1-treated tumors express immunosuppressive and EMT-associated genes (e.g., IL-6 and Snai1), and evade $CD8^+$ T cell-mediated cell death via BIRC2/BIRC3-dependent signaling (395). These findings underscore the multifaceted survival strategies employed by DTP cells.

1.9.1.5. Phenotypic Heterogeneity

Despite comprising a minority of the tumor population, DTP cells are phenotypically diverse. Many exhibit stem-like features, including the expression of markers such as CD44 and ALDH1, and transcription factors like SOX2 and OCT4 (396–400). These traits may reflect a convergence with CSCs, though the relationship between the two populations remains under debate and is discussed further in a later section (1.9.3). In gastric cancer, DTP cells induced by 5-FU treatment expressed various stem cell markers such as Leu-rich repeat-containing G-protein coupled receptor 5 (*LGR5*) and *ALDH1A1* (401). As an indication of their plastic nature, DTP cells are also capable of undergoing EMT, enhancing both their migratory capacity and resistance. EMT-related transcription factors – Snail, Slug, ZEB1/2 – are often upregulated in DTPs following targeted therapies (402–404). Additionally, microenvironmental cues, such as hypoxia or CAF-secreted cytokines, can further promote EMT (405) (406,407). In some contexts, DTP cells undergo lineage plasticity or transdifferentiation (362). For instance, CRC cells resistant to EGFR blockade acquire a Paneth cell-like phenotype, while prostate and lung adenocarcinoma cells adopt neuroendocrine phenotypes under therapy (408–410). Finally, DTP cells exhibit distinct metabolic adaptations to survive therapeutic stress. Shifting from anaerobic glycolysis to oxidative phosphorylation and fatty acid oxidation enables survival under stress, supported by enhanced antioxidant pathways to manage elevated ROS levels (363,411–413). Targeting key

enzymes in these pathways (e.g., ALDH and GPX4) sensitizes DTPs to ferroptosis, providing potential avenues for therapeutic intervention (406).

1.9.2. Embryonic Diapause

Embryonic diapause is a reversible state of developmental arrest observed in over 130 mammalian species, including rodents, marsupials, and carnivores (414). During this state, embryonic development pauses at the blastocyst stage, delaying implantation until environmental conditions become favorable (414). Diapause occurs in two forms: facultative diapause, induced by maternal factors such as lactation-associated metabolic stress, and obligate diapause, regulated by environmental cues like photoperiod and temperature.

Mechanistically, embryonic diapause is characterized by marked reduction in proliferation, metabolic activity, and gene expression, allowing the embryo to remain viable while in a dormant, resource-conserving state. Central regulators of this process include the mTOR signaling pathway and c-Myc, whose inhibition suppresses cell growth and protein synthesis (415,416). A metabolic shift toward lipid metabolism, mediated by factors like FOXO1, supplies energy while maintaining the quiescent state (417). In parallel, increased autophagy helps maintain cellular viability by recycling intracellular components, providing nutrients and removing waste (418).

Striking parallels have emerged between embryonic diapause and the DTP phenotype in cancer. Both states share features of reversible dormancy, suppressed mTOR and c-Myc activity, increased autophagy and metabolic adaptation favoring lipid utilization (419–422). Epigenetic reprogramming – including chromatin remodeling and transcriptional suppression – is another shared mechanism that supports cellular survival under stress (423). Notably, both diapaused embryos and cancer DTP cells can remain under unfavorable conditions and resume proliferation when those conditions improve (354). These functional similarities suggest the cancer cells may repurpose evolutionarily conserved dormancy programs to survive therapeutic pressure. Supporting this hypothesis, seminal work by Dhimolea et al. demonstrated a strong transcriptional overlap between treatment-persistent cancer cells (obtained from organoids, xenografts, and patient tumors) and diapaused mouse embryos (420). Such findings raise the possibility that therapeutic resistance may, in part, reflect a reactivation of embryonic survival pathways in cancer.

1.9.3. DTP cells and CSCs – Two Faces of the Same Coin or Two Coins Altogether?

An ongoing debate that is slowly becoming one of the focal points of oncological research concerns the relationship between DTP cells and CSCs – two populations known to mediate resistance and recurrence. Both DTP cells and CSCs exhibit low proliferative capacity, therapeutic resistance, and the potential to reinitiate tumor growth. While CSCs are defined by specific surface markers and inherent stem-like properties, DTPs are characterized by a transient, reversible drug-tolerant state that may not necessarily (but often does) involve stemness. Notably, some DTP cells do express stem cell-associated markers such as CD133 and ALDH, indicating an overlap between these two populations (379). Despite the similarity between these two populations, there are key mechanistic and functional distinctions whereby DTP cells and CSCs diverge.

CSCs are typically defined by intrinsic stemness, a relatively stable phenotype, and the capacity for self-renewal and differentiation, often demonstrated through serial transplantation assays. They may arise from normal tissue progenitors or differentiated tumor cells (424). In contrast, DTP cells represent a transient, inducible state, emerging in response to therapeutic stress via non-genetic adaptations,

including transcriptional reprogramming and epigenetic modulation. Upon drug withdrawal, DTP cells frequently resume proliferation and regain sensitivity, underscoring the dynamic nature of their phenotype (361,362). Tumorigenicity further distinguishes these populations. While CSCs can initiate tumors at low cell numbers *in vivo*, DTPs are not inherently tumor-initiating unless they acquire additional alterations that confer permanent resistance or induce dedifferentiation into a CSC-like state (424). Additionally, CSCs often rely on developmental signaling pathways such as Wnt/β-catenin, Notch, Hedgehog, and TGF-β signaling to maintain stemness and survival (425). In contrast, DTP cells tend to exploit metabolic reprogramming, oxidative stress tolerance, and suppression of apoptotic machinery (354,361,362,420). That said, more studies – including our own – demonstrate that, similar to CSCs, DTPs can also utilize key signaling cascades such as upregulating Wnt/β-catenin signaling, suggesting shared regulatory programs in certain contexts.

Clinically, the distinction between CSCs and DTP cells has significant therapeutic implications. Targeting CSCs alone may not eliminate DTP cells, which rely on distinct dormancy and survival programs. Conversely, therapies directed at DTP cell-survival pathways may spare CSCs. As both contribute to drug resistance and disease relapse, understanding their overlap and divergence is essential for designing combinatorial treatment strategies that effectively eradicate both populations.

1.9.4. Therapeutic Landscape of DTPs: Opportunities and Challenges

The increasing recognition of DTP cells as drivers of relapse and resistance has catalyzed interest in therapeutic strategies that either eliminate them or prevent their formation. Given their reliance on chromatin remodeling and transcriptional repression, epigenetic regulators are one key avenue. Inhibitors of KDM5 demethylases, HDACs, and EZH2 have shown promise in reversing the DTP phenotype and restoring drug sensitivity (346,426,427). DTP cells are also characterized by heightened dependence on anti-apoptotic proteins, such as MCL1, Bcl-xL and Bcl-2. Targeting these dependencies using BH3 mimetics (e.g., navitoclax/ABT-263) has demonstrated the ability to sensitize DTP cells to apoptosis and has shown efficacy in preclinical models (428). Furthermore, DTP cells exhibit selective vulnerability to ferroptosis, therefore, agents such as GPX4 inhibitors (e.g., RSL3 and ML210) and iron metabolism disruptors have been shown to selectively kill DTP cells in multiple cancer models (390,392). Recent studies have also targeted diapause-associated pathways. Activation of MYC or stimulation of mTOR – both suppressed during the DTP state – can force cells out of dormancy, re-sensitizing them to treatment (429).

Nevertheless, significant challenges remain. The transient and non-genetic nature of DTP cells complicates detection and tracking, especially since they lack consistent surface markers and can revert to a drug-sensitive phenotype upon withdrawal of therapy. Furthermore, phenotypic heterogeneity within the DTP pool – encompassing stem-like, senescent, EMT-like, and metabolically reprogrammed states – underscore the need for multi-targeted therapeutic approaches. Unlike CSCs, whose tumorigenic potential and surface markers enable selective targeting, DTPs require a nuanced understanding of their dynamic transcriptional states, survival circuitry, and overlap with CSCs. In this context, combination therapies that simultaneously disrupt chromatin remodeling, metabolic adaptations, and survival signaling may offer the most effective strategy. Ultimately, advancing therapeutic interventions for DTP cells will depend on precise identification of their shared vulnerabilities and differentiation from CSCs and other resistant cell states.

1.10. Wnt Signaling and Breast Cancer

Dysregulation of developmental signaling pathways is a hallmark of many cancers, highlighting the parallels between embryogenesis and tumorigenesis (430). Among these, the Wnt signaling pathway plays a central role in embryonic development and adult tissue homeostasis and maintenance. Aberrant activation of Wnt signaling contributes to tumorigenesis in several cancers, including BC, where it is reported to be active in over half of cases and correlates with poor prognosis (312,431,432).

The Wnt pathway was first linked to cancer through the discovery of the *wingless* (*wg*) gene in *Drosophila melanogaster* by RP. Sharma in 1973 when performing mutagenesis screening of visual phenotypes (433). *Int1* (Wnt1), later found to be the mammalian homolog of *wingless*, was shown to induce mammary hyperplasia and breast tumors in mice, linking Wnt signaling to tumorigenesis (434–436). Around the same time, mutations in the tumor suppressor gene adenomatous polyposis coli (*APC*) were identified as key drivers of hereditary CRC and later shown to activate Wnt signaling by disrupting intracellular regulation, firmly establishing Wnt as an oncogenic pathway (437–441). Since then, the molecular framework of Wnt signaling has been extensively mapped. It is now broadly classified into two branches: the canonical (β-catenin-dependent) and non-canonical (β-catenin-independent) pathways, each involving distinct ligands, receptors, and intracellular signaling events.

1.10.1. Wnt Ligands

Both signaling branches are initiated by Wnt ligands, a family of 19 conserved secreted glycoproteins acting through autocrine and paracrine mechanisms (442). Prior to secretion, Wnt proteins undergo glycosylation in the endoplasmic reticulum and are lipid-modified by the enzyme Porcupine O-acyltransferase (PORCN), which adds a palmitoyl group to the ligands. The modified ligands then bind Evenness interrupted Wntless (Evi/WLS) and are trafficked to the plasma membrane via the Golgi with the aid of p24 proteins (Fig. 1.12) (443–445). Upon secretion, Wnt ligands bind receptor complexes on target cells to activate the canonical or non-canonical Wnt pathway. Canonical Wnt signaling is activated by receptor complexes consisting of 10 members of the Frizzled (FZD) family of transmembrane receptors and co-receptor low-density lipoprotein receptor-related proteins 5/6 (LRP5/6). In contrast, non-canonical Wnt signaling is mediated by receptor complexes consisting of a FZD receptor and receptor tyrosine kinase-like orphan receptor 1/2 (ROR1/2) or receptor-like tyrosine kinase (RYK) (446).

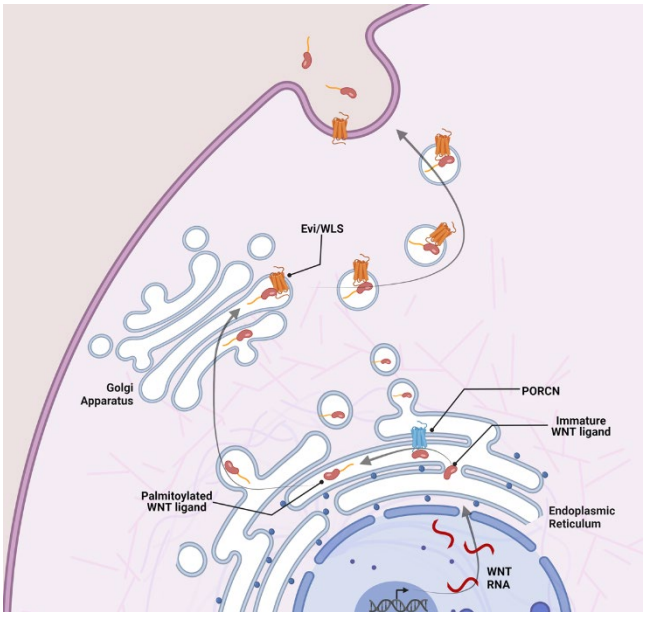


Fig. 1.12: Schematic overview of the Wnt ligand secretory pathway. *This figure originates from the review "Wnt Signaling in the Breast: From Development to Disease" (https://doi.org/10.3389/fcell.2022.884467).*

1.10.2. Canonical Wnt Signaling (β-catenin-dependent Wnt Signaling)

This branch of the pathway is the most studied and generally the best understood and delineated. The activity of the canonical Wnt pathway converges on the central role of β-catenin as the relay between the upstream Wnt ligand-mediated activation of the cascade and the most downstream effectors and regulators of Wnt-dependent gene expression (Fig. 1.13).

In the absence of Wnt ligands, β-catenin is continuously degraded by a multiprotein destruction complex, composed of two scaffolding proteins (tumor suppressors APC and axis inhibition protein 1, AXIN1), casein kinase (CK1), glycogen synthase kinase 3β (GSK3β), and the E3-ubiquitin ligase β-TrCP. Sequential phosphorylation of β-catenin by CK1 (at Ser 45) and GSK3β (at Thr41, Ser37, and Ser33) primes it for proteasomal degradation, reducing its cytoplasmic levels and impeding further signal transduction (443). Ligand binding to the FZD/LRP5/6 receptor complex stabilizes β-catenin levels, allowing its cytoplasmic accumulation and nuclear translocation. In the nucleus, β-catenin binds the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors, displacing repressors such as Groucho to activate the transcription of Wnt-target genes. Wnt target genes are cell type-, tissue-, and developmental stage-specific; however, *AXIN2* is frequently used as a generic target indicator of canonical Wnt pathway-activity (443,447).

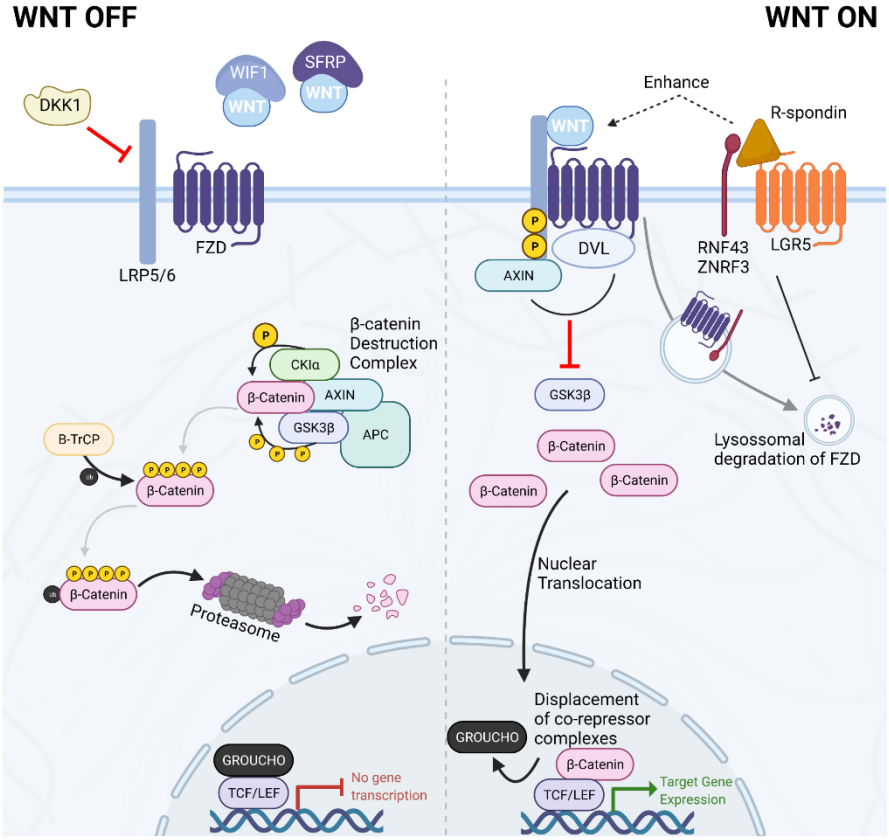


Fig. 1.13: Schematic overview of the Canonical (β-catenin-dependent) Wnt Signaling Pathway. *This figure originates from the review "Wnt Signaling in the Breast: From Development to Disease" (https://doi.org/10.3389/fcell.2022.884467).*

1.10.3. Non-canonical Wnt Signaling (β-catenin-independent Wnt Signaling)

Non-canonical Wnt signaling proceeds independently of β-catenin and involves two well-characterized branches: the planar cell polarity (PCP) and the Wnt/$Ca2^{+}$ pathways. Both are activated by FZD receptors without LRP5/6.

The Wnt/PCP pathway regulates cell polarity, motility, and migration (446,448). Its activation is mediated by the FZD/ROR/RYK/VANGL2 axis, which leads to the phosphorylation of c-Jun N-terminal kinase, which in turn activates gene transcription via activator protein 1 transcription factor (446). The Wnt/PCP pathway is known to negatively regulate the canonical Wnt/β-catenin pathway in a Dishevelled (DVL)-dependent manner (446).

The Wnt/$Ca2^{+}$ pathway is critical in regulating cell adhesion, migration, and embryonic development (446). It is activated upon Wnt ligand interaction with FZD receptors and ROR/RYK. Subsequently, DVL is recruited to the receptor complex in the cell membrane's inner leaflet, leading to activation of phospholipase C and downstream release of intracellular calcium ions. Intracellular calcium fluxes provoke the activation of calcium-dependent kinases (e.g., calpain 1 and calcineurin), which in turn activate the transcription of target genes through the activity of nuclear factor-activated T-cell and NF-κB transcription factors (446).

1.11. Wnt Signaling in TNBC

As previously mentioned, CRC is a classic example of a neoplastic disease driven by mutations in tumor-suppressor genes encoding the Wnt signaling pathway members. In CRC, loss-of-function mutations of the *APC* gene lead to constitutive activation of the canonical Wnt signaling pathway via stabilization of β-catenin (437–441). Alternatively, point mutations in the gene encoding β-catenin (*CTNNB1*) lead to alterations in the N-terminal Ser/Thr phosphorylation sites of the protein, preventing phosphorylation by GSK3β and consequent degradation of β-catenin in some *APC* wild-type CRCs (449). Mutations in other components of the Wnt/β-catenin signaling cascade are known oncogenic drivers of a variety of other neoplastic disorders such as cancers of the liver, stomach, pancreas, and ovaries, among many others (450).

Interestingly, aberrant Wnt/β-catenin signaling is a feature of BCs, specifically TNBCs (> 50% of patients) (451,452). However, oncogenic driver mutations in Wnt/β-catenin signaling components such as *APC*, *AXIN1*, or *CTNNB1* are surprisingly almost undetectable in TNBCs. For instance, alterations in *APC* account for only 2.4% of BC cases, in comparison with a staggering 73% of CRC cases (451). Similarly, the frequency of alterations in *CTNNB1* is approximately 10-fold higher in CRC compared to BC (5% vs. 0.6%) (451).

This suggests that TNBC may activate Wnt signaling through non-mutational mechanisms, a hypothesis supported by recent research (Fig. 1.14). One such mechanism involves the loss of *CDH1* (E-cadherin), which normally anchors β-catenin at the cell membrane. Loss of *CDH1* in advanced and invasive TNBC may liberate β-catenin, promoting its cytoplasmic and nuclear accumulation (453). Another mechanism focuses on alterations in expression levels of upstream regulators of Wnt signaling, such as agonistic and antagonistic ligands, secreted inhibitors, and receptors (451). Genes encoding FZD receptors, *LGR6*, *WNT9A*, R-spondin 2 (*RSPO2*), *SFRP1*, and Dickkopf-related protein 4 (*DKK4*) often show copy number alterations or deregulated expression in TNBC (431). For example, an alternatively spliced isoform of LRP5 that lacks the binding region for the Wnt inhibitor *DKK1* has been identified in BC (454). Furthermore, while *WNT1*, the first Wnt gene linked to mammary tumorigenesis in mice, is rarely expressed in human BC, other ligands such as *WNT10B*, and *WNT7B* are enriched in TNBC and correlated with aggressive features, including higher grade, larger tumor size, and increased recurrence (455–457). Epigenetic silencing of negative Wnt regulators offers another potential route for aberrant Wnt signaling activation. Promoter hypermethylation of genes encoding inhibitors like Wnt inhibitory factor 1 (*WIF1*), *SFRP1/2/4* and *DKK1/3* has been frequently observed in TNBC, contributing to reduced negative feedback and enriched Wnt activity (451).

Additionally, the RSPO family – secreted ligands that potentiate Wnt signaling – appears particularly relevant in TNBC. *RSPO2*, *RSPO3*, and *RSPO4* are often overexpressed, enhancing Wnt signaling by stabilizing FZD receptors via LGR4/5/6 interactions (458). High expression of *LGR5* and *LGR6* is

associated with basal-like BC, poor prognosis, and CSC maintenance (459,460). These findings collectively suggest that TNBC hijacks ligand/receptor modulation and epigenetic silencing, rather than harboring classical Wnt-activating mutations.

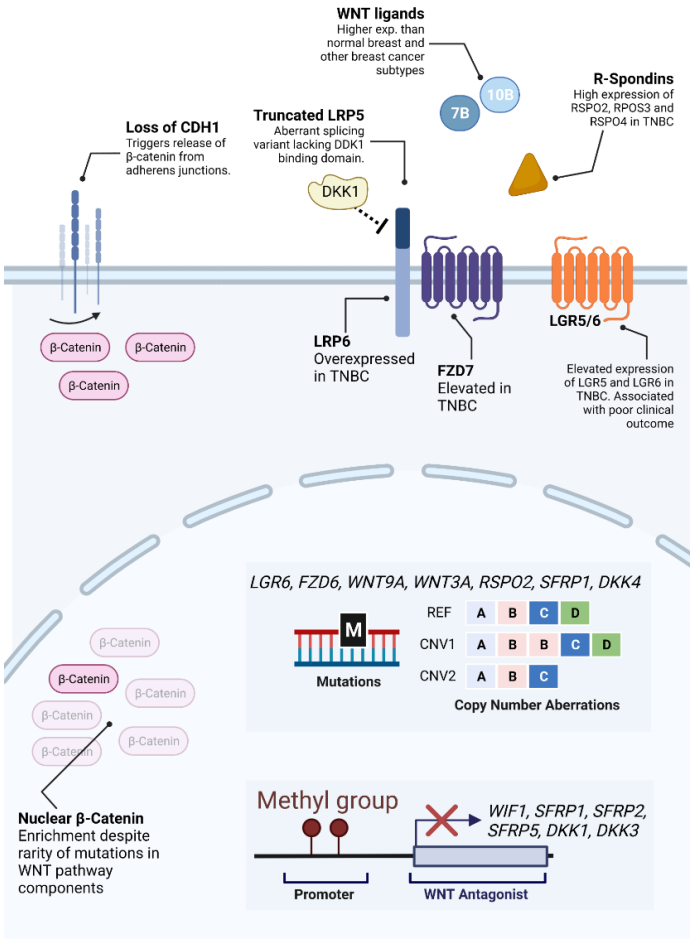


Fig. 1.14: Schematic overview of the know alterations in Wnt signaling pathway components in TNBC. *This figure originates from the review "Wnt Signaling in the Breast: From Development to Disease" (https://doi.org/10.3389/fcell.2022.884467).*

Notably, both canonical and non-canonical Wnt signaling appear active in TNBC pathogenesis (313,461). Immunohistochemical analyses of breast tumor samples have consistently shown nuclear and cytoplasmic accumulation of β-catenin in basal-like and TNBC subtypes (452). Nuclear localization – indicative of active Wnt/β-catenin signaling – was significantly associated with poor prognosis, including shorter metastasis-free survival and OS (311). In particular, nuclear β-catenin correlated with elevated relapse rates and increased risk of brain and lung metastases. Importantly, these findings were independent of *CTNNB1* mutations, suggesting transcriptional or post-translation activation of Wnt signaling (311).

1.12. Wnt Signaling in TNBC Drug Resistance

As discussed in previous sections of this thesis, the development of chemotherapy resistance in TNBC is particularly compelling given the strong initial response of these tumors to NAC. We have already highlighted that chemotherapy resistance in TNBC is mainly driven by progressive adaptations of cancer cell populations to aggressive cytotoxic treatments rather than the selection of subpopulations harboring pre-existing resistance-enabling mechanisms (342,343). Among the key signaling pathways implicated in this adaptation, Wnt signaling plays a multifaceted and context-dependent role in drug resistance.

The Wnt signaling pathway has a wide array of pleiotropic and sometimes paradoxically contrasting functions in both health and disease. For instance, in the context of developmental biology, Wnt signaling plays a role in regulating maintenance of pluripotency and lineage specification in embryonic stem cells and early embryonic development (462). Moreover, while it is commonly regarded as a pro-proliferative signaling cascade, there is emerging evidence that its role in proliferation can be context-dependent (463). In addition to cell cycle regulation and stem cell maintenance, Wnt signaling is known to be involved in a variety of functions with the potential to induce drug resistance.

In *BRCA*-mutated ovarian cancer, PARP inhibitor resistance has been linked to enhanced Wnt/β-catenin signaling through N^6-methyladenosine modification of *FZD10* transcripts, increasing mRNA stability and promoting DNA damage (464). Notably, *FZD10* depletion restored sensitivity, indicating a functional role for Wnt signaling in resistance acquisition via epigenetic adaptation. Induction of Wnt signaling during chemotherapy or radiotherapy has also been in colorectal, pancreatic, breast, and lung cancers, where it correlates with increased resistance (465–469). In these studies, Wnt activation is driven by the regulation of downstream effectors or induction of Wnt-modulation miRNAs. However, the molecular mechanisms regulating this activation and whether upstream ligands are involved remain poorly understood (450). Additionally, crosstalk between Wnt and other key oncogenic pathways such as RAS-ERK and EGFR is particularly relevant in TNBC, where these cascades show elevated activity despite low mutation rates, suggesting convergence on shared downstream resistance programs (470). Mechanistically, Wnt/β-catenin signaling directly contributes to chemotherapy resistance by promoting MDR1/*ABCB1* expression. Co-activators like Pygopus family PHD finger 2 and receptors such as *FZD1* have been shown to be overexpressed in chemotherapy-resistance BC cells, and their inhibition restores drug sensitivity (471,472).

Given its involvement in regulating both embryonic and adult tissue stem cell homeostasis – particularly that of MaSCs – the Wnt/β-catenin signaling pathway has also been implicated in BCSC biology. A central function of Wnt/β-catenin signaling in CSC regulation is the maintenance of self-renewal capacity and pluripotency, as well as the control of differentiation fate. This importance of Wnt/β-catenin signaling in sustaining these key properties is well established, and small molecule modulators of the pathway are commonly used in stem cell maintenance culture systems (462,473–475). Both *WNT5A* and *WNT5B* have been reported to promote basal-like BCSC characteristics by activating both the canonical and non-canonical branches of the Wnt signaling pathway (476,477).

Moreover, Wnt signaling has been shown to maintain the CSC phenotype by directly regulating EMT (478). The TME can also regulate the CSC phenotype, acting through Wnt signaling. Specifically, expression of HIF-2α, which is stimulated by hypoxia in BC cells, significantly induces chemoresistance. Exogenous HIF-2α overexpression is accompanied by stem cell marker expression and elevated Wnt signaling activity (479). Importantly, inhibition of Wnt/β-catenin signaling by DKK1 impacted stemness and reduced paclitaxel resistance (479).

Beyond its role in conferring resistance to conventional chemotherapeutic agents, aberrant Wnt/β-catenin signaling also appears to contribute to resistance against immunotherapy-based treatments. Wnt signaling has been implicated in promoting tumor immune evasion by mediating the exclusion of TILs from the TME (446,480). As previously discussed in earlier sections, high TIL levels in TNBC are associated with favorable clinical outcomes and enhanced response to therapy. In addition, Wnt signaling has also been shown to regulate PD-L1 expression in TNBC, a key determinant of tumor response to ICIs (329). Notably, elevated PD-L1 expression correlates with upregulation of Wnt signaling and stemness associated markers, such as increased ALDH activity (329). Pharmacologic modulation of Wnt activity influences PD-L1 levels, with inhibitors or agonists down- and upregulating its expression, respectively. Furthermore, constitutive PD-L1 expression has been reported in $CD44^+$ CSCs in head and neck squamous cell carcinoma, critically highlighting a crucial link between stemness and immune evasion mechanisms (481).

1.13. Opportunities and Challenges in Inhibiting Wnt Signaling for Cancer Treatment

The impact of Wnt signaling on tumor initiation, progression, and therapy resistance is well established. Naturally, disrupting the activity of the pathway is regarded as a potentially beneficial therapeutic strategy for Wnt-driven malignancies and neoplasia where Wnt signaling is shown to mediate a CSC or DTP cell phenotype, resulting in treatment adaptation, resistance, and disease recurrence. However, given its multitude of functions in normal healthy tissues, the development of efficient therapeutic tools is invariably hindered by the challenge of ensuring minimal pharmacotoxicity and optimal tolerability. Notably, the strong reliance of adult tissue stem cells on Wnt signaling poses a major hurdle in the clinical feasibility of Wnt signaling-inhibition due to the significant hypothetical and demonstrated side effects. One example is the use of tankyrase inhibitors and their severe gastrointestinal side effects (482). Similarly, therapeutic strategies aiming at using agents targeting other critical developmental signaling pathways such as Notch, Hedgehog, or BMP signaling all face the same obstacles (483). As such, despite the high interest in their study and development, so far, no Wnt-targeting treatments have been approved for human use.

Several pharmacological Wnt inhibitors have been developed to target different members or components of the Wnt signaling cascade, starting from ligand biogenesis to transcriptional activation (Fig. 1.15). Inhibitors of PORCN, the rate-limiting enzyme in the Wnt ligand secretory process, have been given substantial attention and have been tested in various cancers. Considering the numerous amounts of different Wnt ligands and their respective FZD receptors as well as the complexity of the ligand-receptor interactions, the possibility of a “shotgun” approach to ligand inhibition is highly desirable. This approach likely prevents compensatory upregulation of alternative receptors or agonist ligands and the acquisition of mutations that alter antibody binding affinity. Notwithstanding, the use of such small molecules are prone to resistance development through other mechanisms such as the upregulation of drug efflux pumps.

Lui and colleagues first reported the efficacy of PORCN inhibitor LGK-974 (WNT974) in a MMTV-Wnt1-triggered rodent BC model (484). Notably, their study reported no significant intestinal toxicity at the therapeutic dose, with significant tumor reduction. The pre-clinical antitumor effect was also confirmed in ovarian cancer and head and neck squamous cell carcinoma (485,486). Importantly, this molecule is currently in phase I/II clinical trials in several solid malignancies, including TNBC (487,488).

Other therapeutic strategies targeting specific extracellular signals, including mAbs, ADCs, decoy receptors, and different small molecule inhibitors, have been developed and investigated with inhibitory activity on Wnt ligands and their respective receptors. These have demonstrated effective repression of Wnt activation, controlling tumor growth in melanoma, sarcoma, CRC, and NSCLC (446).

FZD-targeting mAbs have also shown therapeutic potential in different cancer types. Recently, a novel humanized anti-FZD7 antibody was shown to drastically enhance the activity of bevacizumab, inhibiting Wnt/β-catenin pathway-induced EMT and CSC properties in TNBC (489). Another study showed that the use of Ipafricept (OMP-54F28), a proprietary fusion protein fused to the Wnt-binding domain of the FZD receptor, acting as a decoy receptor for Wnt ligands, alongside chemotherapy, has demonstrated efficacy in ovarian cancer and is currently in phase Ib clinical trials (490).

Downstream inhibitors of the Wnt signaling pathway have also been given extensive attention as potential therapeutic targets. A variety of molecules have been developed to target different

intracellular regulators of Wnt signaling. Tankyrase inhibitors such as XAV939, IWR1, JW55, and G007-LK have shown preclinical efficacy but have failed to advance into clinical trials due to toxicity concerns raised in preclinical studies (487). The inhibition of β-catenin/TCF/LEF-regulated transcription has also been granted some attention (e.g., ICAT, ICG-001, and StAx-35), yet this approach has not yielded very promising results to date (491).

Another important limitation and challenge towards establishing effective Wnt signaling-targeting drugs and therapeutic strategies in general and in BC specifically is the necessity to identify and stratify in which patients the risk-benefit relationship would be reasonable. To that end, the discovery and validation of robust biomarkers to predict response to Wnt-targeted treatments would prove invaluable. Notably, a phase Ib clinical trial has reported a four-gene signature (*FBXW2*, *CCND2*, *CTBP2*, and *WIF1*) as a predictor of response to paclitaxel plus vantictumab (an anti-FZD8 mAb) treatment in HER2-negative BC (492).

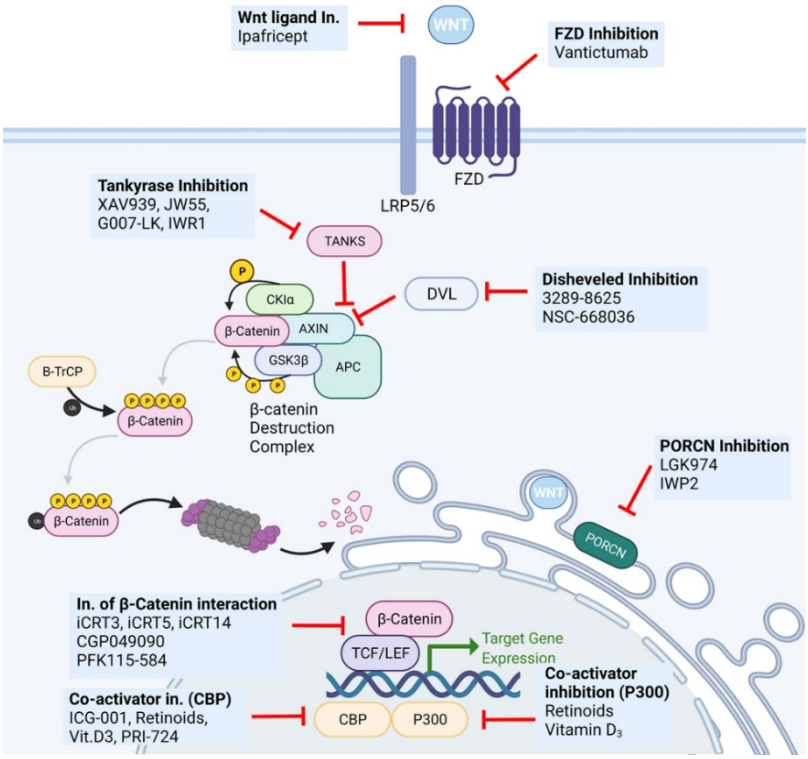


Fig. 1.15: Schematic overview of the Wnt pathway and Wnt inhibiting drugs currently in or previously in clinical development. *This figure originates from the review "Wnt Signaling in the Breast: From Development to Disease" (https://doi.org/10.3389/fcell.2022.884467).*

Additionally, more profound and detailed knowledge of the detrimental effects of Wnt inhibition at the organ and systemic levels would enable the development of more amenable molecules, delivery methods, and formulations that circumvent such hurdles. Alternatively, the use of Wnt-targeted therapies could be accompanied by complementary treatments that compensate for the side effects of Wnt inhibition. For instance, Madan and colleagues have shown, in murine models, that alendronate therapy (approved for osteoporosis) can be used to prevent the skeletal side effects of long-term PORCN inhibition. It is well known that prolonged Wnt inhibition negatively impacts the skeletal system, decreasing bone density, bone mass, bone marrow, and elevating adipocyte counts. The authors showed that co-treatment with alendronate significantly alleviated bone resorption during PORCN inhibition, illustrating that off-target Wnt inhibitor-induced toxicity can be mitigated (493).

2. Chapter 2: Rationale and Research Objectives

The lack of actionable therapeutic targets has long posed a critical challenge in the clinical management of TNBC. While chemotherapy remains the cornerstone of treatment, and response rates have improved through regimen refinement and patient stratification, TNBC continues to carry disproportionately high risks of relapse, metastasis, and mortality.

A well-established predictor of long-term survival in TNBC is the achievement of pCR following neoadjuvant chemotherapy. Patients who fail to achieve pCR – those with residual disease – face significantly worse outcomes, underscoring the need to better understand the mechanisms driving therapeutic failure.

Although much of the historical focus on resistance has centered on pre-existing mutations, accumulating evidence indicates that chemotherapy resistance in TNBC often emerges as an adaptive, non-genetic process during the course of treatment. Residual disease frequently arises not from inherently resistant clones, but from cancer cells that initially respond and subsequently enter a reversible drug-tolerant state. The DTP cells represent a phenotypically plastic subpopulation capable of surviving otherwise lethal treatments through dynamic reprogramming of transcriptional, epigenetic, and metabolic pathways – rather than through fixed genomic alterations.

This thesis explores the emerging concept that cancer cells, much like early-stage embryonic stem cells, may activate evolutionarily conserved signaling pathways and survival strategies to endure therapeutic stress. The parallels between cancer DTP cells and the state of embryonic diapause – particularly the quiescent state, transcriptional repression, and reversible nature – highlight a fundamental shift in how we understand resistance – not merely as a result of Darwinian selection, but as a regulated cellular response to environmental pressure.

Specifically, this work aims to investigate: **(i)** identifying and understanding the early molecular mechanisms driving drug tolerance and (subsequent) chemoresistance in TNBC and **(ii)** whether combinatorial treatment strategies, targeting the mechanisms involved in the acquisition of a drug-tolerant state, improve the efficacy of chemotherapy-based strategies in TNBC (Fig. 2.1).

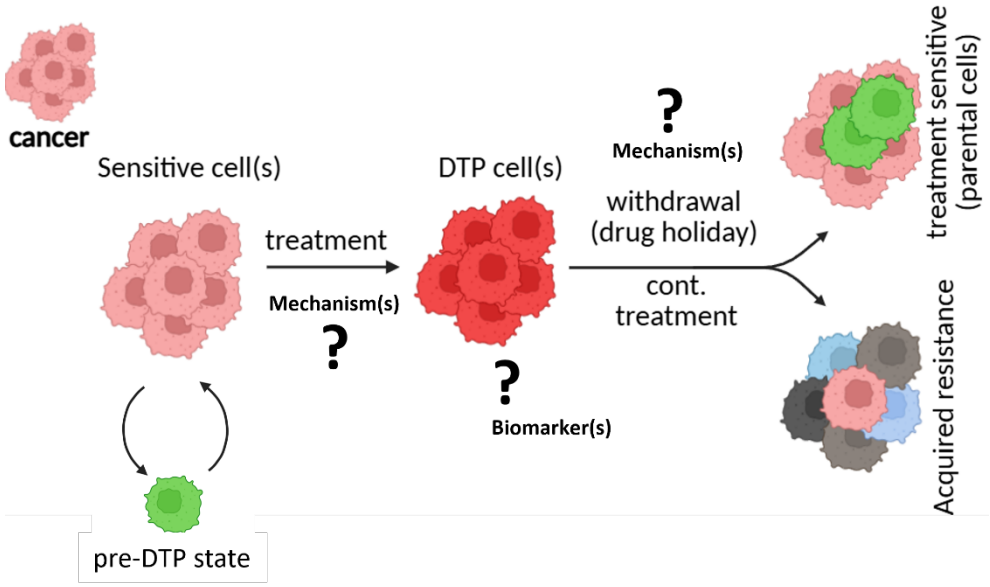


Fig. 2.1: Schematic overview of cancer DTP cells with questions marks highlighting the areas of interest in which our research aims to investigate.

Impact: This body of work will contribute to the growing recognition that non-genetic mechanisms – particularly those involving DTP cells – play a central role in therapeutic resistance and treatment response in TNBC. By elucidating how transient, reversible states governed by rewiring developmental signaling pathways and programs can enable cancer cells to survive therapeutic pressure, this work will contribute to laying the groundwork for novel therapeutic strategies that go beyond targeting mutations. Ultimately, these insights have the potential to inform more effective interventions aimed at preventing relapse, improving patient outcomes, and advancing the broader understanding of therapeutic failure and resistance biology in cancer.

3. Chapter 3: Materials and Methods

3.1. Ethics Declaration

All xenograft animal experiments performed were approved by the Ethics Committee at KU Leuven University under the ethical approval codes P055/2022 and P016/2023.

Patient derived organoid (PDO) models used in this study were established from freshly resected tumor tissues obtained from TNBC patients at the Antoni van Leeuwenhoek Hospital. The study was approved by the institutional review board (NKI-B17PRE) and the subjects provided informed consent.

All cell lines used in this study are approved for use by the Ethics Committee at the KU Leuven University Biobank under the code S65166.

3.2. TNBC Cell Line Culture

MDA-MB-231 (ATCC-HTB-26, RRID: CVCL_0062) and MDA-MB-468 (ATCC-HTB-132, RRID: CVCL_0419) were maintained in DMEM high glucose (Gibco, 41965039) supplemented with 10% (v/v) fetal bovine serum, 1mM sodium pyruvate (Gibco, 11140035), 100µg/mL penicillin-streptomycin (Gibco, 15140163), and 0.01mM 2-mercapthoethanol (Gibco, 31350010). The TNBC status for both cell lines was defined by their IHC profiles, showing negative expression of ER, PR, and HER2 (494). The MDA-MB-231 cell line was originally derived from a pleural effusion of a patient with metastatic mammary adenocarcinoma, while the MDA-MB-468 cell line was derived from a pleural effusion of a patient with metastatic adenocarcinoma (495,496). Both cell lines are widely used *in vitro* TNBC models representing distinct molecular subtypes, with MDA-MB-231 classified as mesenchymal stem-like and MDA-MB-468 as basal-like 1 (497).

PDC-BRC-101 cell line was obtained from collaborators, Daniela Anibali and Stijn Moens (Amant Lab – Gynecological Oncology) – KU Leuven. This cell line was derived from a patient-derived xenograft model, originating from a primary chemotherapy-naïve TNBC tumor (TNBC status assessed by IHC). The PDC-BRC-101 cell line was maintained in OCMI media (498) composed of a composed of 1:1 mixture of Medium199 (Gibco, 31150022) and DMEM F-12 (Gibco, 11320074) supplemented with 10% (v/v) fetal bovine serum, 100µg/mL penicillin-streptomycin, 20µg/mL insulin (Sigma/Merck, I9278), 25ng/mL cholera toxin subunit B (Sigma/Merck, C9903-.5MG), 0.5µg/mL hydrocortisone (Sigma/Merck, H0888-1G), and 10ng/mL epidermal growth factor (Stem Cell Technologies, 78006.1).

All cell lines were cultured in 84mm x 20mm (D x H) tissue-culture treated dishes at 37°C and 5% CO_2 and maintained at 70-80% confluency. For cell line passaging and plating, 1X phosphate-buffered saline (Gibco, 10010-015) was used as a washing solution followed by dissociation using 0.25% trypsin-EDTA (Gibco, 25200-056) and cell-pelleting by centrifugation for 4 minutes at 300G (0.3rcf). Cells were counted manually via the BRAND counting chamber Neubauer improved (Sigma Aldrich/Merck, BR717810-1EA) under a 10X objective lens using a Leica DMi inverted microscope. The same microscope, equipped with a 2.5 Megapixel HD Microscope Camera Leica MC120 HC, was used to obtain images of cultured cancer cell lines. Unless specified otherwise, cancer cell lines were plated according to the following seeding densities: $7.3\ x\ 10^3\ \frac{cells}{cm^2}$ and $10.5\ x\ 10^3\ \frac{cells}{cm^2}$ (MDA-MB-231 and MDA-MB-468/PDC-BRC-101, respectively).

Cell lines were routinely checked (once a month) for mycoplasma contamination using the innuPREP DNA Mini 2.0 (Westburg, 845-KS-1042010) according to manufacturer's instructions (Eukaryotic cell culture protocol).

3.3. Chemotherapeutic and Small Molecule Treatment of TNBC Cell Lines and PDO Models

Cell lines were treated with increasing concentrations of docetaxel (Taxotere, 0–144 nM) and carboplatin (Carbosin, 0–1600 µM) for 72h. Cell metabolic activity, reflecting cell-number and -viability) was assessed using the MTT [Thiazolyl Blue Tetrazolium Bromide] assay (Sigma/Merck, M5655-500mg) according to manufacturer's instructions and sigmoidal dose-response curves were generated to calculate the mean IC50 values of each drug that were used in the subsequent study. Chemotherapeutic agents were obtained from the pharmacy of Universitair Ziekenhuis (UZ) Leuven.

For Wnt pathway-stimulation, CHIR99021 (CHIR – Sigma/Merck, SML1046) and 6-Bromoindirubin-3'-oxime (BIO – Sigma/Merck, B1686-5MG) were used at 8µM and 3µM, for CHIR and BIO, respectively.

3.4. Lentiviral Particle Production and Transduction

Lentiviruses were produced according to the RNAi Consortium (TRC) protocol available from the Broad Institute (https:// portals.broadinstitute.org/gpp/public/resources/protocols). In brief, $7\,x\,10^5$ HEK-293T (RRID: CVCL_0063) cells were seeded per well in 6-well plates and transfected the following day with 750 µg pCMV-dR8.91 (RRID: Addgene_202687), 250 µg pCMV-VSV-G (RRID: Addgene_8454), and 1 µg of the specific lentiviral plasmid/construct using FugeneHD (Promega, E2311) in Optimem (Gibco, 31985070). One day after, the culture medium was refreshed. The same day, lentivirus-recipient cells were plated in 6-well plates at their respective concentrations (see cell line culture). Lentivirus-containing medium was collected from HEK293T cells 48h and 72h post-transfection and added to recipient cancer cells after filtration using a 0.45 µM filter (VWR-Corning, 431220). 48h post infection, recipient cancer cells were washed thoroughly with PBS, medium refreshed, and the appropriate selection antibiotics applied until selection process was completed.

Wnt-transcriptional reporters, TOPGFP (7xTcf-eGFP // SV40-PuroR), TOPFLASH (7xTcf-FFluc), and mCherry-TOPGFP (7xTcf-eGFP // SV40-mCherry) were obtained from Addgene (#24305 – RRID: Addgene_24308, #24308 – RRID: Addgene_24308, and #24304 – RRID: Addgene_24304, respectively). Wnt-transcriptional reporter dTOPGFP (dTGP) was gifted to us from the Moon lab University of (Washington – USA).

For PORCN short hairpin RNA (shRNA) mediated silencing, we used the MISSION® Lentiviral shRNA (Sigma Aldrich/Merck, SHCLNG – clones, TCRN000153848 and TCRN000157366) and the MISSION pLKO.1-puro Non-Target shRNA Control Plasmid DNA (SHC016-1EA) as a negative control in experiments.

3.5. Real-Time Quantitative Polymerase Chain Reaction and Gene Expression Analysis

For real-time quantitative polymerase chain reaction (RT-qPCR), total RNA was extracted (from TNBC cell lines or cryopreserved tumor tissue) using the GenElute mammalian total RNA miniprep kit (Sigma/Merck, RTN350-1KT) according to manufacturer's instructions with an additional step of DNA digestion using the On-Column DNAse I digestion set according to manufacturer's instructions (Sigma/Merck, DNASE70). cDNA was synthesized from 500 ng of total RNA using the BIORAD iScript cDNA synthesis kit (BIORAD, CAT#1708891), according to manufacturer's instructions. RT-qPCR reactions were set up in technical triplicates with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, 11733-046) on a ViiA7 Real-Time PCR System (Thermo-Scientific). Expression levels were normalized to two housekeeping genes (HK) RPL19 and GAPDH to determine ΔCT values. Statistical testing of differences in expression levels between samples was carried out based on relative-expression values ($2^{-\Delta CT}$). In some figures, gene expression values are represented as fold-change for

convenience of interpretation, although statistical testing was performed on relative expression values ($2^{-\Delta CT}$).

3.6. SDS-PAGE and Western Blot Analysis

TNBC cell lines were washed with PBS and collected/pelleted by centrifugation. Whole cell lysates were obtained via mechanical lysis using a needle (VWR-TERUMO, AN2138R1) and RIPA cell lysis buffer (Sigma/Merck, R0278-50mL) supplemented with a cocktail of 1:100 phosphatase inhibitors cocktail 2 and 3 (Sigma/Merck, P5726-1ML and P0044-1ML, respectively) and 1:100 protease inhibitor cocktail (Sigma/Merck, 11873580001). Samples were placed on a rotation wheel for a minimum of 30 minutes at 4 °C after which they were centrifuged at 16,000x g for 10 minutes at 4 °C. The supernatant from the lysates was collected and protein concentration was determined using the Bradford Assay (Biorad, 5000006). For SDS-PAGE 20 mg of protein were mixed with 4x Laemmli buffer (240 mM Tris/HCL pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% 2-mercaptoethanol, 40% glycerol) and denatured for 5 minutes at 95°C prior to electrophoretic protein separation. Resolved protein extracts were transferred to PVDF membranes (BIORAD, 162-0177). Transfer success was assessed with Ponceau S solution, and membranes were blocked with 5% non-fat milk or 5% BSA in TBS-T (0.1% Tween-20®) for 60 minutes. After blocking, membranes were incubated with primary antibodies at 4°C overnight. The day after, membranes were washed 3 times with PBS-T for 10 minutes and incubated with secondary HRP-conjugated antibodies. Immunolabeled proteins were detected with Supersignal West Pico chemiluminescent kit (Fisher Scientific, 34077) on autoradiography film (Santa Cruz, SC-201697). The primary antibodies used were active rabbit anti-non-phosphorylated β-catenin (CellSignaling Technologies, #19807S – RRID: AB_2650576), rabbit anti-PORCN (Novus Biologicals, NBP1-59677 – RRID: AB_11025374), and rabbit anti-Wnt-2b (Abcam, ab178418). Mouse anti-β-Actin (Santa Cruz Biotechnology, sc-47778 – RRID: AB_626632) was used as a loading control.

3.7. Flow Cytometry

For Wnt-activation assessment, cells were washed with PBS and collected/pelleted by centrifugation. Cells were resuspended in PBS2%FBS, counterstained with 5µg of 4',6-diamidino-2- phenylindole (DAPI – 1:1) (Sigma/Merck, D9542-10mg) to eliminate dead cells before running through the flow cytometer. Cell lines lacking any of the previously described Wnt-transcriptional reporters were used as gating controls.

For immunolabeling of CD44 and CD24, cells were detached, washed twice in PBS with 4% FBS, and incubated with CD44-PE (BD Pharmigen, 555479 – RRID: AB_395871) and CD24-APC (Thermo-Life Technologies, 17-0247-42 – RRID: AB_10718833) antibodies according to manufacturer specifications at room temperature. After incubations, cells were washed twice in PBS with FBS and resuspended in PBS containing 4% FBS and 100 nM of DAPI. Cells incubated with PE- and APC- conjugated isotype-antibodies and single-stained cells were used as gating controls.

For Annexin V apoptosis analysis, cells were washed with PBS and collected/pelleted by centrifugation. Cells were resuspended in 1x Annexin V binding buffer (BD Pharmigen, 51-66121E) and incubated at room temperature in the dark for 15 minutes with APC-conjugated Annexin V (Thermo-eBioscience, BMS306APC-100). After incubation, cells were diluted in 1X binding buffer supplemented with 100 nM of DAPI before running through the flow cytometer. Unstained and single-stained (Annexin V-only or DAPI-only stained) cells were used as gating controls.

To obtain chemotherapy-induced Wnt^{High} and Wnt^{Low} cells, cells were washed with PBS and collected/pelleted by centrifugation. Cells were resuspended in PBS4%FBS, counterstained with 5µg of DAPI (1:1) to eliminate dead cells before running through the SONY MA900 Multi-Application Cell Sorter. Depending on the application, $2 - 3\ x\ 10^5$cells were sorted (based on their GFP expression)

into 1.5mL Eppendorf tubs (with 300µl of PBS4%FBS) and either used for RNA-extraction and gene expression analysis or for re-culturing.

For immunostaining of proliferation marker Ki-67, cells were washed with PBS and collected/pelleted by centrifugation. Cells were fixed with ice-cold 70% Ethanol. Samples were washed with PBS2%FBS and blocked with 5% donkey serum (Jackson ImmunoResearch, 017-000-121 – RRID: AB_2337258) at room temperature for 30-60 minutes. Cells were re-pelleted by centrifugation, repeatedly washed with PBS2%FBS and incubated with the Ki-67 Recombinant Rabbit Monoclonal Antibody (SP6) (ThermoFisher, MA5-14520 – RRID: AB_10979488) at room temperature for 60 minutes. Cells were re-pelleted by centrifugation, washed repeatedly with PBS2%FBS and incubated with a conjugated secondary (donkey anti-rabbit – Alexa-647 – Thermo-Life tech, A31573 – RRID: AB_2536183) in the dark at room temperature for 30 minutes. Cells were re-pelleted by centrifugation, washed repeatedly with PBS2%FBS and counterstained with 5µg of DAPI (1:1) before running through the flow cytometer. Unstained and single-stained (secondary antibody-only stained) cells were used as gating and analysis controls.

For immunostaining of active- (non-phosphorylated) β-catenin, cells were washed with PBS and collected/pelleted by centrifugation. Cells were fixed with ice-cold 70% Ethanol. After which samples were with PBS2%FBS and blocked with 5% donkey serum (in PBS) at room temperature for 30-60 minutes. Cells were re-pelleted by centrifugation, washed with PBS2%FBS and incubated with active rabbit anti-non-phosphorylated β-catenin antibody (RRID: AB_2650576) at room temperature for 60 minutes. Cells were re-pelleted by centrifugation, washed with PBS2%FBS and incubated with a conjugated secondary (donkey anti-rabbit – Alexa-647 – Thermo-Life tech, A31573) in the dark at room temperature for 30 minutes. Cells were re-pelleted by centrifugation, washed with PBS2%FBS and counterstained with 5µg of DAPI (1:1) before running through the flow cytometer. Unstained and single-stained (secondary antibody-only stained) cells were used as gating and analysis controls.

Unless specified otherwise, all data were collected on a BD FACS Canto II at the KU Leuven Flow Cytometry Core and analyzed using FlowJo v.10.6.2 (RRID: SCR_008520).

3.8. Growth Rate and Doubling Time Analysis

For Growth rate (µ) analysis, the following mathematical equation was used: $\mu = \frac{\ln\left(\frac{N_t}{N_0}\right)}{\Delta t} \times 24h$, whereby N_0 is the number of cells seeded, N_t is the number of cells harvested/recorded, and Δt is the hours of growth.

For Doubling time (t_d) analysis, the following mathematical equation was used: $t_d = \frac{\ln(2)}{\mu} \times 24h$.

3.9. Conditioned Media and o-culture Analysis

Conditioned media (CM) was collected from TNBC cell lines recovering from chemotherapy treatment (5 days of treatment and 1 week of recovery) and filtered using a 0.45µM filter to ensure removing cell-debris. Filtered CM as concentrated 20x (20mL to 1mL) using Vivaspin centrifugal concentrator column with a molecular weight cutoff of 50kDa (Sigma Aldrich, Z614645-12EA). Filtered media was centrifuged for 45 minutes at 4°C. Concentrated CM was added to chemotherapy naïve TNBC cell lines for 48h in a 1:1 dilution (Concentrated CM:basal culture cancer media) and Wnt-activation levels were evaluated using FACS.

For co-culture experiments, MDA-MB-231 cell line was treated with either chemotherapeutic agent for 72h after which treatment was stopped and an equal number of chemotherapy naïve MDA-MB-231-

TGP.mC cells was plated in the same dish and cultured in basal culture cancer media. After 72h of co-culture, Wnt-activation levels in the MDA-MB-231-TGP.mC cell line was evaluated using FACS.

3.10. Immunofluorescence Staining

TNBC cell lines were plated in µCLEAR® 96 well plates (Greiner, 655090) at their respective seeded densities and treated with chemotherapy at their respective doses. Cells were washed with PBS and collected/pelleted by centrifugation and later fixed with 4% PFA for 15 minutes at room temperature. Samples were permeabilized and blocked in a single step with 0.2% Triton-X and 5% donkey serum. Primary antibodies (active rabbit anti-non-phosphorylated β-catenin antibody – RRID: AB_2650576) were subsequently added and incubated overnight at 4 °C. Next, cells were repeatedly washed with PBS and incubated with secondary antibodies (Donkey anti-Rabbit, ThermoFisher Scientific, A31572 – RRID: AB 162543) for 40 minutes at room temperature in the dark. After 10 minutes of DAPI exposure (used for nuclei staining), cells were imaged using the High Throughput Imager Operetta CLS™ system (Perkin Elmer, HH16000000). Image analysis and quantification were performed using the Harmony High-Content Imaging and Analysis software v.4.2 (Perkin Elmer, HH17000001). Find Nuclei – Method B building block algorithm was used to segment nuclei based on DAPI signal to accurately assess nuclear β-catenin signal. Unstained and single-stained (secondary antibody-only stained) cells were used as gating and analysis controls.

3.11. Cell Line Derived Xenograft Establishment and *In Vivo* Live Imaging Analysis

$1\ x\ 10^6$ MDA-MB-231 TOPFLASH cells were engrafted subcutaneously (1:1 PBS: growth-factor reduced Matrigel) into the right flank of female NMRI-Foxn1 mice (4-6 weeks old) to form a solid tumor. Upon observation of visible/palpable solid growth, tumor volumes were measured using digital calipers (and calculated as $L\ x\ W\ x\ \frac{\pi}{6}$; L: length and W: width). Animals were randomly assigned to one of three (or six) treatment groups (n = 7-8 mice per group) with an average tumor volume of 150mm^3 per group. Docetaxel (15mg/kg) and Carboplatin (100mg/kg) were administered via intraperitoneal (IP) injection once weekly (1 cycle) for a total of three cycles. LGK-974 (2mg/kg) was administered daily via oral gavage for a total of 3 cycles (3 weeks). For assessment of Wnt-activation dynamics, animals were subjected to live-bioluminescent imaging before-, 24 h-, 48h-, and 72h- after chemotherapeutic administration. For live-bioluminescent imaging, animals were injected (IP) with the luciferase substrate D-luciferin (200µL of 15mg/ml - assuming an average animal weight of 24-26gr) (Perkin Elmer, 122799) and incubated for 10 minutes at room temperature before images were taken using IVIS Spectrum In Vivo Imaging System (Perkin Elmer). Wnt-activation signal was calculated as the bioluminescent signal captured by the IVIS Spectrum normalized to the tumor volume recorded per animal. Analysis of bioluminescent images was performed via the Aura software v.4.0.0. Tumor volume was recorded every 48h and body weight was closely monitored throughout the treatment course and recorded every 72-96h using an automatic scale. All animals were euthanized at the end of the treatment course and tumors (when available) were resected/collected for downstream analyses.

Group size was selected based on a power input (0.8), 6 treatment groups (including VEH/control groups), and a minimum of 7 measurements. The software G power (version 3.1.9.2) was used to calculate and determine the sample/group size.

3.12. Tumor Volume-Based Categorical Efficacy Endpoints in CDX Studies

Tumor response was assessed by calculating relative tumor volume (RTV) from measurements obtained at the onset of treatment and at the endpoint (day of sacrifice), as described in section 3.11. RTV was calculated by dividing the final tumor volume by the baseline tumor volume. Based on RTV values, tumors were categorized as Complete Response (CR), Partial Response (PR), Stable Disease (SD), or Progressive Disease (PD) using the following thresholds: CR: RTV = 0, PR: $0 < RTV \leq 0.657$, SD:

0.657 < RTV ≤ 1.728, PD: RTV > 1.728. These categorical endpoints, derived from established mouse-clinical trial methodology (499), were applied in this *in vivo* study to describe therapeutic efficacy based on volumetric changes under treatment.

3.13. Next-Generation mRNA Sequencing

Total RNA was obtained from cells using the GenElute mammalian total RNA miniprep kit (Sigma, RTN350-1KT). RNA-sequencing (RNA-seq) libraries were prepared using 750 ng of total RNA using the KAPA stranded mRNAseq kit (Roche, 8098123702) according to the manufacturer's specifications. 100 nM of KAPA-single index adapters (Roche, KK8702) were added to the A-tailed cDNA and the libraries underwent 10 cycles of amplification. Agentcourt AMPure XP beads (Beckman Coulter, A63880) were used for the 1X library clean-up. The fragment size of the libraries was assessed using the Agilent Bioanalyzer 2100 with the High Sensitivity DNA kit (Agilent, 5067-4626). The concentration of the libraries was measured by the High Sensitivity QuBit kit (Invitrogen, Q33230). Each library was diluted to 4 nM and pooled for single-end 50-bp sequencing on an Illumina Hiseq4000 with 20 – 27 million reads per sample (22 million reads on average).

3.14. Bulk mRNA-sequencing Analysis

FASTQ files generated from the sequencing (Sequencing run Fig. 4.1–4.3 and Fig. 4.8–4.10) were sent for downstream processing. Adapters were trimmed using Trimmomatic v0.39 and the trimmed FASTQ file was aligned to the GRCh38 genome (hg38) using the STAR aligner v2.7.10. Gene counts, gene annotation and sample read characteristics were obtained by applying standard filters within featureCounts from the subread package v2.0.3. Gene counts were then normalized using the variance stabilizing transformation (VST). Z-scores used to describe the gene expression distribution across samples were calculated using median absolute deviation while the heatmaps comparing z-scores between samples were created using pheatmap v1.0.12. Differential gene expression analysis was performed using DESeq2 (500) and batch effects were accounted for in the (Wnt^{High} vs. Wnt^{Low} cohort). Volcano plots were created using EnhancedVolcano v1.18.0 using custom settings of FCcutoff = 0.6 and pCutoff = 0.05. Gene set variation analysis was performed using GSVA v1.48.3. Signature scores for the Caspase 3/Apoptosis (501) and Diapause-DTP signatures were calculated after the gene counts were transformed using both log2(x) +1 and VST methods. Box plots comparing the signature score(s) distribution between Wnt^{High} and Wnt^{Low} samples between treatment conditions were created using ggplot2 v3.4.3. Forest plots for regression analysis were created using forestplot v3.1.3. Analyses following the gene count extraction were all performed in R (502) v4.3.0.

3.15. Gene Set Enrichment Analysis of Publicly Available Datasets

To identify sets of genes associated with a Wnt-active (Wnt^{High}) signature, we performed differential gene expression on the TMM normalized gene counts obtained from the bulk mRNA sequencing analysis using the edgeR R package (version 4.2.2). We included treatment (Docetaxel or Carboplatin) as a covariate within the design matrix and selected genes that were differentially expressed at a log fold change cutoff of 0.5. We tested the enrichment of this Wnt-signature, together with a Diapause-DTP and MYC-hallmark gene set in the GSE123845 GEO dataset, which contains RNA-seq dataset acquired from longitudinally paired breast cancer during neoadjuvant treatment (Fig. 4.30). We retained samples with expression data, which had a pre-therapy sample acquired, and one further sample acquired during treatment. Signature enrichment was performed on log transformed TPM expression data using the single sample singscore R package (version 1.24.0), normalizing against 10 genes known to have a stable expression profile in pan-cancer datasets (RBM45, NRF1, BRAP, WDR33, CNOT2, TIAL1, CIAO1, TARDBP, ZNF207, and HNRNPK).

3.16. Patient Derived Organoid Culture, Treatment, and Analysis

R1-IDC113 and R2-IDC159A PDO lines were gifted by our collaborator, the Laboratory of Colinda Scheele – VIB-KU Leuven. Both models were originally generated at the Netherlands Cancer Institute (NKI) from primary breast tumors that had been previously treated before surgical resection, and both models were classified as TNBC based on IHC staining at the NKI – displaying negative expression of ER, PR, and HER2. R1-IDC113 was derived from a patient who had received NAC (dose-dense adjuvant doxorubicin and cyclophosphamide) prior to tumor sampling. Patient records indicate that despite being classified as TNBC via IHC staining, the R2-IDC159A line was derived from a patient who had been treated with the selective estrogen receptor degrader Fulvestrant, typically used in ER-positive BC. While the rationale for this prescription is unclear from the available records, ER negativity was confirmed by IHC, and the model retained chemosensitivity to both docetaxel and carboplatin *in vitro*.

Both PDO lines were maintained in growth factor reduced type 2 Cultrex (Biotechne/R&D Systems, 3533-010-02) with phenol red-free DMEM/F-12, HEPES (Gibco, 11039021) supplemented with 10mM Nicotinamide (Sigma/Merck, N0636-100G), 1.25mM N-acetyl-L-cystine (Sigma/Merck, A9165-5G), 500ng/mL Hydrocortisone, 100nM β-estradiol (E8875-250MG), 500nM SB202190 (Stem Cell Technologies, 72632), 500nM A83-01 (Stem Cell Technologies, 72022), 5uM Y-27632 (Stem Cell Technologies, 72304), 50µg/mL Primocin (Invivogen, ant-pm-05), 10µM Forskolin (Sigma/Merck, F3917-10MG), 1X B27 (50X – ThermoFisher Scientific, 17504044), 100ng/mL r-Noggin (Stem Cell Technologies, 78060), 5ng/mL FGF-10 (Stem Cell Technologies, 78037), 37.5 ng/mL Heregulin B-1 (Peprotech, 100-03), 5ng/mL EGF, 5ng/mL FGF-7 (Peprotech, 100-19), and 100µg/mL penicillin-streptomycin.

Both PDO lines were cultured in 24 well cell culture microplates – 22 mm x 20 (D x H) – at 37°C and 5% CO_2 and maintained at 70-80% confluency. For passaging and plating, (ice-cold) 1X phosphate-buffered saline (Gibco, 10010-015) was used to wash and dissociate the BME followed by single cell enzymatic dissociation using 0.05% trypsin-EDTA (Gibco, 25200-056) and cell-pelleting by centrifugation for 5 minutes at 1500 rpm (4°C). Cells were counted manually via the BRAND counting chamber Neubauer improved (Sigma Aldrich/Merck, BR717810-1EA) under a 10X objective lens using a Leica DMi inverted microscope. The same microscope, equipped with a 2.5 Megapixel HD Microscope Camera Leica MC120 HC, was used to obtain images of cultured PDO-lines. Unless specified otherwise, both PDO models were plated according to the following seeding density: $5.9\ x\ 10^2\ \frac{cells}{mm^2}$

To determine working chemotherapy drug concentrations, PDO lines were treated with increasing concentrations of docetaxel (Taxotere, 0.0625–512 nM) and carboplatin (Carbosin, 0 – 1600 µM) for 96h. Cell metabolic activity, reflecting cell-number and –viability was assessed using the CellTiter-Glo® 3D Cell Viability Assay (Promega, G9682) and sigmoidal dose-response curves were generated to calculate the mean IC50 values of each drug that were used in the subsequent study. Chemotherapeutic agents were obtained from the pharmacy of Universitair Ziekenhuis (UZ) Leuven.

PDO models were routinely checked (once a month) for mycoplasma contamination using the innuPREP DNA Mini 2.0 (Westburg, 845-KS-1042010) according to manufacturer's instructions (Eukaryotic cell culture protocol).

3.17. Statistical Analysis

All data were analyzed using GraphPad Prism (v8.0.1), except for mRNA-seq derived data and transcriptomic datasets. Unless otherwise specified, comparisons between two groups were tested for statistical significance using unpaired t-tests. Comparisons between multiple groups were performed using a one-way analysis of variance (ANOVA). Comparisons between multiple groups across multiple

time points were performed using two-way ANOVA. All statistical testing was corrected for multiple comparisons, using the Holm-Sidak method when comparing samples based on experimental design. For the reader's convenience, all statistical tests, sample sizes, and corrections are indicated in the figure legends.

For mRNA-seq-derived data, regression analysis was performed to observe associations between outcomes (in-house gene signature scores/GSVA signature scores) and independent co-variate (Wnt^{High} vs. Wnt^{Low}) per treatment condition (CAR or DOC or UNT) using lqmm v.1.5.8 and quantreg v5.97 while accounting for batch effects.

3.18. Data Availability

The bulk mRNA-seq data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) repository under accession number GSE254558 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE254558). For access, please enter the following token into the box to retrieve the dataset: **etsxyqgyrjkbhqt**

The code(s) used in generating the data supporting the conclusions of this article is available in a CodeOcean repository at https://doi.org/10.24433/CO.3286519.v1

Chapter 4: Chemotherapy-treated breast cancer cells activate the Wnt signaling pathway to enter a diapause-like early persister state

This chapter is adapted from the original research article currently in revision in the journal Cancer Research under the title: ***"Chemotherapy-treated breast cancer cells activate the Wnt signaling pathway to enter a diapause-like early persister state"****.*

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4. Chapter 4: Chemotherapy-treated breast cancer cells activate the Wnt signaling pathway to enter a diapause-like early persister state

4.1. Distinct chemotherapy treatments converge on robust Wnt/β-catenin pathway-activation during early persister cell-enrichment

To study and elucidate the common molecular basis underlying the emergence or enrichment of DTP (sometimes referred to as persisters in the manuscript) cells among distinct therapies, we recapitulated early persister cell formation *in vitro*. Three different TNBC cell lines (MDA-MB-231, MDA-MB-468, and PDC-BRC-101) were subjected to two distinct cytotoxic chemotherapeutic agents: docetaxel (DOC) and carboplatin (CAR). These agents operate through different mechanisms of action, with DOC promoting microtubule stabilization and preventing depolymerization, and CAR inducing DNA damage (503,504). Half-maximal inhibitory concentration (IC50) values (at 72h) were determined for each chemotherapeutic agent for every cell line and used in successive studies (see Materials and Methods – 3.2 and 3.3).

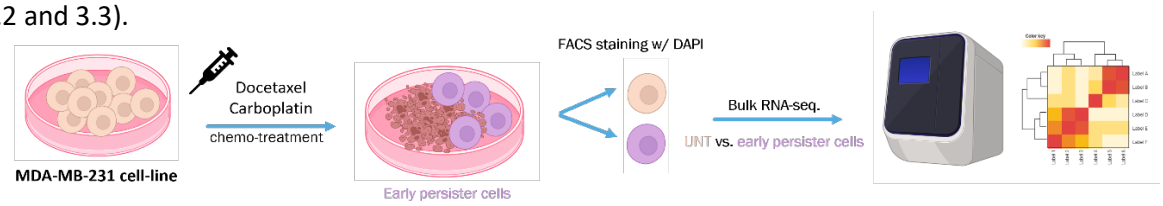


Fig. 4.1: Schematic representation of generation and transcriptional characterization of early persister cells. MDA-MB-231 cells were treated for 72h with DOC or CAR (IC50 concentrations) and viable (DAPI-) cells were sorted and sent for bulk RNA-seq with untreated (UNT) cells as controls.

We next performed bulk transcriptomic analysis on viable/drug-tolerant ($DAPI^-$) MDA-MB-231 cells treated with either DOC or CAR for (72h) (Fig. 4.1). Gene Set Enrichment Analysis (GSEA) (505) using MSigDB (506) datasets on differentially expressed genes (DEGs – Fig. 4.2A, B) between DOC vs. UNT or CAR vs. UNT (FC > 1.5, p-val ≤ 0.05) identified an array of Hallmarks significantly enriched in DOC or CAR treatments, including Apoptosis, p53 Pathway, and Interferon Gamma Response, all of which align with the expected cell stress induced by chemotherapeutic exposure (Fig. 4.2C). Conversely, Hallmarks associated with cell cycle regulation, such as G2M checkpoint, DNA Repair, MYC targets, and E2F targets, were significantly downregulated in DOC- and CAR-treatment (Fig. 4.2D). Interestingly, gene signatures and processes such as EMT and Hypoxia, both associated with tumorigenesis, chemoresistance, and the persister cell-phenotype, were enriched in response to chemotherapeutic exposure (507,508) (Fig. 4.2E).

Fig. 4.2: Bulk RNA-seq analysis of MDA-MB-231 early persister cells. A-B) Volcano plots displaying differentially regulated (down- (left) and up- (right) regulated) genes for MDA-MB-231 cell line treated with DOC (left) or CAR (right). Gene values are reported as Log_2FoldChange. C-E) Normalized GE heatmaps displaying (≈12-20) selected genes (based on DEGs predicting each representative process and/or hallmark) representing different processes and signaling cascades deregulated (relative to UNT cells) in MDA-MB-231 cell line treated with DOC or CAR for 72h. The processes (and subsequent genes selected to represent every process) shown in panel D were based on enriched gene sets from Hallmarks databases analyzed by one-tailed GSEA ranked by Normalized Enrichment Score (NES).

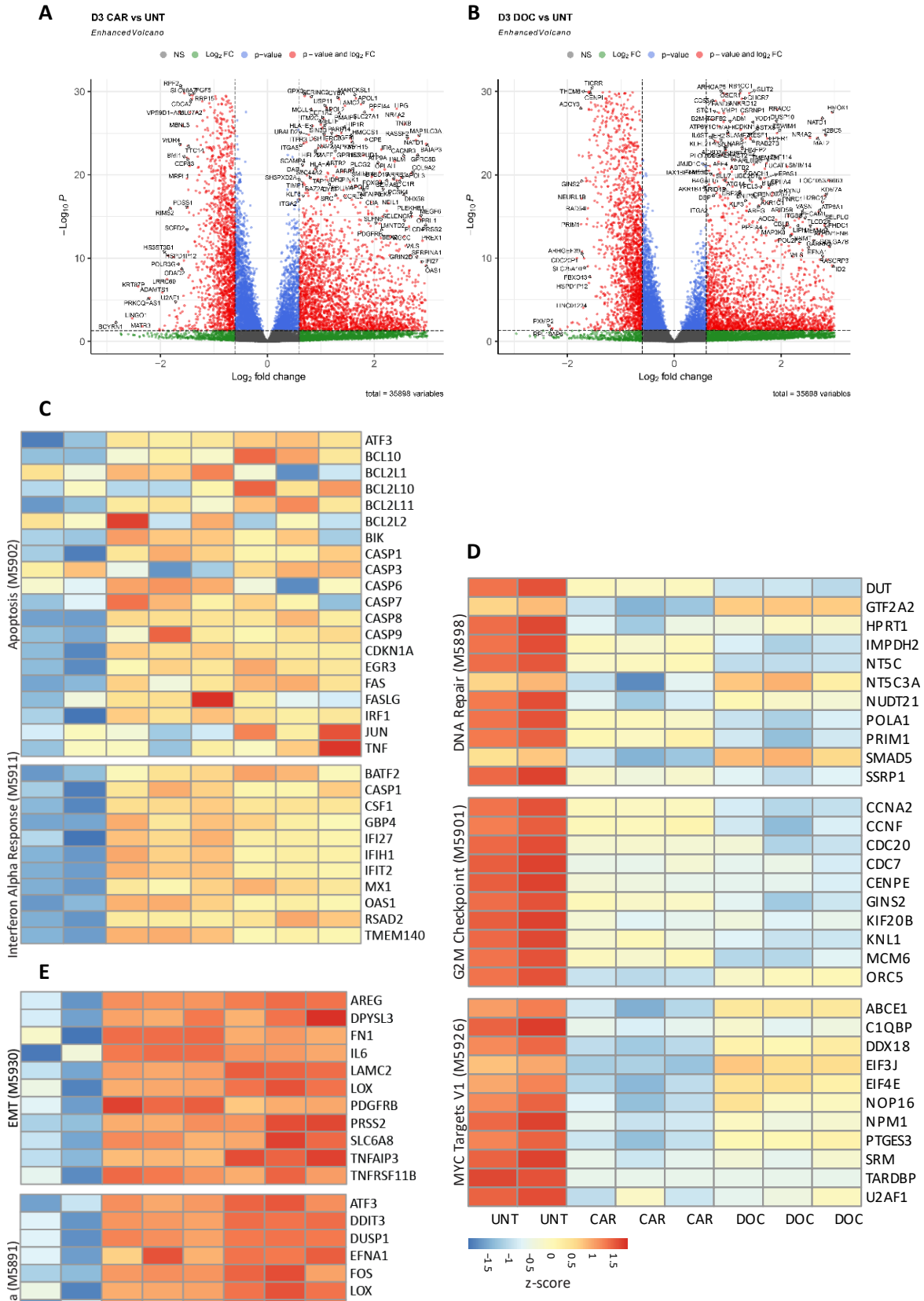


Fig. 4.2: Bulk RNA-seq analysis of MDA-MB-231 early persister cells. Legend on page 60.

Gene Ontology (GO) analysis of common enriched transcriptomic alterations among both chemotherapeutic agents (vs. UNT) (Fig. 4.3A) highlighted a significant enrichment of (positive) regulation of Canonical Wnt signaling, which was corroborated by enrichment in the expression of Wnt targets (*AXIN2* and *LGR5*) and upstream regulators and activators (*WLS* and *WNT2B*) of the pathway (Fig. 4.3B, C). Conversely, GO analysis using commonly downregulated genes shared between both drugs highlighted significant enrichment in processes related to cell cycle regulation and progression (data not shown).

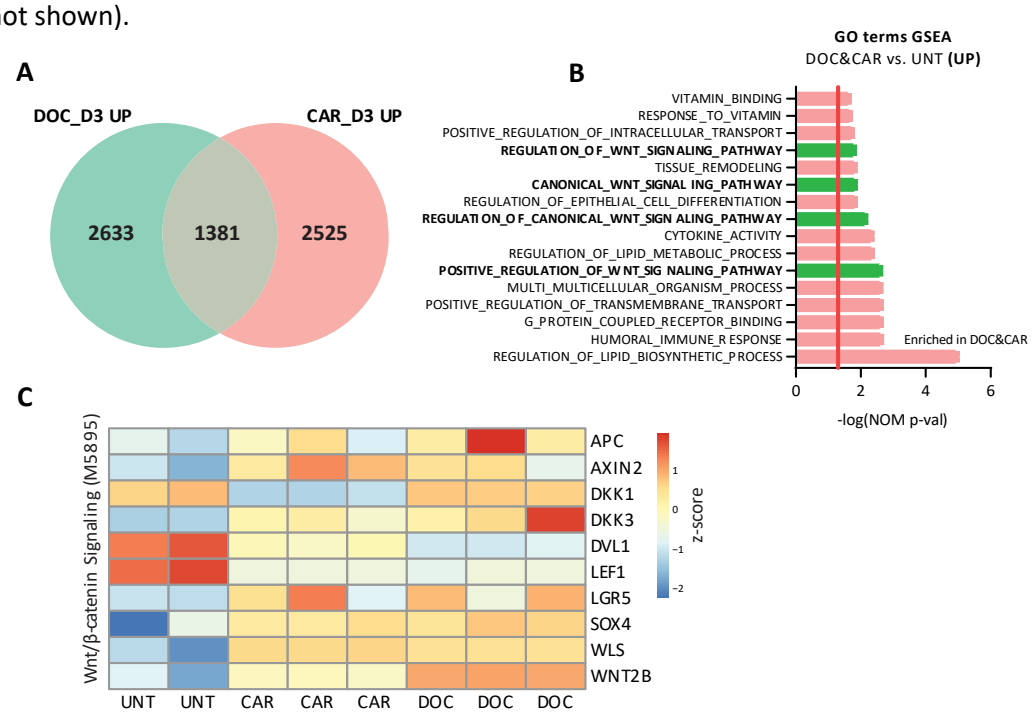


Fig. 4.3: Bulk RNA-seq analysis of MDA-MB-231 early persister cells. A) Venn diagram indicating commonly upregulated (1381) genes between DOC and CAR treatment (vs. UNT). B) Enriched processes from GO terms databases analyzed by one-tailed GSEA ranked by a positive NES and based on commonly upregulated genes between DOC and CAR treatment seen in panel A. Red line indicates significance threshold value, (-log(NOM p-val) = 1.3) and highlighted bars indicate GO terms linked to Wnt/β-catenin signaling regulation. C) Normalized gene counts heatmap displaying (10) selected genes representing Wnt/β-catenin signaling in MDA-MB-231 cell line treated with DOC and CAR for 72h. Genes selected for heatmap C were based on Wnt/β-catenin signaling Hallmark – GSEA.

Western blot analysis of active (non-phosphorylated) β-catenin in TNBC cell lines confirmed increased levels of active β-catenin, as early as 12h post-treatment, and prolonged for up to 72h (Fig. 4.4A-F). Immunofluorescence analysis confirmed increased levels of active β-catenin in the nucleus in response to chemotherapy exposure (Fig. 4.4G, H).

Fig. 4.4: Wnt/β-catenin pathway activation in response to chemotherapeutic exposure in TNBC cell lines. A-C) Representative western blot of active- (non-phosphorylated) β-catenin in TNBC cell lines treated with DOC or CAR for 12h, 24h, 48h, and 72h. D-F) Quantification of western blot analyses shown in A, displayed as fold change (to UNT) of normalized signals (relative to β-actin). Integrated intensity in arbitrary units (arb. units) (n = 3 independent experiments). G) Representative immunofluorescence stainings of active- (non-phosphorylated) β-catenin signal in TNBC cell lines treated with DOC or CAR for 72h. Scale Bar = 50 µm. Representative images are zoomed region of interest reported from full H) Quantification of immunofluorescence stainings shown in G of nuclear active- (non-phosphorylated) β-catenin signal (normalized to DAPI) in TNBC cell lines treated with DOC or CAR for 12h, 24h, 48h, and 72h. Integrated intensity in arbitrary units (arb. units) (n = 3 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

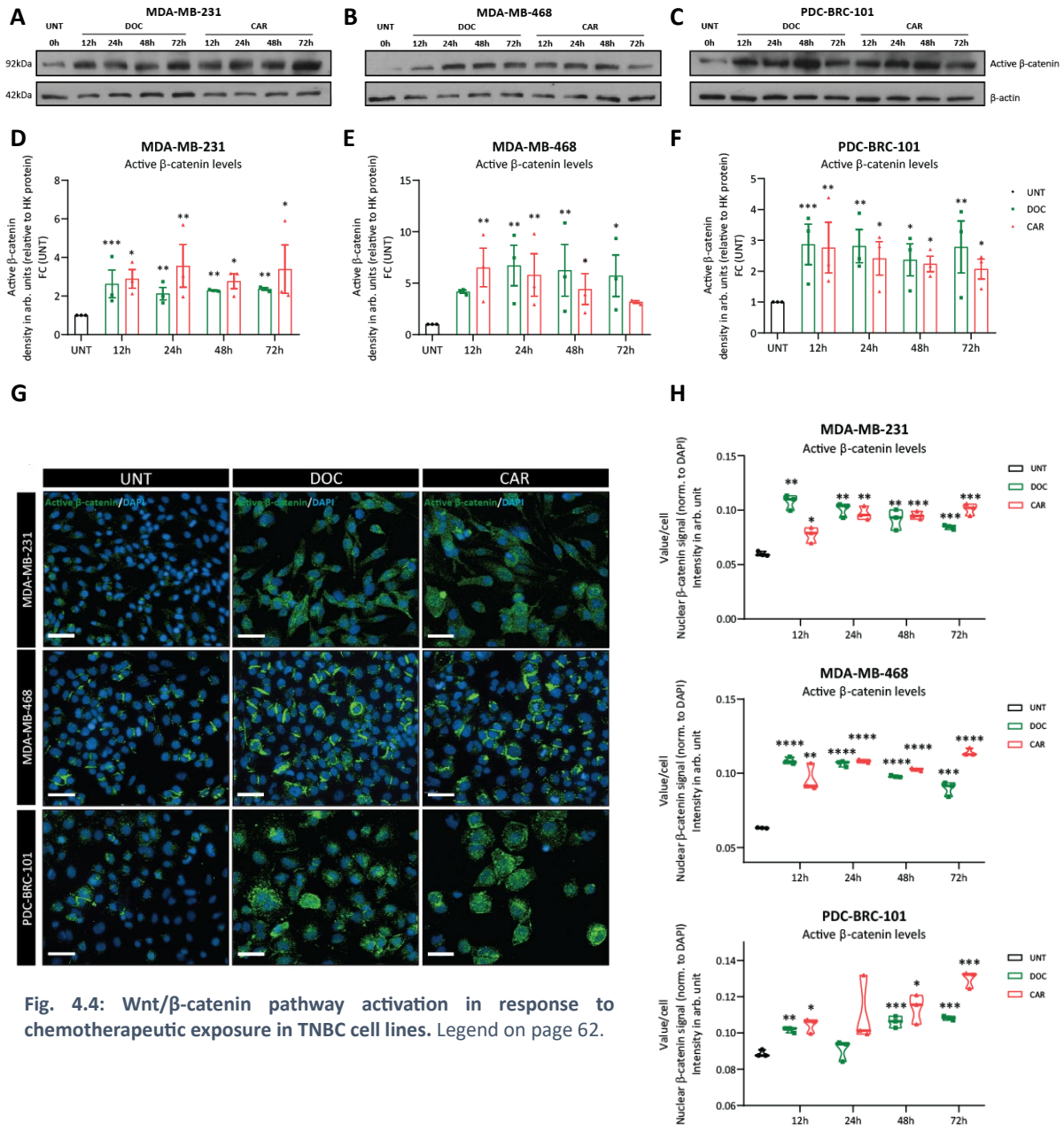


Fig. 4.4: Wnt/β-catenin pathway activation in response to chemotherapeutic exposure in TNBC cell lines. Legend on page 62.

Subsequently, the expression levels of Wnt signaling target genes (*AXIN2* and *LGR5*) showed a significant increase in response to chemotherapeutic treatment (Fig. 4.5).

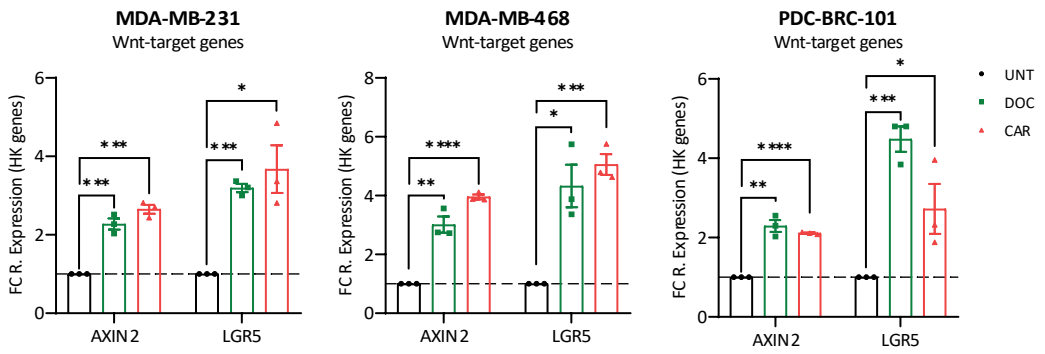


Fig. 4.5: Wnt/β-catenin pathway activation in response to chemotherapeutic exposure in TNBC cell lines. Legend on page 64.

Fig. 4.5: Wnt/β-catenin pathway activation in response to chemotherapeutic exposure in TNBC cell lines. Gene expression levels obtained via RT-qPCR of Wnt-target genes (*AXIN2* and *LGR5*) for TNBC cell lines treated with DOC or CAR for 72h, displayed as fold change (to UNT) of 2^{-dCt} values (relative to HK-genes) (n = 3 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

Previous studies have highlighted the role of Wnt signaling in maintaining breast cancer stem cells (BCSCs) as a mechanism underlying Wnt-mediated drug tolerance (474,504,509,510). Interestingly, although we detected a small population of BCSCs ($CD24^{Low}/CD44^{High}$ cells) in parental cells, we observed no significant enrichment at either 48h or 96h of treatment in MDA-MB-468 and PDC-BRC-101 TNBC cell lines (Fig. 4.6A-D). This aligns with previous findings indicating that chemotherapy does not enrich BCSCs in TNBC (343).

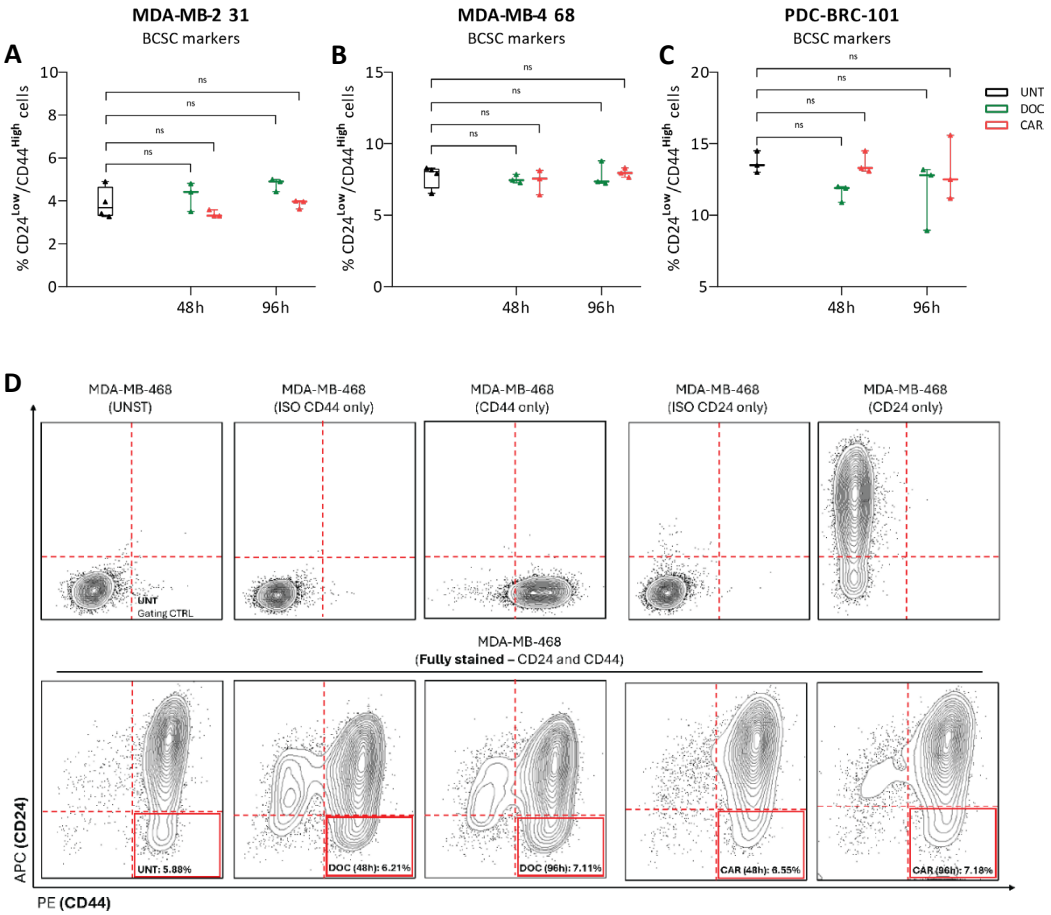


Fig. 4.6: Chemotherapeutic treatment does not enrich for BCSCs in early persister cell-population. A-C) Flow cytometry analysis displaying $\%CD24^{Low}/CD44^{High}$ cells of TNBC cell lines treated with DOC or CAR for 48h and 96h (n = 3 independent experiments). D) Representative flow cytometry contour plots showing appropriate staining controls and gating strategy. Solid red lines (bottom-right quadrant) indicate population of interest; $CD24^{Low}/CD44^{High}$ cells. *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

Our data reveal that upregulation of canonical Wnt signaling following treatment is a consistent phenomenon across various TNBC cell lines and in response to two distinct cytotoxic treatments. Notably, Wnt pathway-activation during early persister cell-enrichment does not correlate with an increase in the BCSC population, suggesting that chemotherapy-induced Wnt activation promotes early persister cell enrichment through alternative mechanisms.

4.2. Parental and early chemotherapy-treated Wnt^{High} persister cells display diapause-like cell-properties

To gain a more comprehensive understanding of the transcriptional and functional features of the chemotherapy-induced Wnt-active population, we exposed MDA-MB-231, MDA-MB-468, and PDC-BRC-101 TNBC clonal cell lines carrying a stable integrated Wnt-transcriptional (β-catenin-TCF/LEF-mediated transcriptional activity) reporter (TOP-GFP/TGP cell lines, Fig. 4.7A) (511) to either DOC or CAR agents.

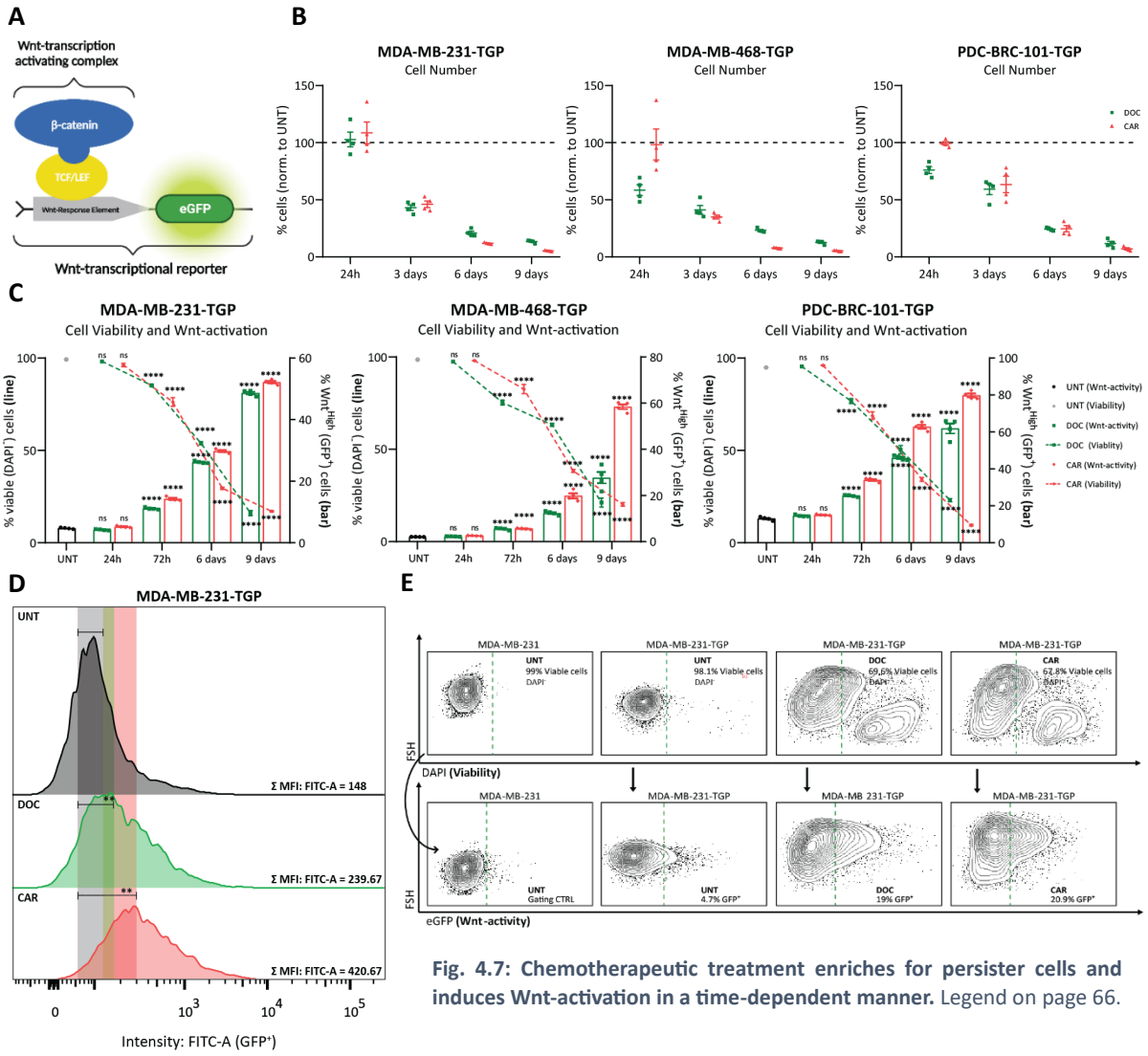


Fig. 4.7: Chemotherapeutic treatment enriches for persister cells and induces Wnt-activation in a time-dependent manner. Legend on page 66.

Chemotherapy treatment significantly reduced both cell number and cell viability at 72h (Fig. 4.7B, C; dashed lines). Conversely, we observed a significant increase both in the percentage and levels (intensity) of Wnt^{High} (GFP^{+}) cells among viable/early drug-tolerant (DAPI^{-}) cells compared to UNT conditions (Fig. 4.7C; bars and Fig. 4.7D, E). Prolonged exposure to either chemotherapeutic agent for 6 or 9 days further increased the levels of overall cell death and concurrently elevated the percentage of transcriptional Wnt^{High} cells (Fig. 4.8C bars), further confirming that the enrichment and/or induction of Wnt^{High} cells is one of the events directing early drug tolerance and highlighting that, among early persister cells, only a subpopulation becomes transcriptionally Wnt-active.

Fig. 4.7: Chemotherapeutic treatment enriches for persister cells and induces Wnt-activation in a time-dependent manner. A) Schematic representation of Wnt/β-catenin-transcriptional reporter (TOPGFP/TGP). B) Cell counts of TNBC cell lines treated with DOC or CAR for 24h, 72h, 6 days, and 9 days. Dashed line represents 100% cell number (i.e., UNT culture conditions) (n = 4 independent experiments). C) Flow Cytometry analysis displaying % of Wnt^{High} (GFP^{+}) within viable ($DAPI^{-}$) cells (bars - right y-axis) and % of $DAPI^{+}$ cells (dashed line - left y-axis) for TNBC cell lines treated with DOC or CAR for 24h, 72h, 6 days and 9 days (n = 4 independent experiments). D) An overlay of representative flow cytometry histograms representing the intensity of recorded GFP-expression (FITC-A channel) levels for MDA-MB-231-TGP TNBC cell line treated with DOC or CAR for 72h. Indicated numerical values denote the average median fluorescence intensity (MFI) recorded by the cytometer per sample (n = 3 independent experiments) comparing MFI of DOC or CAR to UNT. E) Representative flow cytometry contour plots showing FACS gating strategy, displaying % Viable ($DAPI^{-}$) cells (left of the dashed green line – top) and % of Wnt^{High} (GFP^{+}) (right of the dashed green line – bottom) MDA-MB-231-TGP cell line treated with DOC or CAR for 96h. *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

To unravel the transcriptional discrepancies between the Wnt^{Low} and Wnt^{High} early persister populations, we conducted bulk transcriptomic analysis on mRNA-sequenced samples derived from viable/drug-tolerant ($DAPI^{-}$) MDA-MB-231-dTGP (512) sorted populations under chemotherapy-treated conditions (Fig. 4.8A). Gene set variation analysis from DEGs between CAR or DOC (FC > 1.5, p-val ≤ 0.05) sorted Wnt^{High} vs. Wnt^{Low} early persister cells revealed a plethora of MSigDB Hallmark signatures that were differentially up- or down-regulated (Fig. 4.8B, C).

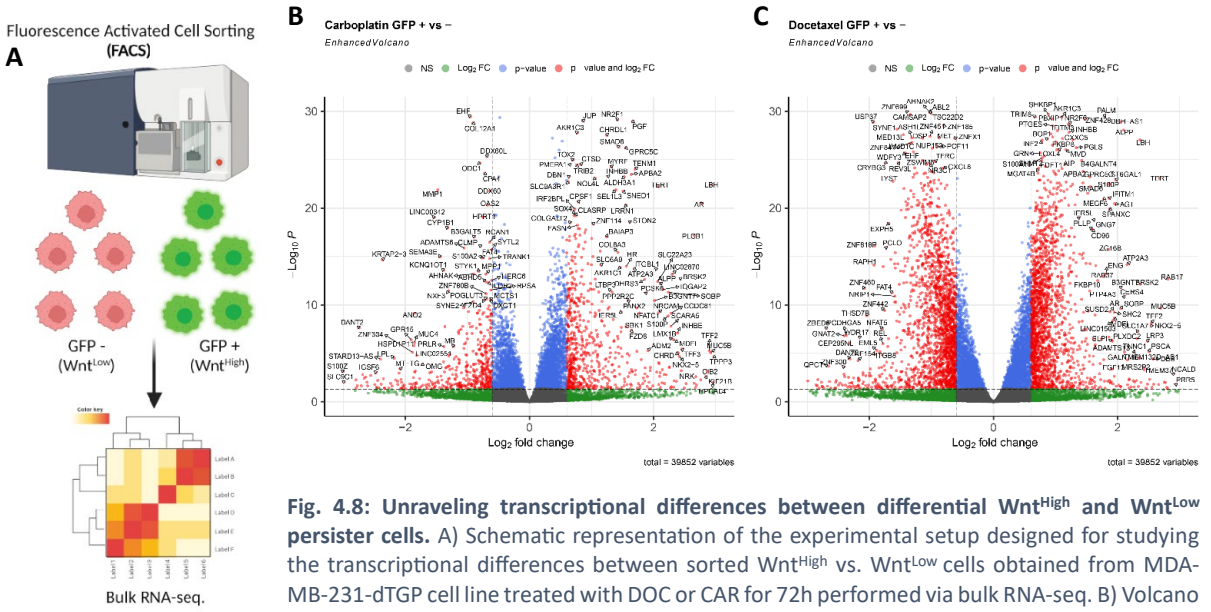


Fig. 4.8: Unraveling transcriptional differences between differential Wnt^{High} and Wnt^{Low} persister cells. A) Schematic representation of the experimental setup designed for studying the transcriptional differences between sorted Wnt^{High} vs. Wnt^{Low} cells obtained from MDA-MB-231-dTGP cell line treated with DOC or CAR for 72h performed via bulk RNA-seq. B) Volcano plots displaying differentially regulated (down- (left) and up- (right) regulated) genes comparing sorted Wnt^{High} vs. Wnt^{Low} for MDA-MB-231-dTGP cell line treated with CAR or DOC. Gene values are reported as Log_2FoldChange. Dot colors are clearly indicated in the plot.

As expected, the Wnt signaling pathway was significantly and positively associated in CAR- and DOC-sorted Wnt^{High} populations compared to Wnt^{Low} populations (Fig. 4.9). While a few signatures exhibited drug-dependent associations, most differentially regulated hallmarks followed a similar associative trend among sorted chemotherapy treated Wnt^{High} and Wnt^{Low} cells observed across both drug treatments (Fig. 4.9). Notably, developmental signaling pathways, including Hedgehog, Notch, IL-6/JAK/STAT3, and TGF-β signaling, along with hallmarks linked to tumor progression, stemness capacity, and metastasis (e.g., Angiogenesis and EMT), displayed a significant positive association with Wnt^{High} populations in comparison to Wnt^{Low} populations (Fig. 4.9). Conversely, the TNF-α signaling pathway via the NF-κB pathway notably exhibited a significant negative association with Wnt^{High} cells (Fig. 4.9), aligning with previous findings suggesting that active β-catenin can attenuate transcriptional NF-κB activity in breast cancer (513).

In contrast to previous reports correlating Wnt pathway-activation with increased proliferation and elevated *MYC* transcription (514,515), our transcriptional analysis revealed that Wnt^{High} cells negatively correlated with transcriptional signatures of proliferation, as evidenced by significant reduction of G2M checkpoint and E2F target signatures across chemotherapy-treated Wnt^{High} samples (data not shown). We, therefore, hypothesized that Wnt^{High}, as opposed to Wnt^{Low} early persister cells, might enter a halted-proliferative state.

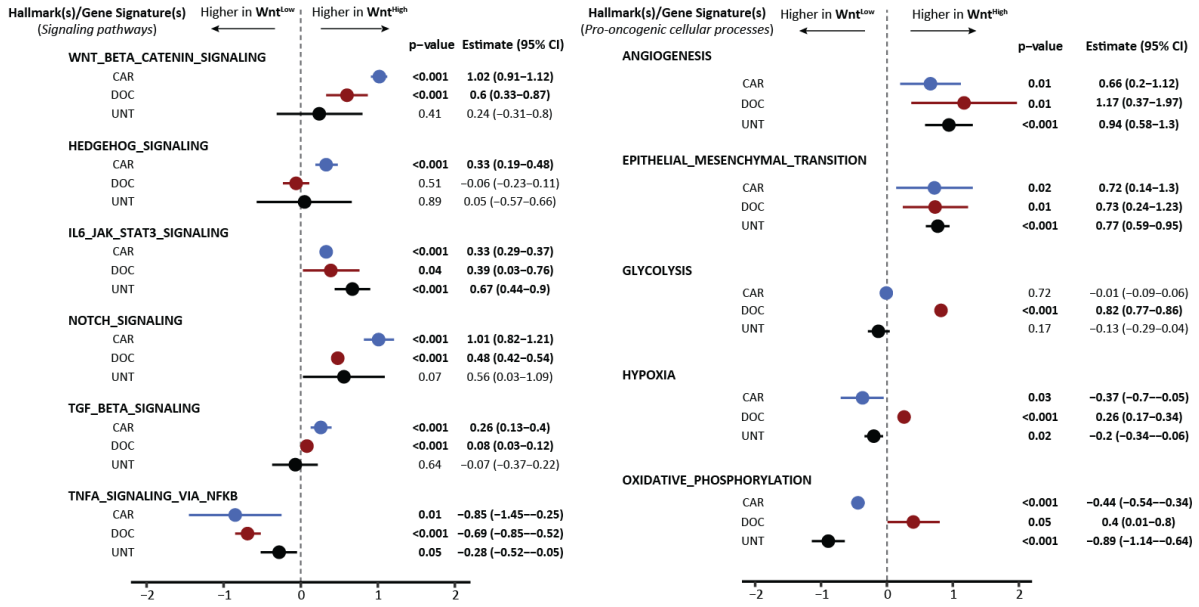


Fig. 4.9: Unraveling transcriptional differences between differential Wnt^{High} and Wnt^{Low} persister cells. Forest plots depicting the association between gene signatures and Wnt-status of sorted Wnt^{High} vs. Wnt^{Low} cells obtained from samples treated with CAR or DOC or UNT samples. Gene signatures consist of in-house gene signatures and Hallmark gene sets from MSigDB analysed using Gene set variation analysis (GSVA). Quartile regression was used to observe the median change in rescaled gene signatures after accounting for batch effects. Signatures having a non-zero positive estimate indicate increased activity in Wnt^{High} cells. Data used to generate this panel was obtained from bulk mRNA-seq of sorted Wnt^{High} and Wnt^{Low} cells from MDA-MB-231-dTGP cell line treated with DOC or CAR for 72h.

Recent studies by Rehman et al. and Dhimolea et al. have provided significant insights into the transcriptional landscape of cancer DTP cells, drawing notable parallels with embryonic stem cells (354,429). These works emphasize that DTP cells suppress MYC activity and exhibit a distinctive gene signature associated with embryonic diapause. Interestingly, our transcriptional analyses revealed that chemotherapy sorted Wnt^{High} cells, but not Wnt^{Low} cells, had a significant negative association with the MYC hallmark signature whilst having a significant positive association with the Rehman et al. embryonic diapause-like gene signature (Fig. 4.10A, B) (354), further providing evidence and highlighting the similarities between the transcriptomes of diapause-like persister cells and chemotherapy sorted Wnt^{High} cells.

Furthermore, the coordinated up- and down-regulation of additional hallmarks and processes (Notch, EMT, and Angiogenesis; upregulated) and (E2F targets, DNA repair, and Apoptosis; downregulated) (Fig. 4.10C) were coequally recorded in diapause-like cells and chemotherapy sorted Wnt^{High} cells.

Fig. 4.10: Unraveling transcriptional differences between differential Wnt^{High} and Wnt^{Low} persister cells. A-B) Forest plot depicting the association between a MYC Hallmark signature (up) and a Diapause-DTP signature (down) vs. Wnt-status of sorted Wnt^{High} vs. Wnt^{Low} cells obtained from samples treated with CAR or DOC and UNT samples. Analysis performed as in Fig. 4.9. C) Binary pattern analysis between transcriptional DTP cells and Wnt-status of sorted Wnt^{High} vs. Wnt^{Low} cells obtained from samples treated with CAR or DOC and UNT samples. Enrichment status score of -1 (light blue) indicates that a given hallmark/process is downregulated in DTPs or sorted Wnt^{High} cells while a score of 1 (red) indicates that a given hallmark/process is upregulated in DTPs or sorted Wnt^{High} cells. Data used to generate panels A-C was obtained from bulk mRNA-seq of sorted Wnt^{High} and Wnt^{Low} cells from MDA-MB-231-dTGP cell line treated with DOC or CAR for 72h.

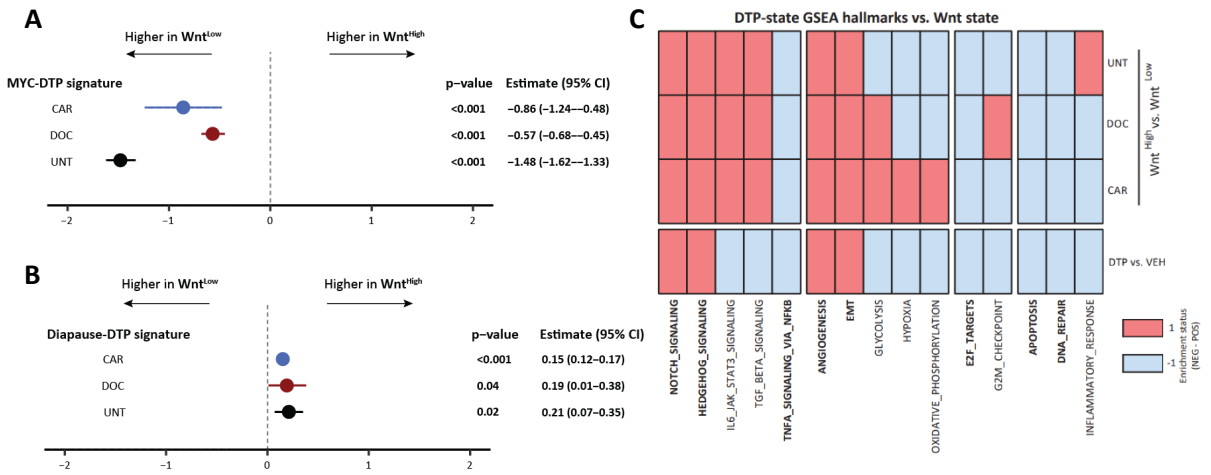


Fig. 4.10: Unraveling transcriptional differences between differential Wnt^{High} and Wnt^{Low} persister cells. Legend on page 67.

We aimed to explore if the observed transcriptional changes in Wnt^{High} cells under chemotherapy pressure were already pre-existing in UNT (i.e., parental cells) conditions. Despite a positive correlation between Wnt^{High} cells obtained from UNT conditions and the Wnt/β-catenin hallmark signature, the association was not statistically significant (Fig. 4.9 – UNT), consistent with our previous data highlighting lower levels of Wnt signaling intensity in UNT samples (Fig. 4.7D). Interestingly, parental-sorted Wnt^{High} cells exhibited overall positive associations with developmental signaling pathways and significant positive associations with EMT, while exhibiting significant negative associations with cell cycle hallmarks, following a similar trend as chemotherapy sorted Wnt^{High} early persister cells (Fig. 4.9 – UNT). Moreover, parental Wnt^{High} cells displayed a significant negative association with the MYC hallmark signature (Fig. 4.10A – UNT), a significant positive association with the Rehman et al. embryonic diapause-like gene signature (Fig. 4.10B – UNT), and other matching hallmarks and processes (Fig. 4.10C – UNT), highlighting transcriptional Wnt-activity as a functional marker for early diapause-like persister cells even in chemotherapy naïve conditions. The significance of these correlations becomes more apparent in chemotherapy-treated conditions, where transcriptional Wnt-activation is strongly exacerbated.

Functional analyses confirmed reduced cell proliferation measured by cell number observed in chemotherapy sorted Wnt^{High} compared to Wnt^{Low} populations (Fig. 4.11A, B). Furthermore, co-staining of GFP-expression (reporter for Wnt-activity) alongside the apoptotic marker Annexin V revealed that Wnt^{High} cells displayed reduced apoptotic activity, confirming that treatment promotes mainly the death of Wnt^{Low} cells while enriching a population of Wnt^{High} persisters (Fig. 4.11C, D).

In summary, our comprehensive data demonstrate that, in both parental drug-naïve cells and early persister cells under chemotherapeutic conditions, only the Wnt^{High} cell-population, as opposed to the Wnt^{Low} cell-population, exhibits a true transcriptional diapause-like cell-phenotype and function as *bona fide* early diapause-like drug-tolerant persister (hereafter referred to as diapause-like Wnt^{High}) cells. This indicates that not all early persister cells correlate with a transcriptional and functional diapause-like cell-phenotype and highlights that Wnt transcriptional activity serves as a distinct transcriptional and functional biomarker for the diapause-like cell-state amongst early persister cells.

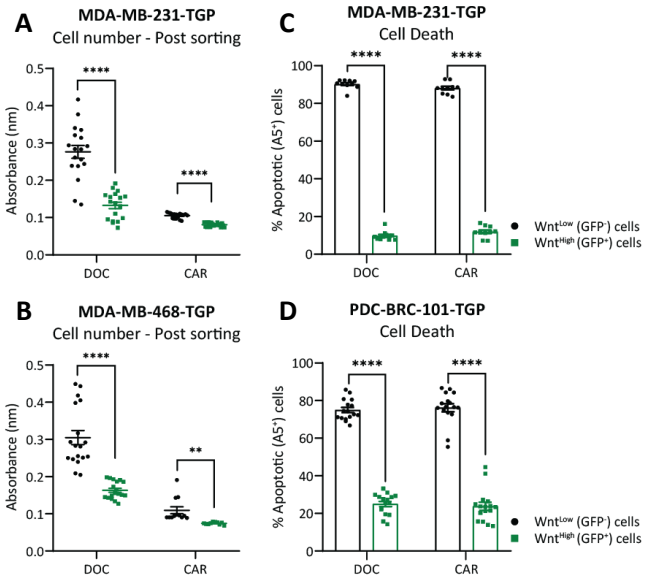


Fig. 4.11: Unraveling functional differences between differential Wnt^{High} and Wnt^{Low} persister cells. A-B) Absorbance values displaying cellular metabolic activity indicating cell number in sorted MDA-MB-231-TGP and MDA-MB-468-TGP cell lines 1 week after sorting (initial treatment before sorting was with DOC or CAR – 72h) (n = 3 independent experiments). C-D) Flow cytometry analysis displaying % of Apoptotic (Annexin V$^+$) and their respective Wnt-status (% of Wnt^{High} (GFP$^+$) cells) of MDA-MB-231-TGP and PDC-BRC-101-TGP cell lines treated with DOC or CAR for 96h (n = 5 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

4.3. Wnt pathway-activation triggers reduced proliferation mimicking a diapause-like state in parental TNBC cells

To investigate the effects of activating the Wnt signaling pathway in parental TNBC cells, we used two distinct GSK3 inhibitors, CHIR99021 (CHIR) and 6-Bromoindirubin-3'-oxime (BIO), which stabilize β-catenin (516). Treatment with either CHIR or BIO resulted in activation of the Wnt signaling pathway, as evidenced by a significant increase (>90%) in levels of Wnt^{High} cells recorded via FACS (Fig. 4.12A, B) alongside a significant increase in transcriptional levels of Wnt target-gene *AXIN2* (Fig. 4.12C).

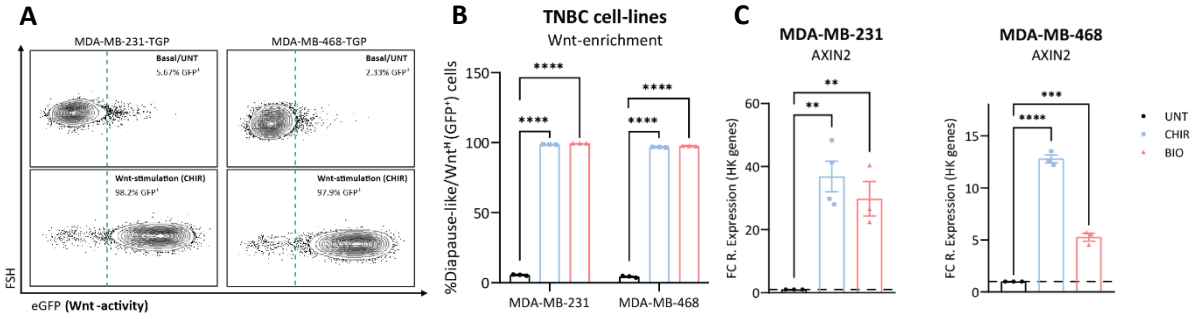


Fig. 4.12: Assessing Wnt pathway-activation on triggering a reduced proliferative, diapause-like state in chemotherapy naïve conditions. A) Representative flow cytometry contour plots displaying % of Diapause-like/Wnt^H (GFP$^+$) within viable (DAPI$^-$) cells for MDA-MB-231-TGP and MDA-MB-468-TGP cell lines in basal (UNT) culture conditions (top) and under Wnt-stimulation conditions (CHIR, 8uM, 72h – bottom). Right of the dashed green line indicates Diapause-like/Wnt^H (GFP$^+$) cells. B) Flow cytometry analysis displaying % of Diapause-like/Wnt^H (GFP$^+$) cells of MDA-MB-231-TGP and MDA-MB-468-TGP cell lines under Wnt-stimulatory conditions, treated with CHIR (8uM) or BIO (3uM) for 72h (n = 3 independent experiments). C) Gene expression levels obtained via RT-qPCR of Wnt-target (*AXIN2*) for MDA-MB-231 and MDA-MB-468 cell lines treated with CHIR or BIO for 72h, displayed as fold change (to UNT) of 2^{-dCt} values (relative to HK-genes) (n = 3 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

GSK3 inhibition significantly inhibited the proliferation of MDA-MB-231 and MDA-MB-468 cells, as observed with cell counts recorded over a 96h time-course, compared to UNT cells (Fig. 4.13A, B). CHIR- and BIO-treated cells exhibited a significant decrease in growth rate at 96h (μ: 0.61 and 0.44 for CHIR and BIO, respectively – Fig. 4.13C – left) and significant increase in doubling time at 96h (27.2h and 40.4h for CHIR and BIO, respectively – Fig. 4.13C – right) compared to UNT cells (μ: 0.77 and doubling time = 21.66h – Fig. 4.14C). These findings were corroborated by additional testing using the MDA-MB-468 cell line (Fig. 4.13D).

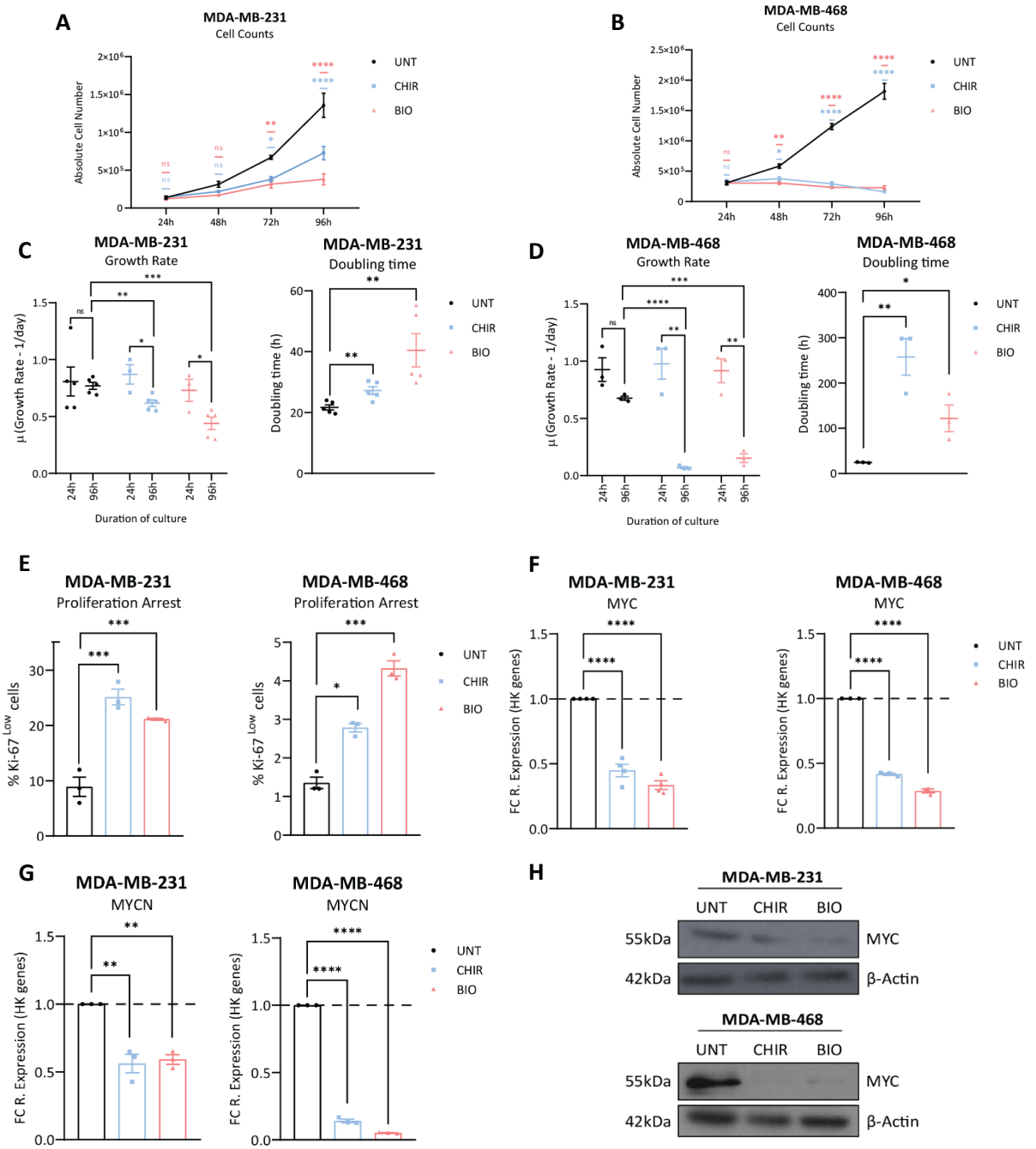


Fig. 4.13: Wnt pathway-activation triggers a reduced proliferative, diapause-like state in chemotherapy naïve conditions.
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Fig. 4.13: Wnt pathway-activation triggers a reduced proliferative, diapause-like state in chemotherapy naïve conditions. A-B) Absolute cell number of MDA-MB-231 and MDA-MB-468 cell lines treated with CHIR or BIO for a time-course of 96h (n = 3 independent experiments). C-D) Growth rate (24h and 96h) and Doubling time (96h) of MDA-MB-231 (left) and MDA-MB-468 (right) cell lines under UNT or under CHIR and BIO treatment conditions (n = 3 independent experiments). E) Flow cytometry analysis displaying % of cells in proliferation arrest (Ki-67^{low} cells) from MDA-MB-231 and MDA-MB-468 cell lines treated with CHIR or BIO for 96h (n = 3 independent experiments). F-G) Gene expression levels obtained via RT-qPCR of *MYC* and *NMYC* for MDA-MB-231 and MDA-MB-468 cell lines treated with CHIR or BIO for 72h, displayed as fold change (to UNT) of 2^{-dCt} values (relative to HK-genes) (n = 3 independent experiments). H) Western blot analysis of MYC in MDA-MB-231 and MDA-MB-468 cell lines treated with CHIR or BIO for 72h. *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

Immunofluorescence-based staining of proliferation marker Ki-67 highlighted a significant induction of growth arrest under Wnt-stimulatory conditions (Fig. 4.13E), suggesting that activation of the Wnt signaling pathway prompts a state of paused proliferation in TNBC cell lines. Treatment with CHIR or BIO resulted in a significant decrease in transcriptional *MYC* and *NMYC* expression and MYC protein levels in MDA-MB-231 and MDA-MB-468 cell lines (Fig. 4.13F-H), highlighting a direct correlation between Wnt-activation and a suppressed *MYC* phenotype.

To evaluate if Wnt-activation could induce a functional drug-tolerant state, we pre-treated TNBC cell lines with CHIR and BIO before administering chemotherapy. The results showed a significant reduction in apoptosis induction in pre-treatment Wnt-stimulated cells compared to those with sole chemotherapy treatment, across both DOC and CAR agents and in both cell lines (Fig. 4.14A, B). Notably, the decline in cell count was not attributed to apoptotic effects exerted via either GSK3 inhibitors (Fig. 4.14C, D).

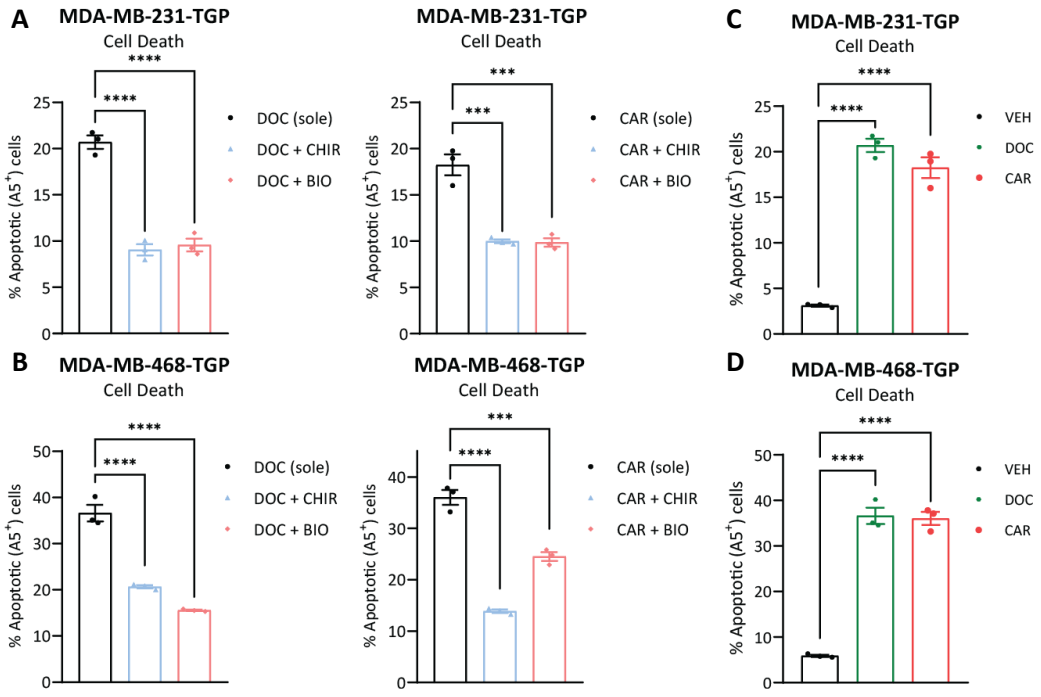


Fig. 4.14: Wnt pathway-activation induces a chemotherapy-resistant state. A-B) Flow cytometry analysis displaying % of Annexin V$^+$ cells of MDA-MB-231-TGP (top) and MDA-MB-468-TGP (bottom) cell lines treated with DOC or CAR for 72h (sole or pre-treated with CHIR or BIO for 48h) (n = 3 independent experiments). C-D) Flow cytometry analysis displaying % of Annexin V$^+$ cells of MDA-MB-231-TGP (top) and MDA-MB-468-TGP (bottom) cell lines treated with chemotherapy (DOC or CAR) for 72h (sole treatments) (n = 3 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

Our results suggest that, in TNBC, Wnt-activation promotes a paused proliferative state, reduced MYC levels, and enhanced chemoresistance, effectively recapitulating major key features of a diapause-like persister cell phenotype.

4.4. Induction of transient *de novo* Wnt signaling transcriptional-activation in response to chemotherapy in *in vitro* TNBC cell lines and in an *in vivo* TNBC xenograft model

The evolution of DTP cells during treatment continues to be a hotly debated topic, with some studies suggesting that a pre-existing DTP cell-population is simply enriched during therapy (406,517,518), while others propose that cells undergo a temporary phenotypic transition due to cellular plasticity (342,343,354,363,429). To better understand the dynamics of this process, we monitored the activation of the Wnt-reporter (TOP-GFP/TGP) in the Wnt^{High} population through live-cell imaging.
While live-cell imaging analysis of the MDA-MB-231-TGP and PDC-BRC-101-TGP cell lines under UNT conditions did not reveal significant changes in the total levels of Wnt^{High} cells (Fig. 4.15A, B – black line), treatment with either DOC or CAR resulted in a gradual enrichment of Wnt^{High} cells over a similar chemotherapy-culture timespan (Fig. 4.15A, B – green and red lines), consistent with our previous FACS-based results.

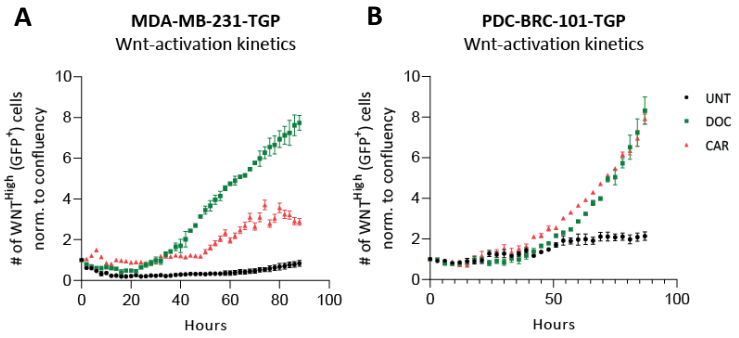


Fig. 4.15: Investigating the kinetics of Wnt signaling transcriptional-activation *in vitro*. A-B) Number of Wnt^{High} (GFP^+) cells detected by live-cell imaging normalized to the confluency of the well (total number of cells recorded) for MDA-MB-231-TGP (left) and PDC-BRC-101 (right) cell lines treated with DOC or CAR.

To visualize Wnt transcriptional-activation dynamics at single-cell resolution, we tracked the original Wnt-state of Wnt^{High} cells (starting at 60h and back to 0h) under UNT or chemotherapy-treated conditions, defining distinct Wnt-activation dynamics (Fig. 4.16A). In UNT conditions, 55% of Wnt^{High} cells in the MDA-MB-231-TGP cell line observed at 60h were initially Wnt^{High} at T_0, while 34% were activated during the culture span (modes #2 and #1, respectively – Fig. 4.16B). Under DOC or CAR treatment, most Wnt^{High} cells at 60h (58% and 55%, respectively) were *de novo*-activated from Wnt^{Low} cells at T_0 (mode #1), while only 27% and 34% were initially Wnt^{High} at T_0 (mode #2) (Fig. 4.16C, D and representative images Fig. 4.16E, F). These findings suggest that chemotherapy-induced Wnt pathway-enrichment primarily arises from *de novo* activation rather than selection of pre-existing Wnt^{High} cells. Additional dynamics (modes #3 and #4) occurred in a minority of cases, while cells that fell out of the imaging frame were considered of unknown origin/state (mode #5). Similar patterns were validated using the PDC-BRC-101-TGP cell line (Fig. 4.16G-I).

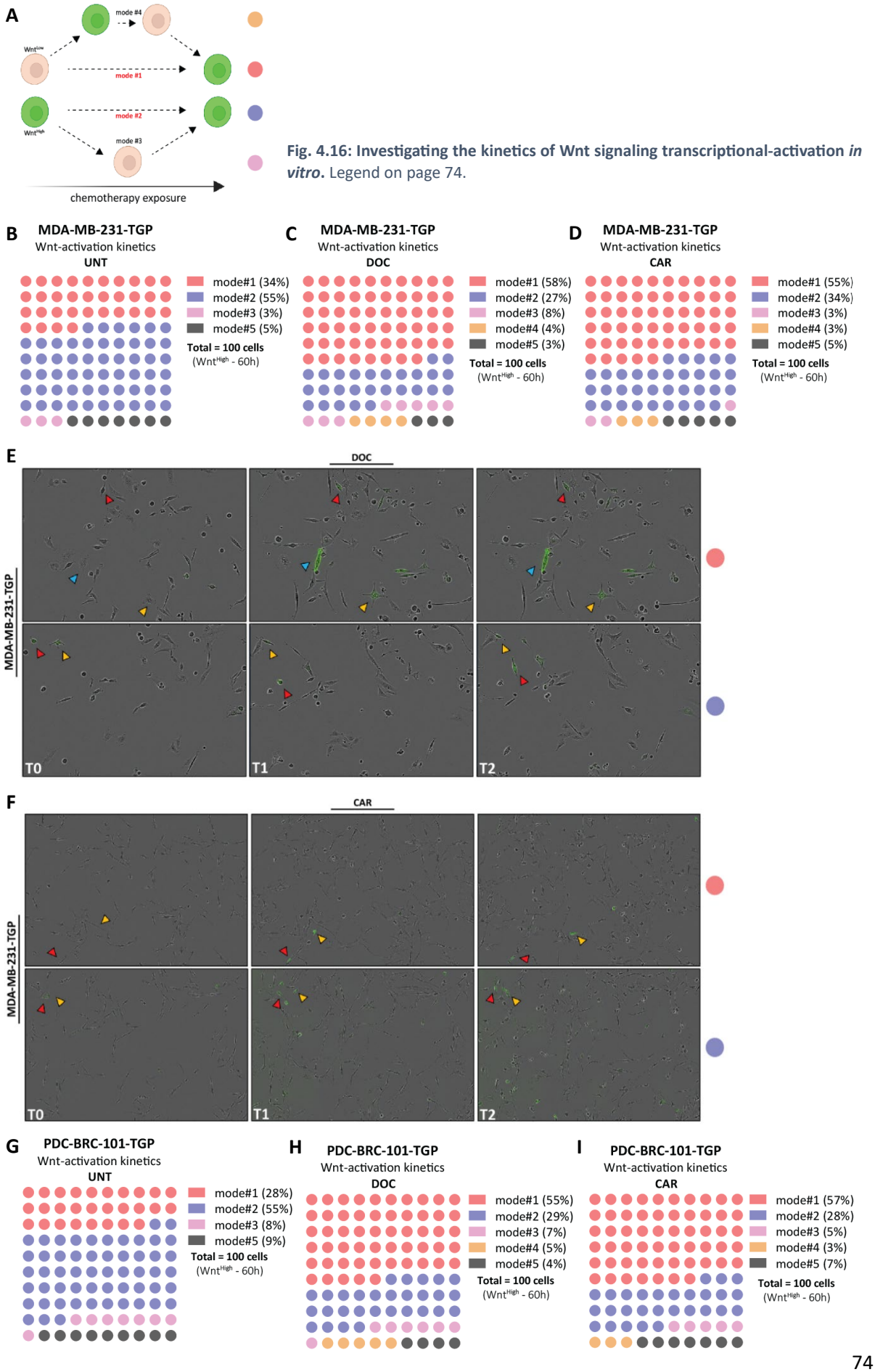


Fig. 4.16: Investigating the kinetics of Wnt signaling transcriptional-activation *in vitro*. Legend on page 74.

Fig. 4.16: Investigating the kinetics of Wnt signaling transcriptional-activation *in vitro*. A) Schematic representation of different fluctuation dynamics of Wnt-activation – mode numbered and color coded. B-D) Live-imaging quantification of different possible mechanisms of chemotherapy-induced Wnt-activation in MDA-MB-231-TGP TNBC cell line UNT or treated with DOC or CAR. n = 100 cells tracked every 2h for 60h, per treatment condition. Every single cell tracked is represented as a circle and color coded with the scheme shown in A. E-F) Snapshots of still-frames from time-lapse live imaging experiments of MDA-MB-231-TGP TNBC cell line treated with DOC (top) or CAR (bottom) presenting mode #1 and mode #2 (color coding in scheme shown in A). T0 indicates 0h, T1 indicates 30h, and T2 indicates approx. 50h. Yellow, red, and blue arrows indicate the same cell followed over the treatment period spanning different images (horizontal). G-I) Live-imaging quantification of different possible mechanisms of chemotherapy-induced Wnt-activation in PDC-BRC-101-TGP TNBC cell line UNT or treated with DOC or CAR. n = 100 cells tracked every 2h for 60h, per treatment condition. Every single cell tracked is represented as a circle and color coded with the scheme shown in A.

Next, we assessed the population dynamics after treatment was halted (Fig. 4.17A). Interestingly, the percentage of diapause-like Wnt^{High} cells was stabilized or even increased upon chemotherapy removal for up to 3 weeks (Fig. 4.17B-E). However, extended chemotherapy-free culture (4 weeks) significantly reduced the percentage of diapause-like Wnt^{High} cells, restoring Wnt-populations to levels similar to that of UNT/basal-cultured cells (Fig. 4.17B-E). MDA-MB-231-TGP cell lines recovering from DOC and CAR treatments (DOC^{REC} and CAR^{REC}, respectively) also reverted to normal (relative to UNT) rates of proliferation and drug tolerance (Fig. 4.17F, G), confirming the transient and reversible nature of the diapause-like early persister-phenotype driven by chemotherapy-induced Wnt-activation.

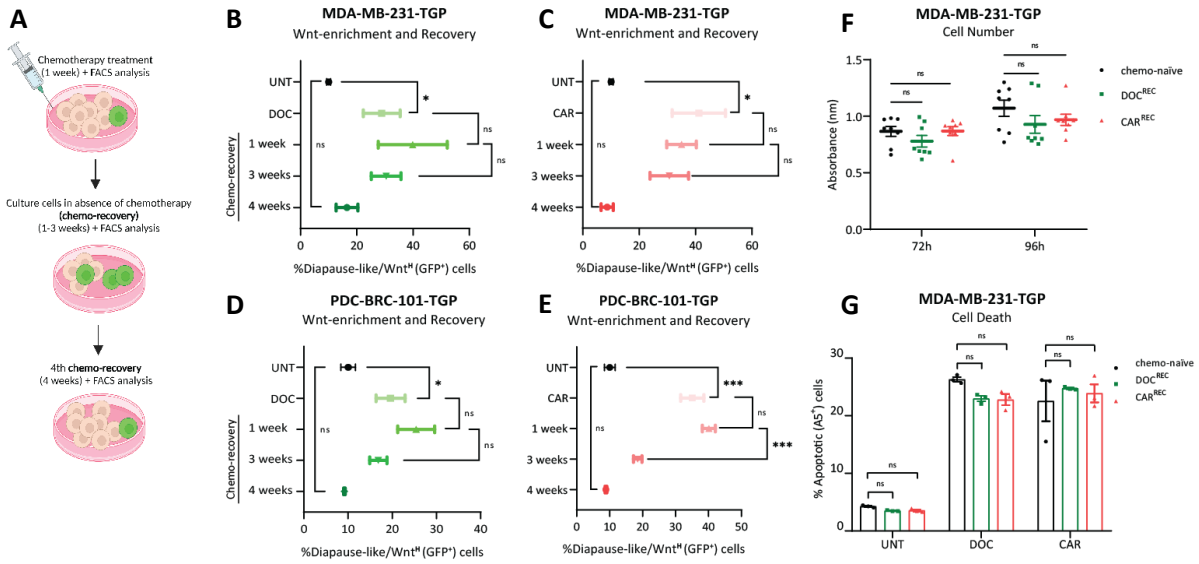


Fig. 4.17: Investigating the transient/reversible nature of Wnt signaling transcriptional-activation *in vitro*. A) Schematic representation of the experimental setup for chemotherapy treatment and recovery. B-E) Flow Cytometry analysis displaying % of Diapause-like/Wnt^H (GFP^+) within viable ($DAPI^-$) cells for MDA-MB-231-TGP and PDC-BRC-101-TGP cell lines treated with DOC or CAR for 1 week followed by removal of chemotherapy for 1-, 3-, and 4-weeks post-recovery (n=3 independent experiments). F) Metabolic activity levels reflecting cell number and proliferation rates of MDA-MB-231-TGP cell lines (chemotherapy naïve vs. DOC^{REC} vs. CAR^{REC}), all in UNT conditions at 72h and 96h (n=3 independent experiments). G) Flow cytometry analysis displaying % of Apoptotic (Annexin V^+) cells of MDA-MB-231-TGP cell lines (chemotherapy naïve vs. DOC^{REC} vs. CAR^{REC}), UNT or treated with DOC or CAR for 72h (n=3 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

Next, we established cell line-derived xenograft (CDX) models using the MDA-MB-231 cell line engineered with the Wnt-transcriptional reporter TOPFLASH (511) and investigated *in vivo* Wnt-activation dynamics using a live imaging system (IVIS) (Fig. 4.18A). Treatment with either DOC or CAR (504,519) resulted in a significant overall decrease in tumor volume when compared to the vehicle (VEH) treated group (Fig. 4.18B, C). Notably, at the administered doses, no significant changes in mouse body weight were observed during the three weeks of treatment (Fig. 4.18D).

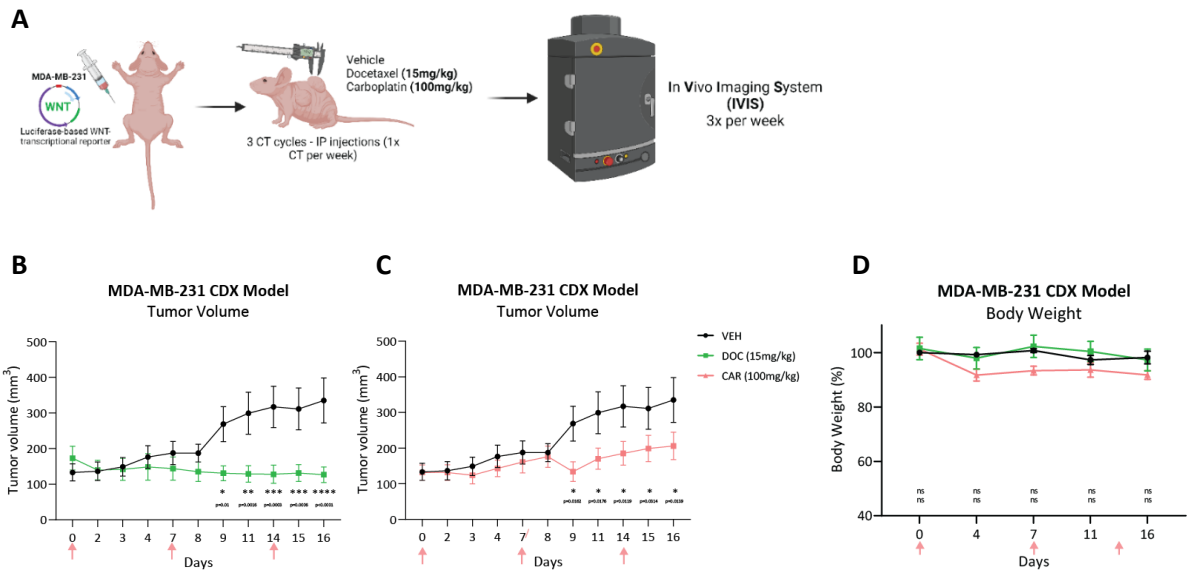


Fig. 4.18: Investigating the kinetics of Wnt signaling transcriptional-activation *in vivo*. A) Schematic representation of the CDX experimental setup to study Wnt signaling pathway kinetics upon chemotherapeutic treatment *in vivo*. MDA-MB-231 cells were engineered with the Wnt-transcriptional reporter TOPFLASH, a β-catenin-responsive firefly luciferase reporter plasmid, compatible for use with the *in vivo* live imaging system (IVIS). B-C) Tumor growth curves of subcutaneous generated xenograft models treated with VEH, DOC (left), or CAR (right) (n = 8,7 mice for all treatment groups). D) % of bodyweight change of initial treatment bodyweight of xenograft models treated with VEH, DOC (15mg/kg/week), or CAR (100mg/kg/week). Pink arrows indicate administration of chemotherapy (n = 8,7 mice for all treatment groups). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

We detected dynamic Wnt-activity characterized by a significant upregulation in transcriptional Wnt-activation as early as 48h and 72h (for DOC and CAR, respectively) upon chemotherapeutic administration (Fig. 4.19A, B and representative image Fig. 4.19C). Notably, this upregulation was followed by a decrease as the week progressed. The same dynamic pattern was observed following administration of the 2nd and 3rd doses of treatment (Fig. 4.19A, B). Gene expression analysis on the resected tumors confirmed elevated expression levels of Wnt-target genes (*AXIN2* and *LEF1*) (Fig. 4.19D) in chemotherapy-treated groups.

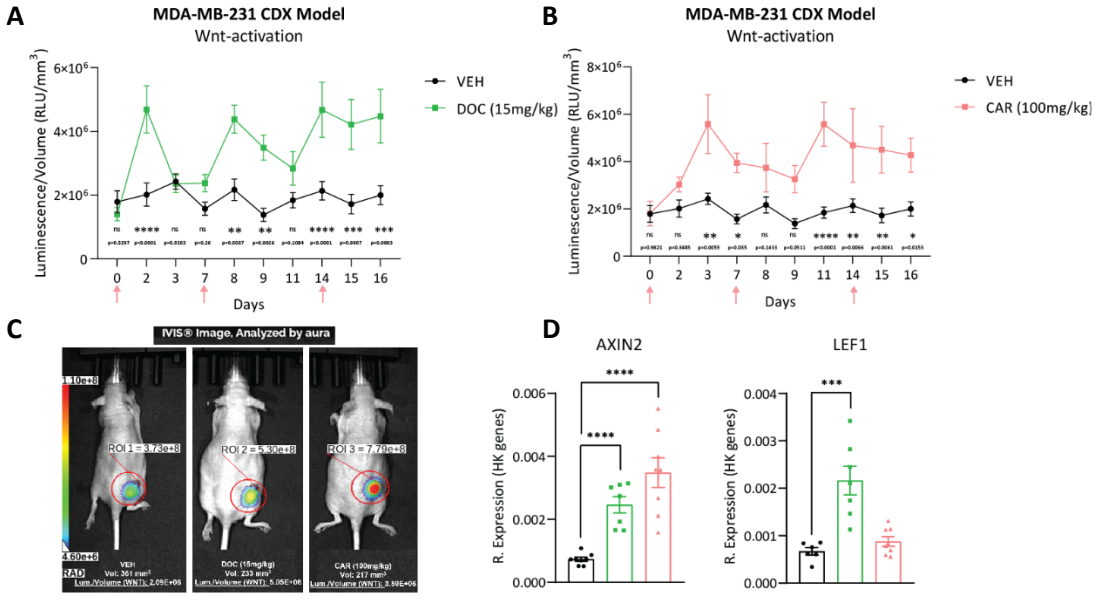


Fig. 4.19 – Legend on page 76.

Fig. 4.19: Investigating the kinetics of Wnt signaling transcriptional-activation *in vivo*. A-B) Levels of Wnt-activation (RLU/mm^3) displayed as luminescent signals (RLU) captured by IVIS Spectrum normalized to tumor volume (mm^3) in xenograft models treated with VEH, DOC (left), or CAR (right). Pink arrows indicate administration of chemotherapy (n = 7 mice for all treatment groups). C) Representative bioluminescent image(s) obtained via IVIS Spectrum displaying one mouse from each of the three treatment groups (VEH – left, DOC – middle, and CAR – right) with supplementary information regarding the tumor volume of each mouse and the calculated Wnt-activation signal D) Gene expression levels obtained via RT-qPCR of Wnt-targets (*AXIN2* and *LEF1*) in samples resected from xenograft models treated with VEH, DOC, or CAR (2^{-dCt}) (n = 7 mice for all treatment groups). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

Altogether, our *in vitro* findings show that the diapause-like Wnt^{High} phenotype results namely from a *de novo* chemotherapy-driven action rather than solely representing a manifestation of an inherently chemotherapy-resistant subpopulation selected under treatment pressure. Notably, upon chemotherapy removal, Wnt-activity levels revert to baseline levels, indicating a transient enrichment of a diapause-like Wnt^{High} cell-state dependent on chemotherapy pressure. Furthermore, we demonstrate the activation dynamics of the Wnt signaling pathway in chemotherapy-treated tumors within an *in vivo* setting, highlighting a transient and dynamic nature.

4.5. Chemotherapeutic treatment induces elevated transcriptional expression of Wnt ligands, Wnt enhancers, and Wnt secretion machinery components

The Wnt signaling pathway is highly conserved and activated via the binding of 19 extracellular Wnt ligands (Wnts) to membrane receptors (510,520). Secretion of Wnt ligands requires the action of the acyltransferase Porcupine (PORCN) followed by a subsequent interaction of the ligands with Wntless/Evenness interrupted (WLS/Evi) which supports transport of Wnts from the Trans-Golgi Network to the plasma membrane. In addition, the RSPO protein family has been shown to enhance Wnt ligand activity to further promote Wnt pathway-activation (510,520).

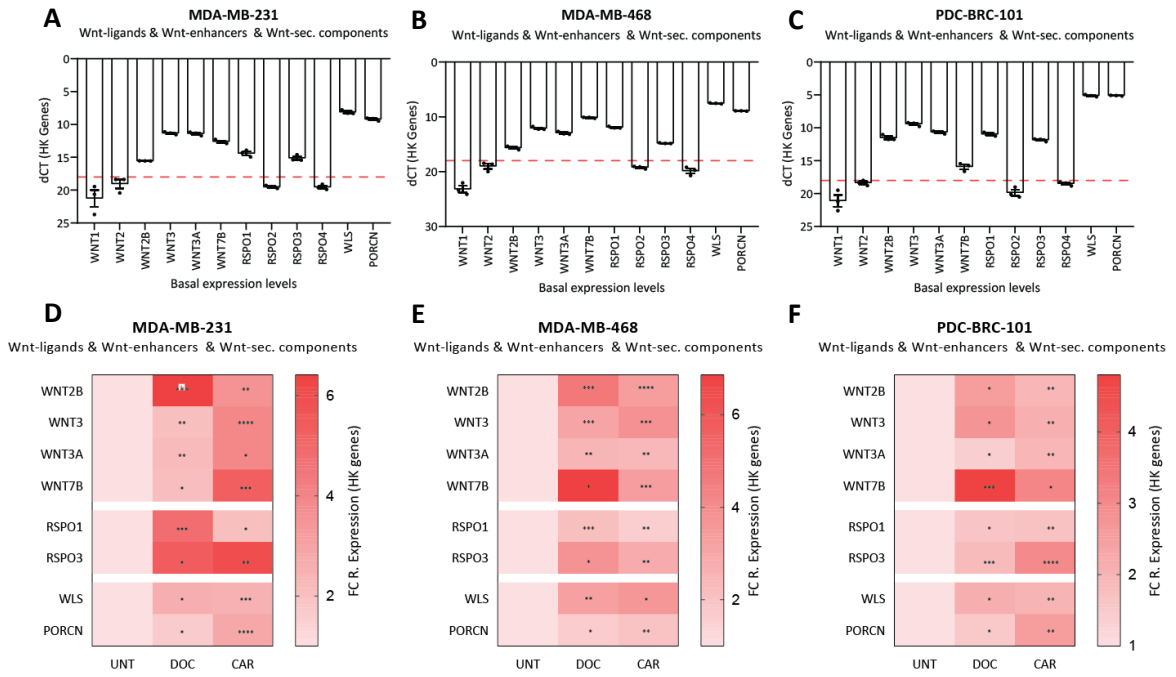


Fig. 4.20: Chemotherapeutic treatment induces elevated transcriptional expression of Wnt ligands, Wnt enhancers, and Wnt secretion machinery components *in vitro*. Legend on page 77.

Fig. 4.20: Chemotherapeutic treatment induces elevated transcriptional expression of Wnt ligands, Wnt enhancers, and Wnt secretion machinery components *in vitro*. A-C) Gene expression levels obtained via RT-qPCR of various Wnt ligands, Wnt enhancers, and Wnt secretion machinery components for TNBC cell lines in basal culture conditions displayed as dCt values (relative to HK-genes) (n = 3 independent experiments). The dashed red line represents a threshold dCt value of 18. Genes that record dCt values above 18 are not considered robustly expressed and are therefore not subject to further analysis in our study. D-F) Heatmaps showing gene expression levels obtained via RT-qPCR of Wnt ligands (*WNT2B*, *WNT3*, *WNT3A* and *WNT7B*), Wnt enhancers (*RSPO1* and *RSPO3*), and Wnt ligand secretion machinery components (*WLS* and *PORCN*) for TNBC cell lines treated with DOC or CAR for 72h, displayed as fold change (to UNT) of 2^{-dCt} values (relative to HK-genes) (n = 3 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

Focusing on established canonical Wnt- and RSPO-ligands, we found *WNT2B*, *WNT3*, *WNT3A*, *WNT7B*, *RSPO1*, *RSPO3*, as well as Wnt-ligand secretion machinery, *WLS* and *PORCN*, to be steadily expressed in basal conditions across all analyzed TNBC cell lines (Fig. 4.20A-C) and significantly increased under chemotherapy-treatment conditions (Fig 4.20D-F), suggesting that chemotherapeutic exposure actively promotes elevated transcription levels of key several key components involved in canonical Wnt pathway-activation.

Furthermore, gene expression analysis performed on the resected tumors from our previous *in vivo* experimental setup (Fig. 4.18) revealed that expression of various Wnt ligands (*WNT2B*, *WNT3A*, *WNT7B*), -enhancers (*RSPO1* and *RSPO3*), and –secretion machinery components (*WLS* and *PORCN*) was upregulated in chemotherapy-treated groups (Fig. 4.21).

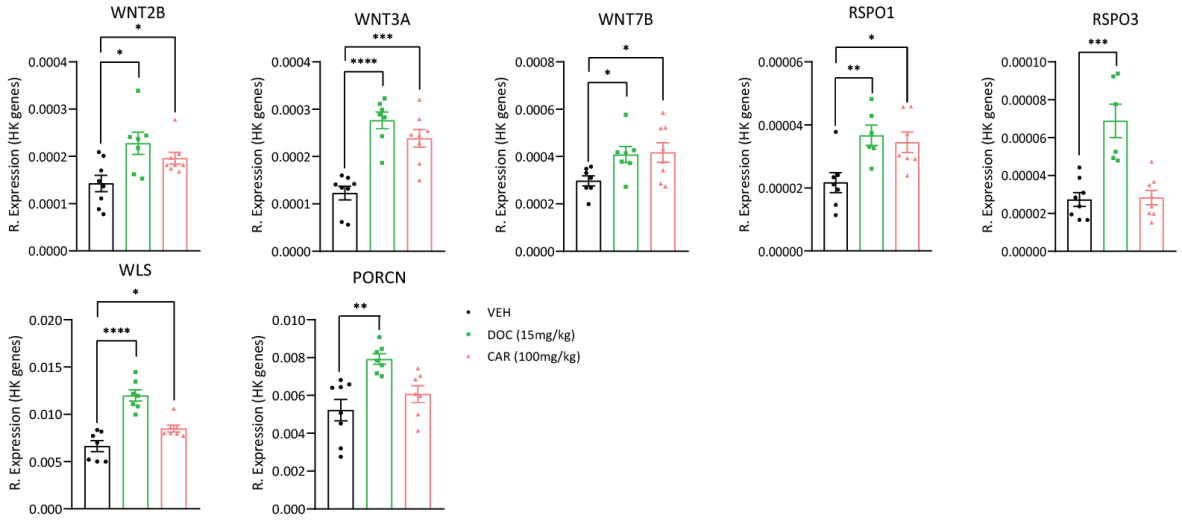
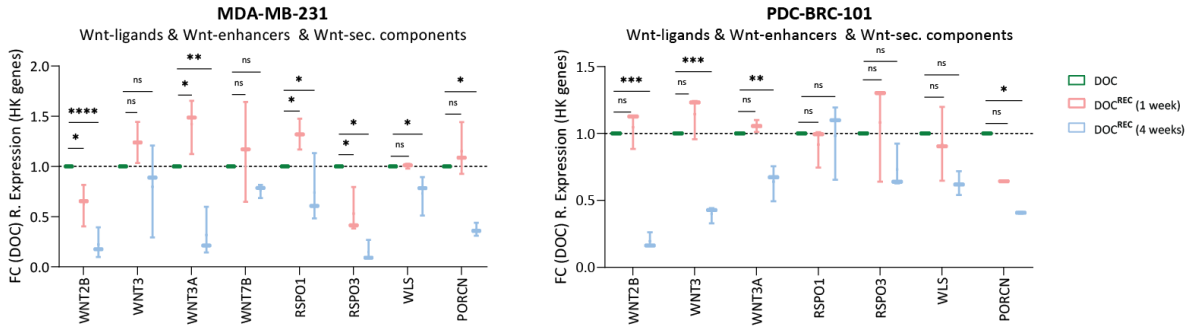


Fig. 4.21: Chemotherapeutic treatment induces elevated transcriptional expression of Wnt ligands, Wnt enhancers, and Wnt secretion machinery components *in vivo*. Gene expression levels obtained via RT-qPCR of Wnt-activators (Wnt ligands, -enhancers, and -secretion machinery components) in samples resected from xenograft models Fig. 4.18 treated with VEH, DOC, or CAR (n = 8 (VEH) and 7 (DOC and CAR) mice). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

Subsequently, following chemotherapy removal and under one-week chemotherapy-recovery conditions, the majority of Wnt-activation components maintained elevated expression levels (Fig. 4.22 – pink bars). However, after four weeks of culture in chemotherapy-free conditions and coinciding with the previous results displaying the return of diapause-like Wnt^{High} cells to basal levels (Fig. 4.17), we observed a corresponding decrease in expression level patterns of Wnt-activation components (Fig. 4.22 – light blue bars), indicating that exit from a diapause-like early persister cell-state correlates with decreased Wnt ligand-expression upon drug holidays.

Fig. 4.22: Expression of Wnt ligands, Wnt enhancers, and Wnt secretion machinery components upon a drug-holiday period *in vitro*. Gene expression levels as in Fig. 4.20D-F for MDA-MB-231 and PDC-BRC-101 cell lines treated with DOC (and recovered) following the treatment scheme shown in Fig. 4.17A, displayed as fold change (to DOC) of 2^{-dCt} values (relative to HK-genes) (n = 3 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.



Western blot analysis confirmed the upregulation of the Wnt ligand Wnt-2b and the acyltransferase PORCN, in response to either DOC or CAR treatments across all analyzed TNBC cell lines (Fig. 4.23A-F). Treatment of chemotherapy naïve cells with concentrated CM derived from MDA-MB-231 and MDA-MB-468 TNBC cell lines (Fig. 4.23G) confirmed increased and functional presence of Wnt ligands in media collected under one-week chemotherapy-recovery conditions, resulting in a significant increase in diapause-like Wnt^{High} cells when compared to chemotherapy naïve cells treated with UNT CM (Fig. 4.23H, I). In a parallel experiment, we co-cultured chemotherapy naïve (MDA-MB-231-mCherry-TGP) cells with chemotherapy-recovering (MDA-MB-231) cells (Fig. 4.23J). This co-culturing approach similarly resulted in a substantial and statistically significant increase in the percentage of diapause-like Wnt^{High} cells within the chemotherapy naïve cell population (Fig. 4.23K).

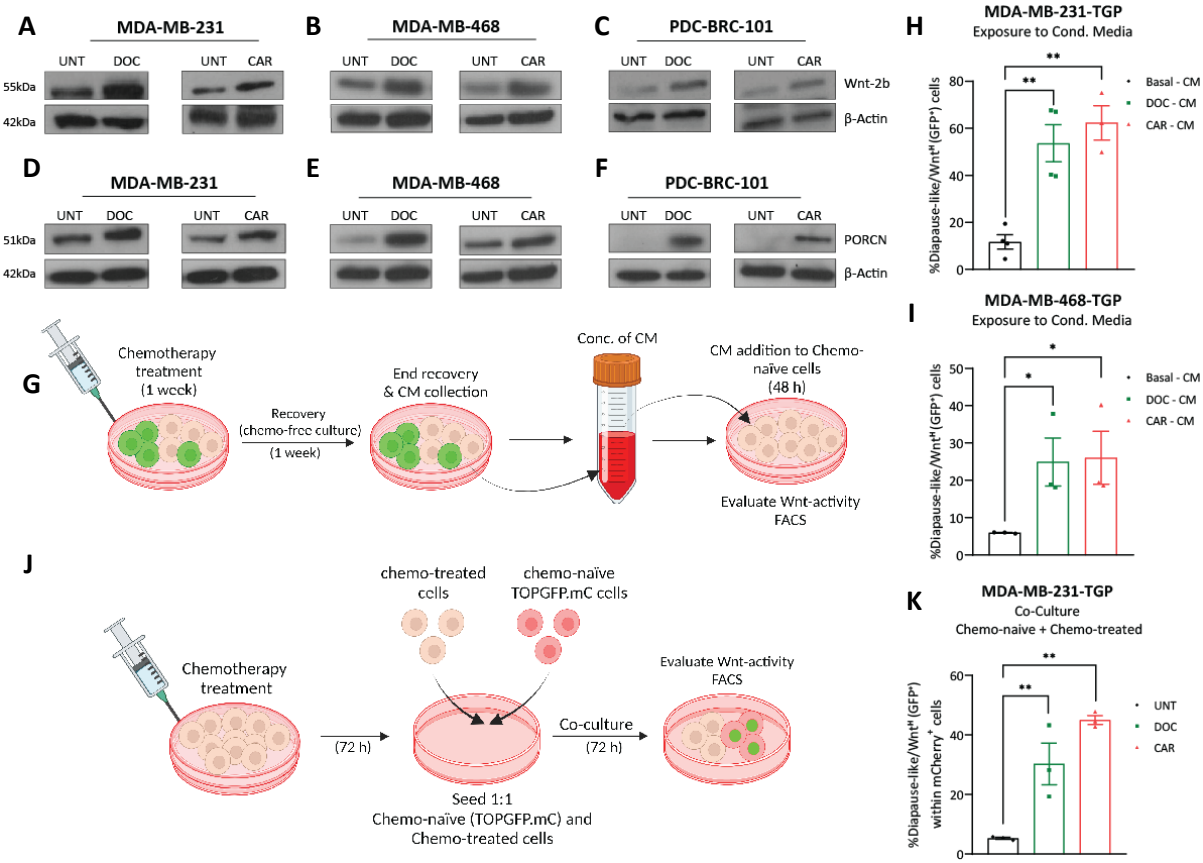


Fig. 4.23: Chemotherapeutic treatment induces elevated expression and secretion of functional Wnt-activators *in vitro*. Legend on page 79.

Fig. 4.23: Chemotherapeutic treatment induces elevated expression and secretion of functional Wnt-activators *in vitro*. A-F) Western blot analysis of Wnt ligand Wnt-2b (top) PORCN (bottom) in TNBC cell lines treated with DOC or CAR for 72h. G) Schematic representation of CM experimental setup. H-I) Flow Cytometry analysis displaying % of Diapause-like/Wnt^{H} (GFP^{+}) within viable (DAPI^{-}) cells of MDA-MB-231-TGP and MDA-MB-468-TGP cell lines cultured with concentrated Basal-, DOC-, or CAR-CM for 48h (n = 3 independent experiments). J) Schematic representation of co-culture experiment setup. K) Flow Cytometry analysis displaying % of Diapause-like/Wnt^{H} (GFP^{+}) within viable (DAPI^{-}) cells of chemotherapy naïve MDA-MB-231-TGP.mC (mCherry-TOPGFP) co-cultured with chemotherapy-treated DOC or CAR MDA-MB-231 cells for 72h (n = 3 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

In summary, our findings demonstrate that chemotherapeutic treatment leads to elevated expression levels of Wnt ligands, -enhancers, and -components of the Wnt secretory apparatus, highlighting a significant role for Wnt-activators in regulating entry into and exit from a diapause-like Wnt^{High} state under chemotherapeutic pressure.

4.6. Wnt ligand secretion-inhibition alongside chemotherapeutic treatment hinders diapause-like early persister cell-enrichment *in vitro* and synergistically sensitizes an *in vivo* TNBC xenograft model

In contrast to our findings, previous studies have demonstrated increased Wnt-activation following therapy through cell-autonomous mechanisms that are independent of Wnt ligand-secretion (469). To test whether the acquisition of a diapause-like early persister cellular-state is Wnt ligand secretion-dependent or -independent, we stably transduced TNBC cell lines with lentiviral shRNA constructs targeting the acetyltransferase PORCN, disrupting the functional secretion of Wnt ligands (521). Chemotherapy-treated PORCN-silenced (shPORCN#1) cell lines (Fig. 4.24A) revealed reduced levels of active β-catenin compared to control (shPLKO) lines, confirming an essential role for PORCN in chemotherapy-induced diapause-like Wnt^{High} cell-enrichment (Fig. 4.24B).

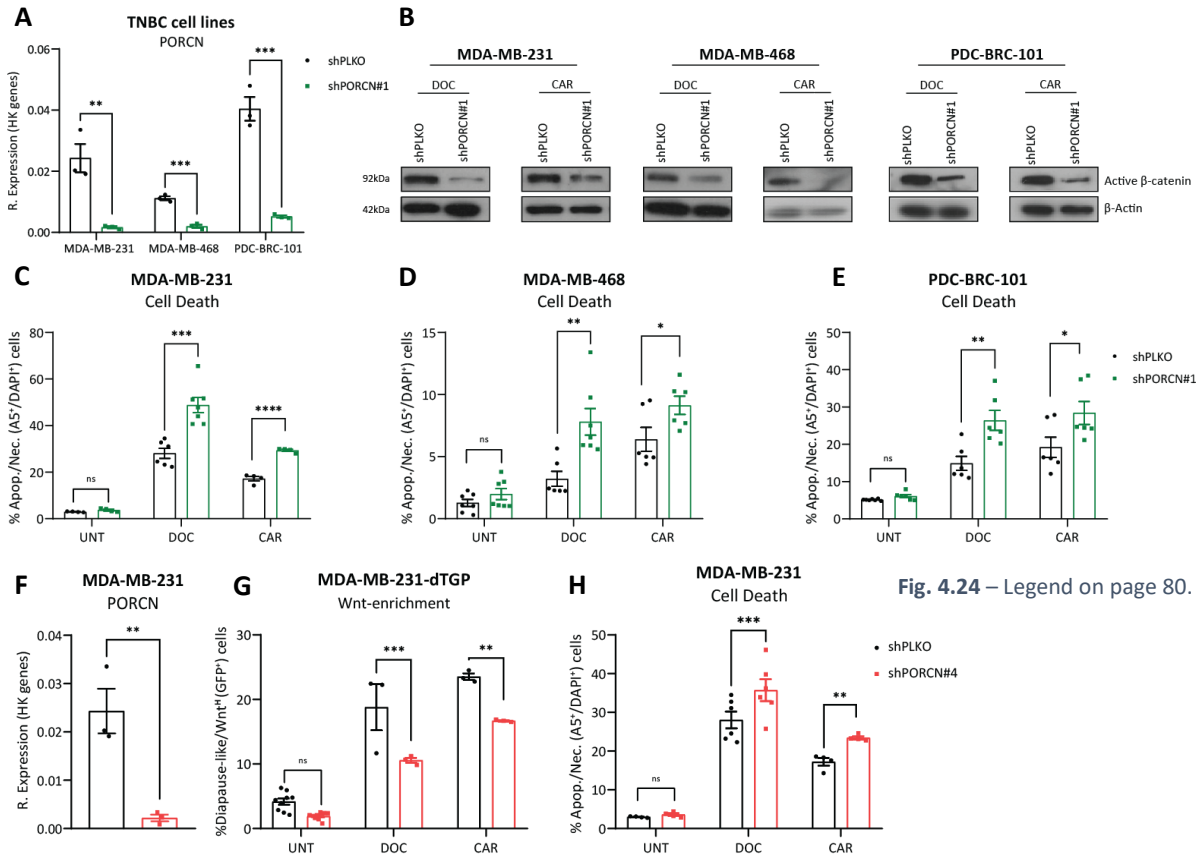


Fig. 4.24 – Legend on page 80.

Fig. 4.24: Interfering with Wnt ligand-secretion via gene-silencing leads to decreased Wnt-activation and increased chemotherapy-sensitization. A) Gene expression levels of *PORCN* obtained via RT-qPCR of PORCN (shPLKO vs. shPORCN#1) TNBC cell lines in basal conditions (n = 3 independent experiments). B) Western blot analysis of active- (non-phosphorylated) β-catenin in (shPLKO vs. shPORCN#1) TNBC cell lines treated with DOC or CAR for 96h. C-E) Flow cytometry analysis displaying % Annexin V^+/$DAPI^+$ cells in (shPLKO vs. shPORCN#1) TNBC cell lines treated with DOC or CAR for 96h (n = 4 independent experiments). F) Gene expression levels obtained via RT-qPCR of *PORCN* of MDA-MB-231 (shPLKO vs. shPORCN#4) cell line in basal culture conditions (n = 3 independent experiments). G) Flow cytometry analysis displaying % of Diapause-like/Wnt^H (GFP^+) within viable ($DAPI^-$) cells of MDA-MB-231-dTGP (shPLKO vs. shPORCN#4) cell line treated with DOC or CAR for 96h (n = 3,4 independent experiments). H) Flow cytometry analysis displaying % of Apoptotic and Necrotic (Annexin V^+ and $DAPI^+$) cells of MDA-MB-231 (shPLKO vs. shPORCN#4) cell line treated with DOC or CAR for 96h (n = 4 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

While the silencing of PORCN had no impact on cell viability in basal (UNT) culture conditions, we observed a marked and significant increase in levels of apoptotic and necrotic (Annexin V^+ and $DAPI^+$) cells in shPORCN#1 TNBC cell lines compared to shPLKO lines upon chemotherapy exposure, indicating a strong sensitization role of PORCN-inhibition (Fig. 4.24C-E). Similar results were obtained using a second independent lentiviral shRNA construct targeting PORCN (shPORCN#4 – Fig. 4.24F-H).

Next, we investigated whether pharmacological inhibition of PORCN could also prove effective in curbing the induction of diapause-like Wnt^{High} cells under treatment pressure. We examined two distinct approaches to therapeutically target treatment-induced diapause-like Wnt^{High} cells. In the first approach, we pre-treated all three TNBC cell lines with the Inhibitor of Wnt Production (IWP-2) (522) for 48h, followed by the application of either DOC or CAR agents (sequential treatment). In the second approach, we applied chemotherapeutic treatment simultaneously and in combination with IWP-2 (combinatorial treatment).

Pre-treatment (sequential treatment strategy – Fig. 4.25A) with IWP-2 led to a notable and significant reduction in the percentage of diapause-like Wnt^{High} cells in parental cells (Fig. 4.25B) with no effects on cell viability (data not shown). However, upon chemotherapy addition, we observed robust chemotherapy-induced Wnt-activation both in IWP-2 pre-treatment conditions and in sole chemotherapy-treatment alike (Fig. 4.25C-E).

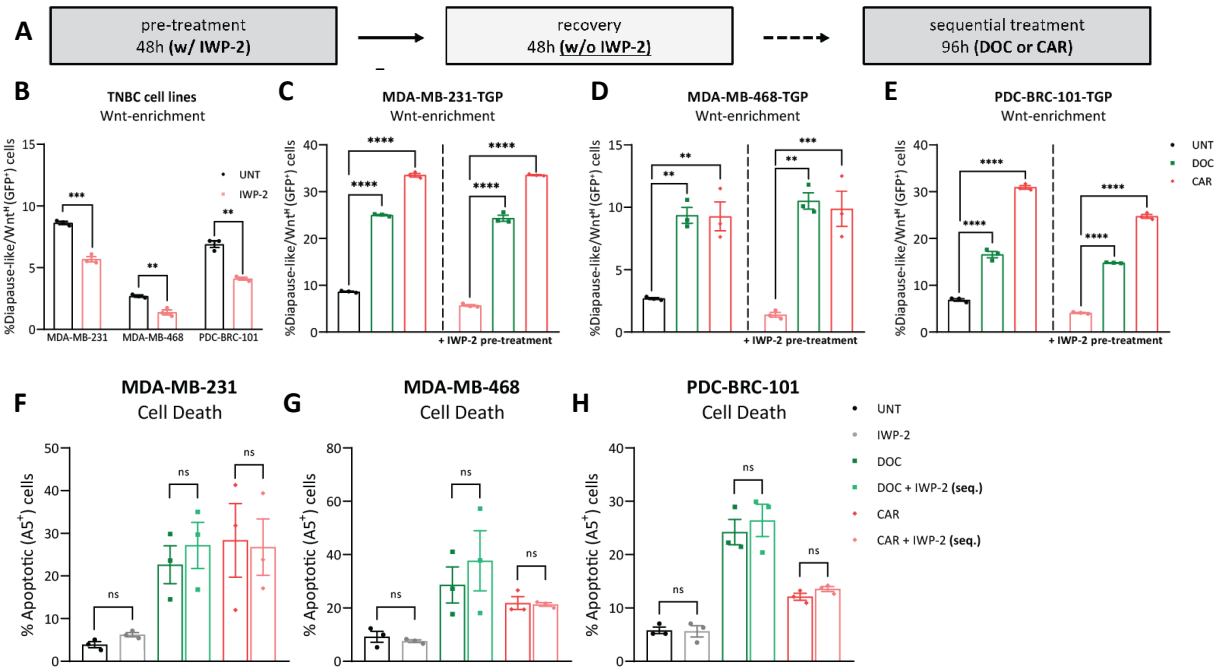


Fig. 4.25: Sequential treatment strategy inhibiting Wnt ligand-secretion alongside chemotherapy *in vitro*. Legend on page 81.

Fig. 4.25: Sequential treatment strategy inhibiting Wnt ligand-secretion alongside chemotherapy *in vitro*. A) Schematic representation of the Sequential Treatment (first IWP-2 pre-treatment followed by a recovery period then chemotherapy) model. B) Flow cytometry analysis displaying % of Diapause-like/Wnt^{H} (GFP^{+}) within viable (DAPI^{-}) cells of TNBC cell lines pre-treated with IWP-2 (10uM) for 48h. C-E) Flow cytometry analysis of displaying % of Diapause-like/Wnt^{H} (GFP^{+}) within viable (DAPI^{-}) cells of TNBC-TGP cell lines treated with DOC or CAR for 96h (with or without IWP-2 pre-treatment (n = 3 independent experiments). F-H) Flow cytometry analysis displaying % of Apoptotic (Annexin V^{+}) cells TNBC cell lines pre-treated with IWP-2 for 48h or treated with DOC or CAR for 96h (sole or sequentially with IWP-2) (n = 3 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

Notably, pre-treatment with IWP-2 followed by chemotherapy addition did not result in increased cell death or sensitization to either agent (Fig. 4.25F-H), suggesting that a sequential treatment strategy to target *a priori* existing diapause-like cell-population might not be sufficient to prevent the *de novo* chemotherapy-induced diapause-like Wnt^{High} phenotype.

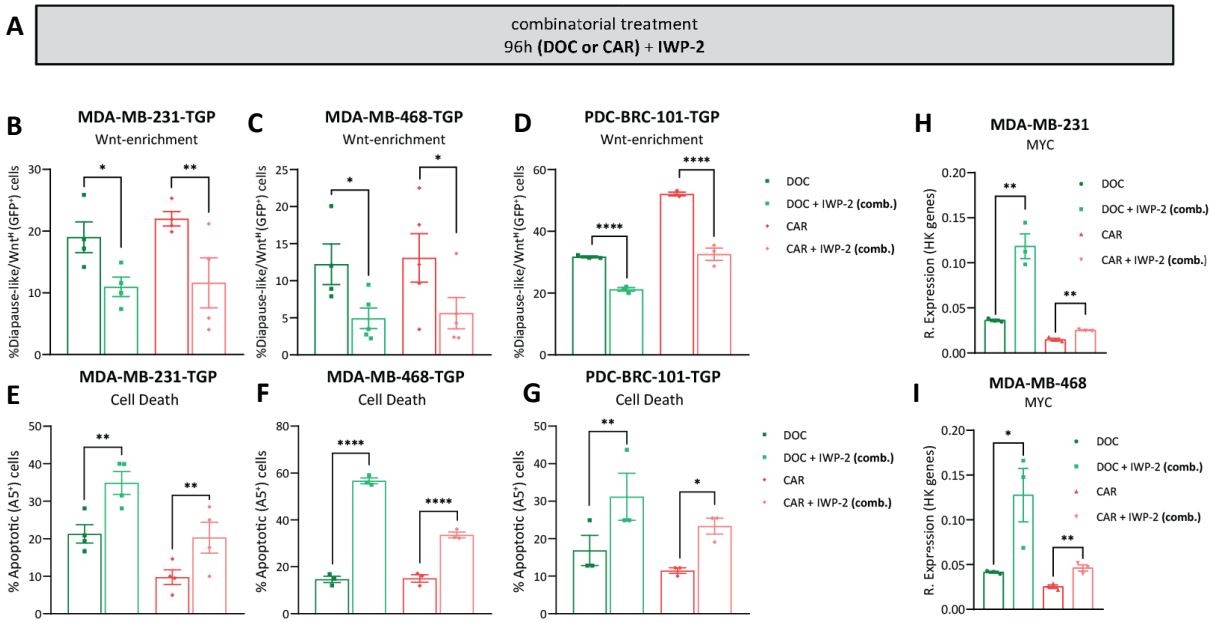


Fig. 4.26: Combinatorial treatment strategy inhibiting Wnt ligand-secretion alongside chemotherapy *in vitro*. A) Schematic representation of the Combinatorial Treatment model (IWP-2 and chemotherapy). B-D) Flow cytometry analysis displaying % of Diapause-like/Wnt^{H} (GFP^{+}) within viable (DAPI^{-}) cells of TNBC-TGP cell lines treated with DOC or CAR for 96h (sole or in combination with IWP-2) (n = 4 independent experiments). E-G) Flow cytometry analysis displaying % of Annexin V^{+} cells of TNBC cell lines treated with DOC or CAR for 96h (sole or in combination with IWP-2) (n = 4 independent experiments). H-I) Gene expression levels obtained via RT-qPCR of *MYC* for MDA-MB-231 and MDA-MB-468 cell lines treated with DOC or CAR for 96h (sole or in combination with IWP-2) (n = 3 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

In the second approach (combinatorial treatment strategy – 4.26A), simultaneous treatment of TNBC cell lines with either DOC or CAR therapeutic agent in combination with IWP-2 led to a significant decrease in the percentage of diapause-like Wnt^{High} cells (Fig. 4.26B-D). Intriguingly, combinatorial treatment resulted in a substantial increase in apoptotic cell death across all analyzed TNBC cell lines compared to treatment with either chemotherapeutic agent alone (Fig. 4.26E-G). Interestingly, we observed that the supplementation of IWP-2 alongside chemotherapy resulted in a significant rescue of *MYC* levels (Fig. 4.26H, I). Furthermore, we validated our findings using a second pharmacological PORCN inhibitor, WNT-974 (LGK-974) (504,523,524) and observed similar outcomes (Fig. 4.27A-G).

Fig. 4.27: Combinatorial treatment strategy inhibiting Wnt ligand-secretion alongside chemotherapy *in vitro*. A) Schematic representation of the Combinatorial Treatment model (LGK-974 and chemotherapy). B-D) Flow cytometry analysis displaying % of Diapause-like/Wnt^{H} (GFP^{+}) within viable (DAPI^{-}) cells of TNBC-TGP cell lines treated with DOC or CAR for 96h (sole or in combination with LGK-974, 2µM) (n = 4 independent experiments). E-G) Flow cytometry analysis displaying % of Annexin V^{+} cells of TNBC cell lines treated with DOC or CAR for 96h (sole or in combination with LGK-974) (n = 4 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

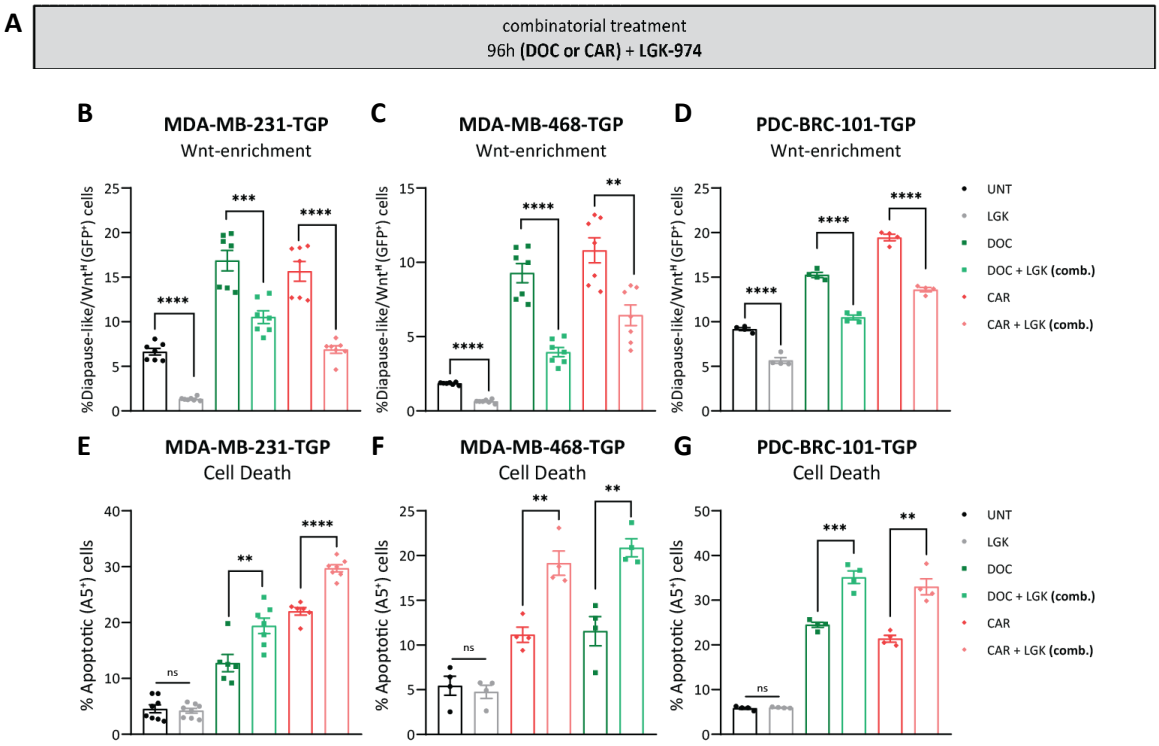


Fig. 4.27: Combinatorial treatment strategy inhibiting Wnt ligand-secretion alongside chemotherapy *in vitro*. Legend on page 81.

Interestingly, the use of LGK-974 in *in vivo* xenograft models (Fig. 4.28A), combined with either DOC or CAR treatment, resulted in a substantial and significant decrease in Wnt pathway-activation, correlating with marked reduction in tumor volume compared to sole chemotherapy-, LGK-treated, or VEH-groups (Fig. 4.28B-E and representative images Fig. 4.28F). Notably, LGK-treatment, sole or in combination with either chemotherapeutic agent, had no significant effect on mouse body weight observed during the three weeks of treatment (525) (Fig. 4.28G).

Categorical efficacy endpoint analysis, based on volumetric tumor measurements, was performed to assess tumor response to treatment, categorizing outcomes into progressive disease (PD), stable disease (SD), partial response (PR), and complete response (CR) (499). In the VEH-group, 75% (6/8) of tumors were classified as PD, and 25% (2/8) were classified as SD (Fig. 4.28H). LGK-974 treatment alone had no impact on tumor response with 75% (6/8) of tumors classified as PD and 25% (2/8) as SD, similar to VEH-conditions (Fig. 4.28H). In sole DOC- or CAR-treated groups, 100% (7/7) of tumors were classified as SD, confirming the efficacy of chemotherapy in controlling tumor growth (Fig. 4.28H). In the DOC+LGK-treated tumors, categorical efficacy endpoint analysis revealed tumor responses as 28.6% (2/7) SD and 71.4% (5/7) PR (Fig. 4.28H), improving outcomes compared to sole DOC-treatment (100% SD). Similarly, in the CAR+LGK-treatment group, tumors were classified as 42.8% (3/7) SD, 28.6% (2/7) PR, and 28.6% (2/7) CR, demonstrating the benefits of combining chemotherapy with Wnt secretion-inhibition (Fig. 4.28H).

In summary, we show that simultaneous/combinatorial treatment, rather than sequential, encompassing chemotherapeutic agents and pharmacological inhibitors of Wnt ligand-secretion, can significantly reduce the induction and/or enrichment of diapause-like Wnt^{High} cells *in vitro,* sensitizing cells to chemotherapy. In agreement, combinatorial treatment in xenograft models is able to significantly enhance the sensitivity of TNBC tumors to chemotherapy.

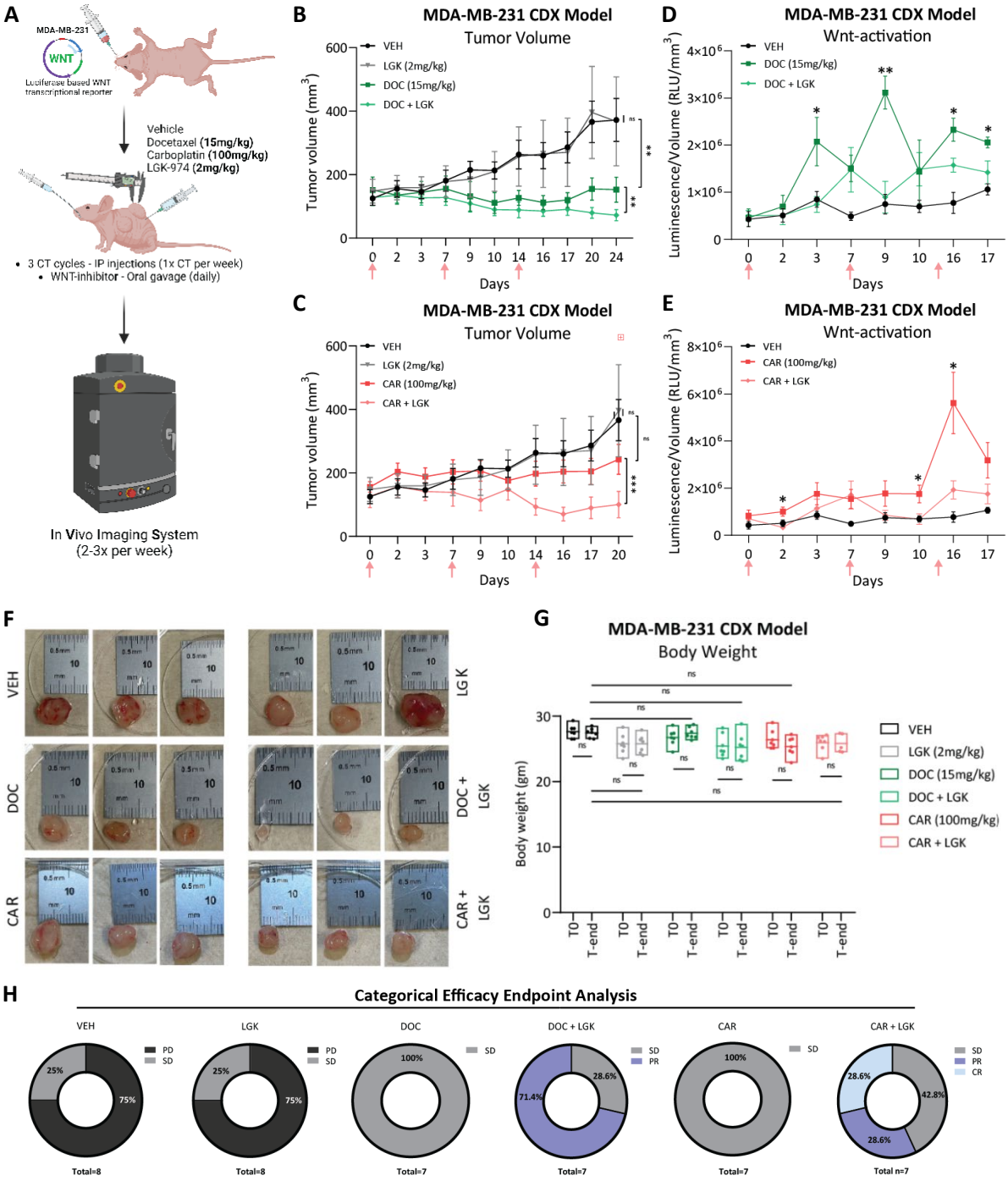


Fig. 4.28: Combinatorial treatment strategy inhibiting Wnt ligand-secretion alongside chemotherapy *in vivo*. A) Schematic representation of CDX experimental setup with Wnt ligand secretion-inhibition *in vivo*. B-C) Tumor growth curves of xenograft models treated with VEH, LGK (2mg/kg/day), DOC (top), DOC+LGK (top), CAR (bottom), and CAR+LGK (bottom) (n = 8,7,6,5 mice per treatment group). Pink arrows indicate administration of chemotherapy. D-E) Levels of Wnt-activation (RLU/mm^3) displayed as luminescent signals (RLU) captured by IVIS Spectrum normalized to tumor volume (mm^3) in xenograft models treated with VEH, DOC (top), DOC+LGK (top), CAR (bottom), and CAR+LGK (bottom) (n = 8,7,6,5 mice per treatment group). F) Representative images (3 per treatment group) of resected tumors from xenograft models treated with VEH, LGK, DOC, DOC+LGK, CAR, and CAR+LGK. G) Bodyweight measurements evaluated at T0 (onset of treatment) and T-end (day of sacrifice) of xenograft models treated with VEH, LGK, DOC, DOC+LGK, CAR, and CAR+LGK (n = 8,7,5 mice per treatment group). H) Categorical Efficacy Endpoint Analysis displayed as percentage of animals per treatment group (VEH, LGK, DOC, DOC+LGK, CAR, and CAR+LGK) classified/assigned to one of four distinct tumor responses to treatment: Progressive Disease (PD), Stable Disease (SD), Partial Response (PR), or Complete Response (CR). For panels B, C, and G the number of animals was as follows: VEH = 8, LGK = 7, DOC = 7, DOC+LGK = 6, CAR = 7, and CAR+LGK = 5. *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

4.7. Preclinical PDO models recapitulate chemotherapy-mediated Wnt-activation and sensitization to synergistic Wnt ligand secretion-inhibition

Transcriptomic analysis performed on RNA-seq-based datasets of longitudinally paired samples of breast cancer patients during NAC treatment (GSE123845 – Fig. 4.29A) (526) showed that a Wnt-signaling signature (derived from chemotherapy-treated Wnt^{High} cells) was significantly enriched in tumor samples obtained from patients undergoing (on-NAC) NAC treatment (Fig. 4.29B). Similarly, a significant enrichment of the Rehman et al. diapause-like gene signature (354) and a significant reduction in the MYC hallmark signature (Fig. 4.29C, D) were seen in samples obtained from patients on-NAC, further highlighting the interplay between the Wnt^{High} and diapause-like cell-phenotypes in a clinical setting.

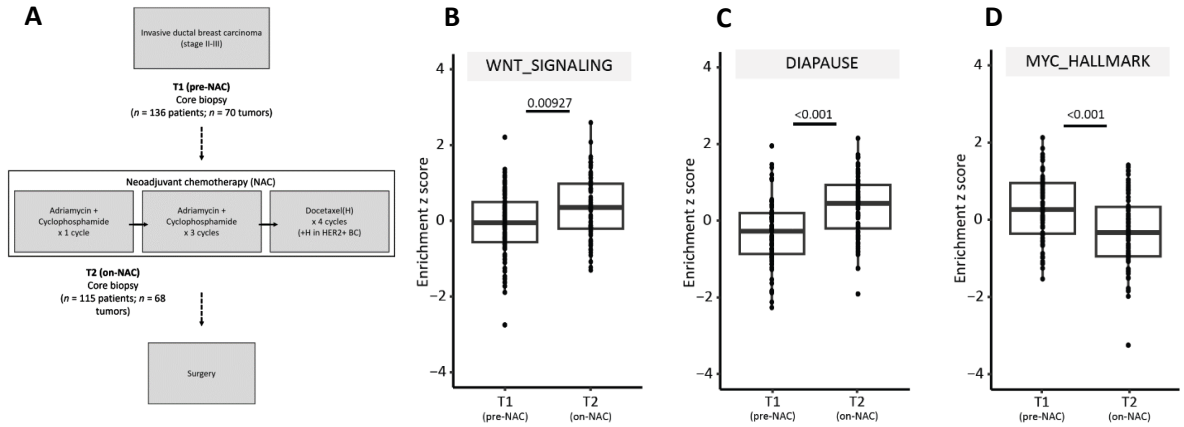


Fig. 4.29: Analysis of BC patient samples under chemotherapeutic exposure. A) Overview of supplementary information of patient dataset (GSE123845) analyzed in this study. B-D) Boxplots showing single sample gene set enrichment scores of our in-house derived Wnt^{High} signature, a Diapause-DTP signature, and MYC GSEA hallmark signature in RNA-seq data of breast cancer biopsies obtained prior to (pre-NAC, n = 70 tumors) and during neoadjuvant chemotherapy (on-NAC, n = 68 tumors). Statistical analysis as performed in Fig. 4.9.

Next, we investigated the effects of chemotherapeutic treatment on pre-clinical 3D PDO models (527). Two different PDO models, R1-IDC113 (113 BCO) and R2-IDC159A (159A BCO) (Fig. 4.30A and representative images Fig. 4.30B) were used. Typically, organoid models are cultured in a growth factor-rich medium (528), of which Wnt ligand (Wnt-3a) and Wnt ligand-enhancer (R-spondin3) are components, possibly influencing studies of Wnt signaling pathway dynamics. Culturing either cancer organoid model for four passages in a $Wnt^{-}/Rspo^{-}$ breast cancer organoid (BCO) medium had no effects on the morphology, proliferation rate, or viability of either PDO model when compared to a baseline ($Wnt^{+}/Rspo^{+}$) BCO medium (529) (Fig. 4.30C).

Fig. 4.30: Overview and culture of pre-clinical 3D PDO models used to assess Wnt-activation under chemotherapeutic exposure. A) Overview of supplementary information of the TNBC PDO models used in the study. B) Phase-contrast images of TNBC-PDO models, 113 BCO (left) and 159A BCO (right) in basal culture conditions at 2.5x (top) and 10x (bottom) magnification. C) Number and viability levels of 113 BCO (left) and 159A BCO (right) models cultured in media not-supplemented with Wnt-Rspo (BCO media$^{Wnt-/Rspo-}$) and media supplemented with Wnt-Rspo (BCO media$^{Wnt+/Rspo+}$) (n = 3 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

A

Sample ID	Tumor	Treatment Status	Treatment	Response	EUSOMA
R1-IDC113	Primary Breast	Pretreated	ddAC	Progression	EUSOMA 3
R2-IDC159A	Primary Breast	Pretreated	AC + paclitaxel + carboplatin + fulvestrant + radiotherapy	Progression	EUSOMA 3

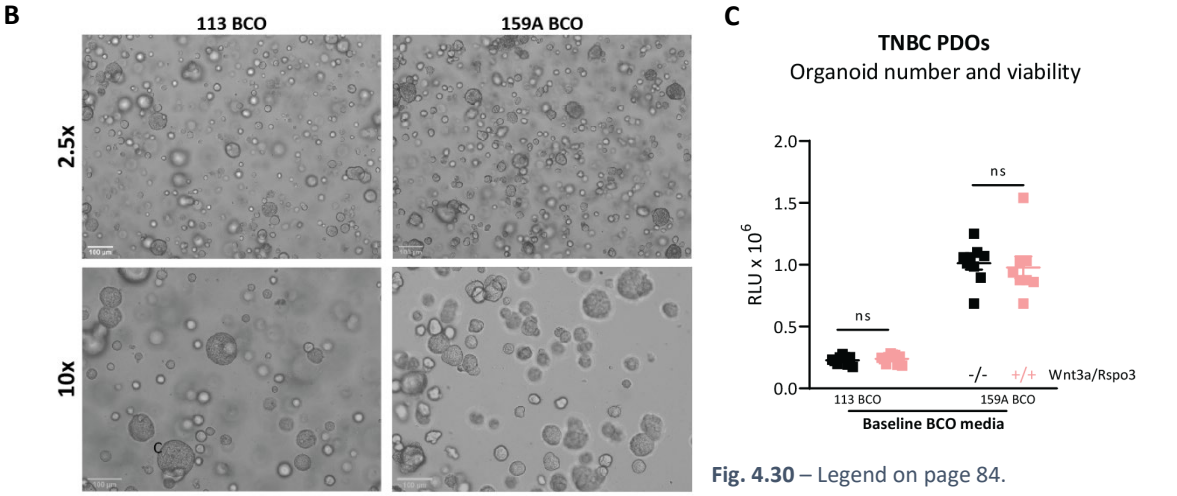


Fig. 4.30 – Legend on page 84.

Upon exposure to IC50 concentrations of either DOC or CAR agents in a Wnt$^-$/Rspo$^-$ BCO medium, both PDO models exhibited a substantial and statistically significant increase of active β-catenin levels (Fig. 4.31A, B) and in expression levels of Wnt-target genes (Fig. 4.31C, D).

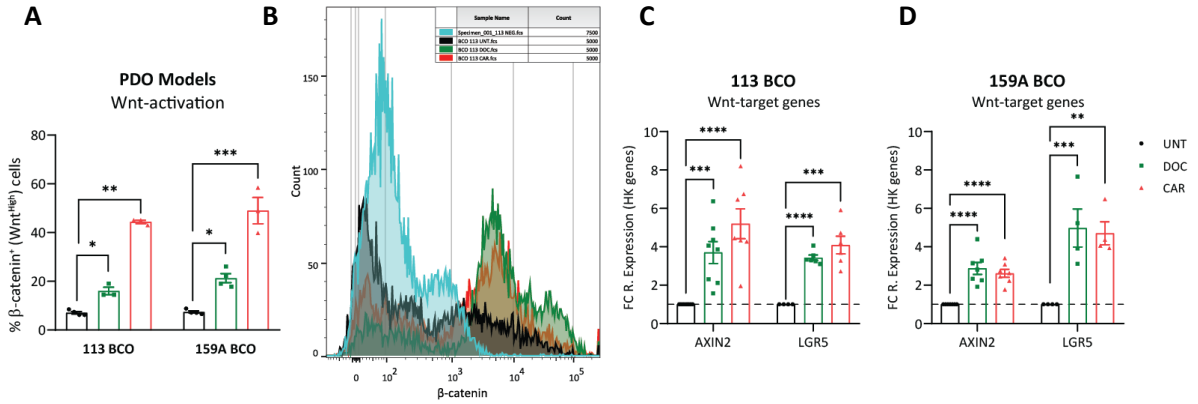


Fig. 4.31: Chemotherapeutic treatment induces Wnt-activation in pre-clinical 3D PDO models. A) Flow cytometry analysis displaying % of Wnt-active (β-catenin$^+$) cells from 113 BCO and 159A BCO models treated with DOC (16nM for 113 BCO and 8nM for 159A BCO) or CAR (50µM for 113 BCO and 125µM for 159A BCO) for 96h (n = 3 independent experiments). B) Representative histograms obtained from flow cytometry-based β-catenin staining displaying counts and intensity of β-catenin signal from 113 BCO model treated with DOC or CAR for 96h. Light blue signal displays background signal obtained from an unstained (NEG) sample. C-D) Gene expression levels obtained via RT-qPCR of Wnt-targets (*AXIN2* and *LGR5*) for 113 BCO and 159A BCO models treated with DOC or CAR for 96h, displayed as fold change (to UNT) of 2^{-dCt} values (relative to HK-genes) (n = 4 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

Treatment with either CHIR or BIO in the 113 BCO PDO model resulted in transcriptional activation of the Wnt signaling pathway, as evidenced by a significant increase in Wnt-target gene expression (*AXIN2* – data not shown). Concurrently, treatment with CHIR or BIO led to a significant decrease in cell number as a possible consequence of growth-arrest (Fig. 4.32A) seen in conjunction with significant suppression of *MYC* and *NMYC* activity (Fig. 4.32B, C). Furthermore, pre-treatment with either CHIR or BIO was able to induce a drug-tolerant phenotype in the 113 BCO PDO model, whereby

we observed a significant decrease in apoptosis induction in cells pre-treated with CHIR or BIO followed by chemotherapeutic treatment when compared to sole chemotherapy exposure (Fig. 4.32D).

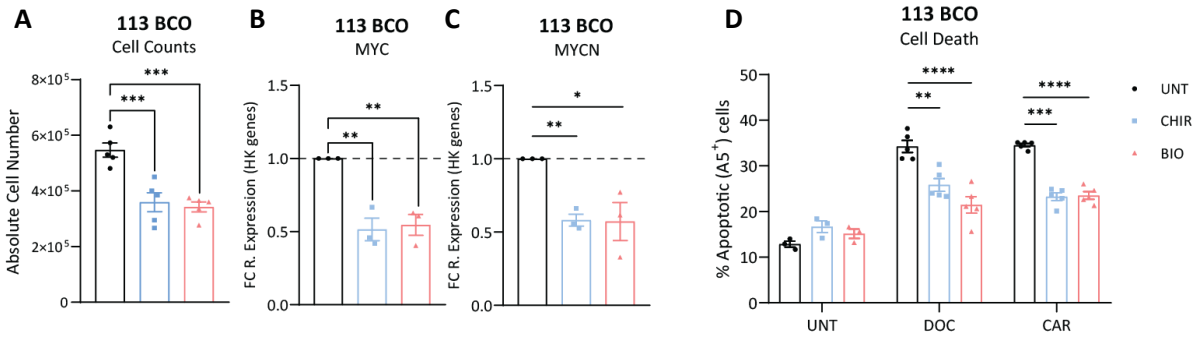


Fig. 4.32: Wnt pathway-activation triggers a reduced proliferative, persister-like state in pre-clinical 3D PDO models. A) Absolute cell number of 113 BCO model treated with CHIR (8µM) or BIO (3µM) for 72h (n = 3 independent experiments). B-C) Gene expression levels obtained via RT-qPCR of *MYC* and *NMYC* for 113 BCO model treated with CHIR or BIO for 72h, displayed as fold change (to UNT) of 2^{-dCt} values (relative to HK-genes) (n=3 independent experiments). D) Flow cytometry analysis displaying % of Annexin V^+ cells of 113 BCO model treated DOC or CAR for 96h (sole or pre-treated with CHIR or BIO for 48h) (n = 3 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

Next, we confirmed significant elevation in the expression levels of Wnt ligands, -enhancers, and -secretion machinery components in chemotherapy-treated PDO models (Fig. 4.33). To investigate the efficacy of the combinatorial treatment strategy, both PDO models were exposed to Wnt ligand secretion-inhibition alone and in combination with increasing concentrations of either chemotherapeutic agent for 96h.

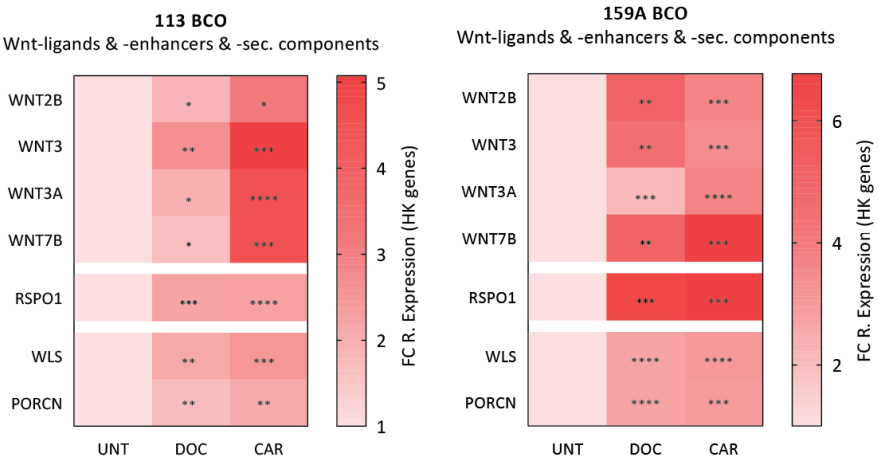


Fig. 4.33: Chemotherapeutic treatment induces elevated transcriptional expression of Wnt ligands, Wnt enhancers, and Wnt secretion machinery components in pre-clinical 3D PDO models. Heatmaps showing gene expression levels obtained via RT-qPCR of Wnt-ligands, -enhancers, and -secretion machinery components for 113 BCO (left) and 159A BCO (right) models treated with DOC or CAR for 96h, displayed as fold change (to UNT) of 2^{-dCt} values (relative to HK-genes) (n = 4 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

At the used concentration, treatment of both PDO models with IWP-2 alone for 96h did not have any effect on cell viability or proliferation (Fig. 4.34A), in accordance with the $Wnt^-/Rspo^-$ culture conditions. However, the exposure of both PDO models to chemotherapy in combination with IWP-2 demonstrated a significant reduction in the percentage of viable cells compared to chemotherapeutic treatment alone (Fig. 4.34B, C). Interestingly, this sensitization effect was most effective when IWP-2

was supplemented with sublethal concentrations of chemotherapy (<16nM DOC and <50µM CAR – 113 BCO | <8nM DOC and <125µM CAR – 159A BCO).

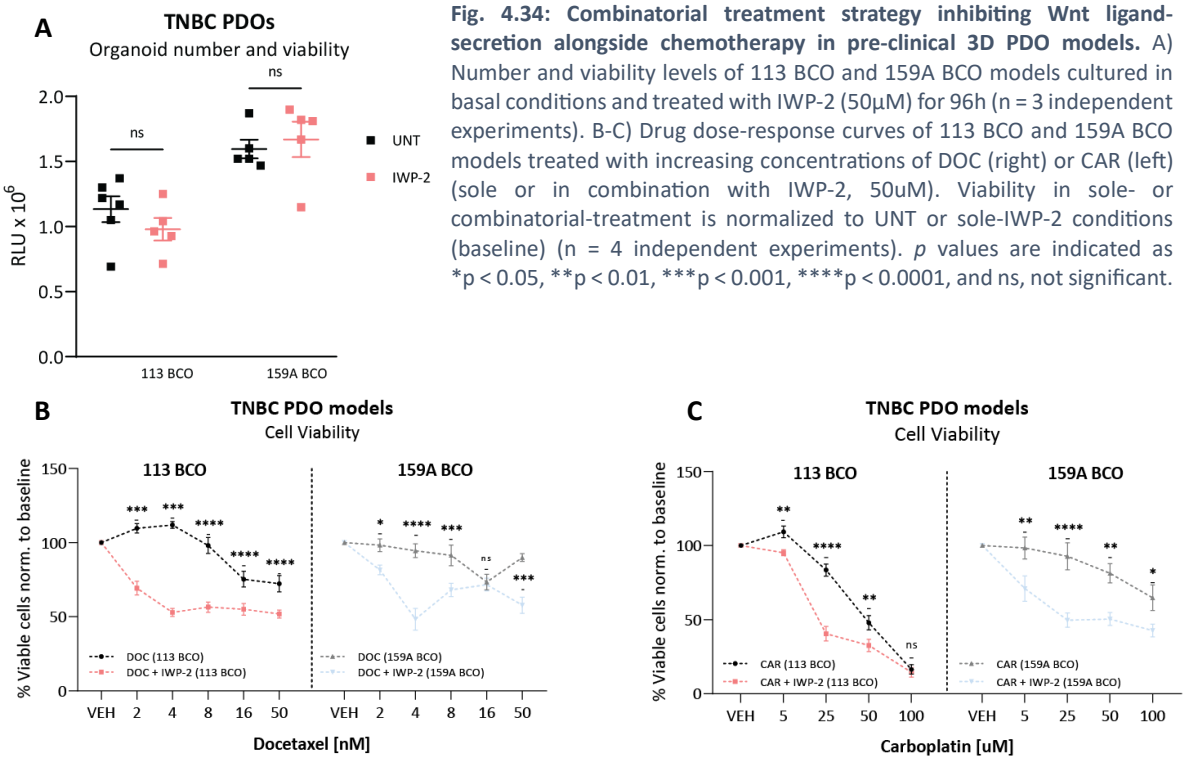


Fig. 4.34: Combinatorial treatment strategy inhibiting Wnt ligand-secretion alongside chemotherapy in pre-clinical 3D PDO models. A) Number and viability levels of 113 BCO and 159A BCO models cultured in basal conditions and treated with IWP-2 (50µM) for 96h (n = 3 independent experiments). B-C) Drug dose-response curves of 113 BCO and 159A BCO models treated with increasing concentrations of DOC (right) or CAR (left) (sole or in combination with IWP-2, 50uM). Viability in sole- or combinatorial-treatment is normalized to UNT or sole-IWP-2 conditions (baseline) (n = 4 independent experiments). *p* values are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns, not significant.

In summary, our data demonstrate chemotherapy-induced Wnt-activation in TNBC pre-clinical PDO models and in transcriptomic datasets derived from patients' samples undergoing NAC. PDO models exhibited a robust and enhanced sensitization to the combinatorial treatment approach comprising Wnt ligand secretion-inhibition alongside sub-lethal (<determined IC50) concentrations of chemotherapy.

Chapter 5: General Discussion and Conclusion

Despite the rapid evolution of therapeutic strategies for TNBC—from conventional chemotherapy and radiotherapy to ICIs, TK inhibitors, ADCs, and DNA repair inhibitors—clinical outcomes remain hampered by the near-universal development of resistance. Drug resistance presents a perplexing hurdle to overcome to improve therapeutic efficacy and overall survival rates. In TNBC, particularly, resistance to chemotherapy, as indicated by an inability to achieve pCR in response to NAC, is associated with significantly higher risks of relapse and recurrence and overall worse survival.

Although much of the historical focus on resistance has centered on pre-existing mutations, accumulating evidence suggests that chemotherapy resistance in TNBC often arises from a complex interplay of ITH, plasticity, and adaptive rewiring in response to therapeutic stress (342,343). The ability of TNBC cells to evade treatment involves not only the selection of pre-existing resistant clones but also widespread transcriptional and epigenetic reprogramming, phenotypic switching, and reshaping of the TME. These dynamic adaptations enable cancer cells to transiently enter drug-tolerant states that are neither terminally differentiated nor fully proliferative, but instead primed for survival under therapeutic pressure. Such reversible states of drug tolerance—marked by altered chromatin landscapes, shifts in lineage identity, and stress response activation—represent a crucial intermediate step before the development of stable resistance. Understanding these non-genetic, reversible processes is therefore essential, as they offer a window of therapeutic vulnerability that may precede irreversible treatment failure.

The emergence of DTP cells during cancer treatment has become a focal point of oncological research due to their pivotal role in drug resistance and subsequent tumor relapse. Recent research has demonstrated that persister cells acquire a slow-growing state, mimicking features of cell dormancy or embryonic diapause characterized by negative *MYC* transcriptional activity (354,420). Importantly, diapause-like persister cells also undergo distinct metabolic reprogramming, often shifting toward oxidative phosphorylation and fatty acid oxidation, enabling survival with minimal proliferative capacity, contrasting with their transcriptionally repressed state (363). Although multiple non-genetic mechanisms have been shown to promote a drug-tolerant state in cancer cells (346,504,530,531), the molecular mechanisms specifically driving a diapause-like persister state remain largely unknown. Moreover, it is also not fully clear whether all persister cells can adopt or reside in a true diapause-like state. Hence, identifying diapause-like cells among other drug-tolerant states within both parental and chemotherapy-treated populations remains challenging. Understanding the early events that lead to a reversible diapause-like persister state could offer new therapeutic strategies to prevent the development of drug-resistant populations before they are firmly established.

Single-cell sequencing technologies, combined with lineage tracing and barcoding approaches, have been proven to be powerful tools in dissecting the complexities of cancer initiation, progression, and drug resistance (532). Studies have shown that single-cell genomic sequencing can track mutational processes and reveal how structural variation contribute to tumor heterogeneity and evolution, as seen in cancers like TNBC (533). Moreover, single-cell lineage tracing in neuroblastoma has revealed how phenotypic plasticity in tumor cells facilitates therapy resistance, while research in hepatoblastoma has identified key plasticity drivers that enhance drug resistance (534,535). Additionally, studies on cancer persister cells have highlighted the importance of distinct lineage programs in maintaining drug-

tolerant states, which enable survival across different cancer types (419). These techniques would allow for precise tracking of individual clones, helping to determine whether diapause-like states emerge from specific clonal lineages or arise stochastically under therapeutic pressure. Single-cell technologies could also provide valuable insights into the dynamic and reversible nature of drug tolerance, further informing strategies to identify potential therapeutic windows before resistance becomes irreversible.

Cancer DTP cells exhibit features of EMT, which are associated with poor drug responsiveness, a senescence-like gene signature, and enhanced stemness (352,536,537). Furthermore, upon discontinuation of treatment—commonly referred to as a drug holiday—diapause-like persister cells resume growth and proliferation, and their progeny retain sensitivity to chemotherapy (538). Only a few biomarkers have been associated with persister cells enriched after chemotherapy, including growth and differentiation factor 15 (GDF-15) as well as developmental and pluripotency-associated factors such as aldehyde dehydrogenase (ALDH), CD133, CD44, and KDM5B (371). While these biomarkers may help identify stress tolerance as a feature of broad drug-tolerant states, specific biomarkers for the diapause-like persister state are still lacking.

While prior studies have shown that cellular persistence *in vitro* typically emerges around day 9 of continuous therapeutic challenge (390), the molecular signals initiating or guiding cells towards a persister state, particularly one resembling diapause, remain poorly understood. In this study, we defined the 72-hour timepoint as an early response to treatment based on observations that it denotes a significant reduction in both cell number (~50%) and viability (~30%), marking the onset of early persister cell development.

Our findings revealed a significant enrichment of the canonical Wnt/β-catenin signaling pathway, highlighted by the percentage of transcriptional (Wnt^{High}) cells, alongside an increase in the intensity of Wnt-activation in various TNBC models subjected to distinct chemotherapeutic agents. Our transcriptional screens and functional analyses revealed that among early persister cells induced in response to chemotherapeutic pressure, only the Wnt^{High} cell-population, as opposed to the Wnt^{Low} cell-population, exhibits a true transcriptional diapause-like cell-phenotype and function as *bona fide* early diapause-like drug-tolerant persister (diapause-like Wnt^{High}) cells. This indicates that not all early persister cells correlate with a transcriptional and functional diapause-like cell-phenotype and highlights that Wnt transcriptional activity serves as a distinct transcriptional and functional biomarker for the diapause-like cell-state amongst early persister cells. Moreover, we highlighted that transcriptional Wnt-activity as a functional marker for early diapause-like persister cells even under chemotherapy naïve conditions. Under chemotherapeutic treatment, activation of the Wnt/β-catenin signaling pathway occurred primarily in a *de novo* manner and slowly diminished upon drug withdrawal with cells returning to normal proliferation and drug-sensitivity, highlighting the transient and dynamic nature of this chemotherapy-mediated transcriptional and functional adaptation. Mechanistically, we showed that chemotherapy-induced Wnt/β-catenin signaling activation was primarily dependent on the expression and functional secretion of various Wnt-ligands and -enhancers, as well as the activity of Wnt-ligand secretion machinery, exerting their effects in an autocrine and/or paracrine manner. Interfering with the functional secretion of Wnt ligands, either via genetic manipulation or pharmacological inhibition, alongside chemotherapeutic treatment was able to significantly reduce activation of the Wnt signaling pathway and the induction of a diapause-like persister phenotype, rendering cancer cells sensitive to the cytotoxic effects of chemotherapy (Fig. 5.1).

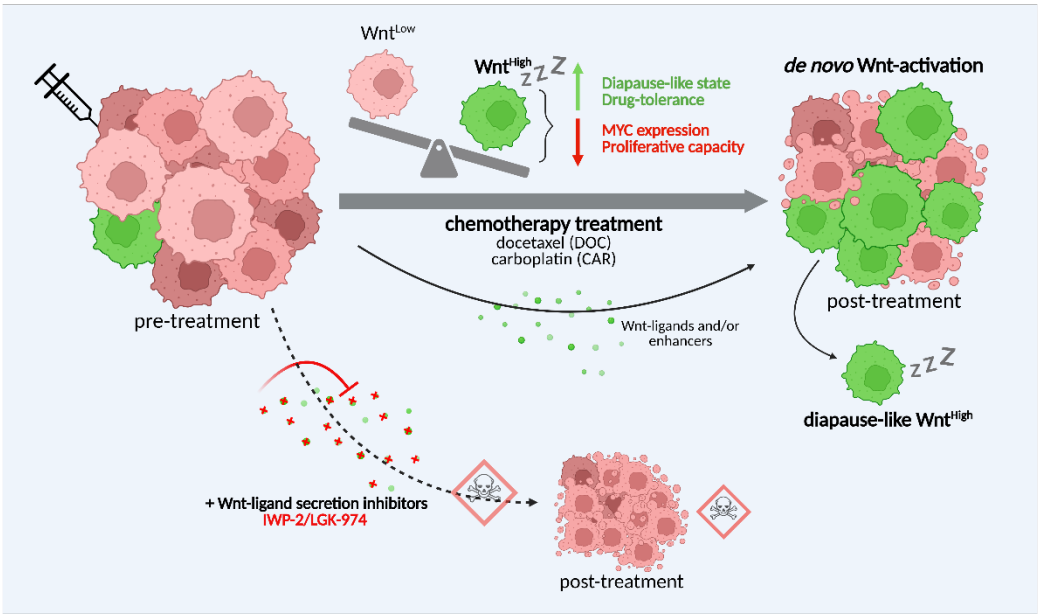


Fig. 5.1: Schematic Overview.

The Wnt/β-catenin signaling pathway regulates a variety of cellular processes, including cell fate commitment, differentiation, proliferation, and stem cell maintenance (539,540). Wnt proteins (Wnts) are a family of 19 secreted, glycosylated, and palmitoylated ligands that primarily interact with FZD receptors and LRP5/6 to promote activation of the canonical (β-catenin-dependent) or non-canonical (β-catenin-independent) pathways (520). Activation of the canonical Wnt signaling pathway leads to stabilization of β-catenin, promoting the transcription of target genes. Wnt signaling is potentiated by the presence of RSPO ligands, binding LGR4-6 co-receptors, preventing FZD receptor degradation (520).

Notably, and despite the well-established role of the Wnt signaling pathway in CSC maintenance, the transcriptional profile of chemotherapy-induced Wnt^{High} cells did not align with a $CD24^{Low}/CD44^{High}$-derived transcriptional profile (284) which is typically associated with TNBC CSCs. Furthermore, this dynamic, transient, and reversible behavior places chemotherapy-treated Wnt^{High} cells closer to the DTP cell criteria and apart from the criteria of CSCs, providing a novel perspective on the poor clinical outcomes in TNBC patients with Wnt-deregulation (311,312,452).

Although mutations in components of the Wnt signaling pathway are rare in TNBC, deregulation of canonical Wnt signaling and β-catenin stabilization in Wnt-addicted tumors have been previously correlated with poor prognosis in TNBC patients (82,311,312,452,510). Unlike previous studies, which primarily explored the role of the Wnt signaling pathway in TNBC under standard (unchallenged) conditions (541,542), our current work shifts the focus to cellular responses under and during chemotherapeutic treatment. Our findings reveal a significant enrichment of Wnt-activity (Wnt^{High} population) in tumors subjected to chemotherapy, suggesting that even tumors with initially low or non-aberrant levels of Wnt-activation may become Wnt-enriched upon treatment exposure, thereby promoting entry into a diapause-like early persister cell-state. Current clinical trials (NCT03447470 and NCT01351103) investigating the effects of PORCN inhibition in Wnt-deregulated cancers are focused on metastatic patients with Wnt-altered tumors and exclude the consideration of chemotherapy (523,524). Our results indicate that inhibiting Wnt ligand-secretion could be beneficial, not only for tumors initially characterized as Wnt-addicted under baseline conditions, but also for all TNBC patients

undergoing chemotherapy, whereby Wnt ligand secretion-inhibition would prevent the enrichment of a diapause-like persister cell-population.

A recent study by Wang et al. demonstrated that Otulin, a linear linkage-specific deubiquitinase, enhances β-catenin activity in a Wnt-ligand-independent manner under genotoxic stress, primarily occurring during the early stages of drug treatment and lasting up to 24h (469). In contrast, our study highlights that chemotherapy-induced Wnt activation relies on Wnt ligands in various TNBC models and persists well beyond the initial 24-hour window following treatment exposure.

While Wnt pathway activation is traditionally linked to **(i)** increased proliferation through elevated *MYC* levels (514,515,543,544) promoting tumorigenic effects and **(ii)** BCSC maintenance to support drug tolerance (82,312,452,504,545), our findings show that chemotherapy induced Wnt-activation does not enrich BCSCs ($CD24^{Low}/CD44^{High}$ cells). Instead, chemotherapy-mediated Wnt-activation promotes a reversible diapause-like early persister cell-state. Furthermore, Wnt-activation – whether triggered by chemotherapy or GSK3 inhibition – induces a slow-proliferating, *MYC*-low transcriptional state resembling that of diapause, suggesting that Wnt-activation is one of the main forces driving cellular diapause.

In mouse embryonic stem cells (mESCs), dormancy or slow proliferation is influenced by the inhibition of mTORC1/2 (546). Alternatively, genetic depletion of c-Myc and n-Myc in mESCs induces a pluripotent dormant state that mimics diapause (416,547). Similarly, in cancer cells, pharmacologic inhibition or depletion of MYC promotes a diapause-like state marked by reduced proliferation and increased therapy resistance. Consistent with this, cancer diapause-like persister cells exhibit a negative correlation with transcriptional MYC hallmark-expression, underscoring MYC's central and pivotal role in this process (429).

Previous studies have shown that, under specific conditions, Wnt-activation can indeed lead to *MYC* downregulation or suppression (548–550). One possible explanation for this context-dependent effect is the complex interplay between Wnt signaling and other pathways, such as TGF-β. Notably, Wnt-activation can induce TGF-β signaling (551,552), which is known to antagonize *MYC* expression by promoting cell quiescence and differentiation, ultimately reducing *MYC* transcription (553,554). Interestingly, the reduced proliferation linked to Wnt-activation in TNBC mirrors findings reported in therapy-treated $LGR5^{+}$ cells in basal cell carcinoma mouse models, where increased Wnt-activity leads to a slow-proliferative phenotype (555). This suggests that Wnt-activation might also promote a diapause-like state in other cancer types.

Enrichment of DTP cells has been observed across various chemotherapeutic treatments (346,358,366,390). In our study, we utilized two chemotherapeutics with distinct mechanisms of action: Docetaxel, which induces microtubule stabilization, leading to cell cycle arrest and apoptosis, and Carboplatin, which forms DNA crosslinks that inhibit DNA replication and transcription, ultimately leading to cell death. In our RNA-seq analysis of MDA-MB-231 cells treated with DOC or CAR, we observed around 2,500 (upregulated) DEGs 72h post-treatment. Notably, more than 50% (1,650) of these DEGs were common between DOC and CAR treatments – highlighting that, despite their different pro-apoptotic mechanisms, both treatments result in an enrichment of diapause-like Wnt^{High} cells, showing that distinct chemotherapies converge on the enrichment of Wnt ligand-expression and activation of the Wnt signaling pathway. Interestingly, in regenerative models, such as *Hydra*, increased Wnt ligand-expression has been noted in cells undergoing apoptosis as a pro-survival mechanism in

response to tissue damage (556). This suggests that, under the stress of chemotherapeutic pressure, cells may activate similar pro-survival mechanisms and signaling pathways. In this article, we propose a mechanistic model for acquired chemoresistance in TNBC mediated by the enrichment of diapause-like drug-tolerant cells, involving two phases. Initially, cells sense environmental changes induced by therapeutic pressure, leading to elevated levels of Wnt ligands and enhancers, along with increased Wnt ligand-secretion. Subsequently, cells adapt to these pressures by transcriptionally activating the Wnt signaling pathway, ultimately resulting in the enrichment of diapause-like Wnt^{High} cells. Interestingly, our RNA-seq data highlighted an enrichment of other developmental and pro-oncogenic signaling cascades including Hedgehog, Notch, IL-6/JAK/STAT3, and TGF-β signaling in chemotherapy naïve and chemotherapy treated Wnt^{High} cells, underlining the role of other signaling cascades in the induction of a persister phenotype.

The molecular mechanisms triggering the initial increased expression of Wnt ligands and Wnt enhancers described in this article, or the transcriptional reprogramming previously described in other works in chemotherapy-treated conditions, remain largely unknown (342,343). Studies in other contexts, such as ovarian cancer, have suggested that chemotherapy-induced stress may induce significant epigenetic changes, indicating a potential role of epigenetic regulation in chemotherapy-induced Wnt pathway-activation (557,558). Recent research shows that depletion of H3K27me3 (trimethylation of histone H3 at lysine 27) promotes a drug-tolerant state in TNBC and highlights epigenetic regulation as an early event involved in promoting chemoresistance (360).

The chromatin architecture of diapause-like persister cells is a critical area of investigation, as it may reveal insights into the cellular plasticity that underpins chemotherapy resistance. One key question is whether these cells share epigenetic features with embryonic stem cells or CSCs, such as bivalent chromatin domains marked by H3K27me3/H3K4me3, which are known to maintain pluripotency and allow rapid activation or repression of genes in response to environmental cues (559,560). The presence of these bivalent domains in diapause-like persister cells could suggest that they retain a stem-like epigenetic memory, enabling them to survive chemotherapy and re-enter the cell cycle once therapy is withdrawn (561). Further investigation into their chromatin landscape is essential to understand how genetic regulation supports both their drug tolerance and the reversibility of this state.

Current research predominantly focuses on targeting established DTP cells, for instance using BET inhibitors (562), rather than preventing the acquisition and emergence of a diapause-like persister cell-state. Our results show that combinatorial treatment involving Wnt ligand secretion-inhibition supplemented alongside chemotherapy reduces diapause-like Wnt^{High} cell-enrichment and consequently sensitizes tumors to treatment, holding significant promise for targeting diapause-like cells and in future clinical trials in TNBC. Our study also underscores the importance of temporal considerations in treatment regimens. Crucially, we show that pre-treatment with PORCN inhibitors does not prevent a substantial increase in diapause-like Wnt^{High} cells once chemotherapy treatment is applied, indicating that patients undergoing chemotherapy might not benefit from an initial treatment with Wnt-ligand inhibitors. Therefore, our findings suggest that simultaneous/combinatorial treatment, rather than sequential treatment, encompassing Wnt-inhibitors and chemotherapy might provide a solution to effectively control and prevent chemotherapy-induced diapause-like cell-enrichment while simultaneously sensitizing tumors to the effects of chemotherapy.

While Wnt pathway inhibition through agents like PORCN inhibitors has shown promise in preclinical models of TNBC, emerging evidence also suggests that resistance mechanisms can develop. Mutations in downstream effectors such as β-catenin can bypass the blockade imposed by Wnt inhibitors, restoring Wnt signaling despite inhibition of Wnt ligand-secretion (563). Additionally, compensatory activation of parallel signaling pathways, like Hedgehog and Notch, as we have shown to be upregulated alongside chemotherapy-mediated Wnt activation and diapause-like persister cells, further complicates treatment and allows tumors to evade Wnt pathway-inhibition. Moreover, ligand-independent activation of Wnt signaling through mechanisms like dysregulated GSK3β activity adds another layer of complexity (563). Delineating and understanding these escape routes is essential for improving the durability and efficacy of Wnt-targeted therapies.

The origin of DTP cells is a subject of ongoing debate. Some research suggests that DTP cells may have a stable clonal origin, while others propose that DTP cells enter a temporary drug-tolerant state under the influence of external factors such as chemotherapy. Our live-cell imaging studies, which allow the tracking and tracing of Wnt-reporter TNBC cell lines, indicate that chemotherapy treatment leads to a significant enrichment of diapause-like Wnt^{High} phenotype, primarily in cells that were initially in a Wnt^{Low} state. This suggests a vital role for *de novo* activation of the Wnt signaling pathway in response to chemotherapeutic treatment. Additionally, a lower but notable proportion of diapause-like Wnt^{High} cells in chemotherapy-treated conditions were initially in a transcriptionally Wnt-active state. These observations imply that both intrinsic and acquired resistance mechanisms, driven by Wnt transcriptional activity, coexist and contribute to early DTP cell-formation. Importantly, our results show that activation of the Wnt signaling pathway through GSK3 inhibition triggers a diapause-like state in parental breast cancer cells, even in the absence of therapeutic pressure. This demonstrates that the diapause-like cell-state is not exclusive to chemotherapy-treated samples and can emerge under non-challenging conditions, explaining its presence in untreated (chemotherapy-naïve/parental) populations (346,358,366). The diapause-like state is a transient and reversible cellular condition, suggesting that the molecular mechanisms and signaling pathways promoting entry into the diapause-like state can be deactivated, allowing cells to exit this state once chemotherapy pressure is removed. Indeed, we associate the exit from a diapause-like state during a drug holiday with a decrease in Wnt ligand-expression, indicating that Wnt ligand-expression regulates both entry into and exit from a diapause-like early persister cellular-state.

The reversibility of the diapause-like state is a critical aspect that warrants deeper molecular investigation. While our data demonstrate phenotypic and proliferative recovery upon drug withdrawal, it remains to be determined to what extent transcriptional and proteomic changes fully revert to a pre-treatment state. Longitudinal single-cell transcriptomic and proteomic profiling could provide key insights into whether “memory” of the diapause-like state persists in persister cells, potentially predisposing them to re-enter tolerance under future therapeutic stress. By tracking changes in gene expression and protein markers related to the cell cycle, stemness capacity, and drug resistance, we can better understand the extent of reversibility and identify strategies to target these cells before they fully re-enter the cell cycle and establish stable resistance to subsequent treatments.

One challenge in translating findings from TNBC to other BC subtypes, such as Luminal B-like tumors, lies in the distinct molecular and genomic profiles between these subtypes. While DTP cells and the diapause-like phenotypes have already been described in various cancers, including NSCLC and CRC (346,354,429), it remains unclear whether the Wnt-mediated diapause-like persister mechanism

described here is unique to TNBC or extends to other BC subtypes, such as aggressive Luminal B-like tumors where chemotherapy remains an important therapeutic component. Furthermore, it is unclear if similar mechanisms operate in other epithelial cancers beyond breast, despite shared therapeutic pressures. The specific involvement of Wnt signaling in this mechanism in TNBC suggests that further investigation is needed to explore whether similar pathways contribute to DTP enrichment in other cancer types. Testing across multiple preclinical models could help determine the degree of generalizability of this mechanism versus their specificity to TNBC.

Our work highlights that the transcriptional activity of the Wnt signaling pathway, in parental (chemotherapy naïve) tumors as well under chemotherapy-treatment conditions, serves as a biomarker and mechanistic player inducing a diapause-like persister cell-state. A combinatorial treatment strategy employing chemotherapeutic agents alongside targeting the Wnt signaling pathway through Wnt ligand secretion-inhibition is able to significantly impact the enrichment and induction of a diapause-like persister cell-population, significantly sensitizing tumor cells to therapy.

Limitations and Future Directions:

While this thesis provides valuable insights into the dynamic role of Wnt signaling in mediating chemotherapy resistance in TNBC through the induction of a diapause-like persister cell-state, several limitations must be acknowledged. First, our experimental models – though various and robust – relied exclusively on cell lines, cell line-derived xenografts, and PDO models, which, despite their utility, do not fully capture the complexity of the TME. The absence of immune and stromal components limits our understanding of how non-cancerous cells contribute to Wnt pathway activation and subsequent chemotherapy resistance. Given the role of immune evasion in Wnt-driven tumors, future studies should integrate immune-competent and stromal-rich models to better mimic the native tumor environment. TAMs, Tregs, and CAFs can influence Wnt signaling, promoting tumor progression and drug tolerance. Incorporating these compounds could deepen our understanding of DTP cell maintenance and enable testing of immune-modulatory therapies that disrupt the TME's protective roles in supporting diapause-like persister cell-states. Another limitation in our *in vivo* xenograft models was the lack of histopathological evaluation for assessing pCR. While categorical efficacy endpoint analysis, based on volumetric measurements, was used to assess tumor response to treatment, this method does not fully capture the cellular and tissue-level changes that pCR would reveal. Future studies should incorporate histopathological analysis to better correlate tumor regression with pCR, providing a more comprehensive assessment of treatment efficiency.

Secondly, while we identified striking transcriptional and functional differences between chemotherapy-induced (and chemotherapy-naïve) Wnt^{High} and Wnt^{Low} cell populations, we did not investigate the epigenetic landscape of these two populations. Since the DTP cell-phenotype is increasingly associated with epigenetic plasticity, investigating chromatin accessibility and histone modifications in these populations using techniques like ChIP-seq or ATAC-seq could reveal whether these cells exhibit CSC-like chromatin features or a distinct phenotype that maintains their drug-tolerant state. Understanding these epigenetic marks could uncover critical regulators of reversibility and therapeutic vulnerability, providing insights into the regulatory mechanisms underlying their emergence and maintenance.

Moreover, the upstream cues triggering Wnt pathway activation remain largely uncharacterized in our system. Although we observed elevated expression of Wnt ligands, -enhancers, and -secretion machinery, the cellular sources and regulatory mechanisms driving the production and release of these activators remain elusive. It is plausible that in response to therapeutic pressure, cancer cells leverage the production and release of pro-survival factors via an apoptosis-driven mechanism such as that shown in the *Hydra* model. Similarly, our data showed consistent downregulation of *MYC* upon Wnt activation, both in response to chemotherapeutic exposure and GSK3 inhibition. However, the molecular mechanisms underlying this phenomenon are not yet fully elucidated. Given MYC's central role in cell proliferation, understanding the relation between Wnt activation and *MYC* suppression could reveal novel regulatory circuits relevant to the induction of a drug-tolerant phenotype.

While Wnt pathway-inhibition shows promise in combination with chemotherapy, the clinical use of Wnt inhibitors is often hindered by systemic toxicity, due to the pathway's involvement in essential developmental processes. Broad inhibition of Wnt signaling can result in off-target effects, affecting normal healthy tissues and organs. Our combinatorial treatment strategy involving pharmacological inhibition of Wnt signaling alongside chemotherapy, proved effective but lacked the specificity required for clinical application. To overcome this, tumor-selective strategies such as nanoparticle-based formulations or ligand-targeted ADCs carrying Wnt inhibitors could help deliver therapy directly to tumor cells, minimizing systemic exposure and reducing adverse effects. Further refinement could involve genetic perturbation techniques, such as CRISPR-Cas9 or inducible shRNA models in *ex vivo*

PDO models or *in vivo* xenografts, to achieve a more precise inhibition of Wnt signaling. This approach could offer a targeted route to disrupt Wnt ligand secretion at its source, but clinical translation still faces challenges due to systemic toxicity. The need to identify tumor-specific vulnerabilities within the Wnt signaling cascade – such as Wnt^{High}-specific transcriptional co-factors or downstream effectors – is critical to improving the precision and safety of future treatments, thereby enhancing the therapeutic window while minimizing side effects.

Lastly, while our work focuses on chemotherapy resistance in TNBC, Wnt signaling is increasingly recognized for its role in resistance mechanisms to other therapeutic modalities, including ICIs, ADCs, and PARP inhibitors. Wnt signaling has been implicated in immune evasion, particularly through the upregulation of PD-L1 expression, which would contribute to resistance against ICIs. Additionally, Wnt-activation is known to influence cellular processes such as DNA repair, potentially impacting the efficacy of PARP inhibitors, especially in BRCA-deficient tumors, although further research is needed to clarify this connection. Given the growing interest in combining ICIs with other therapies, exploring Wnt-targeted strategies alongside immune-based therapies or DDR inhibitors could offer potential benefits in overcoming resistance. Moreover, investigating the role of Wnt signaling in resistance across these therapies, namely through the induction of a diapause-like persister phenotype, will be crucial for developing more effective and durable treatments for TNBC and other cancers.

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Personal Contributions

Chapter 1: This chapter was entirely written by Youssef Ellaithy and corrected by the promotor Prof. dr. Frederic Lluis Viñas. Some sections were adapted from the review "*Wnt Signaling in the Breast: From Development to Disease*" by Abreu De Oliveira, W., El Laithy, Y., et al. published in the journal *Frontiers in Cell and Developmental Biology* under DOI: https://doi.org/10.3389/fcell.2022.884467. Figures directly taken from that publication or adapted from other publications or other sources and used in this chapter are clearly indicated in their respective figure legends.

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Chapter 3: This chapter is completely based on the research article "*Chemotherapy-treated breast cancer cells activate the Wnt signaling pathway to enter a diapause-like early persister state*" by El Laithy, Y., et al. currently under reviewer in the journal *Cancer Research*.

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Listed below are the author contributions of the manuscript:

Y. El Laithy: Conceptualization, data curation, formal analysis, investigation, methodology, resources, validation, visualization, writing–original draft. **W. Abreu De Oliveira:** Conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization. **A. Pabba:** Data curation, formal analysis, software, validation, visualization, writing–review and editing. **A. Qualizza:** Investigation, project administration, resources. **G. Cosemans:** Investigation, formal analysis, visualization. **P. Garcia Diaz:** Investigation, visualization. **F. Richard:** Software, supervision, writing–review and editing. **P. Athanasouli:** Data curation, writing–review and editing. **C. Rios Luci:** Methodology, project administration, resources, supervision, writing–review and editing. **W. De Wispelaere:** Investigation. **L. Mourao:** Resources, writing–review and editing. **S. Hamer:** Validation, writing–review and editing. **S. Moens:** Methodology, resources. **A. De Jaime-Soguero:** Investigation, funding acquisition, methodology, writing–review and editing. **M. Baietti:** Methodology, resources, writing–review and editing. **S. Hutten:** Methodology, resources. **J. Jonkers:** Methodology, resources. **S. Sammut:** Formal analysis, investigation, software, visualization, writing–review and editing. **S. Soenen:** Methodology, Resources. **C. Scheele:** Resources, writing–review and editing. **A. Bruna:** Funding acquisition, methodology, writing–review and editing. **C. Desmedt:** Methodology, supervision, writing–review and editing. **D. Annibali:** Funding acquisition, methodology, resources, supervision, writing–review and editing. **F. Lluis:** Conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing–original draft.

Chapter 5: This chapter was written by Youssef Ellaithy and corrected by the promotor Prof. dr. Frederic Lluis Viñas. The majority of this chapter originates from the research article "*Chemotherapy-treated breast cancer cells activate the Wnt signaling pathway to enter a diapause-like early persister state"* by El Laithy, Y., et al. currently under reviewer in the journal *Cancer Research.*

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Use of Generative AI

I did not use generative AI assistance tools during the research/writing process of my thesis, except for mere language assistance and manuscript structuring. The text/code/images in this thesis are my own (unless otherwise specified) and generative AI has only been used in accordance with the KU Leuven guidelines and appropriate references have been added. I have reviewed and edited the content as needed and I take full responsibility for the content of the thesis.

Curriculum Vitae

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PROFESSIONAL EXPERIENCE

Stamcelinstituut Leuven – KU Leuven **Leuven, Belgium**

Research Associate (PhD Candidate), Department of Oncology *April 2019 – present*

As a PhD candidate, I executed fundamental and pre-clinical research in oncology, focusing on innovative solutions to overcome chemotherapeutic resistance and the strategic development novel drug-targets in triple-negative breast cancer

- Strategic Research Initiatives & Executed Projects:
    - (Co-)designed and led cross-disciplinary research projects, publishing 5 peer-reviewed research and review articles, demonstrating analytical and scientific communication skills (ORCID: 0000-0002-8576-4231)
    - Pioneered the integration of 3D *ex vivo* organoid technology and optimized *in vivo* live imaging workflows, significantly improving pre-clinical study capacity and translational research output
    - Delivered 15+ presentations at national and international scientific conferences, showcasing our novel research findings to diverse expert audiences
- Resource Development & Collaborative Alliances:
    - Drafted successful national (FWO, KOTK, STK) and international (FWO-NSFC) research grant proposals contributing to funding continuity and research scalability
    - Established and coordinated collaborations with national and international research groups including a 2-week collaborative exchange/stay at the Shenzhen Institutes of Advanced Technology (Shenzhen, China; 2025)
    - Active contributing member of the Leuven Kanker Instituut (LKI) and the Multidisciplinar Borstcentrum

EDUCATION

KU Leuven **Leuven, Belgium**

M.Sc. and B.Sc. in Biochemical Engineering Technology – Cum Laude *2014-2019*

- Master Thesis: The role of the WNT-signaling pathway in triple negative breast cancer upon chemotherapeutic treatment

**Score: 17.6/20 – top 10% among 113 students over 3 academic years (2016-2019)*

LEADERSHIP & TEACHING

- Supervised and mentored 15+ students across PhD, MSc, and BSc levels; supported thesis design, data analysis, and scientific communication | Faculty of Biomedical Sciences, KU Leuven
- Coordinated and co-lectured a class (4x; *2022–present*) given as part of the Master course: **Trends and Innovation in the Biomedical Sector (B-KUL-T48IBS)** | Faculty of Industrial Engineering, KU Leuven

STRATEGIC PROJECTS & CASE COMPETITIONS

KBC PhD Winter School – Bright Minds for Social Responsibility **Brussels, Belgium**

Team Member *November-December 2023*

Represented Janssen Pharamaceutica on a case: "How to measure social and human aspects of sustainability?"

- Collaborated with a cross-disciplinary team of PhDs and Postdocs to develop a strategic impact framework to quantify and enhance social sustainability in STEM outreach and workforce diversity

International Genetically Engineered Machine (iGEM) **Belgium & USA**

Team Member *February – November 2017*

- Participated in the 2016/17 edition of iGEM alongside a multidisciplinary team of 12 master's students hosted by the Massachusetts Institute of Technology (MIT). I contributed to wet-lab R&D, fundraising, scientific outreach, youth social engagement, and PR-/collaborative-efforts

Awarded **Best New Application at the 2017 iGEM Giant Jamboree*

SKILLS, ACTIVITIES & INTERESTS

- **Languages:** Fluent in Arabic and English, Intermediate in Dutch (Level B1^{+}; ILT), and Basic knowledge in French.
- **Certifications & Trainings**: Workshop "Presence, authenticity, and resilience in communication" (*2024*), Workshop "Pitching your research" (*2023*), In Vivo Live Imaging Systems (*2022*), Interdisciplinary Program in Healthcare and

Innovation (**Université Libre de Bruxelles**; *2021*), Patent Law in Practice (**B-KUL-E01N8B**; *2021*), Workshop "Introduction to R" (FLAMES; *2021*), Flow Cytometry and Cell Sorting (Machine Automation and Data Analysis; *2019* and *2021*), and Laboratory Animal Science (FELASA B; *2019*)

- **Activities:** Coordinator at Kinderuniversiteit (KU Leuven; *2022–present*), Member of/volunteer at Brussels Ironman Nato (BIN; December *2024–present*), Co-founder of the Egyptian Association in Leuven (EAL; *2017–2021*)
- **Interests:** strong passion for sports, aspiring (non-professional) triathlete, controversial taste in music, and passionate about anything kitchen- and food-related

Personal Acknowledgments

After more than 150 pages of thesis writing, countless rounds of revisions, corrections, and adjustments, the most difficult part remains—writing the last section. Here, I don't need to worry about citing the right references or making sure that I've included the most up-to-date literature—I just need to make sure I don't forget anyone. It feels like just yesterday that I stepped into Fredi's office with Willy by his side, scrambling to find a position and a lab for my master's thesis after my previously agreed-upon internship was canceled last minute. What felt, at the time, like a "making do" or an "alternative" opportunity ended up being one of the most impactful and life-defining choices of my life. For reference, "feels like yesterday" actually refers to August 2018. The plan seemed simple: finish the short master's thesis internship at the Lluis lab, and then back to business as usual—who really does a PhD anyway, right? Fast forward to August 2025, almost six years since I officially started my PhD, and to be honest, I'm still not quite sure I have a definitive answer for that.

Between 2019 and now, I attended many PhD defenses and, as a result, read many acknowledgment sections. I find it fascinating how my perspective on this section has evolved over the course of my own PhD. In the early days, I found it "charming" and "sweet" how graduating students wrote such long and exhaustive sections to thank so many people. Was it mere formality, or did they truly feel that much gratitude? As sweet as I found it, I was also a little surprised. A couple of years into my PhD, the typical struggles started: frustrations, long working hours, failures, loneliness, and more. It was during those low points that my perspective changed. This section, right here, is one of the most important parts of my thesis because, without the support I've received—scientifically, professionally, and personally—I wouldn't be in the position to write or defend this thesis. So yes, this section has very little to do with me, and everything to do with **YOU**.

Prof. Frederic Lluis, **Fredi**, I'm not quite sure where to start, and I highly doubt that a paragraph or two could ever fully convey the gratitude I feel for you or what we've been through these past years. I want to start by thanking you, from the bottom of my heart: thank you for everything. Thank you for granting me that first opportunity as a 20-year-old master's thesis student, and for giving me the chance to embark on this PhD journey as part of your lab. Thank you for your guidance and mentorship throughout it all. Most importantly, thank you for never giving up on me—or the project—even when I was ready to do so myself. The support system you've built in your lab is something I can never fully or properly thank you for. On a personal note, thank you for creating a lab environment that allowed me to be myself—to talk, be expressive, and share my thoughts and opinions, even when it felt like 99.9999% disagreements. I will forever cherish the countless memories of morning coffees, lunch breaks, karaoke happy hours, forcing you into sports conversations, Ronaldo-customized gifts, endless Cola Zeros *(with ice and lemon, please)*, and, most importantly, the simple words: "*Can I please say something?*" I am aware that not everyone gets to experience such a warm and open group environment during their PhD, and I count myself extremely lucky to have been part of the **Lluis Lab**. **Gracias por todo, Fredi!**

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to this day. Thank you for offering your scientific insights into our research project and for the extremely invaluable collaboration we've had – I hope this continues long after I've graduated.

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To the members of the Lluis Lab, my colleagues turned closest friends: words will never be enough to describe how thankful I am for every member of this lab, past and present. Each and every member of this lab has helped shape who I am today and has played a role in this PhD journey. To the two most senior, the foundational members who were there when I started, Anchel and Willy – thank you! **Willy**, you helped ignite my interest in research, guided me as both a master's student and a PhD student, taught me so much of what I know today, pushed me to pursue a better version of myself, and—weirdly enough—subconsciously got me into cycling (I guess). You provided an example I still follow, and most importantly, you conceived the very project that became the focus of this thesis. For all of that, and so much more, thank you. **Anchel**, as I stepped in to start, you were on your way out – our interaction in the lab wasn't long, but it was sufficient for me to know that I wanted to do everything I could to try to replicate your hard work, dedication, work ethic, and scientific prowess. Thank you for setting that example!

Then comes the 2019–2024 crew: Chui, Voula, and Tere. Man, man, man, where do I start? I don't think a couple of lines will ever do you justice or be enough to express my gratitude for you. I am incredibly blessed to have had you as part of this journey; I simply can't imagine having done it without you. We started around the same time, struggled together, laughed together, cried together – creating an eternal bond that I will always carry with me. Thank you for being there for me at all times, through the bad before the good. **Chui**, thank you for always being around when I needed you. I knew I could always look at you and see one step ahead. You've played such a significant role in this journey as both a colleague and a close friend. Thank you for showing me that it's possible to continue getting back up and trying again when things don't go your way time and time again – your resilience has been a huge source of motivation. I know you've always been on my case, pulling on every last nerve during our lunch "discussions," but I would be lying if I said I didn't enjoy every fight we had. To Miss **Voula**, my PhD twin, and the one who made me experience what it is like to have an older sister: thank you for giving me a map to follow and navigate this journey. Even though we started at the exact same time, I've always looked up to you in every aspect. Thank you for trusting me with personal and professional matters and for allowing me to take on that "little brother" role during our shared time. I know that toward the end, things didn't end on the rosiest of terms, but I hope you know that I still hold you in the highest regard, whether professionally or personally. I'm sure we'll manage to get back to that little brother/older sister space again! We still owe each other a proper Greece/Egypt exchange trip so we can finally put this debate to rest. **Tere**, I'm not sure if I've 100% forgiven you for starting one year after me and still defending half a year before me, but I'm going to have to let it go, at least while I'm writing this section for you. Thank you for always being a bright presence in the lab, for always being so kind and supportive, and for always presenting a smile or offering a hug when needed most. Even though I've improved a little in my swimming, I'll always be slightly jealous of your perfect swimming form and that flawless turn at the end of the pool!

For the current Lluis Lab crew, the ones carrying the torch: Gwenny, Tijs, Paula, Xiaoren, and Ale. Ever since all of you started, you marked a new era and chapter in the lab, one that especially made me feel like I had overstayed my welcome and that it was time for me to finish and move on. **Gwenny**, thank you for reinforcing my self-doubt that I have zero creativity… Seeing you combine hard work, friendships, crocheting, salsa, game nights, and more has been incredibly inspiring, and it's something I hope to try to implement in my own life moving forward (not *per se* the crocheting—I'm way too unskilled for that). I'm so grateful that you trusted our lab during the move you made, and it's so precious to see you flourishing in different aspects of life ever since your move to Leuven. I will always be grateful for the memories we shared in Shenzhen and Hong Kong—I don't think I would have survived that trip solo without you! And a huge thank you for always being available whenever something urgently needed to be done for the revision. I want to wish you the best of luck during this

last push. **Tijs**, the moustache man, the hardest-working and smartest kerel in the room. Your hard work, dedication, and commitment speak volumes, and I'm certain you're going to get there. Thank you for bringing that fire and competitive edge to the lab environment—sports speaking, of course. It has been such a pleasure to be around you these past three years, and I can't wait to see you reach your full potential and shine. **Paula**, the newest recruit and the one on whom the future of the cancer work will depend. Thank you for this incredible year; you've shown me nothing but support and understanding during this tough period, and for that I am immensely grateful! I wish you tremendous success in what's to come, whether it's the FWO or the project itself. But I know you've got this! And always remember, you can reach out anytime, and I'll always be around to help as best I can. **Xiaoren**, my "bro," I know the move from China was challenging, but it's extremely special to see how well you are doing now in our lab! It has been such a pleasure to chat and work with you, and to see you contributing to the lab's work. I hope that Yang Hansen does well this year—and so do you! **Ale**, the student turned master, our story is a little more complex relative to the others. We've experienced each other's growth—you as a master's student turned extraordinaire lab tech, and me as a junior PhD student hopefully finishing soon. I'll always be so grateful that you trusted me (and the lab) during your internship; it's through you that I learned a lot about how to guide and what type of supervisor I want to be. And I can say that was quite easy considering that you were the best student I've had the pleasure to work with! Both as a student and a lab tech, your contributions to my (our) project have been huge—from countless western blots, FACS runs, transfections, *in vivo* work, to learning to culture organoids together. This project, and therefore my PhD, wouldn't have been possible without you. Thank you for all your effort.

The Lluis Lab has changed a lot throughout these last six years, with many members coming and going—not just PhD students, but post-docs, master's, bachelor's, and exchange students—all of whom have played a role in shaping this journey of mine and for whom I am extremely thankful. **Martina, Ginevra, Aneta, Joppe, Gert, Matteo, Rita, Valerie, Chara, Francesca, and Ana**, thank you all for being a part of this journey.

Having spent so much time at SCIL, I would also like to thank more senior and ex-members of other groups, all of whom left a strong imprint during my earlier days of this journey and who set an example for me to be inspired by and to follow**. Sherif, Bradely, Ryan, Amitesh, San Kit, Ai, Sam, Joel, Anna, Colin, Ben, Wannes, Lehua, Mariana, Rob, Mostafa, Mady, Manoj, Burak, Arefe, Roya, Tine, Emma, Ellen, Silke, Celine, Amber, Nina, Florian, Annelies, Vittoria, Lorenza** (SUIIUU), **Enrico, Natacha, and Giorgia**, thank you for all your help and support! When I look at SCIL now, it's full of new(er) faces of PhD students, post-docs, and lab managers – all with unique journeys and so much to offer. I would like to thank each and every one of them for the time we've shared over the last years, months, or weeks, and to wish them well with all that's to come. **Mairim, Wilhelm, Jonas, Sandra, Eslam, Margaux, Jarne, Angela, Beau, Lixian, Mengjie, Berke, Ana, Diede, Martina, Ashley, Marga, Jianhao, Zhongpeng, and Jie**. I know these paragraphs aren't as long or as personal, but if we've shared that special connection, I know you know how appreciative I am of you!

No PhD work is complete nor possible without great collaborators to help. **Ani**, I still remember the first time we met during the Breast Cancer Journal Club—you had your face as a sticker on the side of your PPT slides, and I found that hilarious. Little did I know you were going to be such an integral part of this PhD journey, both professionally and personally. Thank you so much for the effort you've put into this project, for the countless times I've come to the 7th floor for a chat about work or personal life, and for sharing with me the struggles of what it's like to integrate. You're right there, so keep pushing! **François** and **Christine**, thank you so much for guiding and supervising this collaboration. To

Daniela and **Stijn**, thank you for helping us out when we were in over our heads with PDX models and foreign cell lines, and for adding that little clinical twist when all Fredi and I could do was think quite fundamentally—your contributions helped advance the project significantly. To **Carla**, thank you so much for guiding me through the mouse work when I had zero clue what I was doing—you were always there when I needed help, even when your working situation wasn't ideal. It's because of you that our *in vivo* work could move forward and progress. To **Colinda** and **Larissa**, thank you so much for sharing with us the organoid models and the knowledge and experience necessary to use them for our research. Thank you, Larissa, for being there time and time again for every little question I had or any trouble I faced.

The fantastic five, **Hava**, **Caro**, **Wout**, and **Vincent**, thank you so much for providing the perfect sweet spot of distant colleagues who turned into super close friends. Fate (aka Hava) managed to bring this tandem together, and I'm so incredibly thankful that we still have it intact no matter where our different paths take us! Memories of the yearly Iftar during Ramadan, potluck dinners, Tomorrowland, and more are something that I will always cherish and hope to continue even after I'm finished. And remember, yes—water, Coke, or midday snacks do break your fast!

Now to the non-science, non-academia aspect of my life. To my closest friends in Egypt, my FAM I should say, my **Everyday Shella**. I left Egypt almost 11 years ago to the month as I'm writing this right now, but you've always been there no matter what. I could be gone for months on end and the minute I land in Egypt, it feels like I've never left you guys. My true day ones, thank you for everything. And I promise (inshallah), this was the last of studying!

For the EGYs in Belgium, we started out as the six boys, **Bibo**, **Bakr**, **Siso**, **Huss**, and **Kiks**. And soon after, the group grew, **Kholio**, **Feeto**, and **Fam**. And more time passed, and Bibs led the way and now (elhamdouleallah), the group has a little flavor with **Silo** and **Menna**. No words would be enough to fully describe how grateful I am for you guys. You've been one of the strongest support systems I've had throughout these last 11 years and will continue for a lifetime to come inshallah. Baheboko aktar men ay haga fel donya kolaha walahi. Thank you for everything boys (+ Silo and Menna). Since we're here then I'm going to have to count in the EGYs (by association and proxy) in Belgium: **Leti**, **Cas**, and **Rein** – thank you guys for always being there for me.

Voor **Johan** en **Hilde**, ik weet echt niet goed waar of hoe te beginnen. Ik kan gewoon beginnen met 'dankjewel' te zeggen. Ik zal nooit echt kunnen uitleggen hoe dankbaar ik ben voor alles wat jullie de afgelopen acht jaar voor mij gedaan hebben. Jullie zijn altijd een bron van steun, warmte en liefde geweest – in elke kant van het leven. De laatste acht jaar zijn jullie er altijd geweest als ik het het meest nodig had, door de moeilijke momenten en de fijne momenten. Ik zal nooit echt kunnen zeggen hoe gelukkig ik mij voel dat ik jullie hier in België als familie heb en dat ik mij hier thuis mag voelen. Ik ben opgegroeid in een kleinere familie in Egypte en heb hier in België ontzettend veel geluk gehad – **Simon**, **Emma**, **oma Anna**, **tante Leen**, **nonkel Luk** en de hele families **Beernaert** en **Verschaeve**, bedankt om mij deel te maken van jullie prachtige grote familie.

بابي ومامي، سامر وغادة، الحقيقة أنا مش عارف أبدأ منين أو إزاي، لأني حاسس إنه مهما قلت ومهما كتبت ومهما اتكلمت مش هقدر أبداً أوفيكو حقكو كله عليا. في السكشينس اللي فاتت كنت بكتب للناس بشكرها على وقفتها جنبي ومساعدتها ليا في الشهور أو السنين اللي فاتت... بس الحقيقة مش عارف أكتب كلام يشكر أهلي اللي واقفين جنبي من يوم ما اتولدت لحد دلوقتي. تلاتين سنة وإنتو مصدر دعم، مصدر حب، مصدر إلهام ما بيتنسيش، ومصدر حاجات كتير من غيرها ما كنتش هقدر أخلص الدكتوراة دي أو أحقق أي حاجة كبيرة في حياتي. شكراً على كل تضحية عملتوها، شكراً على إنكم دايمًا موجودين، شكراً على إني دايمًا حاسس إني في أمان وإنتو حواليا – حتى لو المسافات بعدتنا شوية. طول ما إنتو ورايا، أنا متأكد إني أقدر أعمل وأعمل وأعمل. أنا عارف إن القرار اللي

أخدته من حوالي 11 سنة إني أسافر ما كانش سهل، بس إنتو دايمًا حطتوني في المقام الأول وكنتوا دايمًا عايزينني أنجح. فأتمنى من خلال الدكتوراة دي أبقى حققت حتى لو 1% من الإنجازات اللي تخليني أرفع راسكو زي ما إنتو طول عمركو رافعين راسي.

El **zoooouzzz**, ahla akh del donya dih kolaha. Thank you for always being there through it all, even at times (almost always) when I demanded too much. Forever looking up to you and forever inspired by you. To many big things to come inshallah ya zooz. **Mariouma** el gameela, you have so much ahead of you and I know you got this. Love you so much!

تيتا حبيبة وحنونة، شكراً على وجودكو حواليا وعلى دعمكو المستمر. مفيش إحساس في الدنيا أحلى من إن الواحد بيعمل حاجة ترفع راس عيلتو بيها. **أحمد، حبيبة، انكل عمرو وليلي** الجميلة – بحبكو يا أحلى عيلة في الدنيا كلها.

جدو الليثي، جدو علي، وكوكي – كان نفسي تكونو معانا النهاردة. على طول على بالي وبفكر فيكو دايمًا.

To my best friend, partner, roomie, and for just under one year, my wife, **Leo**—thank you. One of the first sentences I learned in Dutch class was "*Als je iets nodig hebt, sta ik altijd voor je klaar*" – this phrase perfectly summarizes how I feel right now when reflecting on our relationship and what you've meant to me throughout this journey and beyond. You've always been there, never left my side, from the moment we first met until this very moment while I'm writing this. You've been by my side, and I've never once doubted that you wouldn't be. Thank you for the unconditional support, love, sacrifice, effort, and inspiration that you've provided me with during this journey – it has been like nothing else I've experienced in my entire life. You've pulled me through every rough moment, celebrated with me through every happy one, and given me direction and hope when I felt that all was broken. I'm beyond lucky to have you as a companion and partner throughout this little journey of ours – with you by my side, I feel like I can overcome anything that comes my way. Thank you for having my back (and *ma neck*), I love you endlessly, Leo.