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Np63 α in cancer: Importance and therapeutic opportunities

Matthew L. Fisher¹, Seamus Balinth^{1,2}, Alea A. Mills¹

¹Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 11724

²Molecular and Cellular Biology Program, Stony Brook University, Stony Brook, NY, 11794

Abstract

Our understanding of cancer and the key pathways that drive cancer survival has expanded rapidly over the past several decades. However, there are still important challenges that continue to impair patient survival, including our inability to target cancer stem cells, metastasis and drug resistance. The transcription factor p63 is a p53 family member with multiple isoforms that carry out a wide array of functions. Here, we discuss the critical importance of the Np63 α isoform in cancer and potential therapeutic strategies to target Np63 α expression to impair the cancer stem cell population, as well as to prevent metastasis and drug resistance to improve patient survival.

Keywords

p63; cancer; signaling; therapeutic strategy

Cancer stem cells

In adult tissue, stem cells are essential for tissue homeostasis and regeneration. Stem cells are long-lived cells that generate progeny throughout life to regenerate multiple specialized, shorter-lived cells that are essential for various tissue-specific functions [1]. As stem cells are critical to the maintenance of normal tissue, so too are cancer stem cells (CSCs) critical to the maintenance of many tumors. CSCs are broadly defined as cells that possess the ability to initiate tumor growth, self-renew, and differentiate to give rise to the heterogeneous bulk tumor cell population [1]. The existence of CSCs explains many clinical observations and their challenges, such as recurrence following initially successful therapy, as well as metastasis, drug resistance and dormancy. While cancer treatment has made tremendous strides over the years, drug resistance, recurrence and metastasis remain key problems contributing to therapy failure. In many tumor types, these failures can be attributed to the inability to target the CSC population [1]. Therefore, understanding signaling essential to CSC survival and maintenance is of critical importance to improving therapeutic strategies

Corresponding author: Mills@CSHL.edu.

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Declaration of Interests

The authors declare no conflicts of interest.

and patient survival. One protein we believe is at the heart of CSC related signaling is the transcription factor Np63 α . It has long been known that Np63 α is critical for epithelial development and maintenance [2]. Recent advances in the field of p63 biology have demonstrated key roles for Np63 α in cancer progression, metastasis and drug resistance. Despite the importance of p63 in this context, therapeutic strategies to target Np63 α are limited because it is an essential transcription factor with a similar structure to family members with opposing functions to its own [3]. In this review, we look at Np63 α and its role in CSCs, metastasis and drug resistance, and highlight recent advances in our understanding of Np63 α -related signaling that provide exciting therapeutic opportunities in cancer.

Np63 α and stemness

In normal tissue, Np63 α is highly expressed in several stem cell compartments, particularly in stratified and glandular epithelial [5]. The critical role of Np63 α can be seen in p63 deficient mice, which display a lack of all squamous epithelia and their derivatives [2], as well as the severe human developmental defects that occur from germline mutations in p63 (reviewed in [6]). Np63 α is required to maintain the self-renewing capacity of epithelial stem cells and is critical for epithelial stem cell differentiation and proliferation through the regulation of a wide array of downstream targets. Based on its role in regulating normal stem cells homeostasis in epithelial tissues, it is not surprising that Np63 α is also a key driver of CSCs in multiple tumor types [5].

Np63 α in cancer stem cells

Np63 α expression has been linked to a CSC phenotype in a number of epithelial cancers, with increased Np63 α being associated with elevated numbers of tumor initiating cells, tumorsphere formation, invasive potential, and enhanced tumorigenicity [7, 8]. In squamous cell carcinoma (SCC) the gene encoding stem cell factor *SOX2* is co-amplified along with the *p63* locus, and preferentially interacts with the Np63 α protein [9]. The gene encoding the chromatin modifying protein *ACTL6A* is also co-amplified with the *TP63* locus in head and neck squamous cell carcinoma (HNSCC), leading to a CSC phenotype and impaired terminal differentiation [10]. Np63 α and *ACTL6A* cooperate to decrease chromatin accessibility, which results in the repression of the metastasis suppressor gene *WWC1* and the activation of *YAP*, an oncogene that regulates stemness [10]. Furthermore, YAP can bind to Np63 α directly to stabilize it, leading to enhanced cancer stem cell survival in SCC [11]. The Lymphoid-specific helicase (HELLS) is an additional chromatin-modifying protein that is regulated by Np63 α . HELLS expression is important for embryonic development and cellular senescence [12]. Np63 α is capable of binding to consensus p63 binding sites in the HELLS promoter, increasing expression and leading to senescence bypass during tumor initiation in squamous cell carcinoma [12]. Np63 α also induces the expression of genes encoding cell surface proteins involved in establishment of the CSC phenotype. CD44 is a cell surface antigen with roles in migration and adhesion, and is considered a marker of cancer stem cells in various epithelial tumors [13]. Overexpression of Np63 α enhances the CD44⁺/CD24⁻ subpopulation and leads to increased proliferation, colony formation, spheroid formation, and tumor growth in xenografts derived from SCC and MCF-7 cells [14,

15]. Np63 α regulates the expression of not only CD44, but the hyaluronan synthase gene *HAS3*, allowing Np63 α to regulate CD44 expression and activation in both HNSCC and breast cancer cell lines [16, 17]. In addition to *CD44*, Np63 α regulates genes encoding integrins $\alpha 6$, $\beta 4$ and $\alpha 3$ in breast epithelial cells [18]. $\alpha 6\beta 4$ integrin is an essential component of hemidesmosomes, which provide stable adhesion to basal epithelial cells and the underlying basement membrane, and $\alpha 6\beta 4$ integrin has been implicated as a key regulator of cancer stemness in several epithelial cancers [19]. Thus, Np63 α -induced expression of these cell surface markers increases cellular adhesion to the extracellular matrix (ECM) and confers resistance to anoikis [18]. In breast cancer, Np63 α drives WNT signaling, a critical regulator of epithelial stem cell homeostasis, by directly driving the expression of FZD7, a receptor for WNT ligands [20]. Np63 α can also transcriptionally activate *NOTCH1*, leading to enhanced CSC properties [15]. Finally, Np63 enhances stemness through regulation of Hedgehog signaling by directly controlling the expression of *SHH*, *GLI2* and *PTCH1* in mammary CSCs [21].

Resistance to apoptosis is a critical feature of CSCs, and Np63 α plays a key role in that feature as well. Np63 α overexpression protects cells from oxidative stress induced by oxidants, DNA damage, anoikis, and ferroptosis-inducing agents [3, 22]. Np63 α regulates redox homeostasis through transcriptional control of glutathione biogenesis, utilization, and regeneration [22]. Overexpression of Np63 α promotes clonogenic survival of *p53*^{-/-}; *Bax*^{-/-}; *Bak*^{-/-} cells against DNA damage, and coexpression of *BCL-2* and *Np63 α* confers clonogenic survival against matrix detachment and promotes cancer metastasis in lung SCC [22]. Collectively, these unique capabilities clearly indicate that Np63 α is linked to multiple pathways that are central to regulating the CSC phenotype and CSC survival.

Np63 α in metastasis

Metastasis is the result of a multistep process by which cancer cells travel from the primary tumor through lymphatic or blood vessels to invade distant organs. This complex cascade of events involves a number of signaling pathways that allow for local invasion, survival in circulation, extravasation and ultimately proliferation at a distant site. CSCs are widely regarded as key drivers of metastasis, as many pathways involved in the CSC phenotype also contribute to the cells' ability to metastasize, and several reports have indicated the CSC pool is critical for metastatic colonization [1, 23]. In line with this, numerous reports have implicated Np63 α as critical to driving the metastatic cascade at multiple levels.

Early in the metastatic cascade, Np63 α can contribute to local invasion in basal-like breast cancer through regulation of matrix metalloproteinases MT1-MMP and MMP13, important proteases involved in tumor invasion [24, 25]. Additionally, Np63 α directly regulates the transcription of genes encoding two chemokines, *CXCL2* and *CCL22*, which drive the recruitment of myeloid-derived immunosuppressor cells (MDSCs) in triple-negative breast cancer [26]. MDSCs secrete prometastatic factors, including MMP9 to further facilitate invasion [26]. Another important aspect of cancer cell invasion is epithelial-mesenchymal transition (EMT), which confers greater metastatic potential on cells. Endogenous Np63 α induces several markers of EMT, including SNAIL, TWIST and VIMENTIN in esophageal squamous carcinoma cell lines, thereby promoting migration and

invasion in a β -catenin-dependent manner [27]. And in breast cancer, Np63 α enhances cell invasion by transcriptionally regulating genes encoding the EMT-related markers *SLUG*, *FAT2*, and *AXL* [28, 29]. Np63 α also upregulates the TGF- β pathway by activating *SMAD4* and *TGF- β 2*, thus facilitating EMT, invasion and migration in osteosarcoma cells [30]. The ability of Np63 α to regulate matrix metalloproteinases and EMT is likely why Np63 α is so robustly expressed at the edge of invasive tumors, as Np63 α activity might be locally upregulated in the migrating front of cells, enabling ECM degradation and invasion.

In support of this, Np63 α has been shown in breast cancer organoids to control the 'collective invasion' process, a type of cellular invasion in which tumor cells remain connected and invade as multicellular units [31]. These cells display a basal epithelial gene expression pattern that facilitates collective invasion. Particularly, the invading tumor cells activate expression of Np63 α and CK14, which are required for local invasion of breast cancer cells. By maintaining the basal epithelial state, the cells retain enhanced invasive properties characteristic of less differentiated epithelial cells, thus allowing for collective invasion [31].

Another key aspect of the metastatic cascade is survival in circulation. Np63 α contributes to this critical step by suppressing anoikis through regulation of integrins, *BCL-2* and EGFR [18, 22, 32]. When cells reach the metastatic site, they must be able to engage the ECM and proliferate. To facilitate this process, primary tumors actively modify potential metastatic sites prior to dissemination through secretion of various factors [33]. Np63 α contributes to the formation of the metastatic niche by transcriptionally regulating *ANGPTL2* [34]. ANGPTL2 is a secreted glycoprotein and pro-inflammatory and angiogenic factor that is capable of signaling through α 5 β 1 integrins to contribute to metastatic niche formation [34]. When cancer cells arrive at the metastatic site, Np63 α likely further contributes to metastasis through transcriptional regulation of *CYR61*, a matricellular protein linked to extravasation during metastasis through engagement with integrins and heparin sulfate proteoglycans [35, 36].

All together, these data suggest that Np63 α exploits multiple pathways, including the induction of EMT-related factors, metalloproteinases, enhancement of collective invasion, anoikis resistance and metastatic colonization, all of which work together to enhance the metastatic potential of cancer cells. However, there is also evidence suggesting caution should be taken, as Np63 α depletion can have differing impacts under certain conditions. For instance, in certain SCC lines that predominantly express Np63 α , p63 depletion results in increased mesenchymal marker expression associated with invasion [37] and overexpression of Np63 α results in reduced Vimentin and ZEB1 expression [38]. In line with this, in two non-transformed mammary epithelial cell lines (MCF10A and MCF12A) expression of H-RasV12 reduces Np63 α expression and increases EMT and cell migration [39]. Work in the MCF10A cell line also showed that depletion of Np63 α and Np63 β leaving only Np63 γ resulted in TGF β driven EMT [40]. In breast and prostate lines, Np63 α has been shown to impair invasion through the suppression of miR-205, a key regulator of EMT [41, 42] and in prostate cancer cell lines, miR-301 was shown to induce EMT through inhibition of p63 [43]. Beyond the differing impacts on cancer cells, the

key role of Np63 α in senescence and aging in normal tissue should also be considered (reviewed in [44]). Thus, more work is needed to fully understand the cellular context in which Np63 α can suppress EMT and invasive behavior and therefore know when it is appropriate to target Np63 α therapeutically.

Drug resistance

Chemotherapy is one of the principal modes of treatment for cancer, but the effectiveness of chemotherapy is kept in check by drug resistance. Although combination therapies have become the standard for cancer therapy to help circumvent resistance against single-agent treatment, drug resistance continues to be a major obstacle [45], and recent work has linked Np63 α to drug resistance in several cell lines. Np63 α has been implicated in Cisplatin resistance through several mechanisms. In HNSCC, Np63 α has been shown to regulate the transcription of AKT1, leading to Cisplatin resistance [46]. In pancreatic cancer, Np63 α results in Cisplatin resistance through the transactivation of *EGFR* and *14-3-3 σ* [47]. In breast cancer, upregulation of Np63 α leads to an increase in the expression of EGFR and WIP1 to drive cisplatin resistance [48]. Finally, in oral cancer, Np63 α promotes the expression and nuclear translocation of PTEN, leading to Cisplatin resistance [49]. In addition to Cisplatin, Np63 α has been shown to induce resistance to Doxorubicin in hepatocellular carcinoma by downregulating *CD95* and *BAX* gene activation, and Bortezomib resistance in HNSCC through regulation of CYGB-ROS signaling [50, 51]. Therefore, Np63 α is capable of regulating a multitude of targets involved in numerous aspects of cancer progression, including stem cell self-renewal, invasion, anoikis resistance, colonization and drug resistance.

Druggable targets upstream of Np63 α

Because of the difficulties in targeting Np63 α directly, we believe targeting upstream regulators of Np63 α is a potential therapeutic strategy. Below we discuss what we believe are exciting therapeutic targets upstream of Np63 α that could provide a means to reduce Np63 α expression and the CSC phenotype, metastasis and drug resistance associated with it. It is important to note that upstream regulators of Np63 α can vary in differing cell types, and the pathways discussed below may only be present in certain tissues or cell contexts.

Chromatin modifying proteins

BRD4/EZH2

A number of chromatin modifying proteins have been linked to Np63 α (summarized in Figure 2). In pancreatic cancer, loss of *KDM6A* results in squamous-like, metastatic cancers, which are selectively sensitive to Bromodomain and Extraterminal domain (BET) inhibitors including JQ1 [52]. Treatment with JQ1, which predominantly inhibits BRD4, reverses squamous differentiation. It was shown that BRD4 binds to Np63 α -regulating super enhancers, and treatment with JQ1 not only evicts BRD4 from these super enhancers, but also disrupts their long-range interaction with the *Np63 α* promoter [52]. BRD4 has also been linked to Np63 α in SCC, with genetic depletion or pharmacological inhibition of BRD4 using BET inhibitors JQ1 or MS436 reducing Np63 α protein levels and impairing

cancer stem cell phenotypes [53]. In this context, BRD4 transcriptionally regulates *C-MYC*, leading to increased activity of EZH2. EZH2 then binds to STAT3, methylating and activating it, allowing STAT3 to bind to the *Np63α* promoter. Furthermore, treatment with EZH2 or STAT3 inhibitors successfully reduce *Np63α* expression and the CSC phenotype associated with it [53].

In addition to regulating *Np63α* through STAT3, EZH2 can also regulate *Np63α* through RUNX3 in SCC. In multiple cancers, RUNX3 has been shown to be a direct target of EZH2-mediated repression via promoter hypermethylation. Pharmacological inhibition of EZH2 or CRISPR-mediated depletion significantly augments RUNX3 expression at both the mRNA and protein level [54]. This coincides with the loss of *Np63α*. Direct activation of *RUNX3* through either CRISPRa or cDNA overexpression leads to a significant compromise in *Np63α* expression at both the protein and mRNA level.

SETDB1

The histone methyltransferase SETDB1 was shown to physically interact with the C-terminal TID domain of *Np63α* in breast cancer [55]. Depletion of SETDB1 or *Np63α* reduces expression of the other, indicating their reciprocal modes of regulation. SETDB1 depletion leads to upregulation of 30 targets of *Np63α* repression, indicating a possible novel mechanism of *Np63α*-mediated gene repression via SETDB1. Consequently, SETDB1 regulates *Np63α* expression in breast cancer, as well as being a binding partner that may cooperate to repress *Np63α* target genes [55].

The interaction between these proteins is also demonstrated in SCC. The loss of either protein results in a significant disruption of a CSC phenotype [54]. Additionally, the proteins regulate each other's expression and reintroduction of *Np63α* into SETDB1-deficient cells rescues the cancer stem cell phenotype. Likewise, SETDB1 reintroduction rescues CSC phenotypes in *Np63α*-deficient cells, highlighting the intimate connection between these two proteins.

Therapeutic targeting of SETDB1 is a developing area of study that may hold great promise in disrupting *Np63α*-driven cancers with high-level SETDB1 expression. To date, several compounds have been shown to have efficacy in targeting SETDB1, including mithramycin A, the mithramycin analog EC-8042, and a selective inhibitor of SETDB1's tandem Tudor domains [56, 57].

TIP60

The histone acetyltransferase TIP60 activates *Np63α* expression in SCC [58]. Upon TIP60 depletion, *Np63α* is decreased at both the RNA and protein level. This is due to TIP60 directly acetylating *Np63α*, thereby preventing its ubiquitin-mediated degradation. Importantly, the TIP60-selective inhibitor NU9056 produces a similar effect as TIP60 depletion, providing a potential means of targeting *Np63α* in SCCs co-expressing *Np63α* and TIP60 [58].

HDACs

Histone deacetylases (HDACs) play an important role in regulating transcription. HDACs represent potential anticancer targets, as their inhibition can induce apoptosis, differentiation, and growth arrest in cancer cells. In HNSCC, trichostatin A (TSA), an inhibitor of HDAC1 and 6, downregulates the expression of p63 and reduces invasion and migration [49] whereas treatment with suberoylanilide hydroxamic acid (SAHA) reduces EMT and Np63 α [59]. In SCC, Np63 α associates with HDAC1 and HDAC2 to form an active transcriptional repressor complex that can be targeted therapeutically with Vorinostat, which effectively reduces Np63 α expression [60].

Signals from the microenvironment

The tumor microenvironment (TME) consists of diverse cell types and extra-cellular matrix components that surround and support the tumor. There is growing interest in targeting the TME due to its critical role in regulating several aspects of cancer progression. Interleukins (IL) are a key component of the microenvironment, and several have been implicated in regulating Np63 α , including IL-1 β in MCF7 cells, IL-6 in lung cancer, and IL-13, IL-17 and IL-22 in keratinocytes [48, 61–64], Np63 α also induces *IL-6* and *IL-1* in pancreatic cancer cells, providing potential for a positive feedback loop [65]. IL-17A produced by Th17 cells induces Np63 α in keratinocytes through a TRAF4/ERK-mediated pathway [66] and the type 2 interleukins IL-4/13, require Np63 α to block early keratinocyte differentiation [64]. In addition to interleukins, enhanced ECM content augments Np63 α expression, and inhibition of collagen synthesis reduces Np63 α levels. Altered Np63 α levels are also found in keratinocytes grown on different ECM components, with Np63 α levels in epithelial stem cells varying according to the particular matrix composition and stiffness. Activation of the laminin receptor, a key molecule involved in adhesion to the basement membrane, increases Np63 α levels in keratinocytes, as does the ECM component TGFBIp, and integrin-linked kinase (ILK), which is involved in integrin mediated signal transduction [67–69].

These data suggest that Np63 α is capable of regulating and being regulated by various aspects of the TME. With growing interest in targeting the TME and crosstalk between the TME and cancer cells, targeting interleukins upstream of Np63 α potentially represents an opportunity to target not only critical factors of the TME, but also a key regulator of cancer progression that the TME supports.

Cell Surface markers

In addition to the signals released from the TME, cancer cell surface markers are critical in crosstalk with the TME, as they relay those signals to the cancer cells. In line with the importance of signals emanating from the TME in regulating Np63 α , many cell surface markers involved in ‘outside in’ signaling have been linked to Np63 α as well.

EGFR

The tyrosine kinase receptor epidermal growth factor receptor (EGFR) is frequently overexpressed in squamous cell carcinomas, where it has been shown to induce Np63 α .

expression through activation of phosphatidylinositol 3-kinase (PI3K), in turn, activating mTOR-dependent activation of STAT3 [70]. Np63 α is also capable of regulating EGFR expression in cooperation with SOX2 and CCAT1 [71], suggesting a possible feedback loop between EGFR and Np63 α in squamous cell carcinoma. In basal-like triple negative breast cancer, Np63 α expression increases both EGFR mRNA and protein levels, as well as increasing its activity [32]. Silencing of Np63 α in epithelial cells reduces both the total- and phospho-EGFR levels, impairing the activation of EGFR signaling [32, 71].

Integrins/TG2/NRP1

Signaling through $\alpha 6\beta 4$ integrin has also been shown to regulate Np63 α expression. In squamous cell carcinoma, the enzyme transglutaminase 2 (TG2) interacts with $\alpha 6\beta 4$ integrin. This interaction leads to activation of FAK-SRC and PI3K-PDK1 kinases. Signaling through this cascade results in the inhibition of large tumor suppressor kinase 1 (LATS1), an integral component of the Hippo signaling pathway that suppresses YAP [72]. Signaling through this cascade results in the inhibition of large tumor suppressor kinase 1 (LATS1), an integral component of the Hippo signaling pathway that suppresses YAP. This frees YAP to enter the nucleus, where it binds to Np63 α and stabilizes its expression by impairing degradation of Np63 α by the proteasome [11].

Neuropilin-1 (NRP1) is another protein that can activate signaling through $\alpha 6\beta 4$ integrin to regulate Np63 α . NRP1 is a transmembrane protein and co-receptor for a number of extracellular ligands. NRP1 interacts with GAIIP C-terminus interacting protein 1 (GIPC1), a scaffolding protein, and $\alpha 6\beta 4$ integrin. This complex activates a downstream kinase cascade that also leads to suppression of Hippo signaling and increased Np63 α [73]. YAP also mediates stabilization of Np63 α in response to DNA damage-induced p63 phosphorylation by c-Abl, leading to YAP/ Np63 α binding [74]. As mentioned above, Np63 α transcriptionally regulates several integrin isoforms including $\alpha 6$, $\beta 4$ and $\alpha 3$ [18]. This represents another feedback loop that can be targeted therapeutically, as small molecule inhibitors for TG2, NRP1 and YAP are available that have been shown to impair the CSC phenotype in various cancer types [72, 73, 75]. YAP in particular has generated significant clinical interest, with new small molecule inhibitors in development, as well as efforts to repurpose existing drugs like Verteporfin and Digitoxin [76].

Wnt/ β -catenin pathway

Wnt/ β -catenin signaling is a key regulator of stemness through the regulation of self-renewal, pluripotency, differentiation and migration. In cancer, abnormal activation of Wnt/ β -catenin promotes a CSC phenotype and metastasis. [77]. Np63 α is under direct control of the WNT/ β -catenin pathway through binding of lymphoid enhancer binding factor 1 (Lef1) and β -catenin between the promoters of TAp63 and Np63 [78]. Another layer of regulation comes from a β -catenin responsive element within the proximal Np63 α promoter. In addition to direct regulation of Np63 α , WNT/ β -catenin can also regulate the transcriptional co-factor limb-bud and heart (LBH). In mammary epithelial cells, LBH increases Np63 α transcription while downregulating transcription of TAp63 α , resulting in enhanced replicative potential and stemness [79]. Together, these data suggest that in cancers

with elevated Np63 α levels and active β -catenin signaling, targeting the β -catenin pathway may represent a means for impairing Np63 α expression.

STAT3

Of the seven members of the STAT protein family, STAT3 is arguably the most important for cancer progression [80]. STAT3 is not only critical for transducing signals from multiple receptor and non-receptor tyrosine kinases that are frequently activated in cancer cells, but STAT3 is also a transcription factor regulating the expression of a wide range of targets that contribute to tumor progression, most notably Np63 α [80]. STAT3 binds to the promoter of *Np63 α* in several cell types, and the dual-regulatory effect of Np63 α on its own promoter is dependent on STAT3 activation [81, 82]. STAT3 serves as a key mediator of Np63 α for several pathways mentioned above, including IL-6, EGFR, BRD4 and EZH2 [53, 70, 80]. In addition to these, there are likely numerous other activators of STAT3 that can be linked to Np63 α in cancer. Things like VEGFR, PDGFR, CXCR4, and S1PR1 that lead to STAT3 activation and CSC phenotypes, but have yet to be definitively shown to activate Np63 α , all represent interesting areas of investigation [80]. This also leaves STAT3 uniquely positioned directly upstream of Np63 α and important for its activation, while also being downstream of numerous signaling cascades critical for cancer biology. Combine that with a number of STAT3 inhibitors currently at various stages of clinical trials, and STAT3 appears to be a most exciting therapeutic opportunity for targeting Np63 α . The upstream regulators of Np63 α discussed in this section have been summarized in Figure 3.

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Glossary

Tumorsphere

a spherical formation developed from the proliferation of a single cancer stem or progenitor cell in 3D culture.

hyaluronan synthase

enzyme involved in the synthesis of unbranched glycosaminoglycan hyaluronan, or hyaluronic acid, a CD44 ligand.

Hemidesmosomes

protein complexes that facilitate the stable adhesion of basal epithelial cells to the underlying basement membrane.

Extracellular matrix

three-dimensional network of extracellular components including collagens, glycoproteins and proteoglycans, that provide structural and biochemical support to surrounding cells.

Anoikis

apoptosis that results from loss of attachment to the extracellular matrix or neighboring cells

Hedgehog signaling

signaling pathway critical during development for intercellular communication, and is frequently dysregulated in cancer. There are three mammalian Hedgehog proteins including Sonic Hedgehog, Indian Hedgehog and Desert Hedgehog

Ferroptosis

a form of cell death driven by iron-dependent phospholipid peroxidation regulated by multiple cellular metabolic pathways.

Clonogenic survival

an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony, testing the ability of cells to undergo unlimited division. This method is frequently used to determine the effectiveness of cytotoxic agents.

Matrix metalloproteinases

members of the metzincin group of proteases which share the conserved zinc-binding motif in their catalytic active site, and are involved in regulating various components of the extracellular matrix.

Metastatic niche

an environment in a secondary organ that provides favorable growth conditions for cancer cells, allowing for the establishment of metastasis from a primary tumor.

Cisplatin

an anti-cancer, antineoplastic or cytotoxic chemotherapy drug classified as an alkylating agent and works by interfering with DNA replication.

Bortezomib

a dipeptide boronic acid derivative and proteasome inhibitor used to treat multiple myeloma and mantle cell lymphoma.

JQ1

a potent inhibitor of the BET family of bromodomain proteins which include BRD2, BRD3, BRD4, and the testis-specific protein BRDT in mammals

CRISPRa

a variant of CRISPR in which a catalytically dead (d) Cas9 is fused with a transcriptional effector to alter target gene expression. Once the guide RNA navigates to the genome locus along with the effector arm, the dCas9 is unable to make a cut, and instead, the effector activates the downstream gene expression

Mithramycin A

an antibiotic with anti-tumor properties that binds to G-C rich DNA and displaces SP1 transcription factor from its sites in the promoters of selected oncogenes, such as c-Myc and c-Src

Tudor domains

A Tudor domain is a protein region roughly 60 amino acids in length, which folds into an SH3-like structure with a five-stranded antiparallel beta-barrel form. Tudor domains recognize and bind methylated lysine and arginine residues, allowing them to function as histone readers in an epigenetic context

EC-8042

a mithramycin analog (mithralog) with enhanced anti-tumor activity that inhibits SP1 activity.

Vorinostat

an oral histone deacetylase inhibitor and antineoplastic agent that binds to the catalytic domain of the histone deacetylases (HDACs)

Interleukins

a group of cytokines that play essential roles in the activation and differentiation of immune cells, as well as cell proliferation, maturation, migration, and adhesion.

Hippo signaling

an evolutionarily conserved pathway that controls organ size by regulating cell proliferation, apoptosis, and stem cell self-renewal. In addition, dysregulation of the Hippo pathway contributes to cancer development.

Transamidase

an enzyme that catalyzes the transfer of an amide group from one molecule to another

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Box 1.**Additional therapeutic opportunities****Metformin**

Metformin is commonly used to increase insulin sensitivity in patients with type II diabetes, and has numerous known functions such as activating AMP-activated protein kinase (AMPK) and inhibiting glucagon-induced cAMP increases [83]. A recent study in SCC reveals an AMPK-independent mechanism for metformin by which treatment causes an increase in the E3 ubiquitin ligase WWP1, a known Np63 α E3 ligase [83]. Upon depletion of WWP1 in metformin-treated cells, expression of Np63 α protein is rescued. Further, it was shown that in combination with the glycolysis inhibitor, 2-Deoxy-d-glucose, metformin treatment significantly reduces tumor growth [83]. Multiple studies have also shown an effect of metformin on both YAP localization and expression levels [84–86]. This is linked to increased cytoplasmic sequestration and inactivation of YAP by Angiomotin (AMOT) and Angiomotin-like proteins 1-2 (AMOTL1-2), representing another possible mechanism by which Metformin impairs Np63 α expression [84].

Sulforaphane

Sulforaphane (SFN) is a natural isothiocyanate derived from broccoli and other cruciferous vegetables that can act as a cancer preventative [87]. In cutaneous SCC, SFN treatment was shown to increase YAP1 phosphorylation and proteolytic degradation, thereby reducing Np63 α levels [87]. It was later found that SFN covalently and irreversibly binds to TG2 to inhibit transamidase activity and shift TG2 to an open/extended conformation, leading to a partial inhibition of GTP binding [88]. As inhibition of TG2 activity is linked to impaired YAP/ Np63 α levels, this represents a likely mechanism for the SFN-induced reduction in Np63 α expression. Finally, in lung cancer, tobacco smoke is shown to induce a CSC phenotype driven by IL-6-mediated regulation of Np63 α . Treatment with SFN suppresses IL-6/ Np63 α signaling and reduces the CSC phenotype [63].

Thalidomide analogues

Thalidomide, most known for its teratogenic effects, is approved for use in multiple myeloma patients [89]. Cereblon (CRBN), together with DDB1 and Cul4, forms an E3 ubiquitin ligase complex called cullin-ring ligase 4 (CRL4^{CRBN}) [90]. Thalidomide analogues were recently found to alter the CRL4^{CRBN} ubiquitin ligase to target a number of cellular proteins for ubiquitination and proteasome degradation. Np63 α is a neo-substrate of CRL4^{CRBN} in response to Thalidomide treatment, and is targeted for degradation in the presence of thalidomide [90].

While the compounds discussed above have all shown to inhibit Np63 α in various cell lines, whether they will affect Np63 α in patients has yet to be established.

Concluding Remarks

The transcription factor Np63 α is a key regulator of epidermal morphogenesis and epithelial tissue homeostasis. Here, we have discussed evidence supporting the notion that Np63 α regulates various aspects of cancer stemness, metastasis and drug resistance across a number of cancer types. Np63 α regulation of these critical features of cancer biology has been linked to the regulation of several pathways including HELLS, CD44, integrins, WNTs, interleukins and EMT markers. Therefore, impairing Np63 α in certain cancer contexts has the potential to have a profound effect on patient survival. There are a variety of therapeutic targets upstream of Np63 α ranging from chromatin modifying proteins to cell surface receptors, kinases and transcription factors. We believe there are still many regulators of Np63 α with therapeutic potential yet to be characterized. Further characterization of Np63 α interacting partners can allow for the disruption of signaling complexes that either indirectly interfere with Np63 α activity or result in proteasome degradation of Np63 α . In addition, we believe the role of Np63 α in crosstalk with the microenvironment is a particularly exciting area for future research. Several components of the microenvironment have been identified that regulate or are regulated by Np63 α , indicating Np63 α could be a potential hub for crosstalk with the microenvironment. This raises multiple potential interesting areas of investigation (see Outstanding Questions). Although there is evidence indicating Np63 α can transcriptionally regulate some cytokines and interleukins, and interleukins can in turn regulate Np63 α , the impact of Np63 α on modelling the immune-landscape has yet to be characterized. An immunosuppressive microenvironment facilitates cancer progression, and a substantial portion of SCC patients that frequently overexpress Np63 α do not respond to immunotherapies [91]. Understanding if and how Np63 α can contribute to resistance to immunotherapies could lead to better therapeutic options in these patients. It will also be interesting to see how therapeutic targeting of cancer associated fibroblasts (CAFs) alters Np63 α expression. CAFs are capable of stimulating multiple upstream regulators of Np63 α and the growing efforts to target CAF populations may represent an indirect method of reducing Np63 α expression. Therefore, we believe further investigations into how Np63 α crosstalks with the TME will help to continue to identify regulatory pathways with therapeutic potential.

Outstanding Questions

What additional interacting partners of Np63 α have yet to be identified and what are their roles in regulating Np63 α ?

Do upstream regulators of STAT3 like VEGFR, PDGFR, CXCR4 and S1PR1 regulate Np63 α expression through activation of STAT3?

What is the role of Np63 α in the immune-landscape? Does Np63 α regulate or get regulated by the immune-landscape, and can targeting Np63 α improve responses to immunotherapies in patients with high Np63 α ?

Does therapeutic targeting of CAFs impact Np63 α expression in cancer cells? What components of CAF signaling regulate Np63 α ?

Can suppressing Np63 α prevent/overcome drug resistance?

Highlights

- Np63 α is a p63 isoform in the p53 family that is a master regulator of epithelial stemness in normal tissue.
- In cancer, Np63 α regulates a number of key aspects of cancer progression including CSC maintenance, metastasis and drug resistance through regulation of several downstream pathways.
- Np63 α is difficult to target directly, but multiple pathways upstream of Np63 α with druggable targets have been identified that represent potential therapeutic opportunities in cancer.
- Many pathways upstream of Np63 α are involved in crosstalk with the tumor microenvironment. With growing interest in targeting the tumor niche, further investigation into how Np63 α is involved in crosstalk with the microenvironment represents an exciting area of future investigation.

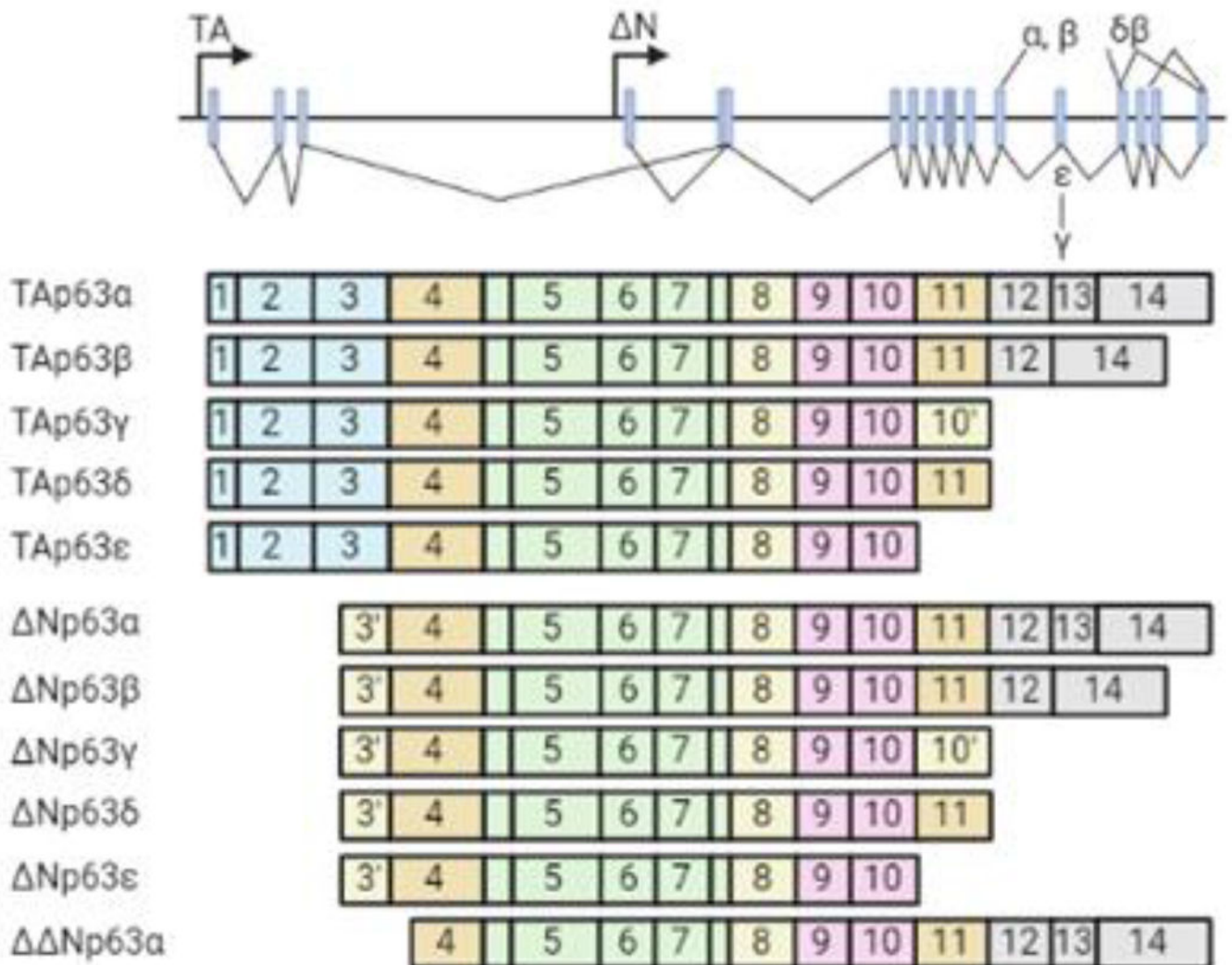


Figure 1. p63 is the primordial member of the p53/p63/p73 family of transcription factors. The human *TP63* gene consists of 15 exons spanning ~270 kb and maps to chromosome 3q27 [3]. It encodes two classes of isoforms generated by alternative promoters: TAp63 transcripts, which possess an N-terminal transactivation domain, and Np63 isoforms that lack the N-terminal transactivation domain but retain the ability to induce genes via a second transcription activation domain. Alternative splicing occurring at the 3' end of *p63* mRNAs generates multiple C-terminal variants (α , β , γ , δ and ϵ) for both TAp63 and Np63 classes [3]. TAp63- and Np63- isoforms have distinct tissue distributions. Np63 but not TAp63 is present in basal and parabasal cells in squamous epithelium and urinary bladder, and in basal cells of breast and prostate. TAp63 is detected in lymphocytes and germ cell precursors and some mesenchymal cells and endothelial cells. The existence of multiple isoforms of *TP63* with differing functions allows *TP63* to regulate a wide array of biological processes such as development and differentiation, senescence, proliferation, stem cell maintenance, and apoptosis [4]. In the context of cancer, TAp63 isoforms are generally regarded as tumor suppressors, [4]. However, Np63 isoforms— Np63 α in particular—frequently act as oncogenes.

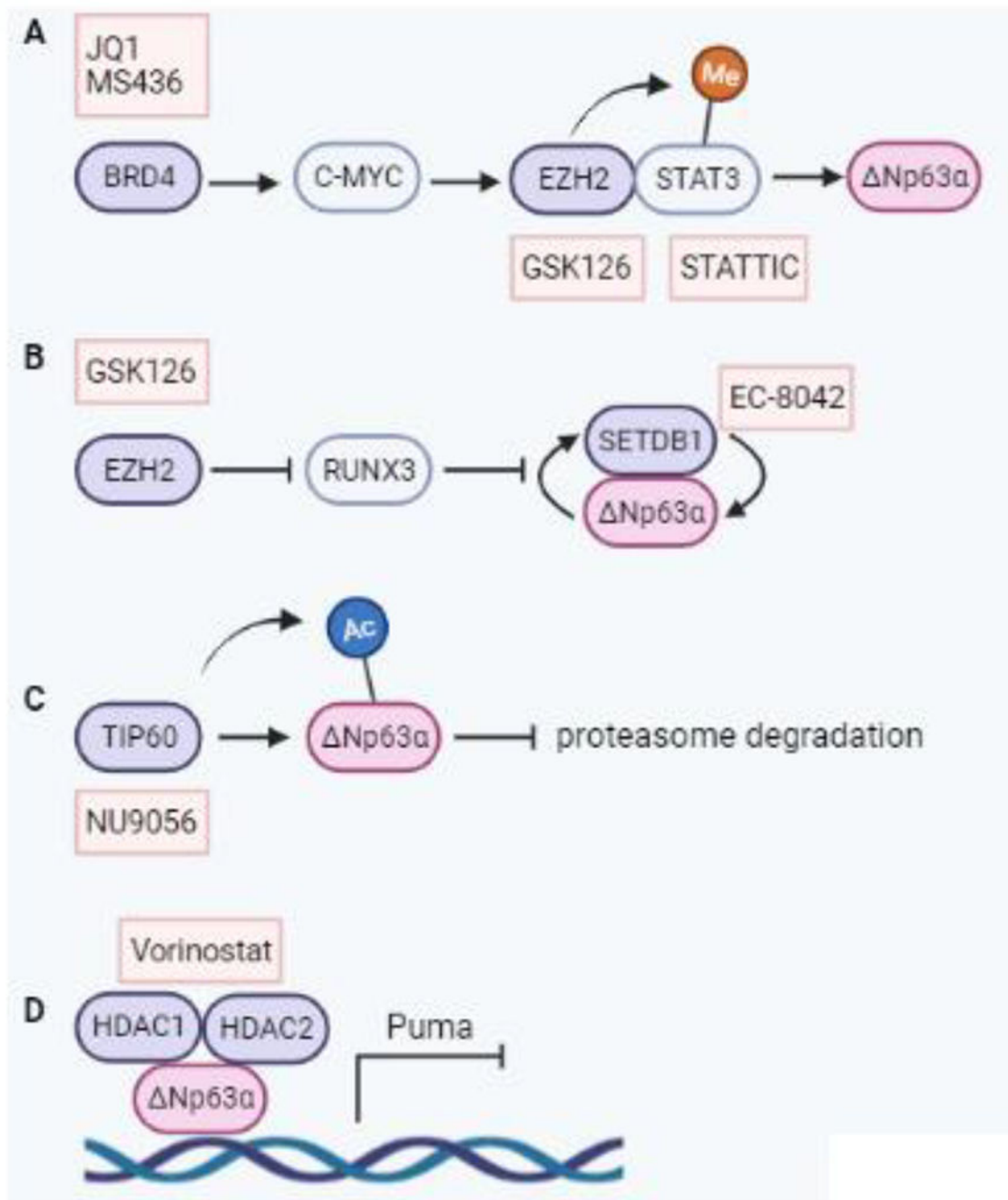


Figure 2. Mechanisms of chromatin modifying protein-mediated regulation of Np63α. **A** BRD4-driven C-MYC leads to EZH2 binding to and methylating STAT3, activating it. This results in STAT3 transcriptionally activating Np63α. Targeting BRD4 with JQ1 or MS436, EZH2 with GSK126 or STAT3 with STATTIC impair this pathway and subsequent Np63α expression. **B** EZH2 suppression of RUNX3 leads to enhanced Np63α and SETDB1 expression, which interact to stabilize expression of the other. The EZH2 inhibitor GSK126 can suppress EZH2 activity leading to increased RUNX3 and reduced SETDB1 and Np63α. The SETDB1 inhibitor EC-8024 is an additional potential means of targeting

this pathway. **C** TIP60 acetylates Np63 α to prevent ubiquitin-mediated degradation. Inhibiting TIP60 with NU9056 reduces Np63 α protein and transcript. **D** HDAC1 and HDAC2 bind to Np63 α to form an active transcriptional repressor complex. The HDAC inhibitor Vorinostat impairs activity of this complex, resulting in increased downstream activation of targets including Puma. Abbreviations: BRD4, bromodomain containing protein; C-MYC, cellular-myelocytomatosis; EZH2, enhancer of zeste homolog 2; HDAC, histone deacytlase; HDAC2, histone deacytlase 2; RUNX3, runt-related transcription factor 3; SETDB1, SET domain bifurcated histone lysine methyltrasferase 1; STAT3, signal transducer and activator of transcription 3; STATTIC, STAT3 inhibitory compound; TIP60 tat interactive protein 60.

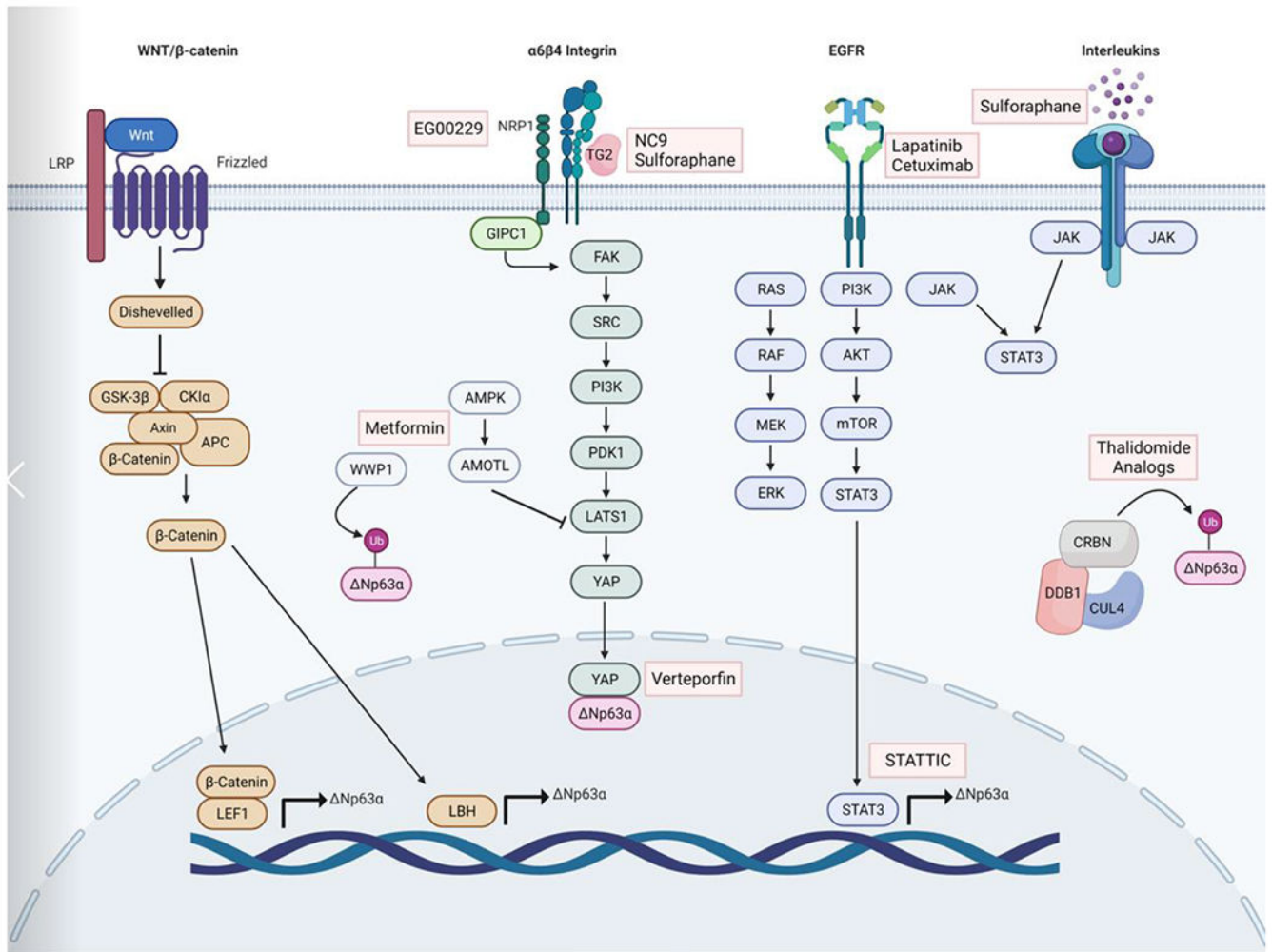


Figure 3. Schematic representation of signaling cascades that regulate Np63α and the drugs that have been shown to target them.

Several signaling cascades have been implicated in the regulation of Np63α. In Wnt/β-catenin signaling, Wnt binds to Frizzled receptors, leading to the formation of a larger cell surface complex with LRP. Activation of the Wnt receptor complex triggers displacement of GSK-3β from the APC/Axin/GSK-3β-complex. β-catenin is translocated to the nucleus where it binds to LEF1 and transcriptionally activates Np63α. α6β4 integrin interaction with TG2 or NRP1 leads to the activation of a kinase cascade that suppresses the Hippo signaling component LATS1, allowing YAP to enter the nucleus where it binds to Np63α preventing proteasome degradation. Several compounds including the NRP1 inhibitor EG00229, the TG2 inhibitor NC9 or the diet derived compound Sulforaphane which inhibits TG2, and the YAP inhibitor Verteporfin can impair Np63α protein expression. In addition to TG2, Sulforaphane can inhibit Interleukin driven JAK/STAT3 activation to suppress Np63α expression. EGFR signaling also regulates Np63α through STAT3. EGFR activation leads to phosphorylation and activation of the PI3K/AKT/mTOR pathway which phosphorylates STAT3, which binds to the promoter of Np63α. CRBN, DDB1 and Cul4, form the E3 ubiquitin ligase complex CRL4^{CRBN}.

Thalidomide analogues alter the CRL4^{CRBN} ubiquitin ligase to target Np63 α , resulting in its degradation in the presence of thalidomide. Abbreviations: AKT, alpha serine/threonine-protein kinase; AMOTL, angiomin-like protein; AMPK, AMP-activated protein kinase; APC, adenomatous polyposis coli; CK1 α , casein kinase 1 α ; CRBN, cereblon; CUL4, cullin 4; DDB1, DNA damage binding protein 1; EGFR, epidermal growth factor receptor; ERK, extracellular signal related kinases; FAK, focal adhesion kinase; GIPC1m GIPC PDZ domain containing family member 1; JAK, janus kinase; LATS1, large tumor suppressor kinase 1; LRP, low-density lipoprotein receptor related protein; MEK, mitogen activated protein kinase kinase; mTOR, mammalian target of rapamycin; NRP1, Neuropilin 1; PDK1, pyruvate dehydrogenase kinase 1; PI3K, phosphatidylinositol 3-kinase; RAF, rapidly accelerated fibrosarcoma; RAS, rat sarcoma; STAT3, signal transducer and activator of transcription 3; STAT3IC, STAT3 inhibitory compound; WNT, wingless and INT1; WWP1, WW domain containing E3 ubiquitin protein ligase 1.