

# Triple-negative Breast Cancer: Identification of circRNAs With Efficacy in Preclinical *In Vivo* Models

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**Abstract.** Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer with insufficient options for therapy. In order to identify new targets and treatment modalities we searched the literature for circular RNAs (circRNAs) which mediate efficacy in TNBC-related *in vivo* preclinical models. In addition to 5 down-regulated circRNAs which modulate tumor-suppressive pathways, we identified 15 up-regulated circRNAs. Down- and up-regulated refers to expression in corresponding non-transformed cells and tissues. The up-regulated circRNAs comprise five transmembrane receptors and secreted proteins as targets, five transcription factors and transcription-associated targets, four cell-cycle related circRNAs and one involved in paclitaxel resistance. In this review article we discuss drug-discovery related aspects and modalities of therapeutic intervention. Down-regulated circRNAs can be reconstituted by re-expression of corresponding circRNAs in tumor cells or up-regulation of corresponding targets. Up-regulated circRNAs can be inhibited by small-interfering RNA (siRNA) or short hairpin RNA (shRNA)-based approaches or inhibition of the corresponding targets with small molecules or antibody-related moieties.

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**Key Words:** CircRNA reconstitution and inhibition, circRNA-related pathways, drug discovery, target identification, review.

Triple negative breast cancers (TNBC) are derived from basal-like cells that line the breast cancer ducts, are negative with respect to estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) and comprise 10-20% of all BC (1). Several molecular subtypes have been defined: two basal subtypes (BL) (1, 2), immune-modulatory subtype (IM), mesenchymal subtype (M), mesenchymal stem like (MSL) and luminal androgen receptor subtype (2). TNBC are more aggressive than other types of BC, are characterized by a high rate of relapse and distant metastasis and poor overall survival after standard chemotherapy (3). Treatment of TNBC consists of a combination of surgery, radiation therapy and chemotherapy (4). The last couple of years has witnessed approval of several target-specific agents for treatment of TNBC, such as poly-ADP-ribose (PARP) inhibitors olaparib and talazoparib in patients with breast cancer gene 1 or 2 (BRCA1, BRCA2) mutations and immuno-therapy-based agents, such as monoclonal antibodies (mAbs) Atezolizumab and Keytruda which are directed against immune-checkpoint protein programmed cell death 1 ligand 1 (PD-L1) (5). They are used in combination with chemotherapy for treatment of unresectable, locally advanced, or metastatic TNBC or in a neo-adjuvant setting (5). However, the therapeutic efficacy is less impressive than in other types of cancer (5). The latest approved agent, sacituzumab govitecan, a human trophoblast cell surface antigen 2 (Trop-2)-directed antibody conjugate with a hydrolysable linker and irinotecan (SN38) payload (6, 7). This agent is approved for patients with metastatic TNBC which have undergone at least two prior treatment regimen (8). Further antibody conjugates are under development. Ladiratuzumab vedotin (LV) targets the Zn-transporter LIV-1 with microtubule disruptor auristatin as a payload and NBE-002, an anti-tyrosine-protein kinase transmembrane receptor-1 (ROR-1) mAb-anthracycline conjugate are under clinical development (9, 10). However, further agents with improved efficacy are urgently needed.



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In order to identify new targets and treatment modalities for TNBC we have searched the literature for circRNAs which mediate efficacy in preclinical *in vivo* models of TNBC. We searched the PubMed data-base and identified 113 references which identify up- and down-regulated circRNAs mediating efficacy in TNBC-related preclinical *in vivo* models. From a therapeutic point of view, the identified circRNAs can be reconstituted by gene transfer for down-regulated circRNAs or inhibited by si-RNA or sh-RNA for up-regulated RNAs. Another option is to modulate the identified targets by up-regulation in case of down-regulated targets or inhibition of up-regulated targets with small molecules or antibody-related moieties.

### CircRNA and Cancer

CircRNAs are products of back-splicing events that splice an exon to a preceding exon rather than to a downstream exon resulting in a covalently closed circ RNA molecule (11, 12). CircRNAs were first discovered as viroids in plants and later on in eukaryotic cells (13, 14). It has been demonstrated that they are involved in mediating brain function in mice (15). At least 30,000 different circRNAs have been identified in mammalian cells (16). They are transcribed by RNA polymerase II and are predominantly located in the cytoplasm, whereas a small part is retained in the nucleus (16). In oncology, circRNA can act as oncogenes as well as tumor suppressors (TS) in a context-dependent manner (17). Their best studied function is the sponging of microRNA (miR) which is in the focus of this review (18). In addition, they can bind diverse RNA binding proteins (18). In the nucleus they can modulate gene expression (18). A minority of circRNAs can encode proteins (19). Their functional contribution in oncology has been demonstrated by inhibition of defined circRNAs in patient-derived xenografts resulting in tumor growth (TG) inhibition in lung adenocarcinoma and gastric cancer-based models (20, 21).

### Down-regulated Circular RNAs

*Circ 0001785 targets suppressor of cytokine signaling 3.* Expression of circ 0001785 (Figure 1) was decreased in BC and TNBC cells T47D, MCF-7, MDA-MB-453, MDA-MB-231 and BT-549 in comparison to immortalized breast epithelial cells MCF-10A (22). Circ 0001785 inhibited proliferation, migration, and invasion of BC cells *in vitro* and TG *in vivo* by sponging miR-942 which led to down-regulation of suppressor of cytokine signaling 3 (SOCS3). The latter inhibits Janus kinase/signal transducer and activator of transcription (JAK/STAT) mediated inflammatory signaling. In a TNBC preclinical model, a SOCS-mimetic peptide caused inhibition of primary TG and pulmonary metastases (23). Decreased SOCS-3 mRNA was noted in BC with lymph node metastasis (24) and down-regulation of SOCS genes has been noted in BC in general (25-27).

*Circ ITCH targets WNT7/β catenin.* Circular RNA Itchy E3 ubiquitin ligase (Circ ITCH) (Figure 1) was down-regulated in TNBC tissues and cell lines and was associated with poor prognosis (28). Circ ITCH inhibited proliferation, invasion of MDA-MB-231 and BT-549 TNBC cells *in vitro* and TG and metastasis *in vivo*. These phenomena were due to sponging of miR-17 and -214 resulting in inactivation of WNT7/β catenin signaling, emphasizing the role of circ ITCH as a TS. Canonical WNT/β catenin signaling is triggered by interaction of Wnt ligands with seven transmembrane frizzled receptors with the assistance of low-density lipoprotein receptor-related proteins as co-receptors (29-31). In TNBC, WNT signaling is essential for maintenance of stem-like cells (32).

*Circ AHNK1 targets Ras GTPase activating protein 1.* Circ neuroblast differentiation associated protein AHNK1 (Circ AHNK1) (Figure 1) was down-regulated in TNBC patients and down-regulation correlated with a poor prognosis (33). Overexpression of circ AHNK1 inhibited proliferation, migration, and invasion of MDA-MB-231 and BT-549 TNBC cells *in vitro* and tumor growth (TG) as well as size and number of lung metastases *in vivo* in nude mice. Circ AHNK1 was shown to sponge miR421 resulting in regulation of Ras GTPase activating protein 1 (RASA1) which acts as a TS. Expression of RASA1 is involved in TNBC and negatively correlates with poor survival (34). RASA1 acts as a TS by inactivating RAS from its active GTP-bound form to its inactive GDP-bound form by enhancing GTPase activity and subsequent inhibition of mitogenic signal transmission to downstream partners, such as mitogen-activated protein kinase (MAPK) (35, 36). In hepatocellular carcinoma (HCC) down-regulation of RASA1 correlates with poor prognosis (37).

*Circ FBXW7 targets FBox/WD repeat containing protein 7.* Circ FBXW7 (Figure 1) was down-regulated in TNBC and down-regulation correlated with worse clinical outcome (38). Overexpression of circ FBXW7 suppressed proliferation and migration of BT549 and 4T1 TNBC cells *in vitro* and TG and lung metastases *in vivo* in nude mice (3). Circ FBXW7 sponged miR-197-3p resulting in down-regulation of FBox/WD repeat containing protein 7 (FBXW7). Circ FBXW7 also encodes a FBXW7 185 AA protein which inhibits proliferation, migration of TNBC cells, increasing the abundance of FBXW7 and induced MYC degradation. FBXW7 constitutes one of the four subunits ubiquitin protein ligase complexes and is the substrate recognition component of the SCF E3 ubiquitin ligase, functions as a TS and controls proteasome-mediated degradation of oncoproteins, such as cyclin E, MYC, induced myeloid leukemia cell differentiation protein 1 (MCL1), mammalian target of rapamycin (mTOR), transmembrane receptor NOTCH and aurora kinase A (AURKA) (39). FBXW7 is down-regulated or inactivated by mutation in many types of human cancer, such as BC (40), HCC (41) and esophageal cancer (42).

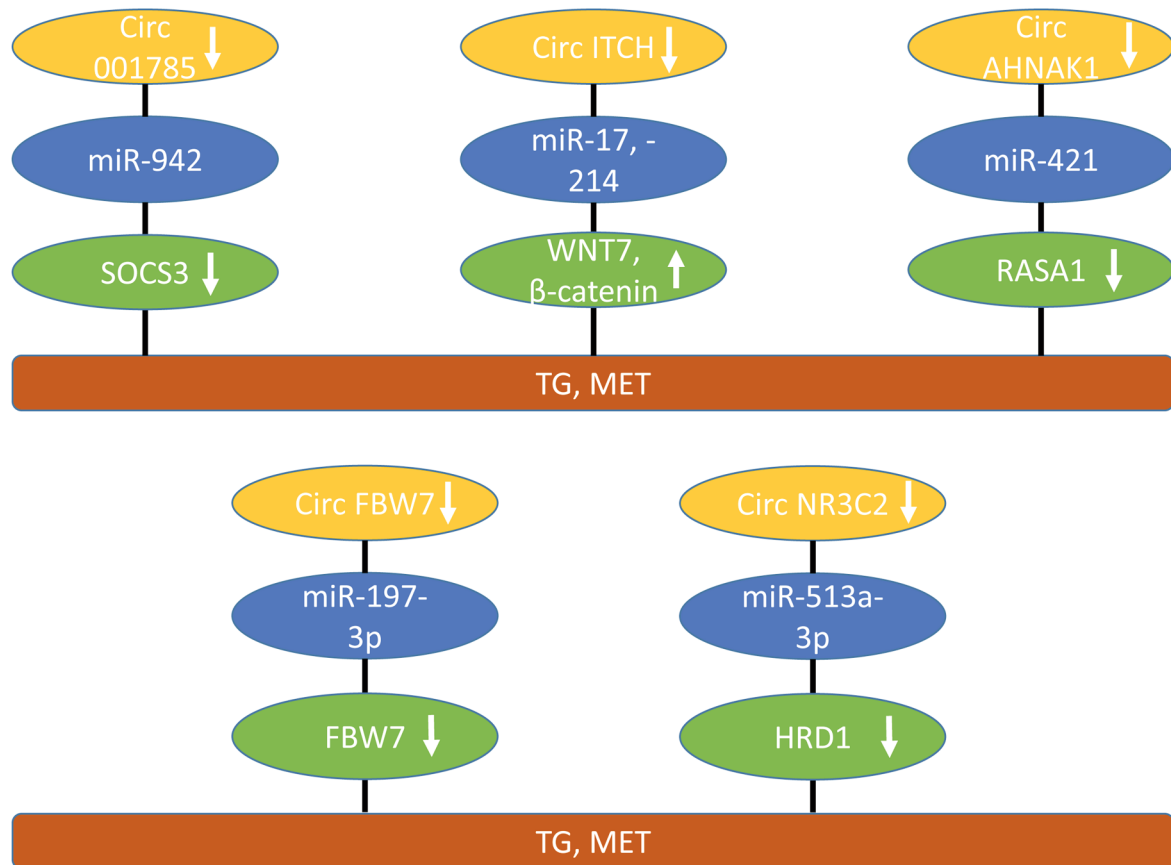


Figure 1. circRNAs down-regulated in TNBC with *in vivo* efficacy in preclinical TNBC-related models. Upward arrows: up-regulated; downward arrows: down-regulated. circ AHNAK1: circ desmoyokin neuroblast differentiation associated protein AHNAK1; Circ ITCH: circular RNA Itchy E3 ubiquitin ligase; FBXW7: F box/WD repeat contain protein X; HRD1: RING-type E3 ligase HRD1; MET: metastasis; NR3C2: nuclear receptor subfamily 3 group C number 2; RASA1: RAS GTPase activating protein 1; SOCS3: suppressor of cytokine signaling 3; TG: tumor growth; WNT7/β catenin: WNT7/β catenin signaling.

Circ NR3C2 targets RING-type E3 ligase. Circ nuclear receptor subfamily 3 group C, number 2 (Circ NR3C2) (Figure 1) was down-regulated in TNBC, sponged miR-513a-3p in MDA-MB-231 TNBC cells and led to up-regulation of endoplasmic reticulum localized RING-type E3 ligase HRD1 (43). Overexpression of circ NR3C2 in MDA-MB-231 xenografts in nude mice reduced TG and metastasis through vimentin degradation. In addition, it has been shown that HRD1 inhibits fatty acid oxidation and tumorigenesis by ubiquitinating carnitin-O-palmitoyl transferase 2 (CPT2) in TNBC (44, 45). It also has been demonstrated that HRD1 suppresses growth and metastasis of BC by promoting insulin growth factor receptor 1 (IGF-1R) degradation (46). Also, HRD1 promotes lung tumorigenesis by inducing sirtuin 2 ubiquitination and degradation (47). One should keep in mind that a single E3 ligase can have opposite effects as TS or oncogene depending on the context or type of cancer involved (45, 46).

### Up-regulated Circ RNAs

*Transmembrane receptors and secreted factors as targets.* Circ IFI30 targets cluster of differentiation 44. Circ  $\gamma$  IFN-inducible thiol reductase (Circ IFI30) (Figure 2) was highly expressed in TNBC and correlated with pathological stage and poor prognosis (48). Circ IFI30 enhanced proliferation, migration and invasion and inhibited apoptosis of MDA-MB-231 and BT549 TNBC cells *in vitro*, facilitated growth, spontaneous metastasis, and angiogenesis of MDA-MB-231 cells subcutaneously implanted into nude mice. This was due to sponging of miR-520-3b which led to up-regulation of cluster of differentiation 44 (CD44). CD44 is a multifunctional cell adhesion receptor which is implicated in tumor progression as well as suppression in a context-dependent and tumor-type specific manner (49). CD44 is also a common marker for cancer stem cells in BC and TNBC (50). In BC and TNBC, CD44 can activate signaling

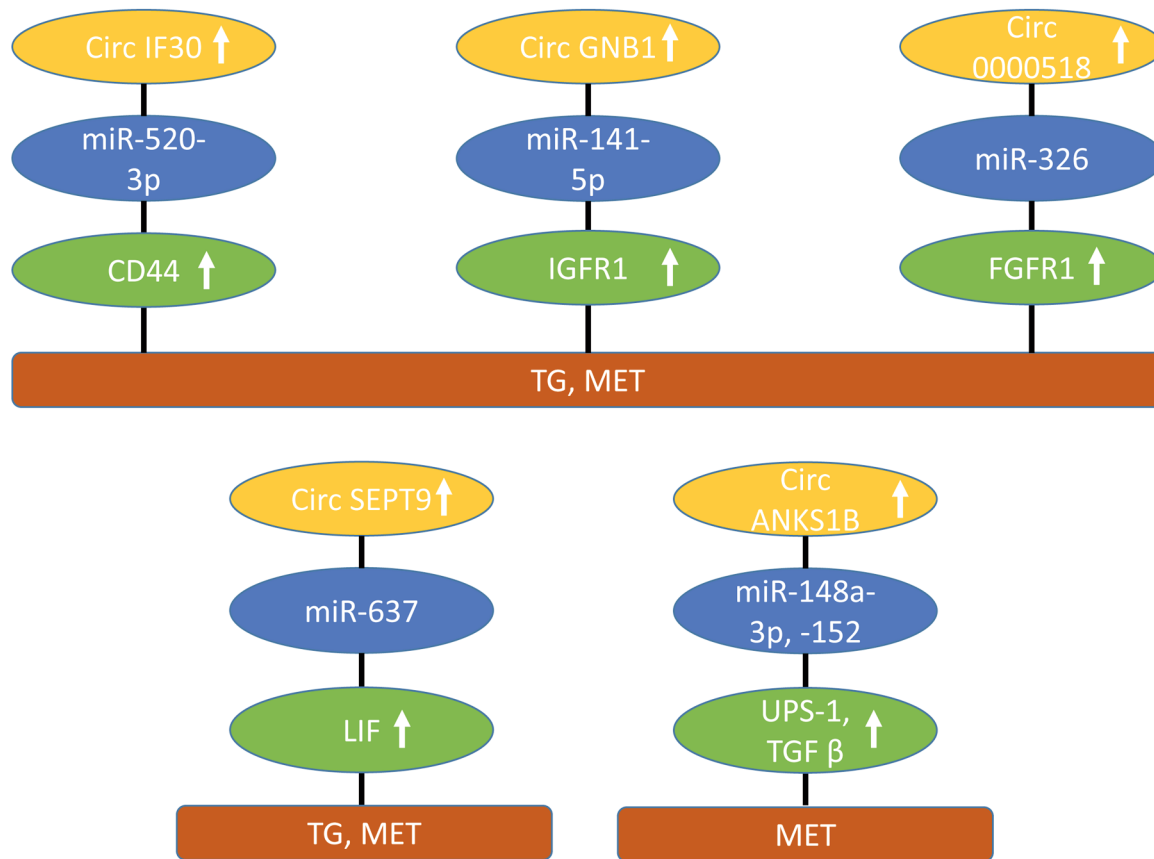


Figure 2. Up-regulated TNBC-related circRNAs increasing transmembrane receptors and secreted factors with efficacy in TNBC-related in vivo preclinical models. Upward arrows: up-regulated; downward arrows: down-regulated. CD44: Cluster of differentiation 44; circ ANKS1B: circRNA ankyrin repeat and sterile alpha motif domain-containing protein 1B; circ IFI30: circ RNA  $\gamma$ IFN-inducible thiol reductase; circ SEPT9: circ RNA septin 9; circ GNB1: circ RNA guanine nucleotide-binding protein subunit  $\beta$ 1; FGFR1: fibroblast growth factor receptor 1; IGFR1: insulin growth factor receptor 1; LIF: leukemia inhibitory factor; MET: metastasis; TG: tumor growth; TGF $\beta$ : transforming growth factor  $\beta$ ; UPS-1: upstream transcription factor-1.

pathways mediated by RHO GTPases, RAS-MAPK, and phosphoinositide-3-kinase, ser-thr kinase AKT (PI3K/AKT) by direct signal transduction or CD44 co-receptor tyrosine kinases MET and HER family of receptors. However, CD44 can also inhibit growth, invasion, and migration of TCs by binding to hyaluronic acid which suppresses epidermal growth factor receptor (EGFR) activation (51, 52). Also, CD44 occurs as numerous splice variants contributing to further functional variety (53). CD44 also links the plasma membrane with the cytoskeleton through binding to ezrin, radixin, moesin (ERM) proteins (54).

*Circ GNB1 targets insulin-like growth factor receptor 1.* Circ guanine nucleotide-binding protein subunit  $\beta$ 1 (Circ GNB1) (Figure 2) was up-regulated in TNBC and correlated with poor prognosis (55). Down-regulation of circ GNB1 suppresses proliferation, migration, and invasion of MDA-MB-231 and BT-549 TNBCs *in vitro*. *In vivo*, circ GNB1 promoted TG and

experimental lung metastases of MDA-MB 231 and BT-549 TNBC cells. Circ GNB1 sponged miR-141-5p resulting in up-regulation of insulin-like growth factor receptor 1 (IGFR1) (55). IGF signaling can promote BC metastasis (56, 57). IGF signaling also promotes tumorigenesis and drug resistance (58). It has been shown that IGF/IGFR1 interaction leads to focal adhesion kinase (FAK) activation and nuclear accumulation of yes-associated protein (YAP) and expression of its target genes in TNBC (59). IGFR1 can be inhibited by antagonizing mAbs directed against its ligands insulin growth factors 1 and 2 (IGF1,2) as well as by small molecules inhibiting its tyrosine kinase function. IGFR1 inhibitor as a single agent gave rise to substantial responses in tumor types, such as Ewing sarcoma and thymoma in a small number of patients, but Phase III studies in other types of tumors have not shown substantial therapeutic benefit (60). This may be due to the complexity of IGFR1 signaling and the lack of appropriate biomarkers for identification of responders.



*Circ 0000518 targets fibroblast growth factor receptor 1.* Down-regulation of circ 0000518 (Figure 2) curbed proliferation, migration, and invasion, arrested cell cycle progression, induced apoptosis in MCF-7 BC and TNBC cells MDA-MB-468 and inhibited TG *in vivo* in nude mice (61). Circ 0000518 acted as a sponge for miR-326 and led to up-regulation of fibroblast growth factor receptor 1 (FGFR1). FGFR1 amplification has been observed in lung cancer and ER+ BC and can activate several signaling pathways, such as RAS-MAPK signaling, canonical and non-canonical WNT signaling, PI3K, hedgehog, Notch and TGF $\beta$  signaling (62). TNBC that display elevated MET and FGFR1 signatures are associated with poor relapse-free survival (63). Targeting of the FGFR pathway is actively pursued in BC (64). Presently, two small molecules inhibiting FGFR signaling are approved by the FDA: pemigatinib for cholangiocarcinoma (65) and erdafitinib for locally advanced bladder cancer (66).

### Circ SEPT9 Targets Leukemia Inhibitory Factor

Circ septin 9 (Circ SEPT9) (Figure 2) was highly expressed in TNBC and its expression was correlated with clinical parameters, such as TNM stage (67). Down-regulation of circ SEPT9 induced cell-cycle arrest, apoptosis, and autophagy in MDA-MB-231 TNBC cells. Circ SEPT9 bound to miR-637 and suppressed miR-637 activity. miR-637 reversed the tumor-promoting effects of circ SEPT9 in TNBC cells. Leukemia inhibitory factor (LIF) was identified as a direct target of miR-637. LIF is a cytokine of the IL6 family which signals through its coreceptor glycoprotein 130 (gp130) and promotes growth and metastasis of tumors (68-70). In BC cancer, LIF functions as a regulator of TG, tumorigenesis, metastasis, EMT, pro-invasive activation of fibroblasts and as a regulator of cancer stem cells (71). LIF activates JAK/STAT3 as immediate effectors and MAPK, AKT and mTOR as further downstream targets (71). MSC1, a humanized anti-LIF mAb and small molecule EC359 are under clinical development in cancer patients (72, 73). EC359 directly interacts with leukemia inhibitory factor receptor (LIFR) to block LIF/LIFR interactions, attenuates STAT3, mTOR and AKT pathways and reduces tumor progression in patient-derived xenografts (73).

*Circ ANKS1B targets upstream transcription factor 1  $\alpha$  and transforming growth factor  $\beta$ .* Circ ankyrin repeat and sterile motif domain containing 1B (circ ANKS1B) (Figure 2) was highly expressed in TNBC and predicted poor prognosis (74). Circ ANKS1B enhanced migration and invasion of MCF-7 and MDA-MB-231 cells *in vitro*. Circ ANKS1B induced epithelial mesenchymal transition (EMT), as indicated by change of a cobblestone shape to fibroblast shape. Overexpression of circ ANKS1B in MCF-7 and MDA-MB-231 cells led to more lung metastases after *i.v.* injection into nude mice and to less lung metastasis in knockdown groups. Circ ANKS1B serves as a

sponge for miRs -148a-3p and -152-3p and up-regulates upstream transcription factor 1 (UPS-1). This led to activation of transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) by binding to the E-box motif of the TGF $\beta$  promoter. Splicing factor epithelial splicing regulatory protein 1 (ESPR1) promoted circ ANKS1B formation and is also a target of USP-1 (74, 75). It has been shown that TGF $\beta$ 1 promotes EMT and migration of BC cells by activation of C-C chemokine receptor 7 (CCR7)/chemokine (C-C) motif ligand 21(CCL21)-mediated chemotaxis (76). Phosphorylation of USP-1 by PI3K/AKT enhances activation of the oncogene WW domain binding protein 2 (WBP2) in BC cells (77). TGF $\beta$  signaling from the membrane to the nucleus is mediated through phosphorylation of SMAD (78). However, it has to be taken into consideration that TGF $\beta$  can act as a TS as well as tumor promoter in a context-dependent manner (79). Numerous preclinical and clinical approaches to inhibit TGF $\beta$  signaling are pursued, but a TGF $\beta$  inhibitor in the indication oncology is not yet approved (80).

### *Transcription factors and transcription-associated factors.*

*Circ RPPH1 targets yes-associated protein 1.* Circ ribonuclease P RNA component H1 (RPPH1) (Figure 3) was overexpressed in BC tissues and its overexpression promoted proliferation in MCF-7 BC and MDA-MB-231 TNBC cells as well as *in vitro* angiogenesis (81). In nude mice, circ RPPH1 increased TG after subcutaneous injection of MDA-MB-231 TNBC cells transfected with circ RPPH1. Circ RPPH1 sponged miR-556-5p which led to up-regulation of yes-associated protein 1 (YAP1), a key regulator of the Hippo signal transduction pathway which controls organ size by regulation of proliferation and cell survival (82). YAP1 is a transcriptional coactivator through interaction with transcriptional enhanced associate domain (TEAD) (82). YAP also interacts with bromodomain-containing protein 4 (BRD4), which binds to acetylated histones and recruits transcription factors to DNA (83). Overexpression of YAP1 facilitates BC cell growth and survival and is associated with poor survival of BC patients (84, 85). YAP transcriptional coactivator with PDZ-binding motif (TAZ) drives TG, metastases, and resistance to therapy (86). The Hippo pathway plays a central role in BC metastasis (87), cancer in general, fibrosis, wound healing and regenerative medicine and several inhibitors of YAP1 signaling, such as verteporin have been identified (88, 89). It remains to be seen whether a therapeutic window for inhibitors of this pathway can be defined.

*Circ EPSTI1 targets B cell CLL/lymphoma 11A.* Silencing of epithelial stromal interaction 1 (EPSTI1) circ RNA (Figure 3) caused inhibition of proliferation and growth and promotion of apoptosis of MDA-MB-231, BT 549 and MDA-MB-468 TNBC cells *in vitro* (90). Circ EPSTI1 inhibited TG of these cell lines in nude mice after subcutaneous implantation. Circ EPSTI1 sponged miR-6809 which led to up-regulation of B cell CLL/lymphoma 11A (BCL11A). circ EPSTI1 positively

correlated with BCL11A and was inversely linked to disease-free survival and overall survival. BCL11A acts as a zinc finger transcriptional repressor and was found to be involved in lymphoid malignancies (91). In BC, BCL11A enhances stemness, promotes tumor progression by activating WNT/ $\beta$ -catenin signaling and promotes metastasis (92, 93). In TNBC BCL11A plays critical functions in stem and progenitor cells (94) and confers cell invasion and migration in androgen receptor positive TNBC (95).

*Circ HIF1A targets nuclear factor IB.* Overexpression of circ hypoxia inducible factor 1 (HIF1A) (Figure 3) in TNBC cells promoted migration and invasion *in vitro* and *in vivo*, whereas its knockdown showed the opposite effects (96). Circ HIF1A increased expression and translocation of nuclear factor IB (NFIB) through post-transcriptional and post-translational modifications, inhibition of cyclin-dependent kinase inhibitor 1 (p21) and activation of AKT/STAT3 signaling. Fused in sarcoma (FUS) was able to up-regulate circ HIF1A and to implement a circ HIF1/NFIB/FUS positive feedback loop (96). FUS has been identified as a DNA/RNA binding protein that is involved in regulation of transcription, splicing, RNA transport as well as DNA repair and plays a role in amyotrophic lateral sclerosis (97, 98). NFIB is part of the NFI family of transcription factors which exhibit oncogenic and TS potential (99). They are differentially spliced yielding up to nine distinct proteins from a single gene (100). NFIB is a potential target for TNBC (101), promotes cell survival by directly suppressing p21 transcription in p53 mutated TNBC cells (102) and stimulates colonization of TNBCs *via* the NFIB- endoplasmic reticulum oxidoreductase 1 (ERO1) axis (103).

*Circ PGAP3 targets transcription factor MYC.* Circ post-GPI-attachment to proteins phospholipase 3 (PGAP3) (Figure 3) was up-regulated in TNBC and its down-regulation in MDA-MB-231 and HS598T TNBC cells inhibited proliferation and invasion *in vitro* (104). In nude mice, circ PGAP3 repressed TG after subcutaneous implantation and lung metastases in an experimental metastasis model. Circ PGAP3 sponged miR-330-3p with the consequence of up-regulation of transcription factor MYC. Deregulation of MYC orchestrates TC proliferation, invasion, apoptosis, ribosomal RNA processing and angiogenesis (105-108). MYC is deregulated in 70% of cancers, modulates 15% of the global transcriptome and has undruggable properties such as lack of pockets which can be bound by small molecules and inaccessibility for mAbs due to its predominant localization in the nucleus (109). Therapeutical options pursued are stabilization of guanine quadruplexes, disruption of MYC/MAX heterodimers and inhibition of bromodomain and extra-terminal motif (BET) factor bromodomain-containing protein 4 (BRD4) (110, 111). A near term clinical MYC inhibitor is recombinant Omomyc, a mutant helix-loop-helix zipper domain, which acts as a dominant

negative inhibitor sequestering MYC in complexes unable to bind to the E-box of MYC-responsive genes (112, 113).

*Circ 0000520 targets zinc finger X-linked.* Knockdown of circ 0000520 (Figure 3) suppressed proliferation, migration, invasion, and induced apoptosis in TNBC cells MDA-MB-231 and BT 549 *in vitro* (114). Circ 0000520 knockdown inhibited growth of MDA-MB-231 xenograft growth in nude mice after subcutaneous injection. miR-1296 was sponged by circ 0000520 and led to increased expression of transcription factor zinc finger X-linked (ZFX) in TNBC cells. ZFX acts as a transcriptional activator in multiple types of human tumors by binding downstream of transcription start sites at the majority of CpG island promoters (115). shRNA-mediated silencing of ZFX attenuates proliferation of BC cells (116). In TNBC, an oncogenic role has been assigned for ZFX (117). In addition, it has been shown that ZFX promotes proliferation and motility of pancreatic cancer cells (118) and up-regulates the extracellular-signal regulated kinase (ERK)/MAPK pathway in gastric cancer *in vitro* and *in vivo* (119).

*Cell-cycle related parameters and paclitaxel (PTX) sensitivity.* *Circ UBE2D2 targets cell division cycle associated 3.* Circ ubiquitin-conjugating enzyme E2 D2 (circ UBE2D2) (Figure 4) was up-regulated in TNBC patients and correlated with poor prognosis (120). In MDA-MB-231 and BT-549 TNBC cells, down-regulation of circ UBE2D2 inhibited proliferation and invasion. Its knockdown also decreased chemo-resistance in these cell lines. Circ UBE2D2 sponged miR-512-3p which led to up-regulation of cell division cycle associated 3 (CDAC3). In nude mice, circ UBE2D2 mediated TG of MDA-MB-231 and BT-549 xenografts after subcutaneous implantation. Tumor suppressive effects mediated by down-regulation of circ UBE2D2 are inhibited by down-regulation of miR-512-3p or overexpression of CDAC3 in TNBC cell lines. CDAC3 contains an F-box motif which can combine with S-phase kinase associated protein1 (Skp1) and cullin of E3 ubiquitin ligases (121). CDAC3 has been shown to promote cell proliferation of gastric cancer (122) and colorectal cancer (CRC) by activating the nuclear factor  $\kappa$ B (NF $\kappa$ B)/cyclinD1 signaling pathway (123) and p21-dependent proliferation by regulating transcription factor E2F1 expression (124) and proliferation and apoptosis in pancreatic cancer (125).

*Circ AGFG1 targets cyclin E1.* Circ RNA Arf-GAP domain and FG repeat containing protein 1 (AGFG1) (Figure 4) was up-regulated in TNBC patients and its level was correlated with clinical stage, pathological grade, and poor prognosis (126). Circ AGFG1 promoted proliferation, migration, and inhibited apoptosis, whereas its down-regulation mediated opposite effects and G1 arrest in MDA-MB-231 and BT-549 TNBC cells *in vitro*. Circ AGFG1 facilitated tumorigenesis, angiogenesis, and metastasis in MDA-MB-231 xenografts in

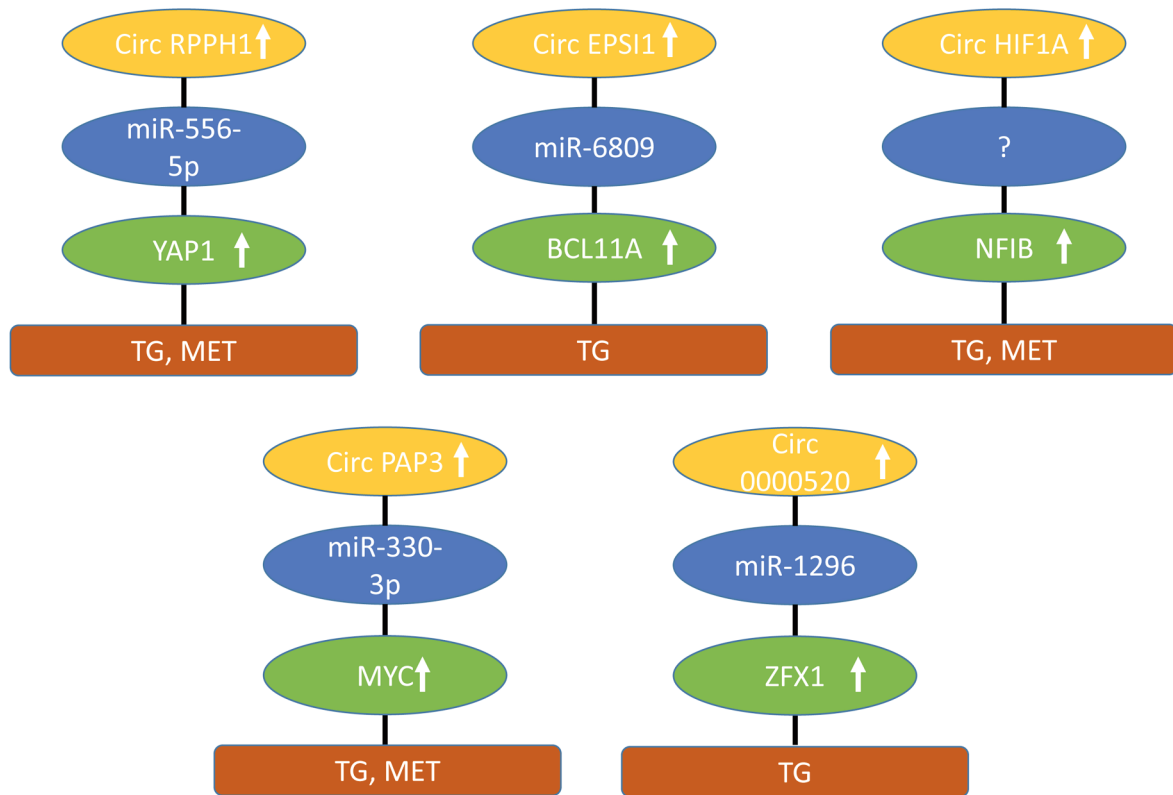


Figure 3. Up-regulated TNBC-related circ RNAs inducing transcription factors and transcription-associated factors with efficacy in TNBC-related preclinical *in vivo* models. Upward arrows: up-regulated; downward arrows: down-regulated. *BCL11A*: B cell CLL/lymphoma 11A; circ RNA *EPSTI1*: circ RNA epithelial stromal interaction; circ *HIF-1 $\alpha$* : circ RNA hypoxia inducible factor 1A; circ *PAPG3*: circ RNA post-GPI attachment to proteins phospholipase 3; circ *RPPH1*: circ RNA ribonuclease P RNA component H1; MET: metastasis; MYC: transcription factor MYC; NFIB: nuclear factor 1B; TG: tumor growth; YAP: Yes-associated protein; ZFX: zinc finger X-linked.

nude mice. Circ AGFG1 acted as a sponge for miR-195-5p and led to up-regulation of cyclin E1 (CCNE1) and its downstream targets cyclin-dependent kinase 2 (CDK2), phosphorylated retinoblastoma (pRb), transcription factor E2F1 and stem cell marker CD44. CCNE1 mainly interacts with CDK2 to regulate cell-cycle progression (127, 128). Overexpression and amplification of CCNE1 is associated with poor prognosis in patients with TNBC (129, 130).

*Circ 10229 targets ser-thr kinase PFTAIRES*. Circ10229 (Figure 4) was overexpressed in TNBC and BC cell lines and strongly associated with negative outcome in patients with TNBC (131). It enhanced proliferation, migration and invasion and suppressed apoptosis in SUM149PT and MDA-MB-468 TNBC cells. In addition, circ10229 sponged miR-152-3p and up-regulated PFTAIRES (PFTK1), a member of the cdc2-related ser/thr protein kinase family, also referred to as cyclin-dependent kinase 14 (CDK14) (132, 133). PFTAIRES functions as a regulator of cyclins and the cell cycle (132, 133). In an orthotopic xenograft mouse model circ 10229 promoted TG after subcutaneous

implantation and increased lung metastases after tail vein injection in nude mice. PTAIRE has been identified as a cyclin B interacting protein and shown to promote BC cell proliferation and migration *via* triggering WNT/ $\beta$  catenin signaling driven by segment polarity protein disheveled homolog 2 (DVL2) (134). Conversely, PTAIRE down-regulation has been shown to attenuate BC cell growth (135).

*Circ PLK1 targets polo-kinase 1*. Circ PLK1 (Figure 4) was up-regulated in TNBC patients and was associated with poor survival (136). Knockdown of circ PLK1 inhibited growth and invasion of TNBC cells *in vitro* and tumor occurrence and metastasis *in vivo*. Polo-kinase 1 (PLK1) is a key initiator of mitosis and cytokinesis (137). PLK1 is overexpressed in TNBC in comparison to normal mammary glands and benign breast cancers and PLK1 inhibitor BI-2536 induces G2/M arrest, creates a polyploid cell population and induces apoptosis in several TNBC cells (137). Inhibition of PLK1 eliminates tumor-initiating cells in BC (138). Together with chemotherapy, PLK1 inhibition is an option for treatment of

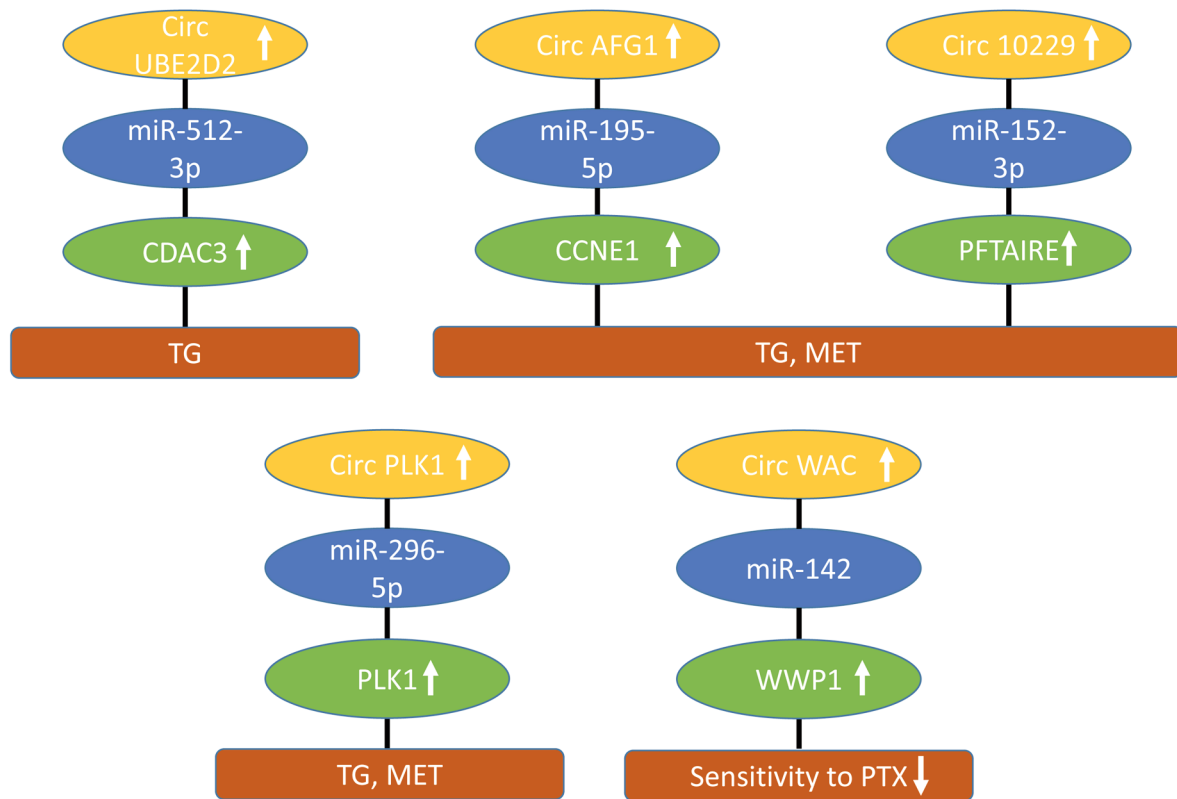


Figure 4. Up-regulated TNBC-related circRNAs affecting cell-cycle-related parameters and PTX-sensitivity with efficacy in preclinical TNBC-related *in vivo* models. Upwards arrows: up-regulated; downward arrows: down-regulated. CCNE1: Cyclin E1; CDAC3: cell division associated 3; CDK14: cyclin-dependent kinase 14; circ AFG1: circ RNA Arf-GAP domain and FG repeat containing protein 1; circ PLK1: circ RNA polo-kinase 1; circ UBE2D2: circ RNA ubiquitin-conjugating enzyme E2 D2; circ WAC: circ RNA ww domain-containing adapter protein with coil-coil; MET: metastasis; PFTAIRE: ser/tre kinase PFTAIRE; PTX: paclitaxel; TG: tumor growth.

TNBC patients (139). In addition, it has been observed that loss of TS retinoblastoma (Rb) induces high levels of PLK1 in TNBC (140). Until now, small molecule inhibitors of PLK1 have only reached limited success in several types of cancers in clinical studies, whereas TNBC remains an indication to be explored (141). It has been shown that PLK1 inhibits phosphatase RP6 resulting in promotion of Aurora kinase A (AURKA), another target for cancer therapy (142).

*Circ WAC targets ubiquitin-protein ligase WWP1.* Higher expression of circ ww domain-containing adapter protein with coil-coil (circWAC) (Figure 4) in TNBC patients correlated with worse prognosis. Down-regulation of circWAC increased sensitivity versus PTX in MDA-MB-231 and MDA-MB-468 TNBC cells *in vitro* and in MDA-MB-231 xenografts in nude mice. Circ WAC was shown to sponge miR-142 which led to up-regulation of ww domain containing ubiquitin-protein ligase WWP1 and activation of PI3K/AKT signaling (143). The latter functions as an oncogene by targeting its substrates for ubiquitination and degradation and is up-regulated in many

types of cancer including BC and its expression correlates with poor prognosis (144-147). WWP1 is amplified in a subset of BCs (148). In a model of MYC-driven tumorigenesis, genetic ablation, or pharmacological inhibition with indole-3-carbinol of WWP1 triggered reconstitution of TS phosphatase and tensin homolog (PTEN) (149).

### Technical Issues

Circ RNA can be down- or up-regulated in tumor tissues in comparison to corresponding normal tissues. Down-regulated circRNAs often exhibit binding sites for miRs which are tumor-suppressive as outlined in the previous chapters. It has been shown that synthetic circRNAs with reiterated binding sites for tumor-promoting miRs can be transfected into tumor cells resulting in decreased tumorigenicity *in vitro* and *in vivo* (150). The corresponding vehicles are plasmid- or viral-based expression vectors. The other alternative is to reconstitute the corresponding target with small molecules, but this approach is hampered by specificity and deconvolution issues.



Overexpressed, oncogenic circRNAs can be inhibited by antisense oligonucleotides (ASO), small interfering RNAs (siRNA) or short-hairpin (shRNAs) RNAs (151, 152).

ASO are based on chemically modified oligonucleotides (12-24 mers) which bind to their RNA targets by Watson-Crick base pairing (151, 152). Resulting DNA-RNA hybrids are degraded by RNase H. This approach has been continuously optimized by medicinal chemistry backbone and sugar modifications (151-152) and the introduction of locked nucleic acids (LNA) (153) and gapmers (154). siRNAs are double-stranded RNAs, 20-24 bp in length which can be transfected into corresponding recipient cells and similarly to miRs induce destruction of target mRNAs *via* the RNA-induced silencing complex (RISC) including endonuclease AGO2 (155). shRNA has a MOA corresponding to siRNA but contain an additional hair-pin loop and can be introduced into cells by plasmid-based or viral vectors (155).

However, several significant hurdles have emerged, which are not discussed in detail in this review. A major issue is immunogenicity mediated by extra- and intracellular receptors (156, 157). Further issues are hurdles of specificity and delivery (156-159). Usage of tiny RNA and combination with other treatment modalities have led to further improvement of therapeutic efficacy (156-159). Design of new formulations and liver specific delivery *via* asialoglycoprotein receptors have marked new milestones in the field (160, 161). But in essence, each approach of therapeutic intervention related to circRNAs has to be optimized according to the criteria as mentioned above including pharmaco-kinetics and pharmaco-dynamics.

## Conclusion

We have identified five down-regulated and 15 up-regulated circRNAs in TNBC in comparison to matching normal breast tissues with efficacy in pre-clinical *in vivo* models. The five down-regulated circ RNAs are shown in Figure 1, and all are involved in TS-related pathways. In experimental models their activity can be reconstituted by gene transfer of the appropriate circRNA into tumor cells with expression vectors. Down-regulation of circ 001785 leads to up-regulation of JAK/STAT signaling, whereas down-regulation of circ ITCH induces WNT7/β catenin signaling which are druggable pathways. However, the targets identified (Figure 1) have to be validated in more detail.

Five up-regulated circRNA induce transmembrane receptors and secreted factors (CD44, IGFR1, FGFR1, LIF and TGFβ (Figure 2). Signaling of CD44, LIF and TGFβ is context-dependent. IGF1R signaling is very complex and due to lack of biomarkers impairs identification of responders. The role of FGFR1 in TNBC needs to be validated in more detail.

Five up-regulated circRNA drive overexpression of transcription factors and transcription-associated factors

(Figure 3). Factors such as YAP1, BCL11A, NFIB, MYC and ZFX exhibit inherent druggability issues (162, 163), but as previously outlined, a MYC inhibitor will enter clinical studies soon (112, 113). Also emerging proteolysis targeting chimeras (PROTAC) agents which interact with the target and E3 ubiquitin ligase leading to proteasomal degradation of the target might be game changers in the near future (164-167).

Further four identified targets are cell-cycle related (CDAC3, CCNE1, PTAIRE, PLK1) and one target (WWP1) mediates PTX-resistance, as shown in Figure 4. The role of PPTAIRE (CDK14) and PLK1 in TNBC should be explored and validated in more detail.

To put circRNA based approaches into clinical practice, hematological malignancies might be preferred target indications due to accessibility of the malignant cells for targeting by corresponding agents. Up-regulated targets are preferred for delivery of si-RNA- or sh-RNA-related entities. However, for TNBC, improvement of delivery techniques is crucial for clinical evaluation of circRNA-based approaches.

## Conflicts of Interest

FB is and UHW was an employee of Roche.

## Authors' Contributions

FB and UHW equally contributed to all aspects of the paper.

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Received November 21, 2022

Revised December 19, 2022

Accepted January 20, 2023