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MED12 Mutations Promote Castration-Resistant Prostate Cancer Through Hyperactivated GLI3/SHH Signalling

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MED12 MUTATIONS PROMOTE CASTRATION-RESISTANT PROSTATE CANCER THROUGH HYPERACTIVATED GLI3/SHH SIGNALLING

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When cells of the prostate gland start growing uncontrollably, it results in cancer due to genetic aberrations. The development and progression of prostate cancer depend on androgenic stimulation. Although prostate cancer is temporarily treated by depriving a tumor of androgen, the patient relapses getting a castration-resistant form of the disease called castration resistant prostate cancer (CRPC). CRPC can specifically develop through crosstalk of androgen receptor signalling pathways. One such pathway that is of specific interest in this paper is the MED12 regulated SHH signalling pathway. Mediator is a signal processor that helps in the transduction of gene-specific transcription factors to RNA Polymerase II (Pol II).The activation of SHH ligand activates a transcription factor called GLI3 which physically targets the MED12 interface within the mediator complex so as to functionally reverse mediator dependent suppression of SHH target gene transcription. In this paper, we have shown that MED12 expression is critical in regulating androgen independent prostate cancer cell and therefore progression towards CRPC. Also, GLI3-dependent SHH signalling is indeed required for the progression of MED12 mutated prostate cancer cells to CRPC, after castration.

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Prostate Cancer Overview

When cells of the prostate gland start growing uncontrollably, it results in cancer. The prostate is an endocrine gland in most mammals which is a part of the male reproductive system.[1] It is a compound tubuloalveolar gland that secrets an alkaline fluid that in human constitutes 30% of the seminal fluid.[3] Prostate cancer might happen due to genetic aberrations that inactivate tumor suppressor genes or activate oncogenes. Most of the mutations are acquired during the evolution of cancer, however, some of them may be inherited.[4] Human prostate is a small walnut-sized organ that is located just below the bladder and surrounds the urethra. It contains a system of branching ducts comprising pseudo-stratified epithelium surrounded by a fibro-muscular stroma. The prostate is a male sex accessory gland that functions by producing and secreting fluids that contribute to the ejaculate, and thereby significantly enhances male fertility. Intriguingly, the prostate is highly susceptible to oncogenic transformation at a frequency significantly greater than that of other male secondary sexual tissues, such as the seminal vesicles.[5] There are different types of prostate cancer based on its cellular origin. The most common type is “adenocarcinomas,” which start with the gland cells that make the prostate fluid, which is added to the semen. Other types are sarcomas, small cell carcinomas, neuroendocrine tumors, and transitional cell carcinomas.[1] Prostate cancer is a progressive disorder (*Figure 1*), that is, it starts as a benign tumor in the prostate gland cell (in case of adenocarcinomas), which is considered stage I. It then progresses to prosthetic intraepithelial layer and nearby tissues, which is considered stage II. Stage III is when the tumor becomes metastatic and spreads to other organs. The disease is considered to have progressed to stage IV when the cancer has spread to distant organs like the bone. At this stage the tumor becomes resistant to most kinds of treatment including certain chemotherapy and hormone therapy.

Finally, the tumor becomes castration resistant and insensitive to androgen and other therapies when it is considered terminal.

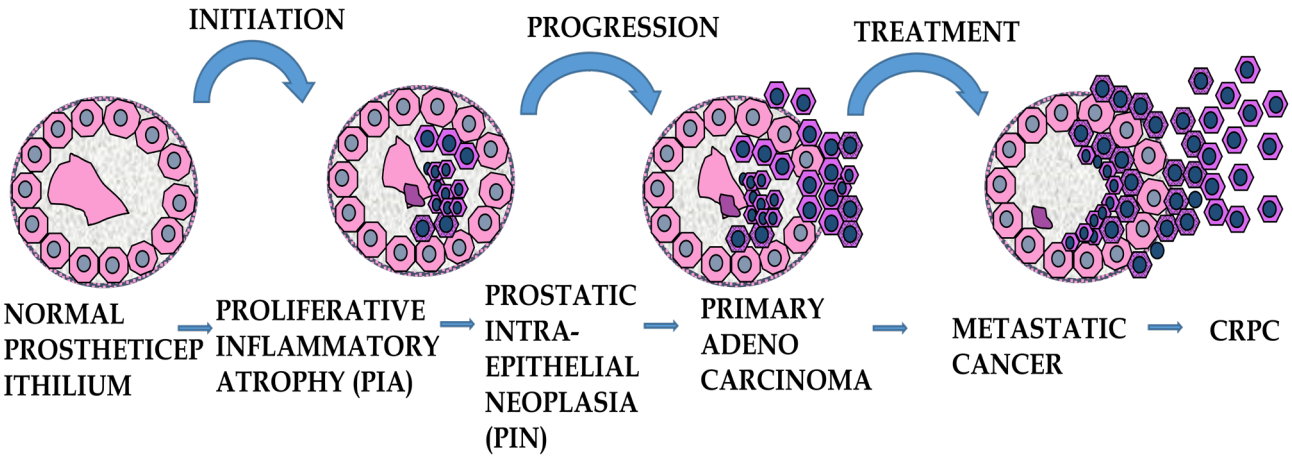


Figure 1. Prostate cancer progression from healthy prosthetic epithelial tissue to castration resistant form. Cells of the prostate start looking abnormal from high PIN stage.

Precancerous Conditions

Depending on how prostate cells look under a microscope, their scope of becoming cancerous is determined. This is done during a biopsy which is an important diagnostic tool. Prostatic Intraepithelial Neoplasia (PIN) is a condition in which some of the prostate cells have begun to look abnormal due to neoplastic growth of epithelial cells within preexisting benign prostatic acini or duct.[6] Depending on how different the intraepithelial cells look under a microscope, they are classified as

- Low-Grade PIN: When the cells look closer to normal
- High-Grade PIN: When the cells look more abnormal

Existence of low-grade PIN does not necessarily lead to cancer, but existence of high grade PIN has a 20% chance that a cancerous condition exists.[6]

Proliferative Inflammatory Atrophy (PIA) is another condition in which the cells of the prostate tend to look smaller than normal and it is usually accompanied by signs of inflammation

in that area. Studies say that PIA might sometimes lead to high grade PIN or prostate cancer and that disease progression is usually initiated by PIN, which then progresses to high grade PIN followed by adenocarcinomas which is the invasion of epithelial cells into the stroma followed by metastasis.[7]

Key Statistics

Approximately one in seven men will be diagnosed with prostate cancer during their lifetime. According to the World Health Organization, per year 1.1 million men are affected by prostate cancer, accounting for 15% of all cancers in men; with an estimated death rate of 307,000 per year, prostate cancer is the fifth leading cause of death due to cancer in men.[8] Hence this disease has been the focus of intense investigation to understand its biology and to provide improved treatment. Since prostate cancer is usually diagnosed in early stages, the treatment options can be decided based on a number of factors like whether prostate-specific antigen (PSA) levels are rising or steady, whether cancer has spread to the bones and health history.

PSA test. Prostate Specific Antigens is a protein that is generally produced by all prostatic cells; but in case of cancerous conditions, this level raises. This is used as a diagnostic tool.[9] This test is usually measured in units of nanograms of PSA per milliliter of blood (NG/ML). This test is usually recommended for men over the age of 50. A score above 4.0 NG/ML is considered abnormal and further examinations are recommended.[10]

Gleason score. The Gleason score is a grading system used to determine the aggressiveness of prostate cancer. Cancer tissue contains cancer cells that are of different stages. Gleason score is calculated by adding together the two grades of cancer cells that make up the largest area of biopsy tissue sample. A score of 8-10 indicates that the cancer is more aggressive.

Treatment Options for Prostate Cancer

Local treatments are aimed at prolonged disease-free survival for many patients with localized prostate cancer.[9] However, the treatment options are decided depending on the stage of the disease, the Gleason score and the PSA level. Other important factors for consideration before treatment are the man's age, his general health and the possible side effects of the treatment. The table below lists the possible combination of treatment options available and commonly suggested for different stages of prostate cancer.

Radical Prostectomy

Surgical removal of the prostate gland and its surrounding tissue usually including seminal vesicle and nearby lymph node is called radical prostectomy. This type of treatment is generally reserved for patients with overall good health and if the tumor is confined to the prostate gland, that is if the patient is in stage 1 or stage 2 of disease. It can be done by retro-pubic approach that is through abdominal incision or by perineal approach that is through an incision in a small space between the scrotum and the rectum. It is important that a pelvic node dissection is definitely done in case of a large, less differentiated tumor. A laparoscopic or a surgical method can be followed depending on other factors. Although this treatment is more effective in 12.7% of the cases than watchful waiting, there are a number of drawbacks.[9] They are,

- Sexual dysfunction
- Fecal and Urinary incontinence or impotence[11]
- Penile shortening[12]
- Inguinal hernia[13]

Radiation Therapy

The use of radiation to induce apoptosis in growing cancer cells by creating an ionic imbalance in the cell microenvironment is called radiation therapy. This type of treatment is most suitable for patients diagnosed with cancer in prostate and surrounding tissues that is if they are in stages 1, 2 and 3. Radiation therapy is often recommended in combination with other treatment methods depending on the Gleason score and metastatic properties.[14] Primary radiation used for cancer treatment is ionizing radiation. Although non-iodizing radiations such as radio waves, microwaves and visible light waves are also available, iodizing waves are considered more efficient.[15] Radiation therapies for prostate cancer can be of two subtypes: brachytherapy or internal radiation and external beam radiation. The unit of measurement for radiation is Gray (Gy) which is defined as the absorption of one joule of radiation energy per kilogram of matter and is often given in small dosages over a period of time.[16]

External Beam Radiation Therapy (EBRT). EBRT is the most widely used method where the source of radiation is from outside the body and photon beams are often used for this kind of treatment.[17] Intensity modulated radiation therapy, volumetric modulated arc therapy, image guided radiation therapy and intensity modulated proton therapy are some of methods used in external beam ration therapy where computerized three dimensional imaging techniques are used to map the tumor along with photon beams from different directions. Stereotactic radio surgery is another technique used to deliver external radiation. It is generally used for large precise dosage of radiation for a small tumor and is generally used when the tumor spreads to the brain. Intra operative radiation therapy is a technique for EBRT used during surgery.[18]

Internal Radiation Therapy or Brachytherapy. In this method, radiations seeds or tubes (catheters) are often placed near the site of tumor which gives out radiation over a period of time. These seeds are then removed. This method is often done to minimize the damage of nearby normal tissues. It is useful in cases which need high dosage of radiation.[17] Patients selected for brachytherapy often have low Gleason score; low PSA levels and are mostly in stage 1 or 2 of prostate cancer.[18]

Complications in radiation therapy include:

- Acute cystitis, Proctitis and Enteritis.
- Sexual and Urinary dysfunction
- Radiation itself being carcinogenic, ration therapy for Prostate Cancer comes with an increased risk of bladder and gastrointestinal cancer

Chemotherapy

The use of therapeutic drugs to reduce the symptoms caused by advanced cancer or to stop the cancer recurrence is called chemo therapy. Some of the most commonly used drugs include Docetaxal (Taxotere), Cabazitaxel (Jevtana), Mitoxantrone (Novantrone), Estramustine (Emcyt) all of which act by affecting one or more steps of androgen receptor mediated signalling. Studies show that a combined treatment with docetaxel and estramustine gives a longer survival and slower progression of the cancer.[19] Major drawbacks of chemo therapy are that patient experience low blood cells count, reduced heart functions, hair loss up to 65%, diarrhea, nausea and vomiting, loss of appetite, shortness of breath, easy chances of infection and fluid retention.[9]

Immunotherapy

Artificial activation or *in vitro* modification of immune cells to employ them towards the treatment of prostate cancer is called immune therapy. Cancer cells have tumor specific antigens which often go unnoticed by the immune cells leading to the development and progression of cancer. In a healthy human, the immune cells recognize any irregularity in cells and induce apoptosis. Immunotherapy utilizes this property to artificially induce apoptosis or cell death of cancer cells.[20] Sipuleucel-T which is the first FDA approved immunotherapy for prostate cancer demonstrates improved survival for advanced, metastatic prostate cancer. There are two subtypes of immunotherapy namely: cellular immunotherapy and antibody therapy.[21-22] Other types of immune therapy called the checkpoint inhibitor therapy targeting CTLA-4, PD-1 and PD-L1, have been approved for the treatment for other types of cancer and are being experimented as treatments for prostate cancer. [20] Side effects include chills, fever, headache, myalgia, sweating, and influenza-like symptoms.[9]

Cellular Immuno therapy. Cellular immunotherapy is of two types namely dendritic cell therapy and car-T cell therapy. In dendritic cell therapy, patient's dendritic cells are removed from the patient's body and are artificially incubated with tumor antigens and activated. These are then returned to the patient's body to induce immune response.[21] In Car-T cell therapy, the patient's T lymphoid cells of the patient are harvested, genetically modified by adding a chimeric antigen receptor thus preparing it to target the cancer cells for destruction.[22]

Antibody therapy. In this type of treatment, in vitro methods are used to make antibodies against the tumor surface antigen and are then injected into the tumor to produce an immune response against the cancer cells. There are different types of antibodies depending on:

- Conjugation: That is if the antibody is naked or is joined with radioactive or cytotoxic molecules.[22]
- FC region: The antibodies come in numerous subtypes and it can be modified based on the immune response that the antibody is designed to trigger.[23]
- Humane/ Non-humane: Antibodies can also be classified based on its source. Antibodies from a different species are called murine; Chimeric antibodies are modified murine antibodies with human counterparts; and human antibodies are derived from human DNA.[24]

Hormone Therapy

Lowering the levels of testosterone hormones to increase the years of survival and to prevent the progression, in case of prostate cancer is the central idea behind hormone therapy. Androgen deprivation therapy or androgen suppression therapy is a standard method of treatment used for all stages of recurrent tumors.[7] Androgen is a male steroid hormone synthesized majorly by the testicles and up to a small quantity by the adrenal glands in males. Androgen has major roles in development and maintenance of male sexual characteristics, development of skeletal muscles, regulating aggression, libido and sexual differences. In prostate cancer, Androgen is the key hormone for therapy since it plays a major role in development and maintenance of prostate cells thus contributing growth and progression of prostate tumors. Studies show that although Androgen Deprivation therapy initially works, in most cases patients relapse with a castration resistant form of the disease.[25] Common methods of hormone therapy are:

Surgical castration or Orchioctomy. This involves surgical removal of the testicles to decrease the level of circulating androgen thereby stopping tumor growth. Unlike other types of treatment, Orchioctomy is permanent. This treatment is often done along with other androgen

blocking techniques since the adrenal and the prostate themselves can produce minor quantities of androgen that will support the tumor growth.

Luteinizing Hormone Releasing Hormone (LHRH) agonist. LHRH agonists are drugs that reduce the amount of testosterone produced by the testicles. These drugs are given as intravenous injections or placed as small implants under the tissue. They are given specific dosages over a period of time. Commonly used LHRH agonists include Leuprolide, Goserelin, Triptorelin and Histrelin. The primarily mechanism behind LHRH agonist is that they persistently release LHRH making the pituitary to LHRH and hence reduce the level of testosterone.[7] A major drawback with this type of treatment is that there is an initial surge in testosterone levels causing severe side effects such as liver pain, urethral obstruction and impending spinal cord compression in some cases.[25]

Luteinizing Hormone Releasing Hormone (LHRH) antagonist. These are another class of drugs that are also considered medical castrating agents. They work by blocking the LHRH receptor thus lowering the level of circulating androgen. Degarelix is the most popular FDA approved LHRH antagonist. It lowers the testosterone levels more quickly and does not cause the initial increase in testosterone level unlike in LHRH agonist. Abiraterone is a drug used for the hormone therapy. This drug works by blocking an enzyme called the CYP17 which stops other cells in the body like the adrenal cells and prostate themselves from making androgen. This drug is generally prescribed for patients who are at a high risk or are castration resistant.[7]

Anti-Androgen. Anti-androgen are drugs that have a high affinity for androgen-receptor. They bind to androgen receptor in place of androgen there by not allowing the downstream signalling by androgen receptor thus stopping tumor growth. Thus they are also called the competitive inhibitors for androgen. Flutamide, Bicalutamide and Nilutamide are some of the

most common FDA approved anti-androgen drugs. Other drugs used for anti-androgen like treatment are: Dutasteride and Finasteride. These work by preventing the conversion of testosterone to DHT ultimately reducing the level of circulating androgen. Galeterone and ASC-509 are drugs under investigation that are targeted for androgen receptor degradation. EPI-506 is another drug under investigation that is aimed at blocking the N-terminal domain of the androgen receptor.

Limitations of hormone therapy. Although androgen deprivation therapy has a number of serious side effects like reduced sexual desire, impotence, penile shortening, hot flashes, growth of breast tissue, osteoporosis, anemia, decreased metal sharpness, loss of muscle mass, fatigue, increased cholesterol levels and depression.[26] The most life threatening one is that, androgen deprivation leads to castration resistant form of prostate cancer over a course of time. Recently there is increasing evidence to show that androgen deprivation could support progression to castration resistant form of prostate cancer.

Castration resistant form of prostate cancer

Growth of tumor that has got adapted to androgen deprived conditions is called castration resistant prostate cancer (CRPC). It is known that effect of hormone therapy is temporary as almost all patients relapse and develop a castration-resistant form of the disease (CRPC).[27] Although the mechanism by which castration resistant prostate cancer (CRPC) develops is multi-factorial, there are four major pathways by which CRPC develops. : 1) Increased sensitivity of the androgen receptor (AR) to its agonists, 2) AR mutations that render the receptor responsive to alternate, non-androgen ligands, 3) ligand-independent AR activation, and 4) AR-independent mechanisms.[28] Recently, reports have shown that CRPC can specifically develop through crosstalk of specific signalling pathways wither signalling; leading to an over expression of AR

protein.[29] About 80% of the CRPC patients had significantly augmented AR mRNA levels and contrasting evidences showed that AR gene amplification was not so frequent in untreated prostate cancers.[30-31] Apart from gene amplification and elevated mRNA expression, increased protein half-life also contributed to elevated level of AR protein in CRPC. In addition, AR co-regulators which are protein factors associated with AR transcriptional activation or inhibition were also found to serve an important role in development of CRPC.[32]

Signalling Pathways Involved In Prostate Cancer

Androgen Receptor signalling

Androgen receptor. Androgen receptor is a cytoplasmic transmembrane G-Protein coupled receptor.[33] It has three main functional domains namely: N-terminal transcriptional regulating domain, DNA binding domain and the ligand binding domain. While the terminal domain is the most variable one, the DNA binding domain is highly conserved consisting of zinc fingers that recognize and facilitate in direct binding of androgen receptor to the target gene. The ligand binding domain has a structure similar to that of a nuclear receptor and is found in association with heat-shock and chaperone proteins. They help in stabilizing the bound androgen.[34]

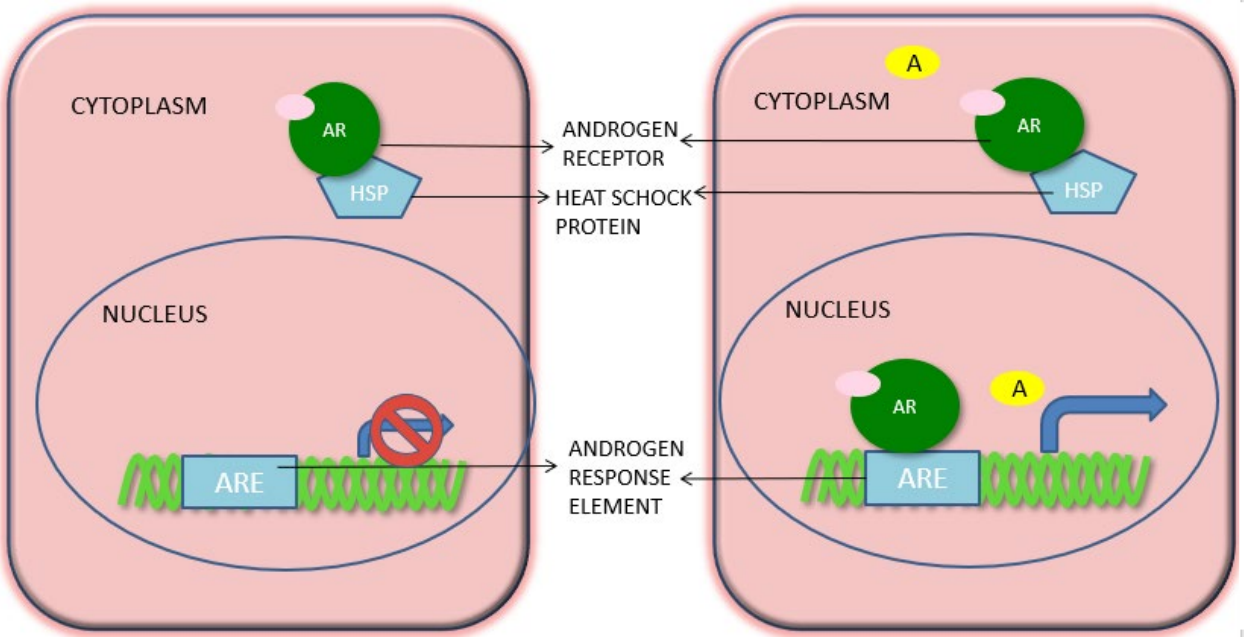


Figure 2 **-**Canonical androgen receptor signalling pathway
In the absences of androgen, the androgen receptor remains bound to the heat shock protein and hence the androgen response element does not get activated leading to no gene transcription; But in presence of androgen, this constraint on androgen receptor gets released due to the conformational changes thus leading to its translocation into the nucleus where it gets bound to the heat androgen response element thereby causing gene transcription.

Canonical AR signalling pathway. It is also called the DNA binding dependent action of AR (*Figure 2*). In this pathway, the binding of androgens to the AR receptor results in conformational change, leading to the dissociation of bound chaperone proteins (Heat Shock Proteins) and exposure of the nuclear localization signal. The androgen complex thus translocates into the nucleus where dimerasation occurs. It then binds to androgen response element for target gene transcription. This process is modulated by coregulators. They bind to the androgen receptor in a ligand dependent manner to upregulate or suppress the transcriptional activity through histone modification and through basal transcriptional machinery.[33]

Hedgehog Signalling

The hedgehog (Hh) was first identified as a segment polarity gene product in *Drosophila melanogaster*.[1] The Hh is a very important signalling pathway contributing to a number of important physiological function and development in a variety of organisms. Drosophila hedgehog has a central role anterior-posterior axis formation through its ability to modulate Cubitus interruptus (Ci), a DNA-binding transcription factor that has bi-functional ability to regulate Hh target gene transcription. Studies show that a morphogenetic gradient controls the pattern of gene expression that in turn determines cell fate.[35]

Mechanism of action. The hedgehog signalling pathway (Figure 3) is initiated by a 19kDa dually lapidated protein derived from autocatalytic processing of full-length Hh translation product. This Hh protein binds to a 12 pass transmembrane protein called Patched receptor (Ptch) thereby hindering Ptch mediated inhibition of Smoothened (Smo) which is a seven pass trans-membrane protein. Activated Smo in turn promote the activation of full length Ci and hence its accumulation in the cytoplasm through a complex series of events. This set of events includes, inhibiting the processing of, suppressor form of Ci. Thus, full length Ci activator translocates into the nucleus, where it binds to and stimulates Hh target gene transcription.[35]

Hedgehog signalling in human. In humans, Hedgehog (Hh) signalling plays an important role in embryonic patter formations, maintenance of adult stem cell and determination of cell fate in significant physiological organs. Dysregulations in this pathway is linked to a variety of pathologies including developmental abnormalities and cancers. Although basic aspects of Drosophila hedgehog have been conserved in human, but due to the enhanced complexity in human, there is an increase in the number of pathway components. Mammals encode three distinct Hh family members namely Sonic Hh, Indian Hh and Desert Hh; two Ptch

homologues Ptch 1 and Ptch 2 and three Ci-related transcription factors GLI1, GLI2 and GLI3 are found. Among the mammalian Hh protein family, SHH is the best characterized and functions to regulate cell fate specification, proliferation, and/or differentiation in a wide variety of target tissues and organ systems.[35]

While GLI1 functions primarily as an activator, GLI2 and GLI3 functionally resemble Ci as a bipartite transcriptional regulator of Hh target genes. Like in Drosophila Hh, activation of SHH signalling pathway suppresses the proteolytic production of GLI3 repressor thereby promoting the accumulation of full-length GLI3 with activator potential. With respect to the activation function of Gli3, previous studies have revealed a physical and functional interaction between mammalian Gli3 and the histone acetyltransferase CBP.[36] The observation that the *C*BP-*b*inding *d*omain on Gli3 (CBD; amino acids 827 to 1132 of the 1,596-residue protein) can function as an independent transactivation domain supports the idea that CBP is a transcriptional co-activator of Gli3.[36] However, more recent studies have identified Gli3 sequences outside the CBD with autonomous transactivation function, suggesting the involvement of additional unidentified activities in Gli3-directed transcription.[37]

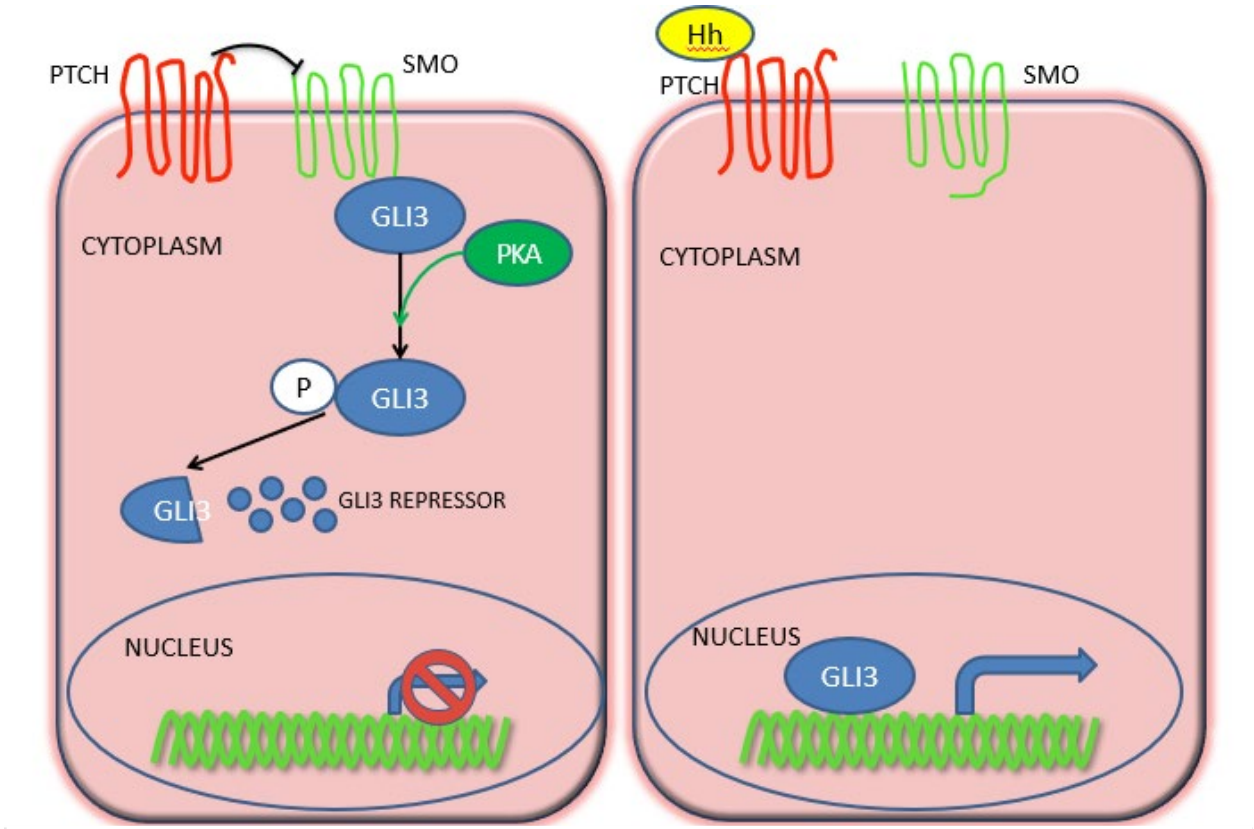


Figure 3- Sonic Hedgehog signalling pathway
In the absence of the Hedgehog ligand, GLI3 gets cleaved into the repressor form which prevents any target gene transcription. Whereas attachment of the Hedgehog ligand to the patched receptor brings conformational changes that releases the bound smoothened thereby preventing it from cleaving the GLI3 protein. Thus full length GLI3 (which is the activator form) is now available to bring about target gene transcription.

Hedgehog signalling in prostate cancer. SHH signalling plays an essential role in development of embryonic prostate. It is also present in the development of epithelium of urogenital sinus from where the prostate derives. In adults, SHH is important in the regeneration of prostate epithelium. Although multiple components of hedgehog signalling pathway fall under the susceptible region of human prostate cancer, however the only known mutation is the loss of function mutation found in Sufu in prostatic tumor.[35] Studies show that when a prostate cancer patient is treated using androgen deprivation therapy, their hedgehog signalling activity in the tumor cells go up.[38] It is important to note theMED12 protein which is a subunit of mediator

complex to which GLI3 is said to attach for the downstream activation of target genes is found to be mutated in 5.4% of the prostate cancer cases.[39] This leads us to our model (*Figure 10*) in which androgen deprivation induces SHH signalling which then activates GLI3. In cells where MED12 expression is normal, MED12 would place a constraint on GLI3 thereby preventing hyper-activation of GLI3 target genes and subsequent cell proliferation. When MED12 expression is low, this constraint on GLI3 gets released thereby inducing expression of GLI3 target genes and cell proliferation.

Mediator complex and MED12 gene

Mediator. Mediator is an evolutionarily conserved multi protein, 26 subunit (in-human) signal processor that helps in the transduction of gene-specific transcription factors to RNA Polymerase II (Pol II). It was originally discovered in a yeast species called *Saccharomyces cerevisiae* and has been found to be conserved in a wide variety of organisms.[40] Functions of Mediator are mainly assembly, activation, and regeneration of transcription complexes on core promoters during the initiation and reinitiation phases of transcription including preinitiation complex (PIC) formation, Pol II recruitment and control in the transcription process.[41] Also, studies show that mediator is required for super-enhancer activity between regulator bound enhancers and basal transcription on core region. It has been shown that Mediator induces conformational changes sufficient enough to impact functional interactions related to its transcriptional process. [36] Mediator is composed of multiple subunits but divided into 4 distinct modules namely head, middle, tail and kinase module (Figure 4). The Mediator core comprising of the head, middle and tail binds to RNA Polymerase II while the kinase module exists in variable association with the core Mediator complex. Transcription factors specific for particular genes bind to tail and kinase modules and this information is then transduced through the middle

and head modules to the RNA Polymerase II. [36] In human, the Mediator Kinase module (which is a 560 kDa) consists of four subunits namely MED12, MED13, CDK8 and CyclinC (cycC) that exist in variable association with the mediator core.[36]

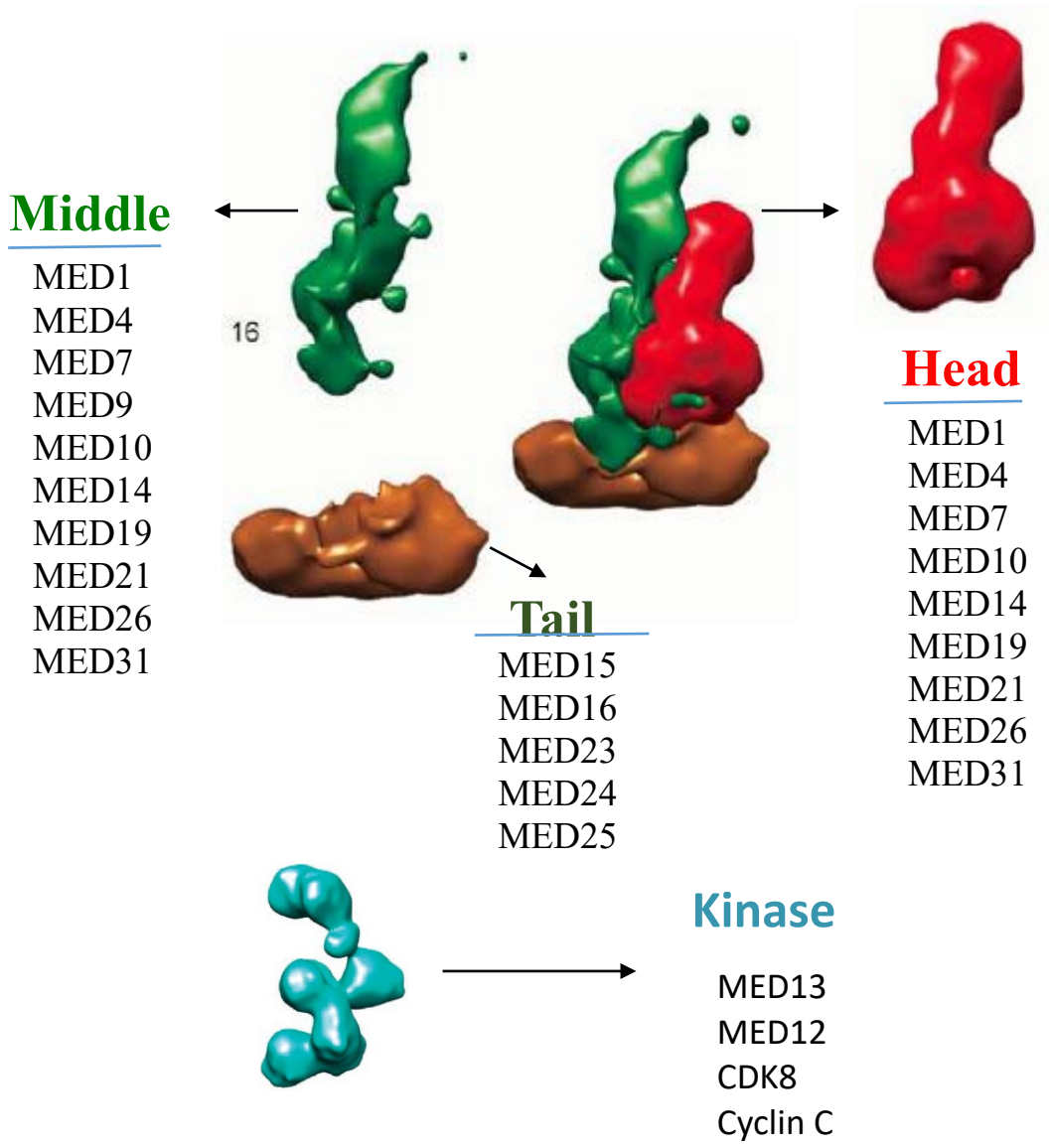


Figure 4- Mediator Complex
It consists of the Head, Middle, Tail and the Kinase subunits. The Head, Middle and Tail together is considered the Core mediator complex. The RNA polymerase II binds to the core and the transcription factor binds to the kinase. The kinase exists in variable association with the core, thus passing on the information from the transcription factor to RNA polymerase II.[42-43]

MED12. MED12 is an Xq13 encoded 230Kda subunit of the RNA polymerase II transcriptional mediator.[29] MED12 plays a role in the formation of the CDK8 kinase which is involved in the nuclear transduction of signalling for several oncogenic pathways.[29] Moreover, MED12 also indirectly affects the p53 and androgen signalling.[44-29] MED12 was found to have direct physical and functional interaction with GLI3 and that, GLI3 binds to isolated MED12 and intact mediator through MED12 or mediator binding domain. It was also shown that, in response to Sonic Hedgehog signalling, there was an inhibition on the disruption of GLI3-MED12 interaction while MED12 depletion enhanced both MBD transactivation activity and GLI3 dependent gene induction in response to SHH signalling. In the SHH signalling pathway, release and binding of SHH ligand to its patched receptor causes activation of smoothened which in turn activates a GLI family protein called GLI3 transcription factor that controls the transcription of the target gene. Studies show that activated GLI3 physically targets the MED12 interface within the mediator complex so as to functionally reverse mediator dependent suppression of SHH target gene transcription. Thus, MED12 is required for the modulation of Gli3 dependent SHH signalling.[30] This study shows that, MED12 which is found mutated in 5.4% of the prostate cancer, disrupts the mediator imposed a constraint on GLI3 dependent SHH signalling causing CRPC.[39] Mutations in MED12 also cause disorders such as X-linked intellectual disability (XLID), multiple congenital anomalies, including craniofacial, musculoskeletal, behavioral defects in humans with FG (or Opitz-Kaveggia), Lujan syndromes and Ohdo syndrome, which is a neuro-developmental disorder characterized by genital abnormalities and missing kneecaps.[45-29]

Methods

Lenti-viral particle production

293T cells were seeded at 5.5×10^6 cells in 10cm dishes, 24 hours prior to transfection.5µg of plasmid pMD2G, 5µg of plasmid pSPAX2 and 5µg of the desired lenti-viral plasmid (namely the CONTROL-pLKO.1 or MED12-pLKO.1) were mixed together in 500µl of serum free media by pippeting followed by a brief spin. In a different tube, ~45µl of XtremeGene9 transfection reagent (3 times by volume to the DNA mixture) was mixed together with 500µl of serum free media. The diluted transfection reagent was immediately added to the DNA solution followed by 15 min incubation. This mixture was then added drop-wise to the 293T cells from the previous day at ~80% confluency. Media from these plates were collected on Day 4 and Day 5 into the same tube and store in 4°C. These tubes were then spun for 5 min in 4°C at 1100rpm to pellet cell debris. The supernatant was then spun in ultracentrifuge at 26000rom for 1:45 hrs. The pellet containing the virus was carefully resuspended in 100µl of serum free media per centrifuge and stored in 4°C overnight. 10µl aliquots were made and stored at -80°C.

Virus Infection

LNCaP cells were seeded at 5×10^5 cells per well in a 6 well plate. Mixtures for infection were prepared using different concentrations (0, 1µl, 2.5µl, 5µl, 7.5µl and 10µl) of MED12 or Control virus with of 500µl of serum free media and 8µg/ml polybrene. Before infection, the plate was washed with serum free media and then the virus mixture was added to the plate drop-wise. 4 hours later, the plate was supplemented with full growth media. On day 4, cells were split depending on its confluence. On day 5, media was replaced with full growth media containing

3µg/ml of puromycin for selection. On day 8, plates with 50-80% cell survival are taken for further experimentation.

Cell Cultures

LNCaP [A], MED12 knock down LNCaP [B], Control knock down LNCaP [C] and GLI3 knock down LNCaP[D] were regularly cultured at 37° C and 5% CO_2 in RPMI 1640 media supplemented with 10% fetal bovine serum (HyClone) and penicillin-streptomycin-L-glutamine (Invitrogen). In androgen deprived conditions cells were cultured in RPMI 1640 media without phenol red supplemented with 10% charcoal stripped fetal bovine serum and penicillin-streptomycin-L-glutamine (Invitrogen). The MED12 knock down LNCaP were further knocked-down with shGLI3 virus [E] and shControl Virus [F] and were regularly cultured in RPMI at 37° C and 5% CO_2 in RPMI 1640 media supplemented with 10% fetal bovine serum (HyClone) and penicillin-streptomycin-L-glutamine (Invitrogen).

Quantitative Real-Time PCR

Cells were seeded at 4×10^5 cells in 60Mm dishes in both androgen replete and androgen deprived conditions, split when ~80% confluent and RNA was extracted on day 3, day 6 and day 9 using Trizol reagent. GLI3 knock down cells were infected with GLI3 specific shRNA expressing lenti-virus and selected with puromycin (3µg/ml) prior to seeding. Cyclopamine (or DMSO) was added 24 hours prior to RNA harvest for all cyclopamine treated cells. RNA was reverse transcribed using oligo (Dt) and superscript III (Invitrogen) following standard procedures and used in quantitative reverse transcription-PCR.

Proliferation Assay

For proliferation assay, cells were seeded at 1×10^4 cells per well original density in 6 well plates in triplets (androgen replete and androgen independent conditions) and media was changed

every other day. Growth rate was calculated by dividing cell confluence value on days 3, 6, 9 and 12 by the initial confluence value. Results are the mean of three independent experiments.

Colony Formation Assay

For Colony formation assay cells were seeded at 2000, 4000 and 6000 cells/well in a 6-well plate in regular and androgen deprived conditions. Media was changed every 3 days and cells were stained with crystal violet on day 15. Wild type LNCaP cells [A], MED12 knock-down LNCaP [B] and control knock-down LNCaP cells [C] were used in one set of experiment. GLI3 knock-down LNCaP cells [D], GLI3 knock-down MED12 knock-down LNCaP cells [E] and control knock-down MED12 knock-down LNCaP cells [F] were used in the other set of experiment. The cells were selected with puromycin (3µg/ml) prior to seeding. Over the course of the assay, cells were kept at 2µg/ml selection.

Results

MED12 Knockdown Promotes Androgen Independent Growth

In order to study the effect of MED12 mutations on CRPC cell growth we knocked down MED12 expression in LNCaP cells and proliferation assay was done with both the parental and MED12 knockdown LNCaP cell, as described in the protocol above. Since MED12 mutations inactivate the protein we assume that MED12 knockdown mimics the MED12 mutant setting. MED12 knockdown does not dramatically effect LNCaP cell growth in androgen replete conditions, but we observe an increase in cell growth specifically in the absence of androgens (Figure 1). Therefore, it appears that MED12 expression is critical in regulating androgen independent cell growth and therefore progression towards CRPC.

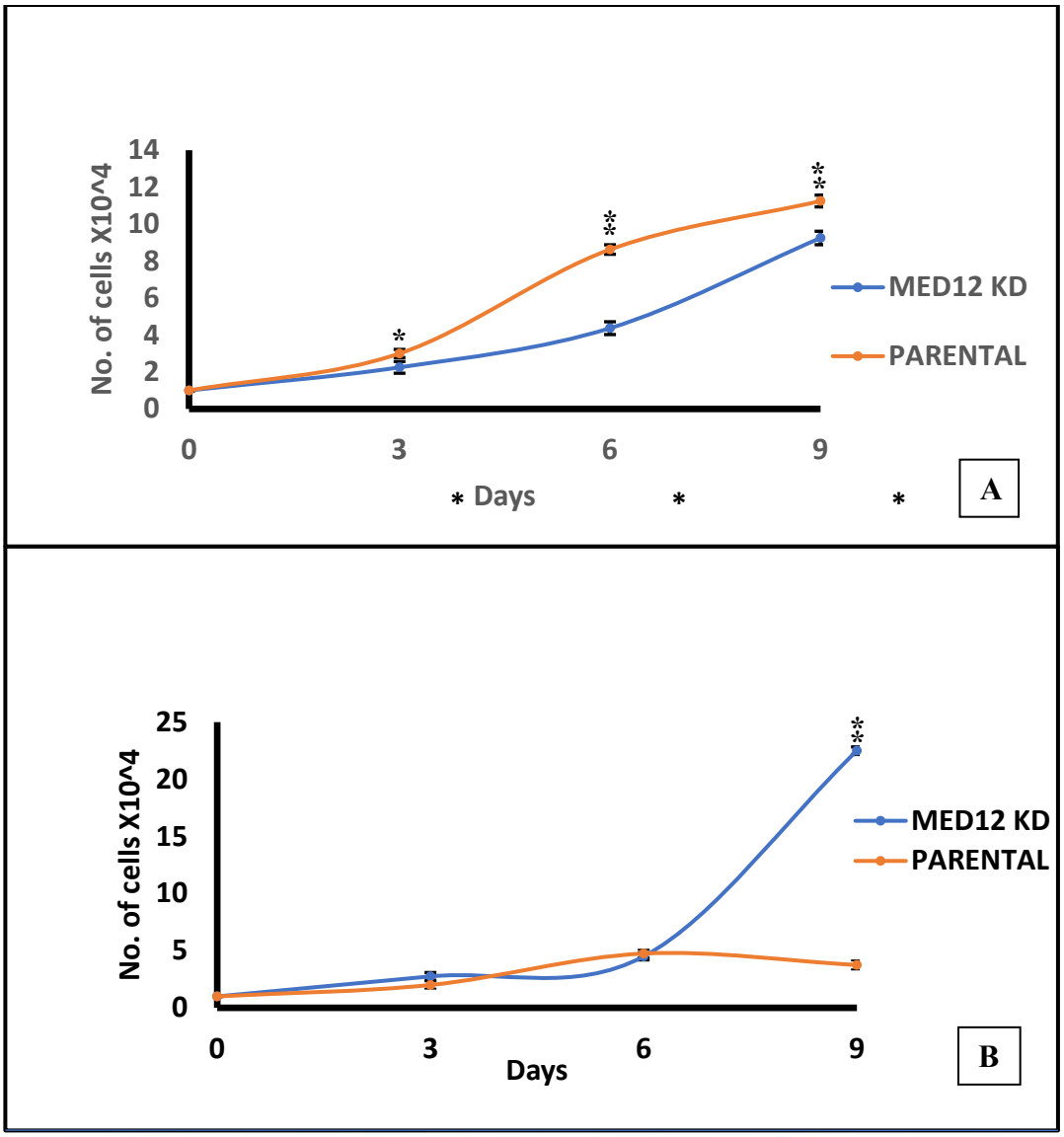
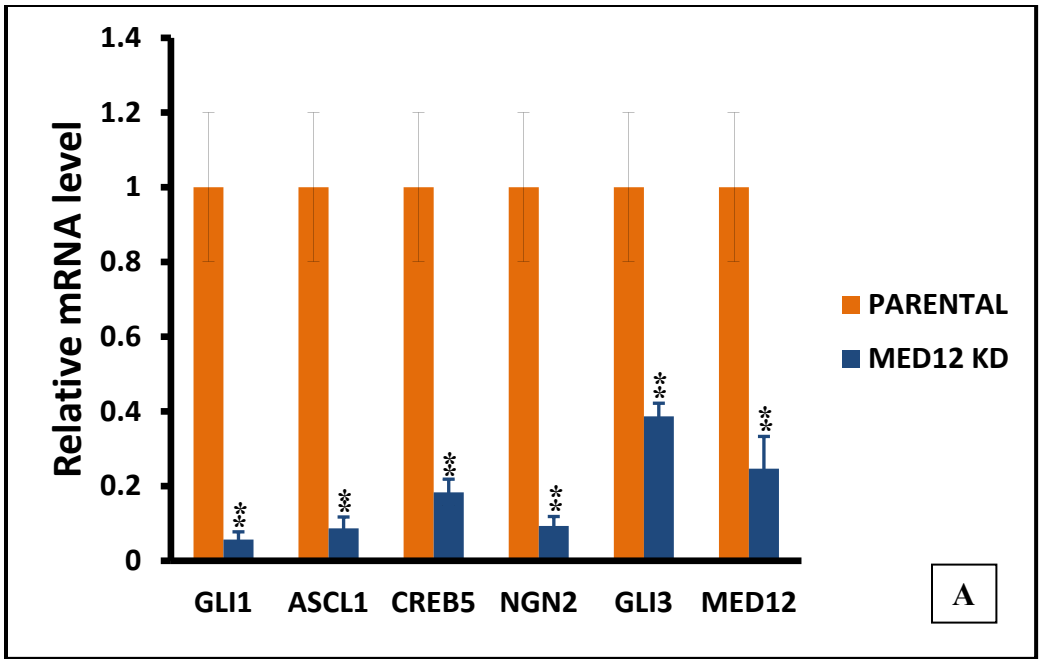


Figure 5- MED12 promotes androgen independent growth.
Proliferation assay was done by seeding cells in triplicates in androgen replete (A) or androgen deprived (B) on 24 well plates and harvested on days 3, 6 and 9. Counting was done using hemocytometer. Parental cells are untreated LNCaP cells and shMED12 cells are subjected to MED12 knockdown by shRNA mediated lenti virus infection. This experiment was repeated 4 times and the average of best 3 was taken for results and the standard deviation between the 3 sets of data was taken for error calculation.
Student's *t*-test: *$p < 0.05$; ** $p < 0.01$

GLI3 Target Genes Are Upregulated in the Absence of Androgen

In order to determine the mechanism behind increased cell growth in MED12 knockdown cells and since MED12 modulates GLI3-dependent SHH signalling we decided to investigate expression of GLI3 target genes. It is known that androgen deprivation induces SHH signalling which in turn activates GLI3. MED12 places a regulatory constraint on GLI3 thereby preventing its hyper activation. Due to the mutation in MED12, this constraint is removed. Thus leading to the over expression of GLI3 target genes in the absence of androgen [Figure 7(C)]. Interestingly, our data shows a downregulation of GLI3 target genes upon MED12 knockdown in androgen replete conditions [Figure 6(A)] and a dramatic upregulation in the absence of androgens [Figure 7(C)]. In result 1[Figure 5(B)], MED12 knock-down cause a dramatic cell proliferation in androgen deprived condition. In result 2 [Figure 7(C)], GLI3 target genes are up regulated upon MED12 knock-down in androgen deprived conditions. These two results are consistent showing mutation in MED12 causes hyperactivation of GLI3 dependent SHH signalling, in androgen deprived conditions consequently resulting in increased cell growth.



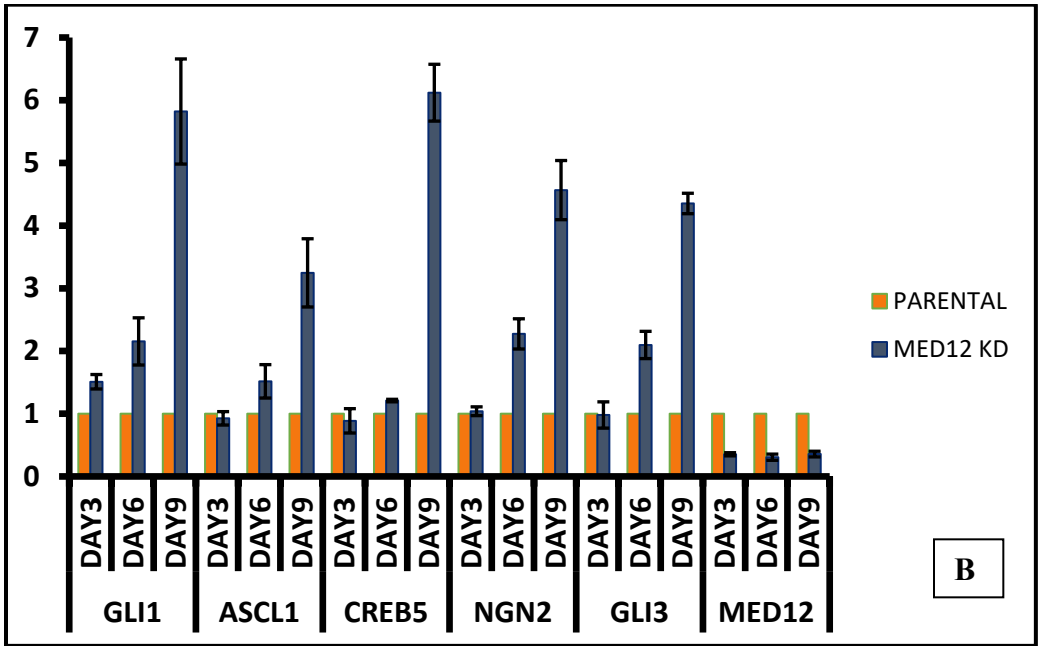


Figure 6. (A) & (B) - GLI3 target gene transcription under androgen replete and androgen independent conditions. (A) Cells were seeded in Androgen Replete media and harvested for RNA followed by quantitative PCR. Results indicate that GLI3 target genes were down regulated upon MED12 knockdown.(B) Cells were seeded and grown in androgen deprived media and harvested for RNA on days 3, 6 and 9. Results indicated that GLI3 target genes were significantly up regulated to drive androgen deprived cell growth in MED12 knockdown cells. This experiment was repeated 3 times and the average was taken for results and the standard deviation between the 3 sets of data was taken for error calculation.
Student's t-test: *$p < 0.05$; ** $p < 0.01$

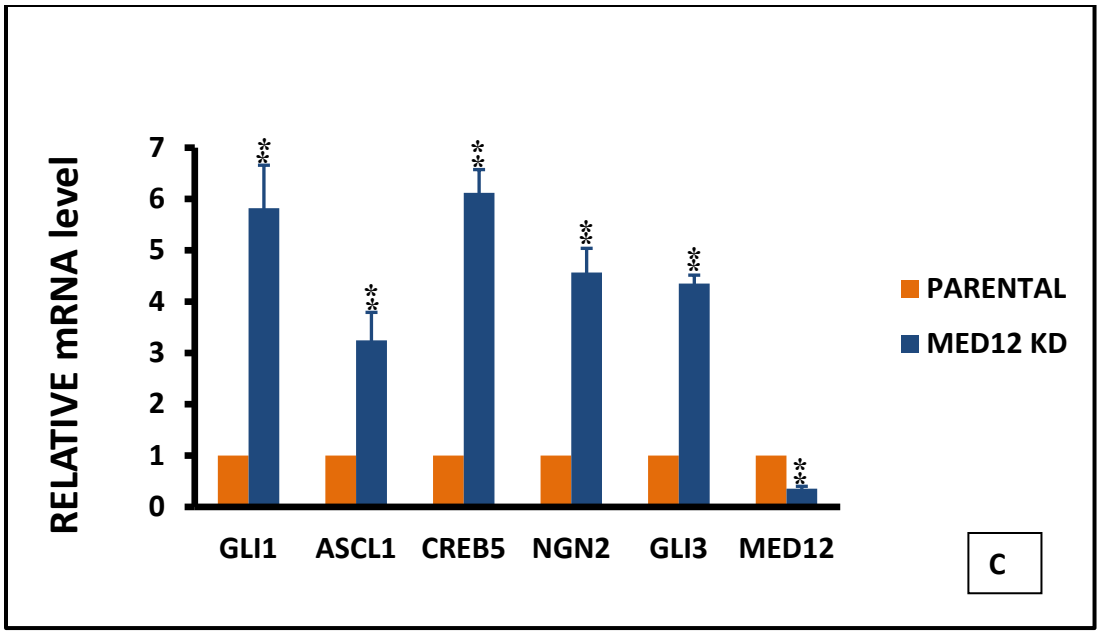


Figure 7 (C) - GLI3 target genes were up regulated in the absence of androgen. This graph shows the result of QPCR done forGLI3 target gene using RNA extracted on day 9 from MED12 knockdown cells grown in androgen independent media. On day 9, GLI3 target genes were significantly up regulated to drive androgen independent cell proliferation. Hence a separate graph was made to show that. This experiment was repeated 3 times and the average was taken for results and the standard deviation between the 3 sets of data was taken for error calculation. Student's t-test: *$p < 0.05$; ** $p < 0.01$

GLI3 is Required for Androgen Independent Growth of Med12 Knockdown Cells

To further investigate if GLI3-dependent SHH signalling is indeed required for the increased cell growth, we repeated the proliferation assays by knocking down MED12 and GLI3 simultaneously. Consistent with our hypothesis, we observed that the increased cell growth of MED12 knockdown cells is dependent on GLI3. As can be seen in Figure 8(B), when GLI3 is simultaneously knocked down, the increase in cell proliferation is reversed. According to our model (Figure 10), since mutational inactivation of MED12 induces hyperactivation of GLI3 after castration, GLI3 plays a major role in the hyperactivated proliferation of castration resistant prostate cancer cells. Thus when GLI3 is knocked down, the sonic hedgehog pathway does not get activated and hence the hyperactivation of MED12 mutant LNCaP cell in androgen deprived condition is reversed.

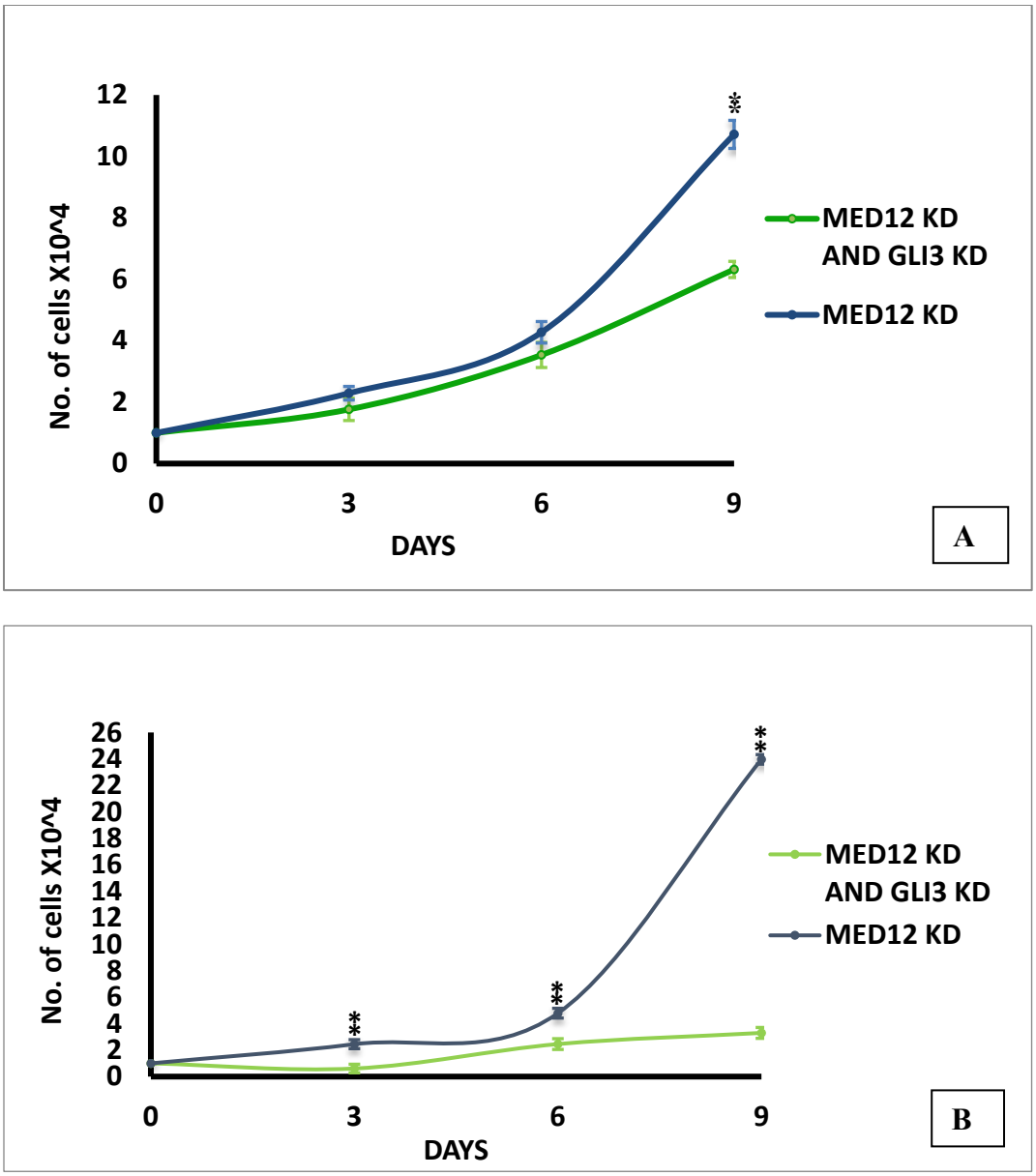


Figure 8- GLI3 is required for androgen independent MED12 knockdown cell growth.
Cells were seeded in triplicates in androgen replete (A) or androgen deprived (B) media on a 24-well plate and harvested on days 3, 6 and 9 to be counted using a hemocytometer. MED12 KD are parental LNCaP cells subjected to MED12 knockdown using shMED12 mediated lentiviral infection. GLI3 KD and MED12 KD are LNCaP cells subjected to a double knockdown of both MED12 and GLI3 by shRNA mediated lentiviral infection.

(A) Results indicate that GLI3 knockdown does not affect cell growth of MED12 knockdown cells in androgen replete media.

(B) Results indicate that GLI3 knockdown significantly blocks androgen independent cell growth of MED12 knockdown cells.

Student's t-test: *$p < 0.05$; ** $p < 0.01$

MED12 Knockdown Promotes In-Vitro Colony Formation

To investigate the ability of a single cell to form colonies and to create an in-vitro model for androgen independent growth of MED12 knockdown LNCaP cells, Colony formation Assay was performed. Consistent with our hypothesis, we observed that MED12knock-down LNCaP cells could form colonies in androgen deprived conditions [Figure 9(C)] and the cells in which MED12 and GLI3 were simultaneously knocked down, could not form colonies [Figure 9(D)]. It was also observed that the regular LNCaP cell could not form colonies in androgen deprived conditions [Figure 9 (B)].

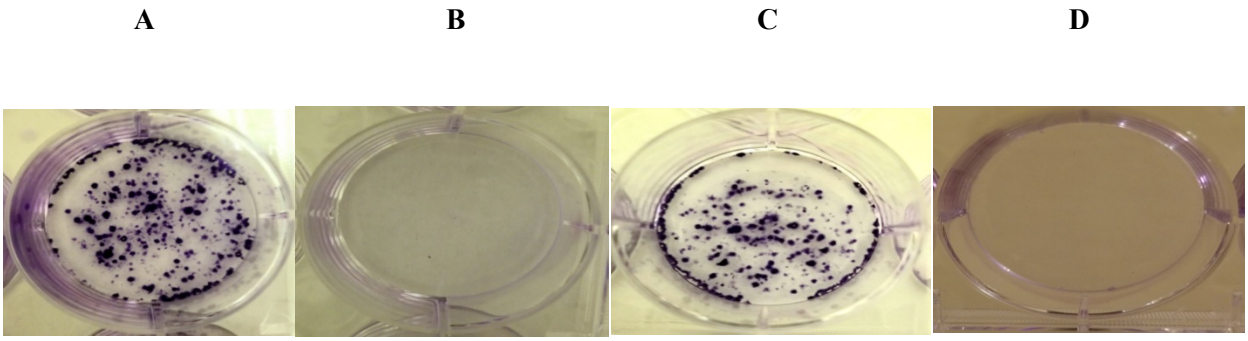


Figure 9- In-Vitro model for androgen independent MED12 knockdown cell growth.
Cells were seeded at densities of 2000cells/well, 4000cells/well and 6000cells/well in duplicates in 6 well plates. They were stained with crystal violet (Procedure from Yang, X. (2012). Clonogenic Assay. *Bio-protocol* 2(10): e187) on day 35 for observation of the colonies formed.

A: Parental LNCaP cells in Androgen deprived condition.
B: Parental LNCaP cells in Androgen Replete conditions.
C: MED knock-down and GLI3 knock-down LNCaP cells in Androgen independent condition.
D: MED12 knock-down cells in Androgen independent condition.

It was observed that MED12 knock-down in LNCaP induces cell growth in androgen independent conditions. No colonies were formed on plates D in which had LNCaP cells with a double knock-down of both MED12 and GLI3 in androgen deprived conditions.

Summary

The development and progression of prostate cancer depend on androgenic stimulation. Although prostate cancer is temporarily treated by depriving tumor of androgen, the patient relapses getting a castration-resistant form of the disease called castration resistant prostate cancer (CRPC).CRPC can specifically develop through crosstalk of Androgen Receptor signalling pathways. Recent work has shown that androgen deprivation induces SHH signalling, which reciprocally activates AR-dependent gene expression and prostate cancer cell growth in the absence of androgens. Mechanistically, this crosstalk appears to involve direct interaction between AR and GLI proteins, downstream transcriptional effectors of SHH signalling. Together, these findings suggest that activated SHH signalling in response to androgen deprivation could support the reactivation of AR-dependent prostate cancer cell growth and progression to CRPC. In this study, we have showed that, prostate cancer associated mutations in MED12 inactivates its constraining activity on GLI3 target genes thereby promoting its hyper activation. Since androgen deprivation therapy induces GLI3 dependent sonic hedgehog pathway, we conclude that mutations in MED12 promote castration resistant form of prostate cancer through hyperactivated GLI3 dependent sonic hedgehog signalling.

Conclusions and Discussion

MED12 knock-down in LNCaP cells (Assuming that MED12 knock down mimics the MED12 mutant setting since MED12 mutation inactivate the protein) did not affect its growth in androgen replete condition but we observed a dramatic increase in cell growth in the absence of androgen (*Figure 5*). That leads us to our first conclusion that MED12 expression is critical in regulating androgen independent cell and therefore progression towards CRPC.

GLI3 target genes were down-regulated upon MED12 knockdown in androgen replete conditions, but dramatically up-regulated in the absence of androgen (Figure 6). This leads us to the second conclusion that increased cell growth that we observe in MED12 knockdown LNCaP cells in the absence of androgen could be due to hyperactivation of GLI3 dependent Sonic Hedgehog signalling.

Hyper activated cell proliferation observed in MED12 knockdown LNCaP cells in androgen independent condition was reversed when MED12 knock down LNCaP cells were further knocked down with shGLI lentivirus (*Figure 8*). Further this helps us conclude that GLI3-dependent SHH signalling is indeed required for the increased cell growth observed in MED12 knock-down LNCaP cells in androgen independent conditions.

In-vitro model of MED12 knock-down tumor growth showed that parental LNCaP cells could not form colonies in androgen deprived conditions as against the control which was parental LNCaP cells grown in androgen replete media [Figure 9(B) and Figure 9(A) respectively]. This shows that androgen is important for the growth and survival of prostate cells; but when MED12 was knocked down, the parental LNCaP cells could form colonies in androgen deprived condition [Figure 9(C)] showing that MED12 knockdown induces sonic hedgehog signalling in the absence of androgen. Interestingly, parental LNCaP cells that were subjected to a double knockdown with both shMED12 lentivirus and shGLI3 lentivirus did not form colonies under androgen deprived conditions [Figure 9(D)], showing that GLI3 is required for the proliferation of MED12 knockdown cells in androgen deprived conditions.

This leads us to our model (Figure 10). It is known that androgen deprivation induces SHH signalling which then activates GLI3. In cells where MED12 expression is normal, MED12 would place a constraint on GLI3 thereby preventing hyper-activation of GLI3 target genes and

subsequent cell proliferation. When MED12 expression is low, this constraint on GLI3 gets released thereby inducing expression of GLI3 target genes and cell proliferation.

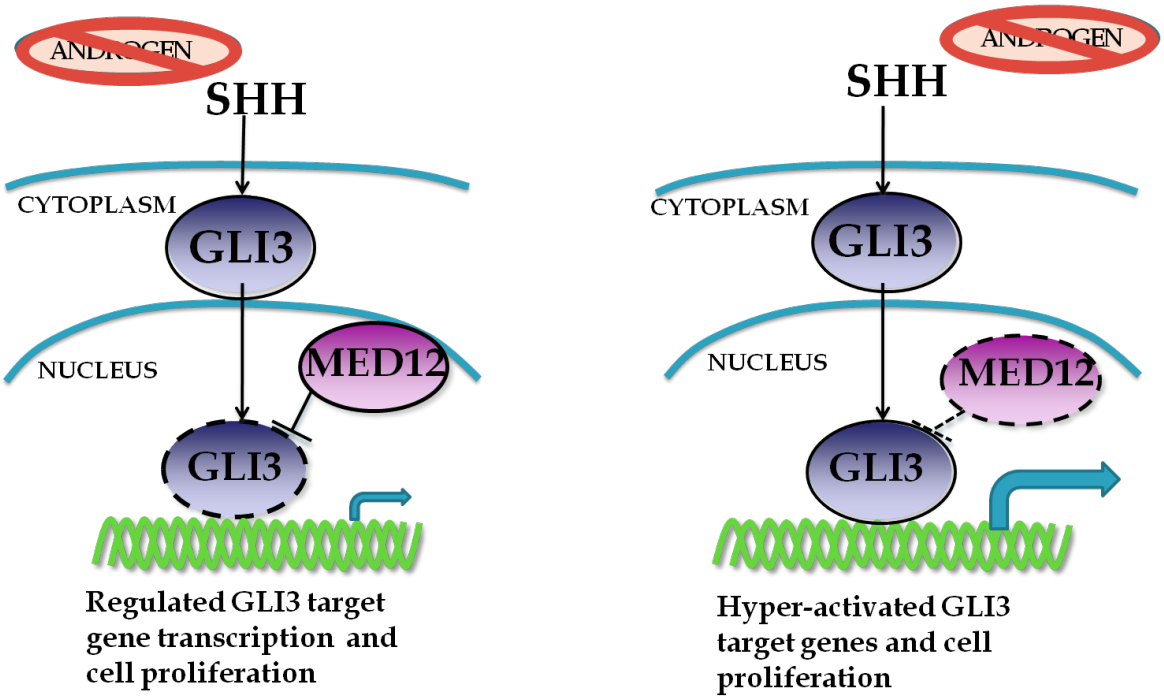


Figure 10 - Model

In case of healthy human, MED12 places a constraint on GLI3 target genes and hence regulated the expression of GLI3. Also, in the presence of androgen, full length GLI3 is reduced to its repressor form and hence regulating target gene transcription. In case of androgen deprivation therapy, full length activator form of GLI3 gets accumulate in the cytoplasm. Also mutation in MED12 leading to its inactivation, prevents MED12 from placing a constraint on GLI3 thereby hyper activating GLI3 target gene transcription and cell proliferation.

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