UNIVERSITÀ
DEGLI STUDI
FIRENZE

DOTTORATO DI RICERCA IN

SCIENZE BIOMEDICHE

CICLO XXIX

COORDINATORE Prof. Persio Dello Sbarba

INDUCTION OF STEMNESS MARKERS IN MELANOMA CELLS BY CRISPR/CAS9 uPAR GENE KNOCKOUT RESULTS IN TUMOR GROWTH INHIBITION

Settore Scientifico Disciplinare MED/04

Dottorando

Dott. Biagioni Alessio

_____

(firma)

Tutore

Prof. Del Rosso Mario

_____

(firma)

Coordinatore

Prof. Dello Sbarba Persio

_____

(firma)

Anni 2013/2016 *(di inizio e fine corso)*

Urokinase Plasminogen Activator Receptor (uPAR)

The urokinase receptor was originally identified as the receptor for the **serine protease urokinase (uPA)**, involved in the plasminogen activation cascade and the regulation of metalloproteinases-dependent proteolysis. It is a very peculiar molecule since it has no intracellular domain thus exerts its signalling through lateral interactions with other plasma membrane components. For this reason, uPAR could be defined "a very sociable molecule" and its interactome is so large that more than 42 proteins can directly interact with uPAR. Moreover, it is important to remark that uPAR can interact with members of three major families of membrane receptor i.e. G protein-coupled receptors, receptor tyrosine kinases, and integrins [1]. uPAR belongs to an exclusive class of proteins equipped with a **glycosylphosphatidylinositol (GPI) anchor** that influences both the subcellular localization and obviously, the function of many membrane proteins such as enzymes, adhesion molecules, surface antigens and receptors. Through this ability, it plays a central role in progression, metastasis, angiogenesis and stemness of numerous solid tumors [2-7].

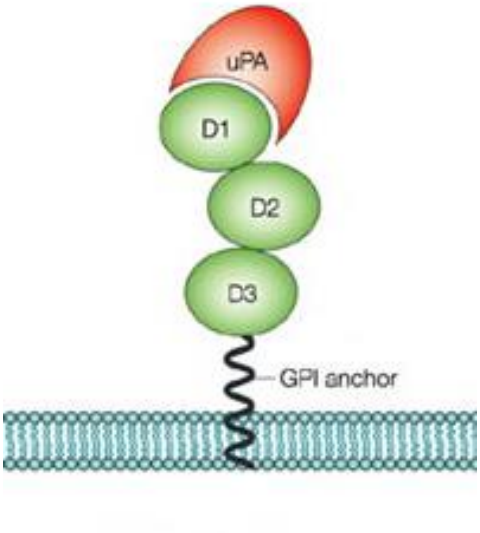


Figure 1 – uPAR domains and globular-like structure

The urokinase receptor binds uPA. uPAR lacks a cytosolic domain but is composed by a GPI-anchor and three protein domains. [7]

By binding uPA at the leading edge of the migrating cell, uPAR organizes a cascade of extracellular proteases that facilitate cellular penetration in tissues [8] and it also laterally associates with integrins in the plasma membrane, regulating the state of integrin activation [9]. GPI anchoring is a ubiquitous process, taking place in the endoplasmic reticulum. Such a link, between the functional protein and the plasma membrane, shows variations on a common theme, which consists of a phosphoethanolamine linker, a glycan core, and a phospholipid tail [10]. This lipid anchor determines uPAR localization and distribution on the plasma membrane, it confers high

mobility to move on the membrane and facilitates the redistribution of uPAR in lipid rafts and caveolae [11]. Besides the GPI-anchor, uPAR is composed by three protein domains, each one forming a globular-like structure (Figure 1). The GPI moiety is added post-translationally at the C-terminus of domain III while there are two linker regions located between domain I and II and domain II and III. So formed, uPAR has a large external surface, particularly suitable for lateral interactions [12-15]. Together with its ligand uPA and the specific inhibitor **Plasminogen Activator Inhibitor-1 (PAI-1)**, uPAR forms the so-called membrane-bound **Plasminogen Activation System (PAS)**. After about 30 years of intensive research, PAS overexpression has reached the level of evidence 1 as an indicator of poor prognosis in node-negative breast cancer [16;17], and a pro-tumoral role has been shown in all the malignancies where uPAR is over-expressed [18], thus eliciting clinical interest for its control in human cancers [19]. As a tumor promoting machinery, the PAS acts at two distinct levels: a) by regulation of cell invasion and motility [20], and b) by orchestrating receptor tyrosine kinases (RTK) signalling that results into cell proliferation and survival [7;21] (Figure 2).

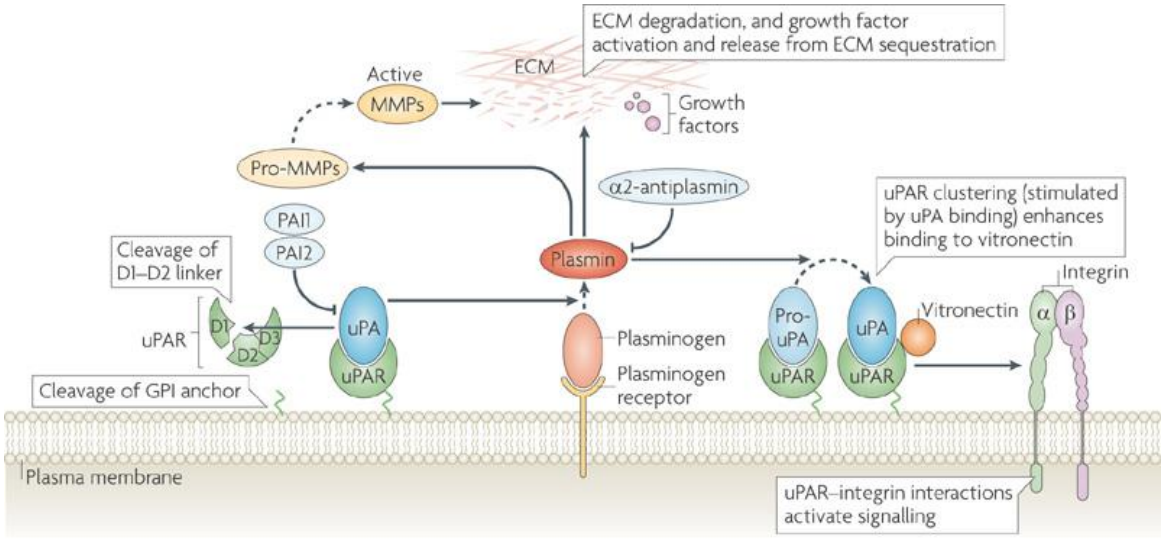


Figure 2 – The Plasminogen Activation System

uPAR binds the protease urokinase-type plasminogen activator uPA in its active and zymogen (pro-uPA) forms. uPA cleaves plasminogen, generating the active protease plasmin. Plasmin can reciprocally activate pro-uPA. Increased cell surface concentration of uPA or pro-uPA (by binding to uPAR) and plasmin or plasminogen (via multiple receptors) accelerates their mutual activation. Plasmin cleaves and activates matrix metalloproteases (MMPs). Both plasmin and MMPs degrade many extracellular matrix (ECM) components and activate growth factors or liberate them from ECM sequestration. The proteolytic activities of uPA and plasmin are antagonized by the serine protease inhibitors (serpins) plasminogen activator inhibitor 1 (PAI1; also known as SERPINE1) and PAI2 (also known as SERPINB2) and α2-antiplasmin. uPA–uPAR binding promotes clustering of uPAR in the plasma membrane, possibly in cholesterol- and sphingolipid-enriched domains referred to as 'lipid rafts', and increases its ability to bind the ECM protein vitronectin. uPA also cleaves uPAR in the linker between its first and second domains (D1 and D2), generating a soluble D1 fragment and a membrane-associated D2–D3 fragment. uPAR cleavage abrogates uPA binding, thereby inactivating the function of uPAR in proteolysis, and also inactivates or modifies the signalling functions of uPAR. [22]

uPA, the main uPAR ligand, is produced as a catalytically inactive precursor, **pro-uPA**. This single-chain zymogen is activated into the two-chain form, by the cleavage from a variety of proteases. When activated, it can exert its major biologic function, that is, to convert **plasminogen** to the active **plasmin**. In a reciprocal fashion, plasmin activates the single-chain pro-uPA [2;3;22;23]. Although a secreted protease, uPA can be tethered to the cell surface through binding of its **growth factor-like domain** to uPAR [7;24]. Both pro-uPA and uPA bind uPAR with similar affinities; however, cell surface-bound uPA is significantly more potent in the catalytic conversion of plasminogen to plasmin [25]. Plasminogen can be localized to the cell surface as well by binding to several membrane proteins, including annexin II and Plg-RKT [26;27]. Once activated by uPA, plasmin has a broad substrate collection. Whereas the canonical function of active plasmin is fibrin clot lysis, it can also cleave several non-collagenous components of the extracellular matrix (ECM), such as fibronectin and laminin [28-30]. In addition, plasmin is involved in proteolytic activation of additional proteases, for example, **matrix metalloprotease (MMP) zymogens**, including pro-

MMP-1 and pro-MMP-3. uPA physiological inhibitor is the serpine PAI-1. It is released in an active S or Stressed conformation, which then is rapidly inactivated through the transition into an R or Relaxed form. This change of conformation naturally occurs rapidly unless PAI-1 associates with Vitronectin, which can be considered a PAI-1 cofactor and another important uPAR ligand [1]. PAI-1/uPA complex is formed with high affinity and leads to PAI-1 cleavage. As a uPAR ligand, this complex, is catalytically inactive and moreover it cannot initiate signalling nor promote cell migration. At last, PAI-1 is fundamental for uPAR internalization through direct interactions with the **LDL receptor-related protein-1 (LRP-1)** which is a scavenger receptor [33-35]. uPAR internalization inhibits cell migration and provokes cell detachment through the inactivation and internalization of the integrins interacting with uPAR. Another important factor in the PAS is the **Vitronectin** (VN), which exerts important physiological and pathological functions including cell adhesion, migration, proliferation, fibrinolysis, hemostasis, immune response, thrombosis, atherosclerosis, restenosis and cancer [106]. VN is an adhesive glycoprotein that acts by binding to αvβ3, αIIβ3, αvβ5, αvβ1, αvβ6 and αvβ8 integrins, which control intracellular signalling pathways, cytoskeleton organization and gene expression. VN is also an important uPAR ligand that act promoting uPAR redistribution on the cell surface influencing pericellular proteolysis and focal adhesions [37;38]. uPAR binding to VN induces the formation of uPAR dimers promoting cell anchorage, but prevents interaction with G protein-coupled receptors and so uPAR-dependent signalling [39-42]. VN has been described as single- and two-chain forms. The latter, as it happens for pro-uPA, is generated through proteolytic cleavage and the two chains are connected by a single disulphide bond. This structure imposes that PAI-1 binding to VN blocks uPAR, inhibiting uPAR-mediated cell anchorage and integrin-dependent cell migration and adhesion [41;43;44]. It is also present a collagen-binding and one heparin-binding site that is especially responsible for VN-protein interactions mediating the binding of proteins such as heparin, collagen, proteoglycans, osteonectin, tenascin, complement complexes, thrombin-serpin complexes, plasminogen, and finally VN itself. As a result, this site is responsible for the oligomerization of VN. Moreover, VN is the cofactor of PAI-1, stabilizing the S conformation of this serpin thereby preserving its biological activity. The other side of the coin is that PAI-1 affects the adhesion- and migration-promoting activities of VN, and conversely VN inhibits PAI-1-dependent signalling and cell migration by impeding PAI-1 binding to LRP-1 [45-47]. All these complex interactions illustrate the way by which not only uPAR but also the other components of the uPAR system work together to exert a myriad of cellular effects.

Lateral uPAR partners

Most of the side interaction that uPAR has are with half of the members of the integrin family (that includes 24 membrane receptors) that consist of heterodimers of one α and one β subunit. Integrins exert a wide array of functions in embryonic development, tissue homeostasis, inflammation, cell differentiation, adhesion, migration, cell cycle progression, angiogenesis and tumor metastasis. Their interaction with uPAR let this one to join further indirect connections with the integrin interactome or adhesome [48]. Moreover, uPAR is also involved in cell-cell contacts through trans-interactions with integrins [49]. uPAR is a regulator of integrin activity acting on affinity and avidity, but the reverse relationship is also true [50]. In line with this observation, uPAR occupancy by uPA is required for the correct functioning of most integrins [51-55]. The importance of the influence of uPA on uPAR-integrin interaction may also reside in the fact that uPA is a ligand of integrins [56-62]. Indeed, uPA may thus promote uPAR-integrin interaction by bringing together these receptors. Moreover, anti-uPAR antibodies effectively blocks αvβ3 activity inhibiting VN-induced cell migration [63]. uPAR also increases integrin-dependent adhesion and migration [25;27;29;44;45]. Therefore, the influence of uPAR on integrins is functional exerting a broad effect on the cellular localization of integrins [64-68]. Furthermore, it is important to remark in this relationship, that uPAR upregulation enhances integrin activation, while downregulating uPAR results in integrin deactivation [26;31;44;50;57;63-66].

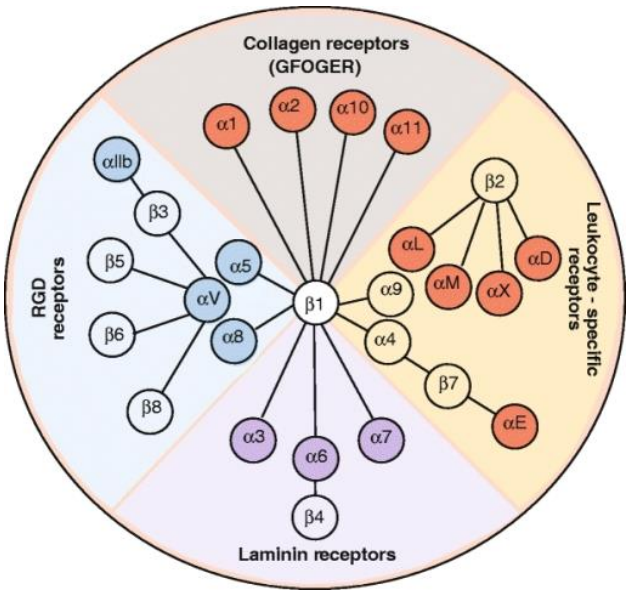


Figure 3 – The Integrin Family

Representation of the integrin family. [71]

Finally, integrin activity and number are regulated via the LRP-1-dependent internalization of uPAR. By binding to uPA, PAI-1 promotes the internalization of the LRP-1/PAI-

1/uPA/uPAR/integrin complex that leads to concurrent deactivation and internalization of the integrins associated with uPAR [35]. This process is particularly relevant in the context of cell motility when cell is moving and require the disorganization of adhesion sites. On the other hand, integrins regulate uPAR activity, distribution and expression. In addition, uPAR-integrin interaction and localization are governed by integrin ligands VN, Fibronectin and Laminin [69]. Interestingly, the complexity of the relationship between uPAR and integrins is reinforced by the property of uPAR to transduce signals leading to activation of integrins; uPAR is used by many integrins as transducer for other integrins activation, showing that uPAR could regulate their activity serving as a membrane messenger [70]. Other important partners interacting with uPAR on the plasma membrane are many growth factor receptors or **receptor tyrosine kinase (RTK) family**. These are a very large family of 58 membrane receptors, which share a common structure: an extracellular domain where the ligand binds, a single transmembrane domain, and an intracellular domain that harbors the tyrosine kinase activity and the sites of phosphorylation. The mechanism of RTK activation is also similar. The binding of ligand induces in most cases the dimerization of the RTK and the autophosphorylation of its cytoplasmic domain that initiates the downstream signalling cascade. Several RTKs interact with uPAR: EGFR, PDGFR-β, and most likely IGF-1R and c-Met, which serve as membrane transducers mediating uPAR-induced signal to the cell [72-76]. The relationship between uPAR and EGFR is the most studied [72-77]. EGFR, integrins and uPAR together with other membrane and cytoplasmic components constitutes a large membrane signalling complex implicated in the regulation of downstream signalling such as the MAP kinase pathway, and the stimulation of cell growth and other cellular effects [72;77-85]. Direct interactions of uPAR with integrins and EGFR have been reported suggesting that uPAR may simultaneously bind to both integrin and EGFR. According to the present literature, uPAR-induced signal is mediated by the integrin that in turn transactivates EGFR showing that uPAR is using the integrin as membrane messenger [72;81]. Interestingly, both uPA and uPAR expression are EGF/EGFR-dependent [86-91] suggesting that this system is capable of auto-amplification: uPA/uPAR transactivate EGFR which then enhances uPA expression, in a continuous positive loop.

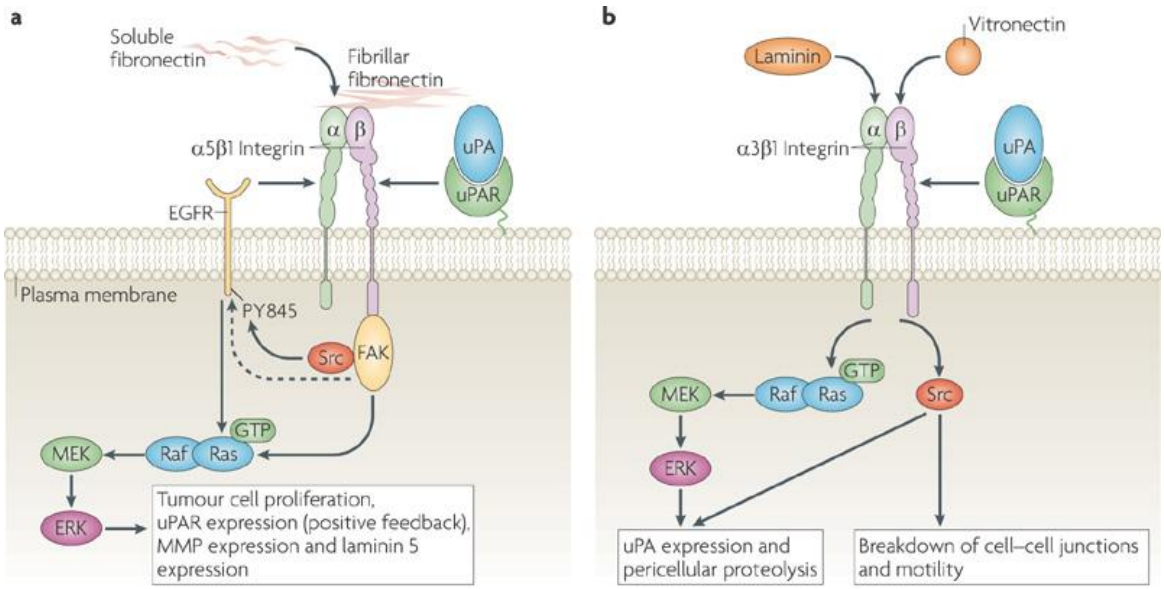


Figure 4 – The EGFR and the uPAR-mediated ERK cascade

uPAR signals through α5β1 and α3β1 integrins in fibroblasts, kidney epithelial cell lines and some carcinoma cells. uPA binding to uPAR is required for signalling through β1 integrins. a) A physical association of uPAR and α5β1 integrin is implicated in fibronectin fibrillogenesis. Subsequent adhesion to insoluble fibronectin fibrils and the association of uPAR and α5β1 integrin with the epidermal growth factor receptor (EGFR) causes activation of focal adhesion kinase (FAK). This leads to downstream activation of Ras and the mitogen activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK). b) uPAR–α3β1 integrin complexes are proposed to bind laminin and vitronectin. Signalling downstream of uPAR and α3β1 integrin is associated with the disruption of cell–cell junctions and the development of an epithelial–mesenchymal transition-like phenotype through Src activation. The Ras–MAPK kinase pathway is also activated by uPAR– α3β1 integrin signalling, and enhances pericellular proteolysis through the transcription of uPA. [21]

uPAR in Melanoma Progression

Cutaneous melanoma, the deadliest of skin cancers, is caused by the transformation of melanocytes (pigment-producing cells) that accumulate genetic alterations, leading to abnormal proliferation and dissemination [92]. Melanomas remain a significant cause of mortality in Caucasians and tumors consist of heterogeneous populations whose biological properties still remain poorly characterized. Melanomas are believed to arise from a mature, differentiated melanocyte [93].

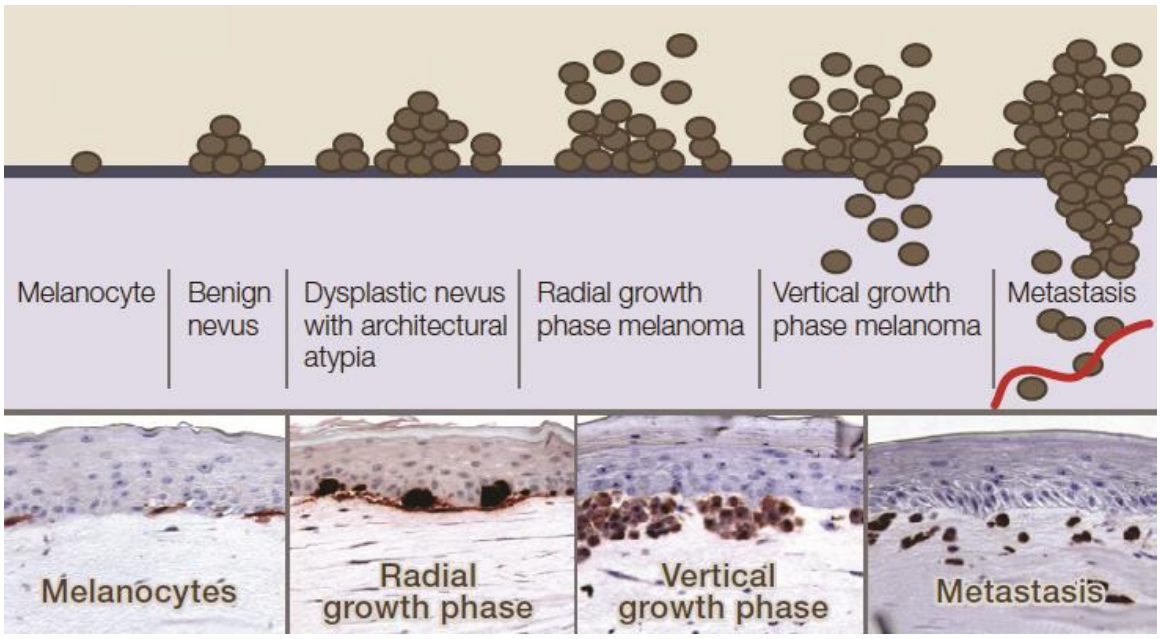


Figure 5 – Melanoma Evolution

Briefly the evolution of melanoma, from the melanocyte to the transformation in the benign nevus until the development of a vertical growth melanoma. The last stage ends with the formation of distant metastases. [92]

Melanocytes transformations require many genetic and environmental factors that enable their escape from the regulation of keratinocytes; for instance, the loss of E-cadherin and gain of N-cadherin is a fundamental step for escaping from keratinocytes and starting interactions with dermal stromal cells, such as fibroblasts and endothelial cells [93]. So transformed melanocytes proliferate and spread into a benign nevus, lesions that rarely progress to melanoma probably due to oncogene-induced cell senescence [94]. Aberrant growth and further alterations can lead to transform into dysplastic nevus, which can either stay latent or progress to a more malignant stage. The majority of these do not give rise to malignant disease but a subset will begin to spread. Melanoma growth is well distinguished in a first radial growth phase, when cells proliferate and spread laterally mostly within the epidermis, and the vertical-growth phase, invading derma and leading to melanoma cell intravasation in the circulatory or lymphatic system. The transition to the vertical stage is associated with the acquisition of metastatic potential as the cells have

penetrated the basement membrane and the increasing vascularization of these lesions means that the cells have an available route for distant spread [95]. While most of the genetic alterations associated with melanoma development result from sporadic somatic mutations, only 10% of melanomas are familiar, due to familiar germline mutations in genes involved in regulating cell cycle progression, like CDKN2A and CDK4, or pigmentation, such as melanocortin receptor MC1R [96]. 50-70% of melanomas carry mutation in BRAF [97], a member of Raf family of serine/threonine kinases, which are effectors of the small GTPase Ras in the ERK/MAPK pathway. It is activated by lots of membrane-bound receptors, like receptor tyrosine kinases and G-protein-coupled receptors, and it is involved in cell growth, survival and differentiation [98]. uPAR, influencing their activity, can lead to ERK/MAPK activation as yet described above. Also gain-of-function mutation in Ras family members have been reported in melanoma which generally lead to enhanced ERK-mediated proliferation; NRAS is mutated in 15-30% of the cases, HRAS activation has occasionally been detected in melanoma, while KRAS mutations have not been described in human melanocytic lesions [99]. Anyway, to date the most common mutation in melanoma is the substitution of a glutamic acid with a valine at position 600 (V600E) of BRAF, which accounts for 90% of BRAF mutated melanomas [97]. Further BRAF mutation have been discovered in melanoma that either activate ERK signalling directly (e.g. G465A, T598, K600E, A727V) or indirectly, towards CRAF in a RAS-independent manner (e.g. G466E/V, G469E, G596R, D594G) [98;100;101]. Besides the constitutive ERK signalling, $BRAF^{V600E}$ also contributes to neo-angiogenesis by stimulating autocrine VEGF, invasion by promoting the expression of fibronectin and the component β3 of its receptor αvβ3, by inducing MMP-1 and MMP-2 activity and IL-8 secretion [95;102-104], *in vitro* extravasation, *in vivo* lung metastasis development [105] and last it is also involved in metastatic spread and distant organ colonization [106]. Many studies reported the presence of a particular subpopulation of cancer cells characterized by self-renewability and the capacity to initiate, replenish and expand human tumors. These cancer cells with stem cells features and defined as **cancer stem cells (CSCs)** or **tumor-initiating cells** have been isolated from different human solid tumors, including melanoma [107]. This discovery has allowed a better understanding of the biology and neoplastic transformation of normal melanocytes, and the possible mechanisms by which melanoma cells acquire tumorigenicity. Several potential bio-markers of CSCs have been described as expressed by human solid tumors including melanoma [108] even if none has been shown to be truly CSC-specific [109-113]. Morover, it should be considered the possibility that different subpopulations of CSCs may exist within single tumors (intratumoral heterogeneity) including melanoma [114-116] and/or among different tumors (intertumoral heterogeneity). Nevertheless, tumour-initiating cells have been successfully

isolated using appropriate cell surface markers, including CD44, CD24, CD133, epithelial cell adhesion molecule (EPCAM) and ATP-binding cassette sub-family B member 1 and 5 (ABCB1-5) [113;117-122]. These tumour-initiating cells can produce phenocopies of the original primary tumours when transplanted into NOD–SCID mice [123].

CRISPR/Cas9 genome editing system

CRISPR (Clustered Regularly Interspace Short Palindromic Repeats) system is a prokaryotic adaptable immune mechanism used by many bacteria and archaea to protect themselves from foreign nucleic acids, such as viruses or plasmids [124-127]. The first CRISPR description was from Osaka University researcher Yoshizumi Ishino in 1987 [128], who accidentally cloned part of a CRISPR together with the iap gene, his target of interest. The organization of the repeats was uncommon because repeated sequences are typically arranged consecutively along DNA. The function of the interrupted clustered repeats was not known at the time [129]. Later on, in 1993 researchers of Mycobacterium tuberculosis in the Netherlands published two articles about a repeat cluster in this bacterium that was named "direct repeat (DR)" region. These researchers recognized the diversity in the composition of repeat cluster spacers [130]. At the same time, the later called CRISPR was also observed in the archaeal organism Haloferax mediteranii and its function was studied by Francis Mojica at the University of Alicante, Spain [131]. However the real begin of CRISPR history, is in 1997 when Ruud Jansen at the University of Utrecht, recognized a similarity among the structure of the iap repeats of E. coli, the DR region of M. tuberculosis and the repeat cluster of H. mediteranii, defining these as members of the CRISPR family. From that time on numerous CRISPR's were recognized in the whole genomes of bacteria and archaea that were published, indicating that CRISPR is a universal feature of prokaryotes. A major addition to the understanding of CRISPR came with the observation that the repeat cluster was accompanied in the prokaryotic genomes by a set of homologous genes, the **CRISPR associated or Cas genes**. Four Cas genes (Cas 1 to 4) were recognized and the Cas proteins showed helicase and nuclease motifs, suggesting a role of these proteins in the dynamic structure of the CRISPR loci. For many years CRISPR remain a “mystery item” until 2005, when three independent research groups showed that some CRISPR spacers are derived from phage DNA and extrachromosomal DNA such as plasmids [132-134]. In effect, the spacers are fragments of DNA gathered from viruses that previously tried to attack the cell. The source of the spacers was a sign that the CRISPR/Cas system could have a role in adaptive immunity in bacteria. The first publication proposing a role of CRISPR/Cas in microbial immunity, by Mojica et al., already predicted a role for the RNA transcript of spacers on target recognition in a mechanism that could be analogous to the RNA interference system used by eukaryotic cells. Koonin et al. extended that hypothesis proposing mechanisms of action for the different CRISPR-Cas subtypes according to the predicted function of their proteins [135]. The first experimental evidence that CRISPR could be an adaptive immune system was in 2007 but we had to wait till 2014 to see the first example of use as a tool for editing the genome, when Zhang et al. [129] manipulated the resistance of S. thermophilus to phage by adding and

deleting spacers whose sequence matched those found in the phages tested. In 2008, Brouns et al. identified a complex of Cas protein that in E. coli cut the CRISPR RNA within the repeats into spacer-containing RNA molecules [126], which remained bound to the protein complex. In the same year, Marraffini [137] showed that a CRISPR sequence of Staphylococcus epidermidis targeted DNA and not RNA to prevent conjugation. All these findings confirmed the proposed RNA-interference-like mechanism of CRISPR-Cas immunity. A 2010 study provided direct evidence that CRISPR-Cas cuts both strands of phage and plasmid DNA in S. thermophiles [124;138].

CRISPR System

The CRISPR system can be found on both chromosomal and plasmid DNA. Type II CRISPR incorporate sequences from invading DNA (often derived from nucleic acid of viruses and plasmids) between CRISPR repeat sequences. These regions that are complementary to the foreign DNA are called **Protospacers**. Transcript from part of these regions are processed into **CRISPR RNAs (crRNAs)** and hybridizes with a second RNA called **transactivating CRISPR RNA (tracrRNA)** [139]. All togheter this complex of crRNA and tracrRNA binds the nuclease Cas9. Protospacer-encoded portion of the crRNA directs Cas9 to cleave complementary target-DNA sequences, if they are adjacent to short sequences known as **protospacer adjacent motifs (PAMs)**. Protospacer sequences incorporated into the CRISPR locus are not cleaved presumably because they are not next to a PAM sequence (Figure 6a).

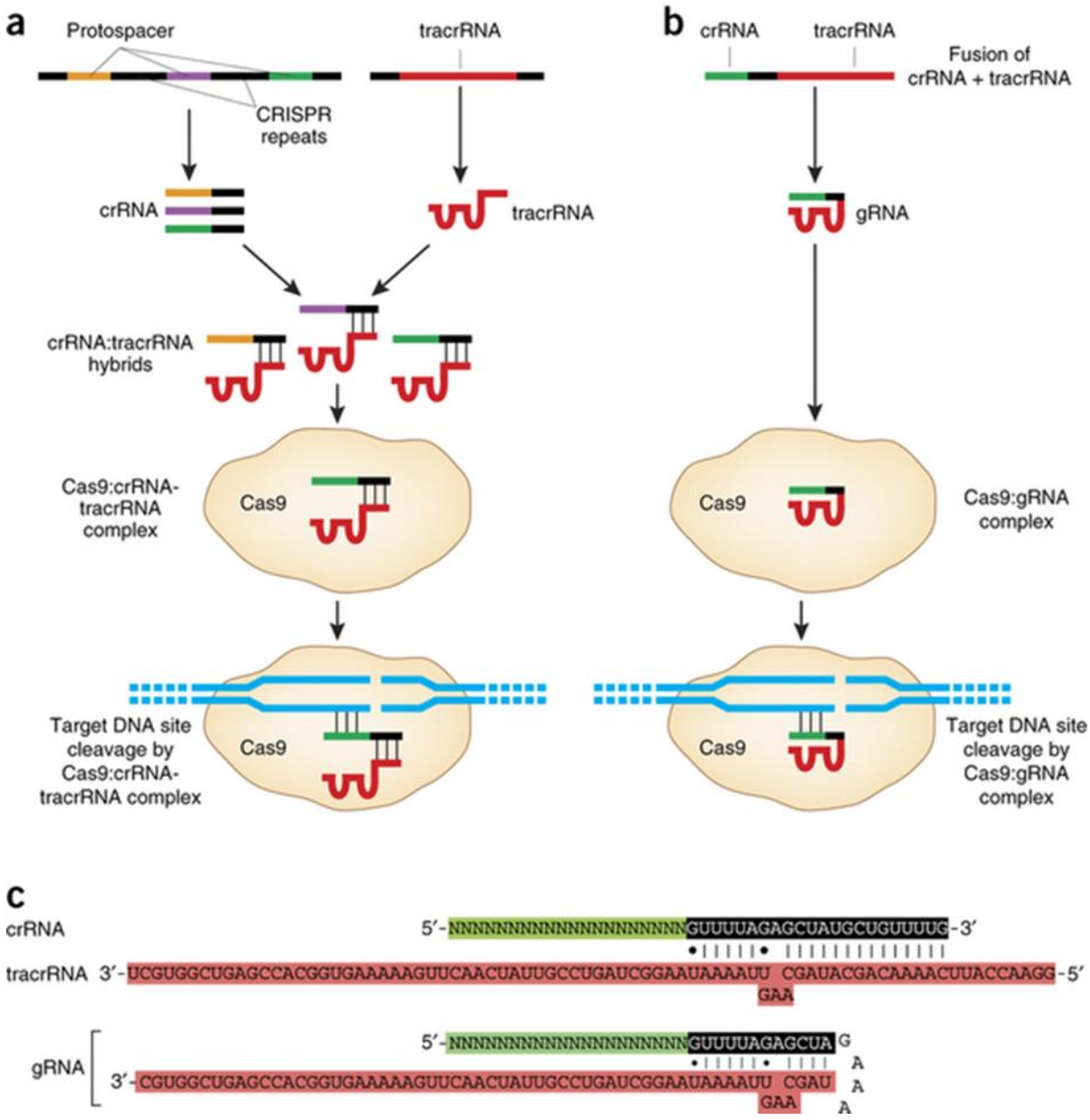


Figure 6 - CRISPR-Cas platform.

(a) Natural bacterial system is composed of incorporate foreign DNA sequences into CRISPR arrays, which then produce crRNAs bearing "protospacer" regions that are complementary to the foreign DNA site. crRNAs hybridize to tracrRNAs and this pair of RNAs can associate with the Cas9 nuclease. crRNA-tracrRNA:Cas9 complexes recognize and cleave foreign DNAs bearing the protospacer sequences. (b) The most used engineered CRISPR-Cas system utilizes a fusion between a crRNA and part of the tracrRNA sequence. This single gRNA complexes with Cas9 to mediate cleavage of target DNA sites that are complementary to the 5′ 20 nt of the gRNA and that lie next to a PAM sequence. (c) Example sequences of a crRNA-tracrRNA hybrid and a gRNA. [141]

This prokaryotic system has been adapted to be used in vitro, merging the crRNA with a part of the tracrRNA in a hybrid called guide RNA (gRNA) (Figure 6b). So, in the simplest and most widely used form of this system only two components must be introduced into and expressed in cells to

perform genome editing: the Cas9 nuclease and the gRNA. Twenty nucleotides at the 5' end of the gRNA (corresponding to the protospacer portion of the crRNA; Figure 6c) direct Cas9 to a specific target DNA site using standard RNA-DNA complementarity base-pairing rules. The site to be cleaved must lie immediately 5' of a PAM sequence, although recognition at sites with alternate PAM sequences could be possible, albeit at less efficient rates [140;142;143]. Thus, with this system, Cas9 nuclease activity can be directed to any DNA sequence by altering the first 20 nt of the gRNA that correspond to the target DNA sequence. Thanks to these extreme variability, this platform also functions efficiently in a variety of cells and organisms, not only for genes in bacteria but also for cultured transformed human cancer cell lines and human pluripotent stem cells in culture [144-147], as well as in a whole organism such as the zebrafish [148]. Cas9-induced double strand breaks (DSBs) have been used to introduce NHEJ (Non Homologous End Join)-mediated indel mutations as well as to induce HDR (Homologous Directed Repair) with double-stranded plasmid DNA and single-stranded oligonucleotide acting as a donor template (Figure 7). Being able to introduce DSBs at multiple sites in parallel with Cas9 is a unique advantage of this RNA-guided genome editing platform relative to meganucleases, ZFNs or TALENs. Moreover, this platform is easier to be used than previous editing systems because is based on RNA-DNA complementarity base-pairing rules and not on recognition between proteins and DNA.

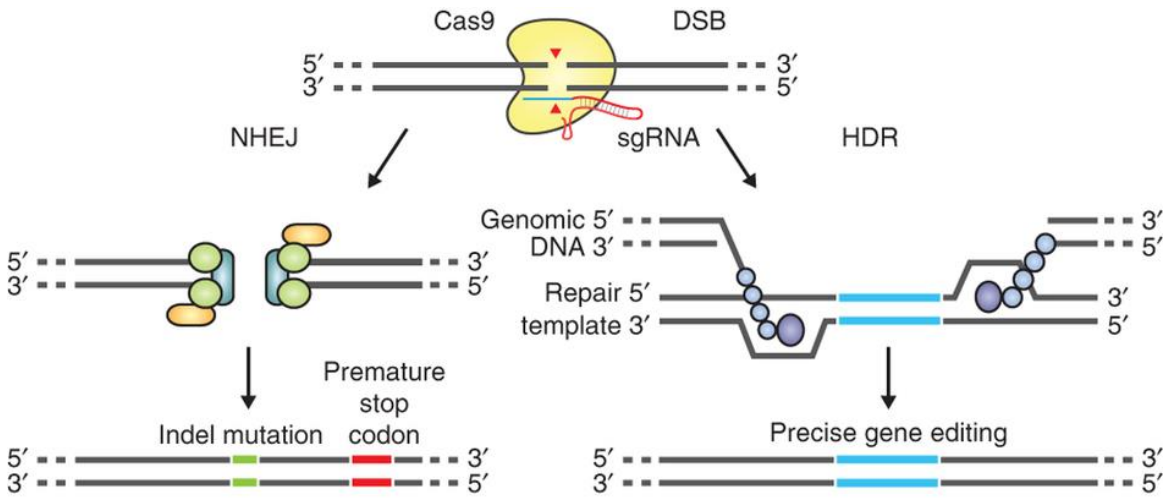


Figure 7 – Recombination systems

DSBs induced by Cas9 can be repaired in one of these two ways. In the error-prone NHEJ pathway, the ends of DSB are processed by endogenous DNA repair machinery and rejoined, which results in random indel mutations at the site of junction. Indel mutations occurring within the coding region of a gene can result in frameshifts and can cause the creation of a premature stop codon, resulting in gene knockout. Alternatively, a repair template in the form of a ssODN can be supplied to active the HDR pathway, which allows high fidelity and precise editing. [149]

Cas9 variants

Cas9 is a bi-lobed architecture protein with the gRNA nestled between the alpha-helical lobe and the nuclease lobe. These two lobes are connected through a single bridge helix.

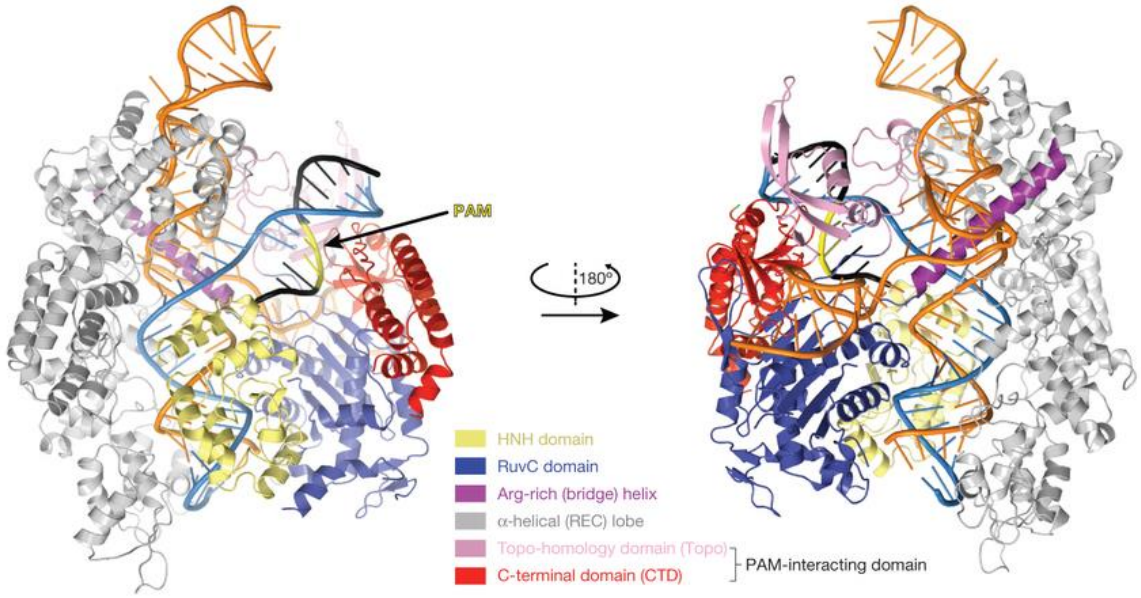


Figure 8 – Cas9 Structure and Motifs

Front and rear views of the Cas9–sgRNA–DNA complex. [152]

There are two main domains located in the multi-domain nuclease lobe: the **RuvC**, that shares an RNase H fold structure with other nucleases in the retroviral integrase superfamily [150], which cleaves the non-target DNA strand, and the **HNH** nuclease domain, that has a ββα-metal fold that comprises the active site, which cleaves the target strand of DNA [151]. Cas9 in complex with the crRNA and the tracrRNA recognizes and degrades the target dsDNA [149]. The gRNA base paired with target ssDNA is anchored by Cas9 as a T-shaped architecture. The nuclease also consists of a **recognition lobe (REC)** that match the target sequence in the host DNA. Several Cas9 mutants including REC domain deletion and residues mutations in the **bridge helix (BH) domain** have been tested. REC and BH mutants show lower or none activity compared with wild type, which indicate these two domains are crucial for the gRNA recognition and stabilization of the whole complex (Figure 9e and f). Normally Cas9 performs a double strand break in the target DNA site, while introducing a **D10A** or **H840A** mutation into the RuvC- or HNH-like nuclease domains results in the generation of a single cut (Figure 9b and c) [135;152]. These mutants also known as **Nickase** have also been shown to be useful for genome editing. Nickases cut either the complementary or noncomplementary DNA target strands, respectively, in vitro [140;153;154]. The choosen DNA repair pathways, by which these various alterations are introduced, remain undefined; one

potential mechanism that has been postulated is that passage of a replication fork through a nuclease-induced nick site might result in a DNA DSB.

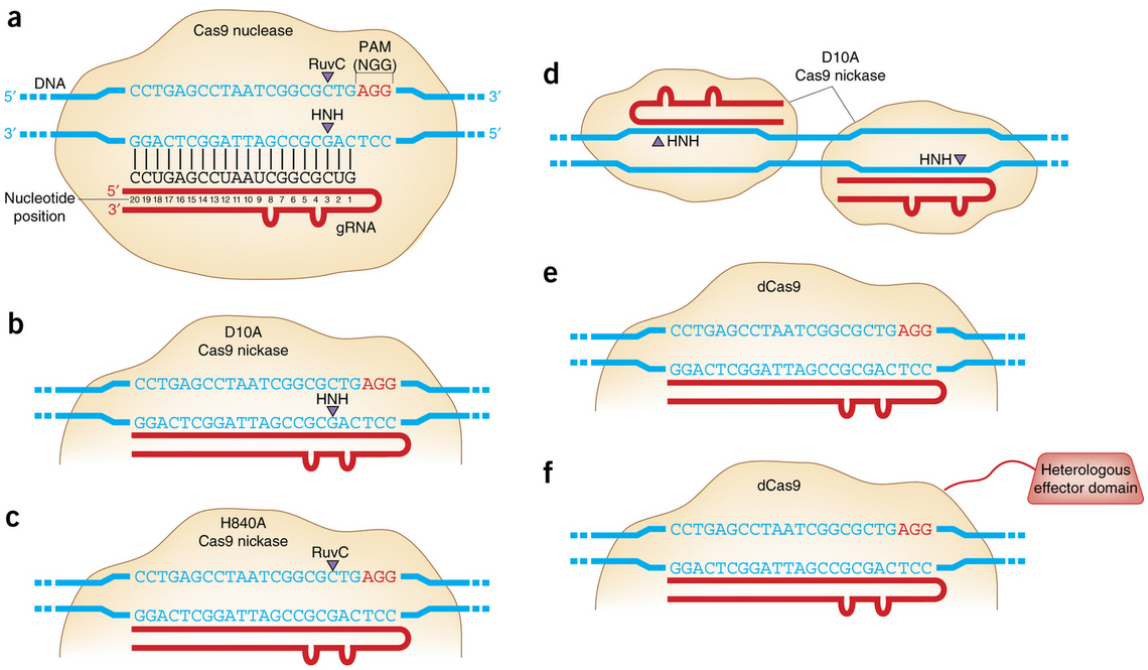


Figure 9 – Cas9 Mutants

(a) Cas9 wt nuclease creates double-strand breaks at DNA target sites. (b) Cas9 nickase created by mutation of the RuvC nuclease domain with a D10A mutation. This nickase cleaves only the DNA strand that is complementary to and recognized by the gRNA. (c) Cas9 nickase created by mutation of the HNH nuclease domain with a H840A mutation. This nickase cleaves only the DNA strand that does not interact with the gRNA. (d) Paired nickase strategy for improving Cas9 specificity. Two D10A Cas9 nickases are directed by a pair of appropriately oriented gRNAs. This leads to promote two single nicks that, if introduced simultaneously, simulate a DSB. (e) Catalytically inactive or 'dead' Cas9 (dCas9) (e.g., with mutations in both the RuvC and HNH domains). This can be recruited by a gRNA without cleaving the target DNA site. (f) Catalytically inactive dCas9 can be fused to a heterologous effector domain. [141]

Off target effect risks and possible solutions

Even if very efficient, this system is not completely immune to errors. Understanding the possible weak sides could be helpful in order to prevent all potential off-target effects. Recently, a number of studies have examined potential off-target sites that differ from one to six positions from the on-target site in human cells [143;149;155-157]. These reports have found cases of off-target mutations at sites that differ by as many as five positions within the protospacer region [156] and that have an alternative PAM sequence [158]. Interestingly, indel mutation frequencies at these off-target sites can be so high (>2–5%) that sometimes can be comparable to the on-target site mutation frequency [156;159;160] even if more sensitive deep sequencing assays have identified lower frequency off-target mutations [149;155;157]. To prevent these effects, it has been suggested that higher GC content at the RNA:DNA interface might potentially help to stabilize binding the hybridization, indeed high rates of mutagenesis have been observed for off-target sites with as little as 30% matched GC content [143;156]. A recent published paper reported that using whole-exome sequencing did not find evidence of Cas9-induced, off-target mutations in three modified human K562 cell line clones [147] even if the high false-negative rate associated with exome sequencing analysis, limits interpretation of these data. However, these results suggest that with careful target selection, it may be possible to isolate Cas9-edited cells with a good successful rate. Overall, the various published studies strongly suggest that off-target sites of RNA-guided Cas9 nucleases can be variable in frequency and challenging to predict. For any given target site, it is not currently possible to predict how many mismatches can be tolerated, nor do we fully understand why some sites are cleaved whereas other are not. We also do not know how genomic and/or epigenomic context might affect the frequency of cleavage. Although some initial evidence suggests that DNA methylation does not inhibit Cas9-based genome editing [157], it seems plausible and likely, that chromatin structure could play a role in off-target site accessibility. One potential strategy to reduce off-target mutations is to reduce the concentrations of gRNA and Cas9 expressed in human cells. Another one is to use a modified gRNA architectures with truncated 3’ ends (within the tracrRNA-derived sequence) or with two extra guanine nucleotides appended to the 5’ end (just before the complementarity region) which yields better on-target to off-target ratios but generally with lower efficiency on on-target genome editing [143;147]. Morover, studies using single gRNAs have reported substantially higher editing rates than those using dual gRNAs [144;145;148;161]. These findings suggest that the single gRNA system may be more active than the double gRNA system, presumably because two components can assemble more efficiently than three components. However the best approach for improving specificity involves the use of paired nickases in which adjacent off-set nicks are generated at the

target site using two gRNAs and Cas9 mutant (see above Figure 9d). In contrast to single Cas9 nickases (Fig.9b and 9c) (which can at some sites more favorably induce HDR events relative to NHEJ indels), paired Cas9 nickases targeted to sites on opposite DNA strands separated by 4 to 100 bp can efficiently introduce both indel mutations and, in case a single-stranded DNA oligonucleotide donor template is used can induce HDR events in mammalian cells [159;162;163]. Importantly, paired nickases can reduce Cas9-induced off-target effects of gRNAs in human cells; the addition of a second gRNA and substitution of Cas9 nickase for Cas9 nuclease can lead to lower levels of undesired mutations. However, the second gRNA can itself induce its own range of off-target mutations in the genome as multiple studies have shown that single monomeric Cas9 nickases can function on their own to induce indel mutations at certain genomic loci, perhaps because an individual nick might be converted to a DSB when a replication fork passes through the locus [164;165]. The existence of these off-target effects and our inability to identify these alterations on a genome-wide scale mean that researchers need to account for the potentially confounding effects of these undesired mutations. One strategy to rule out off-target mutations is the use of complementation, reintroducing a wild-type gene, which can be used to confirm the effects of knockout. Another possibility is to use gRNAs targeted to different sites. Presumably, each gRNA will be expected to have a different range of off-target effects and therefore if the same phenotype is observed with each of these different gRNAs it would seem unlikely that undesired mutations are the cause.

CRISPR delivery possibilities

There are several methods to delivery all the components of CRISPR system. In cultured mammalian cells, can be used electroporation, nucleofection and Lipofectamine-mediated transfection of nonreplicating plasmid DNA to transiently express Cas9 and gRNAs [144;145;156;161]. Lentiviral vectors have also been used to constitutively express Cas9 and/or gRNAs in cultured human and mouse cells [167-169]. RNAs and/or plasmid DNA transcribed in vitro have been injected directly into the embryos of zebrafish, fruit flies, mice, rats and into the gonads of adult roundworms [148;166;169-178]. In addition to animal models and cell lines, it has been successfully used in multiple plant species including wheat, rice, sorghum, tobacco and thale cress [173-183]. For most applications, transient expression of gRNAs and Cas9 is typically sufficient to induce efficient genome editing. Indeed, after expression and selection plasmid expressing CRISPR machinery is usually lost. Although constitutive expression of these components may potentially lead to higher on-target editing efficiencies, extended persistence in

the cell might also lead to increased frequencies of off-target mutations, a well-known phenomenon for the ZFNs technology [184].

Other uses and perspectives

In addition to the use described above, CRISPR-Cas system has the potential to be used to regulate endogenous gene expression or to label specific chromosomal loci in living cells or organisms. Catalytically inactive or "dead" Cas9 (dCas9) (Figure 9e) can be recruited by gRNAs to specific target DNA sites [140;173]. Targeting of dCas9 to promoters was initially shown to repress gene expression in both Escherichia coli and human cells [186;187] (Figure 10a).

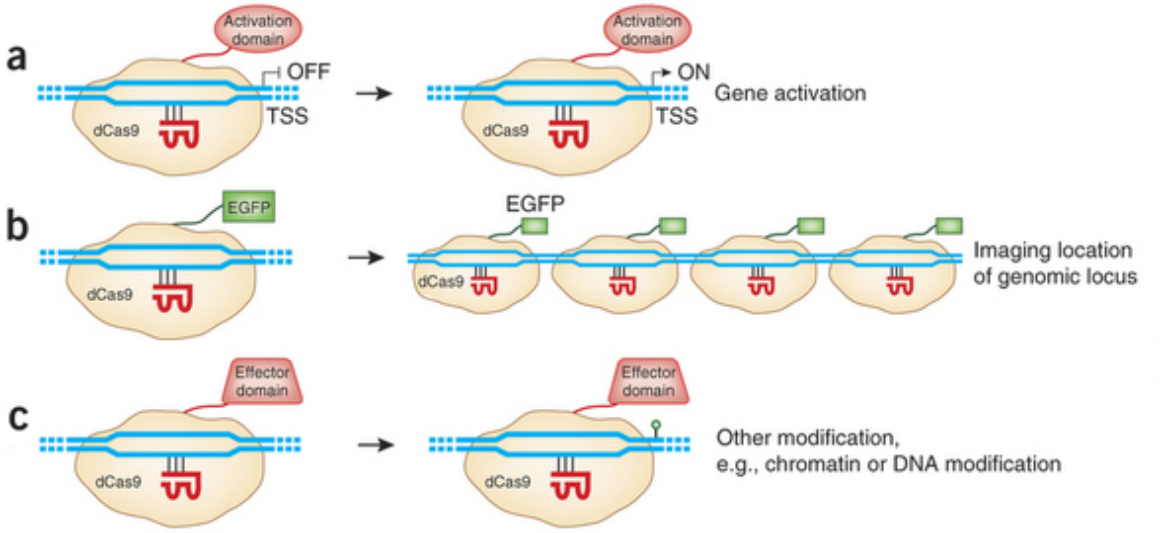


Figure 10 – Applications

gRNA-directed dCas9 can be fused to activation domains (a) to mediate upregulation of specific endogenous genes, heterologous effector domains (b) to alter histone modifications or DNA methylation, or fluorescent proteins (c) to enable imaging of specific genomic loci. [141]

Interestingly, dCas9 repressed a bacterial promoter efficiently when recruited with gRNAs that interacted with either strand of sequences upstream of the promoter. However, when targeting sites downstream of the transcription start, only gRNAs that interacted with the non-template strand induced dCas9-mediated repression. dCas9 also provides a general platform for recruitment of heterologous effector domains (Figure 4f) to specific genomic loci. For example, dCas9 fused to a transcriptional activation domain or a transcriptional repression domain have been shown to regulate the expression of endogenous genes in human and mouse cells [187-191]. However, multiplex recruitment of dCas9-based activators using between 2 and 10 sgRNAs targeted to the same promoter can result in substantially higher levels of human gene activation and this capability of dCas9-based activators to function synergistically is consistent with previous

observations for TALE-based activators. It has also been demonstrated that an EGFP-dCas9 fusion can be used to visualize DNA loci harboring repetitive sequences (Figure 10b), such as telomeres, with a single gRNA or non-repetitive loci using 26 to 36 gRNAs covering a 5-kb region of DNA [192]. This imaging system provides a powerful tool for studying chromosome dynamics and structure. In future, it will be interesting to see whether dCas9 fusions to histone modifiers and proteins involved in altering DNA methylation, can also be used to perform targeted “epigenome editing” (Figure 10c). Methods for expanding the targeting range of RNA-guided Cas9 will be important for inducing precise HDR or NHEJ events as well as for implementing multiplex strategies, including paired nickases. As noted above, the targeting range for Cas9, paired Cas9 nickases and dCas9 fusions is restricted mainly by the need for a PAM sequence matching the form NGG. Other gRNA:Cas9 platforms with different PAM sequences isolated from Streptococcus thermophilus, Neisseria meningitidis and Treponema denticola have also been characterized [145;193;194]. Morover it is necessary to improve the reliability of the system in order to reduce off-target effects. It is likely that further improvements will be needed, particularly for therapeutic applications. Examples of such improvements might involve using protein engineering to modify Cas9 and/or modifying the nucleotides used by the gRNA to mediate recognition of the target DNA site. Alternatively, the construction of inducible forms of Cas9 and/or gRNAs might provide a means to regulate the active concentration of these reagents in the cell and thereby improve the ratio of on- and off-target effects [141]. Methods for efficient delivery and expression of CRISPR-Cas system components will undoubtedly need to be optimized for each particular cell-type or organism to be modified. Collectively, these advances will be important for research use and therapeutic applications. Lastly, strategies for shifting the balance away from NHEJ-mediated indel mutations and toward HDR-driven alterations remain a priority. Although high rates of HDR can be achieved with the CRISPR and single-stranded DNA oligonucleotides, competing mutagenic NHEJ also occurs simultaneously. One of the drawbacks to developing an approach to improve the HDR:NHEJ ratio is that inhibition of NHEJ is likely to be poorly tolerated by most cells, given its central role in normal DNA repair. For therapeutic applications seeking to exploit HDR, reduction or elimination of competing NHEJ will be crucially important. The simplicity, high efficiency and broad applicability of the RNA-guided Cas9 system have positioned this technology to transform biological and biomedical research. The ease with which researchers can now make changes in the sequence or expression of any gene means reverse genetics can be performed in virtually any organism or cell type of interest. Although the off-target effects of Cas9 remain to be defined on a genome-wide scale, much progress has already been made toward improving specificity, and further advances will undoubtedly come rapidly, given the intensity of research efforts in this area.

All of these recent advances—and those to come—in developing and optimizing Cas9-based systems for genome and epigenome editing should propel the technology toward therapeutic applications, opening the door to treating a wide variety of human diseases.

Aim of the Project

Recurrence is the main cause of treatment failure after surgery and chemotherapy. In the last decades, many resources were spent in order to find a way to detect that small number of cancer cells that remain quiescent and after an unspecified time, they recur forming a new tumor, sharing many features with the past one. Indeed often chemotherapy, although effective, does not destroy all tumor cells leaving a pool of chemo-resistant cells that are commonly invisible to all the detection methods. This pool of cells is referred as Cancer Stem Cells (CSC) that may generate tumors through the stem cell processes of self-renewal and differentiation [196-198]. In tumors, they form a distinct population and can cause relapse and metastasis giving rise to new tumors. CSCs are the core contributors that affect therapy failure, regardless of whether these determinants are present within a transitory state or in well-defined CSC populations. Therefore, we need to develop specific therapies targeting CSCs in order to disrupt the pool of cells that sustain tumor growth and recurrence. In this study, we focused on the Urokinase Plasminogen Activating Receptor (uPAR), a well-known molecule that hide a new unexpected role in tumor recurrence. Its expression increases with disease progression, correlates with poor prognosis and therefore was considered a good target for anti-tumoral therapy [199]. These chemotherapies with the addition of anti-uPAR impairing agents are aimed to block its protease- and anchorage-dependent property but also its function to activate the cellular signalling of some receptor such as EGFR, IGFR, PDGFR and integrins receptors [1]. As many studies demonstrated, even if these therapies are effective they also showed that they are not capable to eradicate all the tumor cells. We have shown that uPAR is strongly over-expressed in human melanoma cells and that its presence controls either the mesenchymal (protease-dependent) and the amoeboid (protease-independent) type of cell invasion [200]. We have also cleaved uPAR expressed in human melanoma cells by the uPAR-degrading enzyme MMP12, properly engineered within shuttle Endothelial Progenitor Cells (EPCs). Once delivered within experimental human melanomas xenografted in nude mice, such EPC-MMP12 inhibited tumor growth, angiogenesis and lung metastases [201]. Further, we have observed that uPAR expression is proportional to the phase of tumor progression of melanoma cells and that the TGFβ of conditioned medium of mesenchymal stem cells (MSC) induces an epithelial-to-mesenchymal (EMT) profile in melanoma cells, characterized by uPAR and TGFβ/TGFβ-receptor upregulation: TGFβ gene silencing in MSCs downregulates uPAR expression and EMT in melanoma [202]. Therefore, we decided to investigate what happens to melanoma cells when uPAR is completely Knock-Out, through the recent and innovative technique called CRISPR. Mounting evidence suggests that cancer may arise from a transformed stem cell, which is able to self-renew, differentiate into diverse progenies,

and drive continuous growth [203]. Indeed many characteristics suggest the presence of stem-like cells in melanomas like their phenotypic heterogeneity both in vivo and in vitro [4], their ability to express developmental genes [204] and their capacity to differentiate into a wide range of cell lineages, including neural, mesenchymal, and endothelial cells [205;206]. Given that all, we speculated that destroying or inactivating all uPAR in melanoma cells can lead to slow their cell cycle, showing an acquired chemo-resistance and a quiescent status that can cause the loss of all the possibilities to strike the tumor with the conventional chemotherapies.

Materials and Methods

Cell lines

The melanoma cell line A375 (MITF wild type, BRAF V600E, NRAS wild type) was obtained from American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM) High Glucose with 10 % FBS (Euroclone, Milano, Italy).

Transfection and Plasmid

The plasmids for CRISPR-Cas9 were obtained from Santa Cruz Biotechnology (Santa Cruz, California). Plasmid were diluted into Plasmid Transfection Medium at optimized concentration and transfected using UltraCruz Transfection Reagent according to the manufacturer's instructions. Medium was replace after 48h of incubation at 37°C in 5% CO2 humidified incubator and cells were selected using 1ug/mL puromycin (Sigma-Aldrich, Saint Louis, Missouri, USA). Limiting dilution was then performed in order to select indivudual clones that were characterized using WB, PCR, Flow Citometry and Immunecitochemistry.

Western blot analysis

Cell pellets obtained by centrifugation were incubated with RIPA lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mMEDTA) and proteinase inhibitor cocktail (Roche, Mannheim, Germany) for 30 minutes on ice. Lysates were then centrifuged at 14,000 r.p.m. for 10 minutes. Aliquots of lysates (40 µg) of A375 and M6 cells were subjected to Western blotting. The primary antibodies were anti-uPAR R-3 (1:1000; mouse monoclonal antibody, MON-R3 (Life Technlogies, Monza, Italy), which recognizes full-length uPAR; anti-uPAR R-4 (1:1000; mouse monoclonal antibody, MON-R4(Life Technlogies, Monza, Italy), which recognizes both uPAR full-length and truncated form; anti-Cyclin A/D1/E(Santa Cruz, California); anti-α Tubulin (Sigma-Aldrich, Saint Louis, Missouri, USA); anti-LC3A/B (Cell signalling Technology, Danvers, MA, US); anti-GAPDH (mAbcam, Cambridge, UK); anti-PARP (Cell signalling Technology, Danvers, MA, US); anti-p53 (Santa Cruz, California); anti-γH2AX (Cell signalling Technology, Danvers, MA, US); anti-pmTOR (Cell signalling Technology, Danvers, MA, US); anti-pAkt (Sigma-Aldrich, Saint Louis, Missouri, USA); anti-Akt (pan) (Cell signalling Technology, Danvers, MA, US); anti-pERK (Cell signalling Technology, Danvers, MA, US); anti-ERK (Cell signalling Technology, Danvers, MA, US). Membranes were incubated in a blocking solution consisting of phosphate-buffered saline (PBS)/Odyssey Blocking Buffer 1:1 (PBS/OBB) (Lycor Bioscience) for 1 hour at room temperature. Membranes were then incubated overnight at 4 °C with the

appropriate antibody, washed four times with PBS-Tween 0.1% solution, and probed with the secondary IRDye antibodies according to the manufacturer's instructions. The protein bands were analyzed by the Odyssey Infrared Imaging System (Lycor Bioscience) using software for protein quantification.

RNA extraction, semiquantitative and quantitative PCR

Total RNA was prepared using Tri Reagent (Sigma-Aldrich, Saint Louis, Missouri, USA), agarose gel checked for integrity, and reverse transcribed with cDNA sintesys kit (BioRad, Milano, Italy) according to manufacturer's instructions. Selected genes were evaluated by qualitative PCR using Blue Platinum PCR Super Mix (Life Technologies, Monza, Italy) or Real-Time PCR using SsoAdvanced Universal Green Mix (BioRad, Milano, Italy) with 7500 Fast Real Time PCR System (Applied Biosystems, Waltham, Massachusetts, USA). For Real Time PCR, fold change was determined by the comparative Ct method using β2-Microglobulin as the normalization gene. Amplification was performed with the default PCR setting: 40 cycles of 95°C for 10 seconds and of 60°C for 30 seconds using SYBR Green–based detection. Primer sequences (IDT, TemaRicerca, Bologna, Italy) are reported below.

Primer	Sense	Antisense
GAPDH	CAATGACCCCTTCATTGACCTC	AGCATCGCCCCACTTGATT
β2-Microglobulin	GCCGTGTGAACCATGTGACT	GCTTACATGTCTCGATCCCACTT
UPAR (910bp)	GGTCACCCGCCGCTG	CCACTGCGGGTACTGGACA
UPAR	CAGAGTTGCCCTGGTTGCA	AAGGAGAAGAGCTGGAGCTG
EGFR	GGTGCGAATGACAGTAGCATTATGA	AAAGGTGGGCTCCTAACTAGCTGAA
Cyclin A	GAGCTGGTTAGTTGAAGTA	CTCCATTCTCAGAACTTG
Cyclin B1	GAAGATCAACATGGCAGGCG	GCATTTTGGCCTGCAGTTGT
Cyclin C	TGGGCCAAGAAGACATGTTG	GATCCGTTCTGTAGGTATCATTCACTAT
Cyclin D1	GGATGCTGGAGGTCTGCGAGGAAC	GAGAGGAAGCGTGTGAGGCGGTAG
Cyclin E	GGAAGGCAAACGTGACCGTT	GGGACTTAAACGCCACTTAA
Cyclin H	GAGGAGCAGCTGGCAAGACT	ACGGCTTTGCATCTGAATTTG
PKM2	CAGAG-GCTGCCATCTACCAC	CCAGACTTGGTGAG-GACGAT
LDHA	AGGGAATGTACGGCATTGAG	CCTCATCGTCCTTC-AGCTTC
G6PD	TGGAACCGGGACAACATC	CAACACCTTGACCTTC-TCAT
PDK1	CCAAGACCTCGTGTTGAGACC	AATACAGTCTCAGGTCTCCTTGG
PGC1α	GGGAAAGTGAGCGATTAGTTGAG	CATGTAGAATTGGCAGGTGGAA
CytC	TTGCACTTACACCGGTACTTAAGC	ACGTCCCCACTCTCTAAGTCCAA
COX5b	TGCGCTCCATGGCATCT	CCCAGTCGCCTGCTCTTC
COX4 1i	GGCCCGGCATTTTACGA	TCACCGTGGAGCGGAAA
ATPS	TGCAAGGAACTTCCATGCCTC	CGCCCAGGTTCTTCAAGATCAA
GLS1	TGCTACCTGTCTCCATGGCT	CCTAGATGGCACCTCCTTT
GLS2	TGCCTATAGTGGCGATGTCTCA	GTTCCATATCCATGGCTGACAA
GLUT1	CGGGCCAAGAGTGTGCTAA	TGACGATACCGGAG-CCAATG
GLUT3	CGAACTTCCTAGTCGGATTG	AGGAGGCACGACTTAGACAT
MCT-1	GTGGCTCAGCTCCGTATTGT	GAGCCGACCTAAAAGTGGTG
ASCT2	GGTGGCTGGCAAGATCGT	CCAAGGCGGGCAAAGAG
KLF-4	GCAGCCACCTGGCGAGTCTG	CCGCCAGCGGTTATTCGGGG
Nanog	ACCTTGGCTGCCGTCTCTGG	AGCAAAGCCTCCCAATCCCAAACA
OCT3/4	TTTTGGTACCCCAGGCTATG	GCAGGCACCTCAGTTTGAAT
SOX2	GAGCTTTGCAGGAAGTTTGC	GCAAGAAGCCTCTCCTTGAA
c-Myc	AATGAAAAGGCCCCCAAGGTAGTTAT	GTCGTTTCCGCAACAAGTCCTCTTC

Table 1 - Primer List

All the primers are suitable for Real-Time PCR except Cyclin E, GAPDH and uPAR (910bp).

Immunofluorescence analysis

Cells were grown on glass coverslips, washed twice with 1 ml of PBS, fixed for 20 minutes in 3.7% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes. The cells were incubated in blocking buffer (3% BSA and 0.1% Triton X-100 in PBS) for 1 hour at room temperature and then stained with the appropriate antibody overnight at 4° C. Successively, the cells were incubated at room temperature for 1 hour with the specific secondary antibody. Nuclei were stained with DAPI dye (4',6-diamidino-2-phenylindole; Life Technologies). Then, the cells were dried, mounted onto glass slides, and examined with confocal microscopy using a Nikon Eclipse TE2000-U (Nikon, Tokyo, Japan). A single composite image was obtained by superimposition of 20 optical sections for each sample observed. The collected images were analyzed by ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsbweb.nih.gov/ij/index.html). To calculate the correct Fluorescence values we draw an outline around each cell and circularity, area, mean fluorescence were measured, along with several adjacent background readings. The Corrected Total Cellular Fluorescence (CTCF) = integrated density – (area of selected cell * mean fluorescence of background readings), was calculated.

Flow cytometry analysis

Melanoma cells were harvested with Accutase (Euroclone, Milano, Italy), washed once with cold PBS and then stained with fluorochrome-conjugated mAbs anti-CD44 (Immunotools GmbH, Germay), -CD87 (Life Technologies, Monza Italy), -CD133 (eBioscience), CD243 (eBioscience), ALDH1 (Abcam) for 1h on ice in dark. The externalization of phosphatidylserine was determined by flow cytometric analysis of cell staining using an Annexin V-FITC conjugate (Immunotools GmbH, Germay) and PI according to the manufacturer's protocol. After washing in PBS plus Bovine Serum albumin 0.5% (BSA), cells were analyzed by flow cytometry BD-FACS Canto with DivaSoftware (BD Biosciences).

Cell cycle analysis

Cell cycle distribution was analyzed by the DNA content using PI staining method. Cells, starved for 24 h, were harvested, washed 2 times in PBS by centrifugation and stained with a mixture of 100 µg/mL propidium iodide (PI), 20 µg/mL RNase A, 1 mg/mL trisodium citrate and 0.3 % (v/v) Triton X-100 in dark at room temperature for 30 min. The stained cells were analyzed by flow cytometry (BD-FACS Canto) using red propidium-DNA fluorescence.

CFSE Proliferation assay

Cells were harvested with Accutase (Euroclone, Milano, Italy), wash once with cold PBS and then incubated with CFSE (Life Technologies, Monza, Italy) for 20 min at 37°C protected from light. After washing with cold PBS, five times the original staining volume of culture medium was added to the cells and incubate for 5 minutes. This step removes any free dye remaining in the solution.
Cells were pelleted by centrifugation and then plated again in three P100 plates. T0 were harvested after 4 hours to let the reagent undergo acetate hydrolysis while the other two were harvested after 24 and 48 hours. Cells were then fixed and analyzed by flow cytometry (BD-FACS Canto).

Tumor Spheroid Formation

Tumor cell monolayers were washed with PBS and then harvested using Trypsin, collected and centrifuged at 500 x g for 5 min. Supernatant was removed and resuspended cell pellet in 1mL complete growth medium. 500 cells/well were seeded dispensing 200 µL per well into a 96-well flat-bottomed plate precoated with 1.5% Agar. The plate was centrifuged at 500 x g for 5 min and then transfered to an incubator (37°C, 5% CO2, 95% humidity). Four days later tumor spheroids formation was visually confirmed and we proceeded with the 3D assays.

Tumor Spheroid Growth Kinetics and Treatment with Drugs

Spheroids were generated as described above. Growth kinetics and inhibition assays were performed as previously reported [207].

Spheroid-based Migration Assay

Matrigel (BD Biosciences) was diluted to 125 µg/mL in serum-free medium using pre-cooled pipette tips and dispensed 50 µL per well into the inner 60 wells of a 96-well flat-bottomed plate and incubated for 2h at RT. Wells were washed twice with PBS and then were distributed 200 µL per well of medium supplemented with 2% FBS. The 4 days old spheroids were transferred into the migration plate into a final volume of 300 µL/well. Spheroids were allowed to adhere to the surface for 30-60min before imaging the t=0 time point.

2D Invasion assay

Invasion of A375 melanoma cells was determined in vitro on Matrigel-coated polycarbonate filters (8µm pore size, 6.5 mm diameter), 12.5 mgMatrigel/filter, mounted in Boyden's chambers. 1 x 10^4

cells suspended in 200 µL of DMEM with 2% FBS as in the lower chamber. Cells were incubated for 6 hours at 37°C, 10% CO2 in air. After incubation, filters were removed and the non-invading cells on the upper surface were wiped-off mechanically with a cotton swab. Cells on the lower side of the filters were fixed overnight in ice-cold methanol, then stained using a Diff-Quick kit (BD Biosciences) and pictures of randomly chosen fields were taken.

Lactate Assay

Lactate was measured in cultured media with Lactate Assay kit (Vinci-Biochem, Florence, Italy) according to the manufacturer's instruction. In brief, samples were prepared in 50 µl/well with Lactate Assay Buffer in a 96-well plate. 50 µl Reaction Mix containing 46 µl Lactate assay buffer, 2 µl Probe, 2 µl Enzyme Mix were added to each well and the reaction was incubated for 30 minutes at room temperature, protected fromlight. Lactate reacts with enzyme mix to generate a product, which interacts with probe to produce fluorescence.

2-NBDG Uptake Assay

Cells were incubated with 10 µM 2-NBDG (Life Technologies, Monza, Italy) for 60 min at 37°C, then harvested with Accutase (Euroclone, Milano, Italy) and washed with cold PBS. NBD fluorescence typically displays excitation/emission maxima of ~465/540 nm and can be visualized using optical filters designed for fluorescein. So cells were immediately analyzed by flow cytometry (BD-FACS Canto).

Radiation Treatment

Cells were irradiated with UVc at a dose of 60 J/m^2 (254 nm; UV Stratalinker 1800, Stratagene). Damaging agent was applied at doses experimentally established to induce apoptosis rather than necrosis. The treatments proceeded for 24 h.

Statistics

Results are expressed as means ± SD. Multiple comparisons were performed by the Student test or One-way or Two-way ANOVA using GraphPad Prism 6. Statistical significances were accepted at * $p<0.05$.

Results

Genomic engineering of melanoma cells using CRISPR-Cas9

We transfected A375 melanoma cells with CRISPR-Cas9 D10A Mutant Plasmid in order to generate a complete PLAUR gene knock out. We then selected uPAR KO cells (thereafter called A375 PL1 and A375 PL25) and evaluated the success of transfection with PCR, Western Blotting, Flow Cytometry and confocal analysis (Fig. 11a-d). We immediately noticed morphological changes, as they showed a round shape and bigger dimensions, respect to the cells transfected with CRISPR-Cas9 Control Plasmid (A375 Control) (Figure 11e).

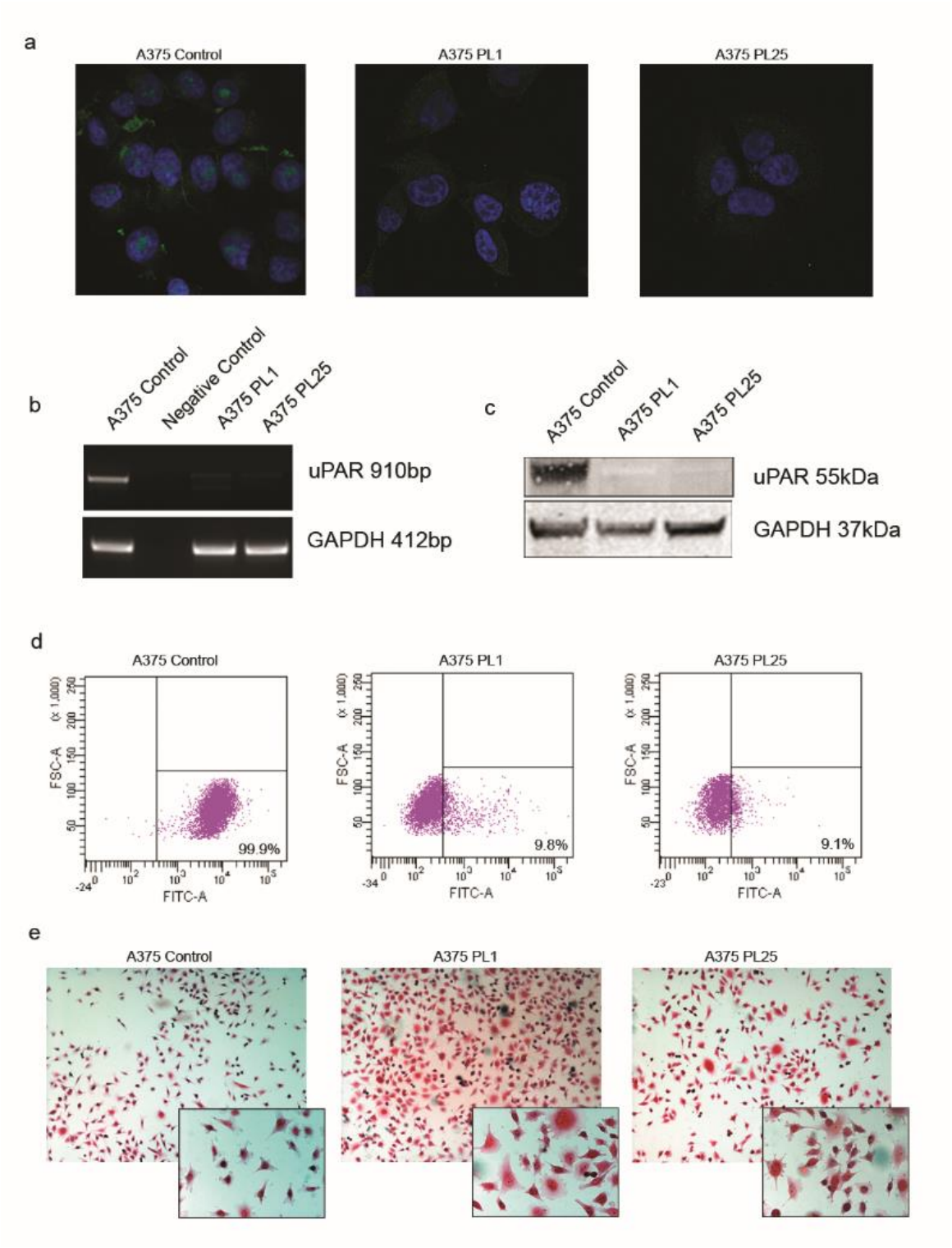
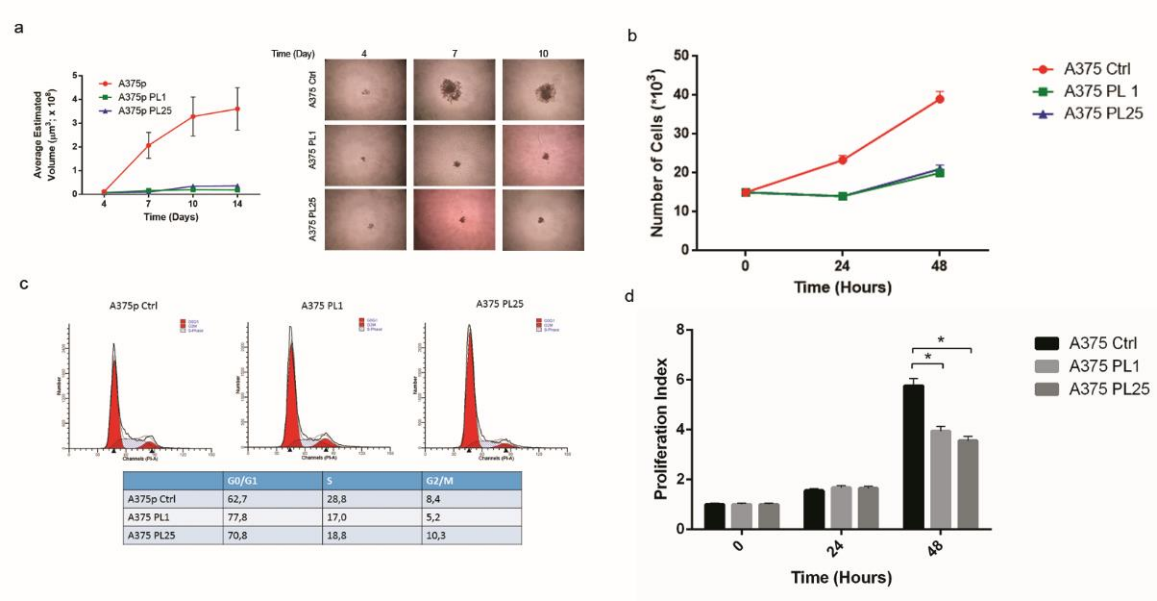


Figure 11 - Characterization of uPAR KO melanoma cells.

(a) The expression of uPAR under confocal microscope. Cells were fixed and immunostained with uPAR MON-R4 in green. DAPI was used for nuclear staining. (b) Total RNA isolated using Trizol reagent was subjected to RT–PCR analysis and (c) immunoblot analysis was performed on total cell lysates, to determine the expression of uPAR in A375 Control and uPAR KO CRISPRed cells. (d) Flow citometry analysis of uPAR. (e) Images of A375 Control and uPAR KO cells 2 weeks after transfection. Cells were fixed and stained with Hematoxylin and Eosin.

Lack of uPAR causes growth inhibition

A few days after uPAR KO, we observed a slower growth rate and, to assay that, we generated spheroids of A375 uPAR KO and A375 Control. These cells form particular spheroids commonly called “Loose Aggregate Spheroids” due to their melanocytic origin, as they grow as a compact mass at the center with friable aggregate cells all around like a sort of crown [17]. We monitored them for 14 days and we demonstrated that there is an evident growth inhibition in our uPAR KO cells (Figure 12a). We decided to repeat this also in a 2D assay simply counting plated cells at 24 and 48 hours and the results were the same (Figure 12b). We further confirm these data by cell cycle analysis, observing a shorter S phase and an accumulation in G0/G1 and G2/M phases in uPAR KO cells, which is compatible with a cell cycle arrest (Figure 12c).



	G0/G1	S	G2/M
A375p Ctrl	62,7	28,8	8,4
A375 PL1	77,8	17,0	5,2
A375 PL25	70,8	18,8	10,3

Figure 12 - Lack of uPAR causes growth inhibition.

(a) Agar-coated 96-well flat-bottomed plates were used to generate spheroids (a single spheroid per well). Starting from day 4 post generation, images were obtained at intervals using an inverted microscope. Analysis was carried out using ImageJ software and growth curves were obtained. Values are mean±SD (n = 20 spheroids/timepoint);(b)Cellular growth counting the total number of cell 24 and 48 h after the initiation of the culture; (c) DNA histograms were made by PI staining and FACS flow cytometry: the initial and last red peak represent G0-G1, G2-M stage respectively, middle is S stage. DNA histograms were analyzed by ModFitLT cell cycle analysis software. Percent cells in G2/M phase of the cell cycle are indicated in the table below; (d) Proliferation Index: fold expansion during culture (ratio of final cell count to starting cell count) as defined in ModFitLT.

Treating cells with CFSE labelling dye, we were able to confirm previous results and assessed that lack of uPAR led to a slower cell cycle. As we can see in Figure 12d there were no evident changes after 24 hours, probably due to the latency phase of these cells, but after 48 hours we observed a

decreased proliferation index (the average number of divisions that all responding cells have undergone since the initiation of the culture) in the uPAR KO cells. This particular feature has been yet observed by Gopinath S. et al. [208] showing that uPAR is fundamental for tumor cell proliferation. By the way, uPAR plays also a major role on the plasma membrane physically engaging Vitronectin, EGFR, IGFR, PDGFR and integrins receptors regulating their functions. Indeed, a competent factor, such as the ones listed above, can initiate signalling events (i.e. extracellular signal-regulated kinase (ERK-1/-2) activation and c-myc induction) that is sufficient to drive cells out of G0 and lead them through the early portion of G1[209]. As we evaluated through PCR and flow cytometry, EGFR expression is strongly downregulated and is plausible that this loss leads the cells to remain in G0 (Figure 13).

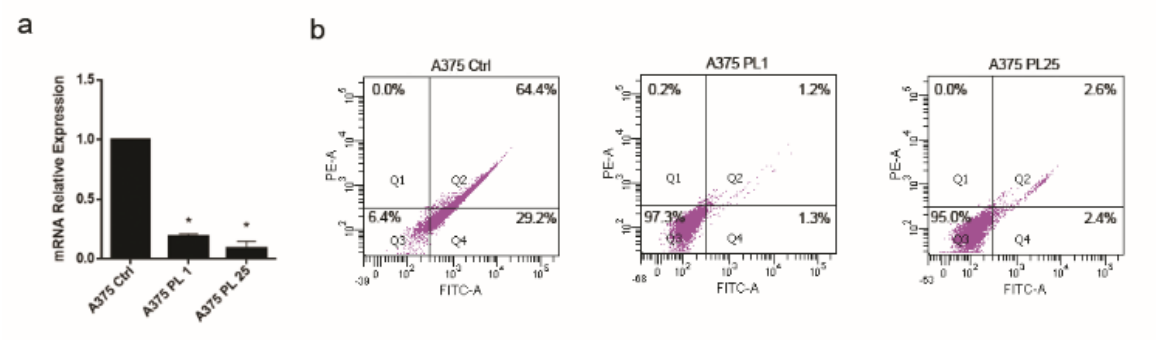


Figure 13 - Lack of uPAR causes growth inhibition.

(a) EGFR mRNA expression was evaluated using total RNA isolated using Trizol reagent and then subjected to RT–PCR analysis and quantitative PCR. The results are means and SD (b) Flow citometry analysis of uPAR stained with FITC and EGFR stained with PE.

As reported by Lui VW et al [210] the EGFR plays an important role in cell cycle. Indeed, the relationship between EGFR and the cell cycle has been primarily elucidated by examining the effects of specific EGFR-targeting agents on cancer cells. Attenuation of EGFR growth signalling by various therapeutic agents (i.e. EGFR antisense, monoclonal antibodies against EGFR, or specific tyrosine kinase inhibitors) results in cell cycle arrest in many tumor systems [211;212]. To better understand this phenomenon, we also evaluated Cyclins pattern through Western Blotting (Figure 14a) and PCR (Figure 14b) and we assessed that, while there was not a noticeable change in Cyclines A and E protein expression, we observed a substantial upregulation of Cyclin D1 and a significant Cyclin A, B1, C, E and H downregulation in PCR. As demonstrated by Leslie K. Diaz et al. [213] Cyclin D1 has a role in the development of a metastatic phenotype, even if partly unclear, and is commonly correlated with poor prognosis. Cyclin C peaks in the G1 phase of the cell cycle and has been implicated both in G0 to G1 and G1 to S phase regulation [214;215]. It is particularly

important during the exit from G0 phase as it let the cells to stop resting and go in an active proliferation status. Cyclin H is closely related to EGFR as reported by Solmi R et al. [216] who reported that by treating cells with the EGFR inhibitor, Cetuximab, Cyclin H results downregulated. Cyclins E, A and B1 are involved in the transition from, in order, G1 to S, S to G2 and G2 to Mitosis.

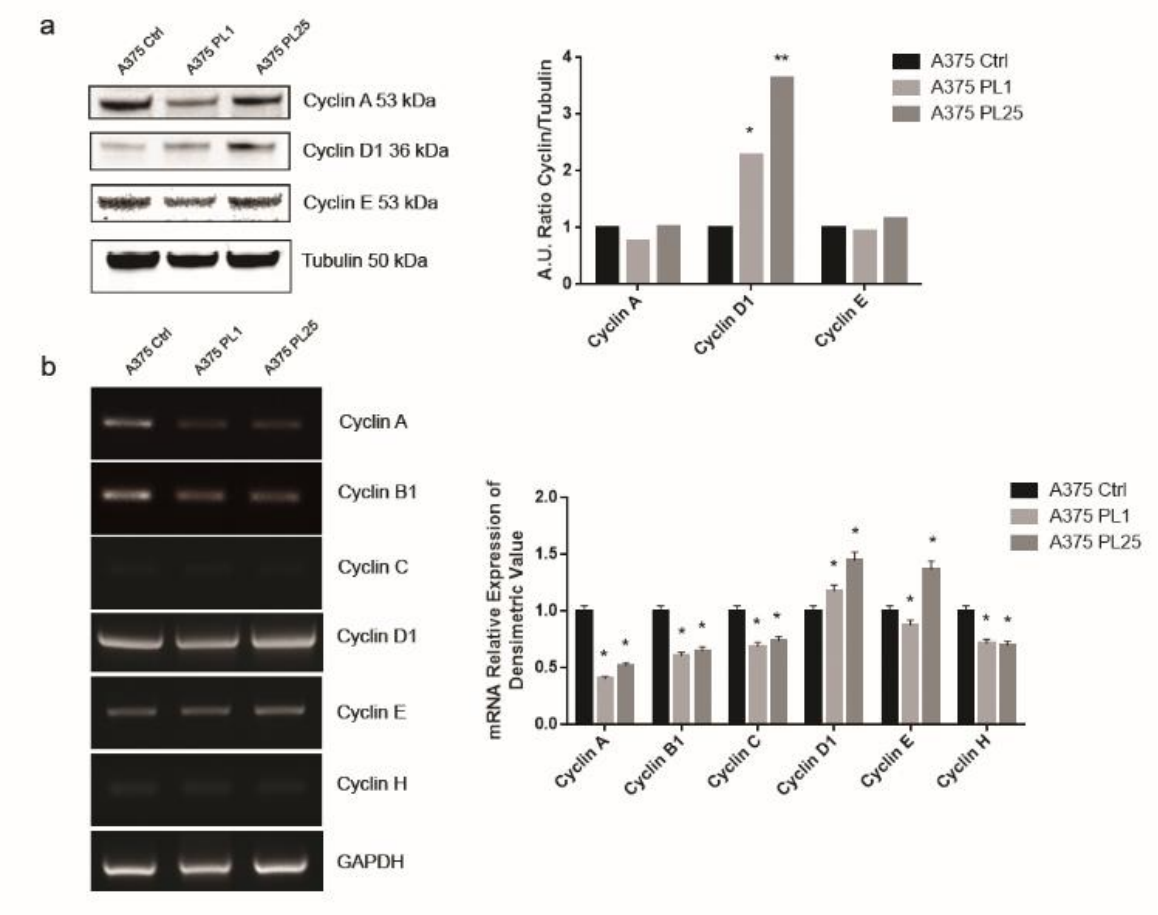


Figure 14 - Lack of uPAR causes growth inhibition.

(a) Immunoblot analysis of total cell lysates, to determine the expression levels of Cyclins in A375 cells subjected to CRISPR KO. Expression levels of Cyclin A, D1 and E were checked by loading equal amounts of total protein. Tubulin was used as a loading control. Densitometry analysis was performed using ImageJ software; (b) Cyclins A, B1, C, D1, E and H mRNA expression was evaluated using total RNA isolated using Trizol reagent and then subjected to RT–PCR analysis and qualitative PCR. Densitometry analysis was performed using ImageJ software.

uPAR KO cells metabolic switch

Apart the Cyclins pattern abnormalities and the loss of EGFR expression, we decided to investigate if a metabolic change can be responsible for the growth inhibition. Indeed, cell growth, especially for tumor cells, has a huge energetic requirement but when some metabolic disorder occurs, cells are forced to inhibit or arrest their growth. As we can see in figure 15a, after uPAR KO there is an evident downregulation of glucose, lactate and glutamine importers genes, GLUT1, GLUT3, MCT-1 and ASCT2, which are responsible for the acquisition of the main metabolic sources for the ATP production. The upregulation of Pyruvate kinase isozymes M2 (PKM2) and Lactate Dehydrogenase A (LDHA) is commonly correlated with poor prognosis and with an advanced cancer progression [217]. Expression level of glucose-6-phosphatedehydrogenase (G6PD) is upregulated as well, indicating that there is also a greater use of the pentose phosphate way in order to get more NADPH to be used in many anabolic processes (an effect that is potentiated also by PKM2 overexpression). We speculate that their increment, together with the transporters downregulation and the decreased oxidative phospotilation (OXPHOS) genes expression, could be due to a predominant glycolytic metabolism. The Lactate assay confirms that uPAR KO cells secrete more lactate in the medium than their uPAR positive counterpart (Figure 15b). Contrary to the downregulated expression of GLUT1 and 3, the Glucose Up-Take Assay (Figure 15c) demonstrated that uPAR negative cells are able to introduce glucose with the same efficiency as A375 Control cells. Indeed, even if glucose receptors are downregulated in uPAR KO cells, they have a very low Km (Michaelis-Menten constant), indicating that are capable to import glucose with a very high efficiency. The higher Glutaminases (GLS) expression in uPAR KO cells, which hydrolyze the amide group obtaining NH4+ and glutamate, that may fuel the intracellular pool of the Krebs cycle intermediates, could be due to the loss of the Amino-acid Transporter 2 (ASCT2). Indeed glutamine supply is a central factor for tumor cell proliferation, being a source of nitrogen for DNA and RNA synthesis. As reported by Bolzoni M et al. some types of human tumor cells exhibit a high requirement for glutamine (“glutamine addiction”) and use large amounts of the amino acid as an anaplerotic substrate [218]. However, all the other metabolic genes are downregulated meaning that there could be other ways which, with the tumor cells that lost uPAR expression, could feed themselves and could find the sources that they need to produce ATP. Therefore, we decided to investigate the autophagy process evaluating the expression of LC3 A/B protein isoforms. The cytosolic form of LC3 (LC3-I, 16 kDa) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II, 14 kDa), which is recruited to autophagosomal membranes. Autophagosomes fuse with lysosomes to form autolysosomes, and intra-autophagosomal components are degraded by lysosomal hydrolases

producing a moderate resource of ATP [219]. As assessed with the protein analysis (Figure 15d), there is a significant increase in LC3A/B isoform II which was sustained by co-treatment with Bafilomycin A, which is an inhibitor of the lysosomal proton pump and may also inhibit the fusion between autophagosomes and lysosomes preventing autophagic flux. To confirm this, we visualized LC3A/B using confocal microscopy (Figure 15e) and we confirm that, after 24 hours of starvation we could observe an increased level of LC3A/B in uPAR KO cells compared to control cells.

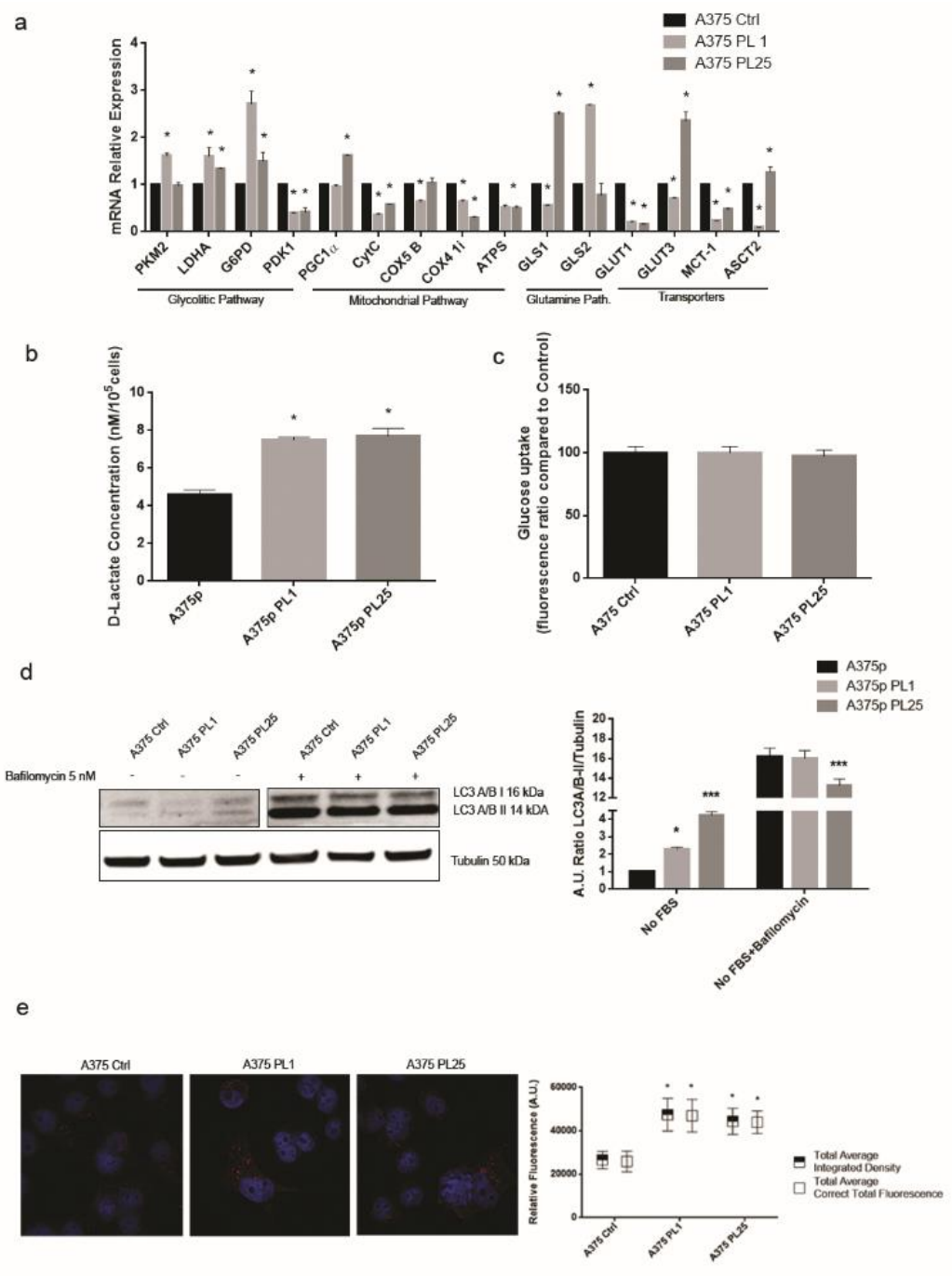


Figure 15 - uPAR KO cells metabolic switch.

(a) Total RNA isolated using Trizol reagent was subjected to RT–PCR analysis and quantitative PCR was performed. The results are means and SD (b) Measurement of Lactate in medium conditioned for 24h; (c) The level of uptake of this glucose analogue after 1 hour incubation was determined by FACS as the geometric mean of fluorescence intensity of the cell population for each sample;(d) Immunoblot analysis of total cell lysates, of A375 uPAR KO cells with or without Bafilomycin, to determine the expression of LC3 in A375 cells subjected to CRISPR KO. Expression levels of LC3 A/B isoforms I and II were checked by loading equal amounts of total protein. Tubulin was used as a loading control. Densitometry analysis was performed using ImageJ software. (e) The expression of LC3A/B under confocal microscope. Cells were fixed and immunostained with LC3A/B in red. DAPI was used for nuclear staining. Densitometry analysis was performed using ImageJ software.

uPAR-mediated acquired chemoresistance

These cells were also assayed for the capacity to resist chemotherapy. Indeed, chemotherapy is particularly effective on cells in a replicative status while, as seen above, uPAR KO cells are in a G0 steady state. We tested two typical melanoma drugs, the Vemurafenib, which is a B-Raf enzyme inhibitor used on unresectable or metastatic melanoma bearing V600E mutation, and the Trametinib, which is MEK inhibitor that is commonly used on B-Raf ^{V600E} metastatic melanomas. As we can see in figure 16, uPAR KO cells are much more resistant to both drugs than A375 Control cells.

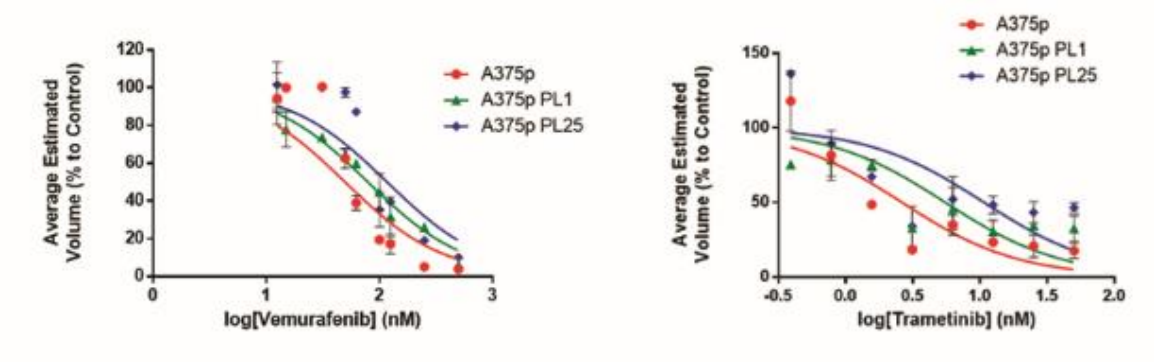


Figure 16 - uPAR-mediated acquired chemoresistance.

Day 4 A375 uPAR KO spheroids were treated with Vemurafenib (B-Raf enzyme inhibitor) or Trametinib (MEK1 and MEK2 inhibitor) with 1:2 serial dilutions (final concentrations 0 to 500 nM for Vemurafenib and 0 to 50 nM for Trametinib). Controls spheroids were treated with vehicle. Values are mean±SD (n=12) and a representative of three separate experiments for each agent is shown.

This is only one mechanism used by multi drug resistance cancer cells to reduce sensitivity to anticancer drugs. Other mechanisms include an increase in drug efflux, the reduction of drug uptake, the activation of survival signalling, the dysregulation of DNA repair, and the inhibition of apoptosis signalling. To evaluate if other mechanisms are involved in the enhanced chemoresistance to these drugs we decided to observe the CD243 expression level. This cluster of differentiation is also known as ATP-Binding Cassette subfamily B member 1 (ABCB1) or Multi Drug Resistance Protein 1 (MDR-1) and is commonly linked to a stem-like phenotype and great metastatic potential [220;221]. As we can see in figure 17, ABCB1 is not expressed in A375 Control cells while there is an increased expression in uPAR KO cells demonstrating that this one could be one of the mechanisms, together with the slower rate proliferation, that led these cells to resist chemotherapies. In order to better understand these phenomena, we decided to evaluate the apoptosis resistance using UVc treatment, which is the gold standard to assay apoptosis in melanoma cells.

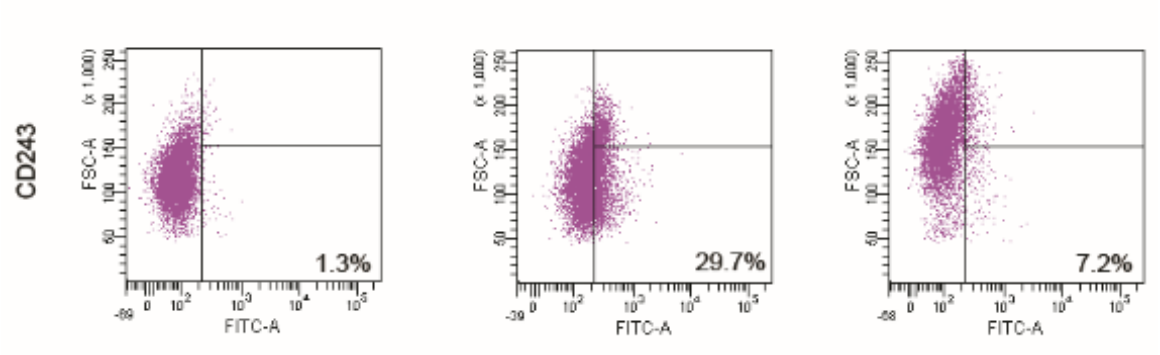


Figure 17 – ABCB1 expression uPAR-mediated.

A375 uPAR KO cells were tested for CD243 (ABCB1; MDR-1) expression by FACS analysis; positive cells percentage are shown in the dot plots.

uPAR KO melanoma cells are resistant to UV-induced apoptosis

To induce apoptosis, we treated cells with UVc and after 24 hours, we evaluated the number of apoptotic and necrotic cells with Annexin V/PI staining. As we can see in figure 18a, A375 uPAR KO cells are more resistant than A375 Control, demonstrating that uPAR loss leads to a more apoptotic-resistant phenotype. This apoptotic insult led the cells to overexpress and activate p53 machinery in order to repair the damaged DNA. In additon, PARP is more expressed in A375 Control than in uPAR KO cells mediating a more resistant phenotype in the last (Figure 18b). Moreover, a part the total level of PARP in its uncleaved form, we can also see a minor activation in uPAR KO cells meaning that these cells are incapable of repairing and, in case of ATP-deprivation, inducing apoptosis. The desensitization, of uPAR negative cells compared to A375 Control, is also validated by the phosphorylation of H2AX that is phosphorylated on serine 139 (called gamma-H2AX) as a reaction on DNA Double-strand breaks. Indeed, uPAR KO cells have a minor activation of this UV-induced biomarker, meaning that after this kind of hurt, cells are less prone to find and repair the damage. By the way, as Malla et al. demonstrated few years ago, uPAR downregulation induces apoptosis through PI3K/Akt pathway [222]. In accordance, uPAR KO cells are composed from an evident part of cell population in early and late apoptosis. These could be due also to the propensity to use the autophagy pathway inducing, sooner or later, apoptosis. As we expected from previous studies, pAkt and pmTOR are downregulated in uPAR negative cells while pERK is strongly up-regulated (Figure 19). uPAR loss in fact, downregulating PI3K/Akt pathway, forces the cells to use ERK as an alternative pathway to grow and survive [223]. ERK up-regulation could also be another responsible for the uPAR-induced chemoresistance, at least, to Vemurafenib and Trametinib.

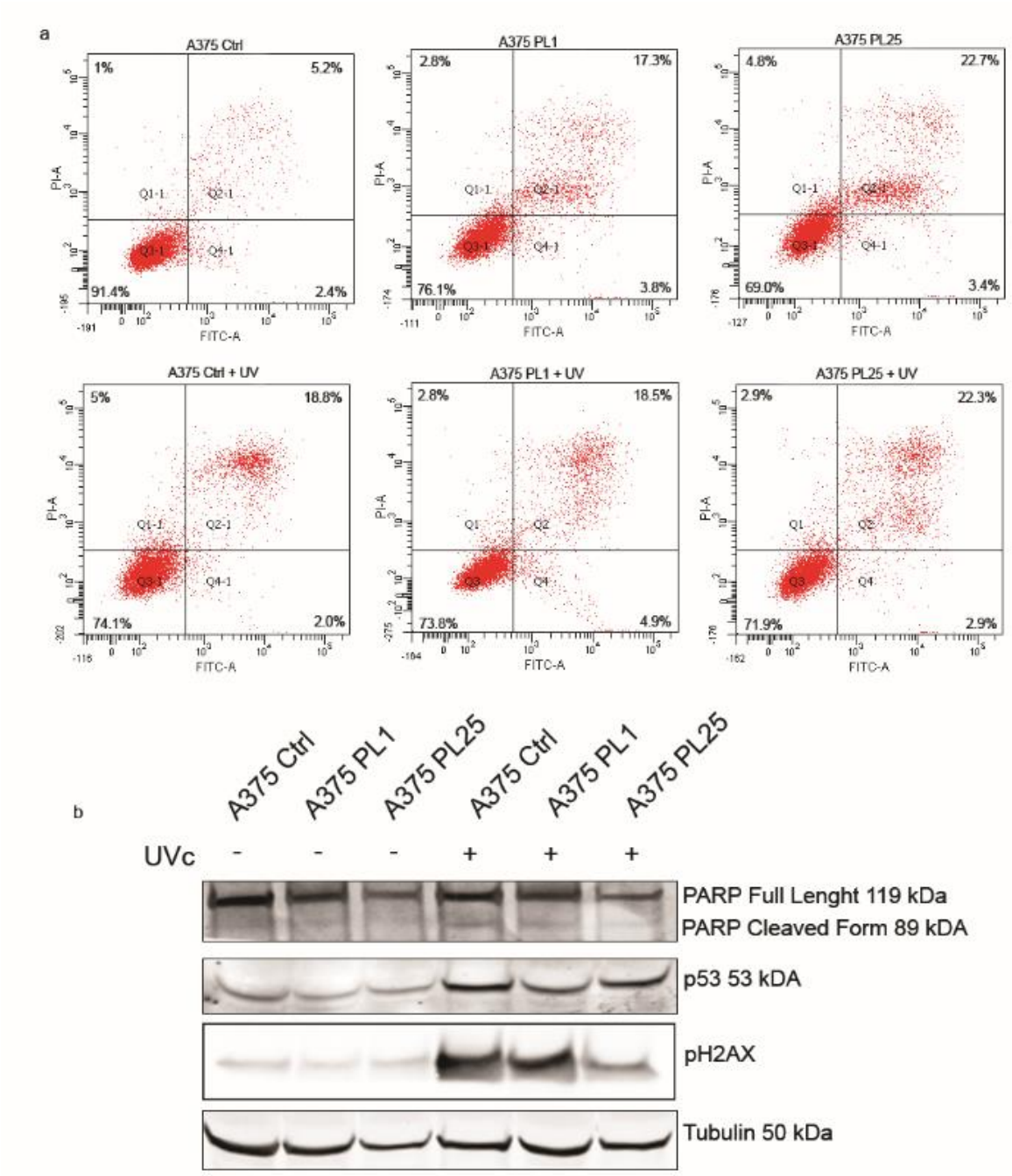


Figure 18 - uPAR KO melanoma cells are resistant to UV-induced apoptosis

(a) Annexin-V/PI FACS analysis of the three cell lines either untreated or treated with 60J/m². (b) Immunoblot analysis of total cell lysates, of A375 uPAR KO cells treated with or without UVc, to determine the expression of PARP, pH2AX and p53. Expression levels were checked by loading equal amounts of total protein. Tubulin was used as a loading control.

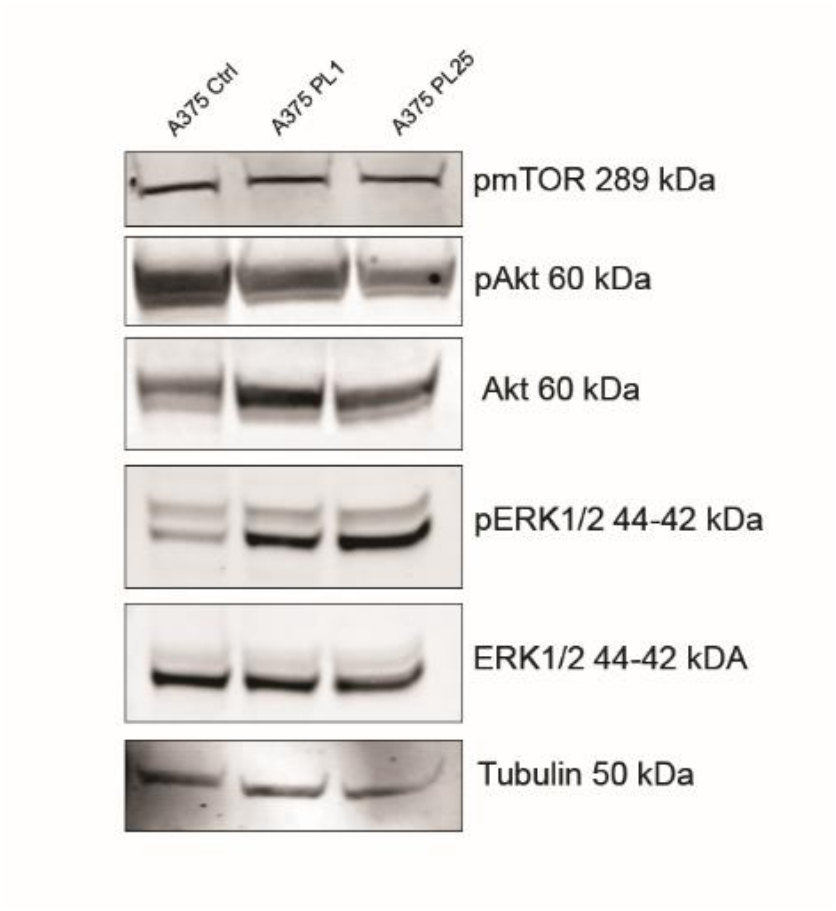


Figure 19 – uPAR negative cells transduction pathway

Immunoblot analysis of total cell lysates, of A375 uPAR KO cells, to determine the expression of pmTOR, pAkt and pERK. Expression levels were checked by loading equal amounts of total protein. Tubulin was used as a loading control.

uPAR deficiency leads to the acquisition of stem-like features

The morphological changes, the slower growth rate, the glycolytic metabolic phenotype, the acquisition of the autophagic capacity, the chemoresistance and the apoptosis resistance are all typical features of cells who gained a stem-like phenotype [224]. Cancer Stem Cells (CSCs) often express distinctive markers like CD133, CD44, CD24, ABCB1/5, ALDH1 and many others, though many of them are tissue and tumor related. Even if a universal marker for CSC identification remains undiscovered, multiple malignancies have been shown to contain stem-cell like population capable of initiating tumors in xenograft model [225]. As shown in figure 20a, performing a cytometer analysis, we discovered that our uPAR negative cell lines express two stem cell markers otherwise not expressed in control A375, while there are no changes in CD44 expression in both control and uPAR KO cells (data not shown). Moreover, as demonstrated above, also CD243 is expressed in these uPAR negative cells. To further analyze uPAR-mediated stem-like phenotype acquisition, we performed a Real-Time PCR for the Yamanka's factors [226] and for Nanog, one of the gene that is involved in the maintenance of the stem state [227-230]. As we can see in figure 20b the expression of SOX2 and Oct3/4 is upregulated while KLF-4 and c-myc are substantially not expressed. Regarding Nanog expression, we reported different levels in our two clones.

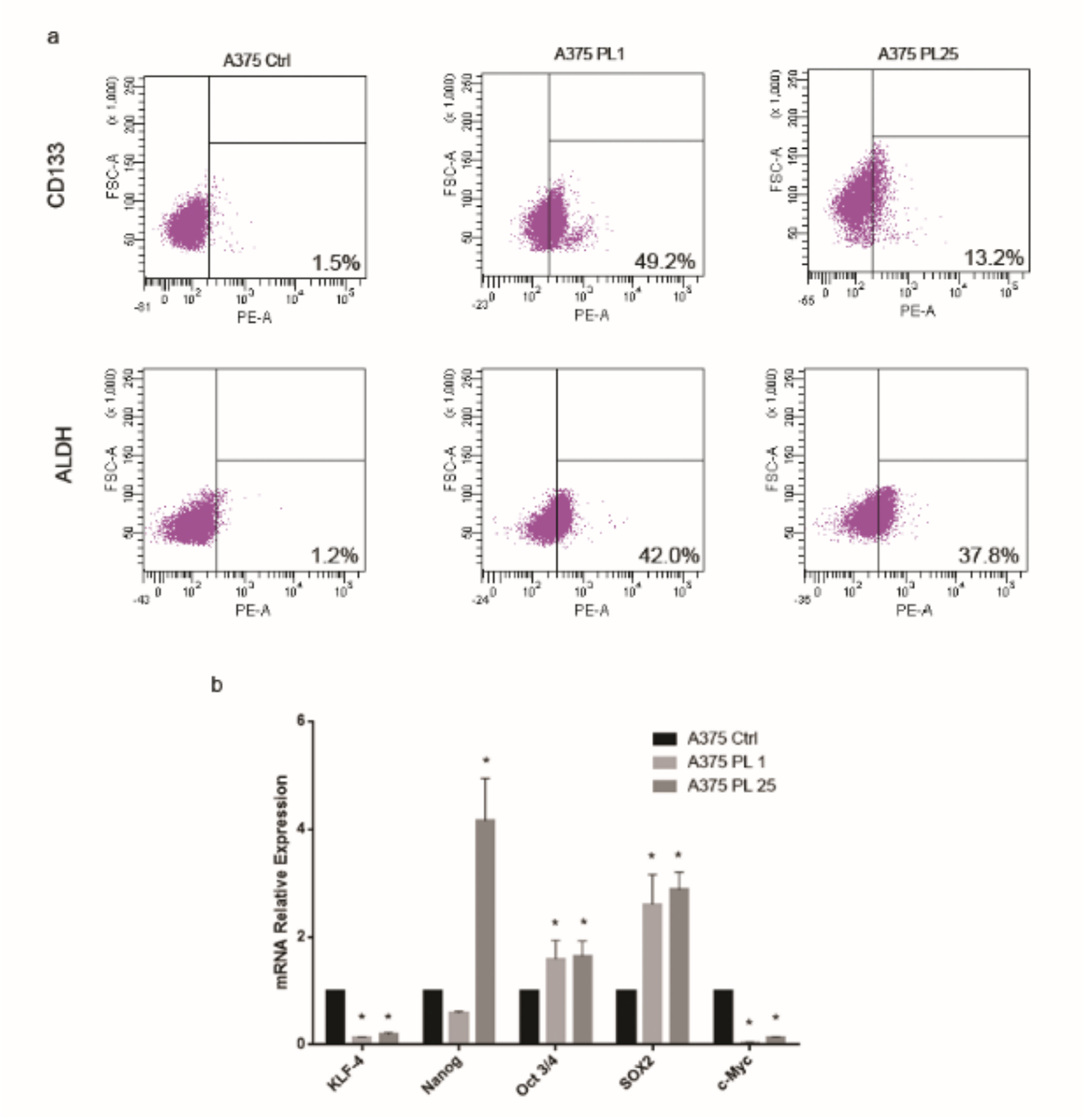


Figure 20 - uPAR deficiency leads to the acquisition of stem-like features.

(a) A375 uPAR KO cells were tested for CD133 and ALDH1 expression by FACS analysis; positive cells percentage are shown in the dot plots. (b) Total RNA isolated using Trizol reagent was subjected to RT–PCR analysis and quantitative PCR was performed. The results are means and SD; P < 0.05

Discussion

Overall, our study demonstrated the deep impact that loss of uPAR can have on human melanoma cells. uPAR is fundamental for their growth as for their invasive and migratory ability. As we previously demonstrated [200-202] uPAR mediates both the amoeboid movement, through Rho GTP-ases, and the mesenchymal movement through its action on the plasminogen system and the metallo-proteases (MMPs) cascade. Therefore, the inhibition or complete knock-out of uPAR gene leads to a great reduction in melanoma cell invasion and migration. Indeed, uPA/uPAR system is actually considered the main system involved in tumor invasion and metastasis, as well as in tumor angiogenesis [18]. Endogenous levels of uPA and uPAR increase with disease progression, correlate with poor prognosis and outcome in patients, and therefore uPAR can be considered a promising target for anti-tumoral therapy [199]. uPAR plays a major role in invasivity of malignant cells since it is involved both in ECM degradation and in cell adhesion through a "grip-and-go" mechanism [231]. It has been previously shown that uPA-uPAR interaction induces endothelial cell chemotaxis [232] and stimulates angiogenesis in the rabbit cornea [234]. Inhibition of uPAR expression blocks tumor cell invasion, angiogenesis and metastasis [200;234], and MMPs activation [235]. Also, uPAR induces EMT in cancer cells [236] and is involved in hypoxia-induced EMT [237]. Tumor progression is also characterized by increased expression of uPA/uPAR system, and many data indicate a regulative role of TGFβ on uPA/uPAR system in normal and cancer cells [238-240]. However, uPAR is transiently expressed whenever cell movement is required, as during inflammatory events, wound repair and tumorigenesis, while its expression is low in normal quiescent tissues. In the tumor microenvironment uPAR is expressed not only in tumor cells but also in endothelial cells, fibroblasts, inflammatory and mesenchymal stem cells [241;242]. We speculate that this opportunistic expression might be also used by tumor cells to shift from a proliferative and invasive phenotype to a quiescent steady state in order to escape chemo- and radiotherapy and finally generate new neoplastic lesions at local or distant sites. Indeed, when a sub-population of cancer cells within tumor mass become resistant to therapy, it can generate, after the clinical treatments, a new tumor causing recurrence. Presumably, these cancer cells are those that survive therapy and lead to relapse [225]. Even if every cell within the tumor mass possesses tumorigenic potential, the presence of a discrete subset responsible for treatment resistance would have an incontestable clinical significance. Cells who gained stem-like phenotype are commonly defined as **Cancer Stem Cells (CSC) or Tumor Initiating Cells (TIC)**. The CSC concept potentially explains not only the low clonogenic capacity of most malignancies but also why treatment responses rarely translate into complete remission for cancer patients: initial responses in cancer represent therapeutic effectiveness against the bulk cancer cells, while rarer but more

resistant CSCs theoretically are responsible for the **minimal residual disease (MRD)** and tumor relapse. Considering that CSCs are more resistant to therapy than the bulk tumor cells and thus responsible for cancer relapse then, minimal residual disease after treatment should be enriched of these cells. Furthermore, the presence of CSCs after therapy should predict recurrence. Indeed, it has been recently found that residual breast tumor cell populations persisting after conventional treatment are enriched for phenotypic breast CSCs [243]. Similarly, patients with myelodysplastic syndrome continue to have a population of phenotypically distinct myelodysplastic stem cells (CD34+ CD38lowCD90+), even in complete clinical and cytogenetic remissions [244]. These cells appear resistant to lenalidomide, a Thalidomide derived, treatment and may account for disease relapse. It is very important to improve our understanding of the mechanisms through which MRD acts because it may lead to the way for better defining CSCs. While the identification and characterization of CSCs from hematologic malignancies was founded on decades of biologic experience in human hematopoiesis, limited understanding of the biology of their normal counterparts has disadvantaged the study of solid tumor CSCs. Thus, initial research of CSCs in solid tumors was based on findings in liquid malignancies. Accordingly, breast CSCs, initially described as CD44+ CD24low, were identified by their ability to generate tumors in immunodeficient mice [245]. This description was followed quickly by the discovery of CSCs expressing CD133 in brain cancers [113]. Since then, although the importance of any specific marker for CSC identification remains unclear, multiple malignancies have been shown to contain a stem-cell like population capable of initiating tumors in a xenograft model (Table 1). Back in 1953, Nordling [246] was the first to formulate the theory that cancer results from an accumulation of DNA mutations. This was further refined by several researchers including Ashley [247], Knudson [248] and Nowell [249]. In this model of carcinogenesis, inherited mutations and/or environmental carcinogens lead to the development of premalignant clones. These cells further accumulate genetic hits until one single cell reaches a critical genetic or epigenetic state that confers a growth and/or survival advantage over the other normal cells. Over time, if it can evade the immune system, this abnormal cell would give rise to a malignant tumor. The cell that suffered the “critical insult” is the primordial cancer-initiating cell and the tumor, that will develop from that cell, will represent its clonal expansion. As postulated by Ashley, a cancer-initiating cell must survive long enough to accumulate three to seven genetic mutations necessary to generate cancer [247].

Cancer Type	Phenotype	Xenograft model used
Breast	CD44+CD24−Lin−	NOD/SCID
	ALDH1+	NOD/SCID
Brain	CD133+	NOD/SCID
Glioblastoma	CD133+	nu/nu
Lung	CD133+Ep-CAM+	NOD/SCID
Prostate	Side population	NOD/SCID
	CD44+	NOD/SCID
	CD44+/α2β1+/CD133+	Methylcellulose progenitor assay
Colon	CD133+	NOD/SCID
	CD44+/Ep-CAM+	NOD/SCID
Melanoma	ABCB5+	NOD/SCID
	1:4 unselected cells	NOD/SCID/IL2Rγ−
Liver	CD90+CD44+	SCID/Beige, BALB/c
Pancreas	ALDH1+	NOD/SCID
	CD133+	NMRI-nu/nu
	CD44+CD24+ESA+	NOD/SCID
Head and neck	CD44+Cytokeratin 5/14+	NOD/SCID

Table 2 – CSC Markers of Identification

ABCB5, ATP-binding cassette subfamily B member 5; ALDH1, aldehyde dehydrogenases 1; CD, cluster of differentiation, Ep-CAM, epithelial cell adhesion molecule; ESA, epithelial specific antigen; IL2Rγ−/−, interleukin 2 receptor gamma knock out; Lin, lineage; NOD/SCID, nonobese diabetes/severe combined immunodeficiency; NMRI, Naval Medical Research Institute; nu/nu mice, homozygous nude mice. [226]

Nowell [249] hypothesized that the inherent longevity and extensive proliferative capacity of a stem cell within a normal tissue make it the ideal candidate to be the cancer-initiating cell. On the other hand, most terminally differentiated cells are neither long-lived nor possess the ability to produce tumors with the limited number of divisions remaining in their differentiation program. The way through which a tumor generates from a single cell that suffered a series of insults would explain why only a minority of cells from most hematologic malignancies and solid tumors are

clonogenic in vitro and in vivo. In this CSC model, the cancer-initiating event, while conferring some advantages to the original cancer cell, does not completely alter its differentiation program; the malignant tumor would thus consist of a heterogeneous population of cells including the differentiated progeny of the original cell, simulating the hierarchical structure of the normal tissue of origin. Since the primordial cancer-initiating cell or one of its progeny in this model possesses self-renewal capability and at least some differentiation potential, this cell naturally came to be called a CSC. Alternatively, it is also conceptually possible that the low clonogenicity of cancer is the result of all cells within a cancer retaining the capacity to proliferate but only at a low rate. Which of these two scenarios account for the low clonogenicity of most cancers has been debated for years. As we demonstrated above, melanoma cells that through CRISPR/Cas9 technique loss uPAR gene (PLAUR) undergo a series of alterations that confer them a stem-like phenotype. First of all, the growth inhibition, caused by cyclins pattern alteration and maybe by the metabolic dysregulation, is the main imprint of the cancer stem cells. uPAR itself, binding to uPA leads to the downstream activation of plasminogen and matrix metalloproteases, which lyse the extracellular matrix, releasing and activating matrix-bound growth factors which are important for supporting tumor growth [250;251]. As reported by Nathan Moore and Stephen Lyle [252], heterogeneous tumors are predicted to contain a population of slow cycling cells. Conventional radiotherapy or chemotherapies target and kill rapidly proliferating cells, while does not affect quiescent cells. Moreover, resistance to cytotoxic treatments may also be attributable to that lower proliferative rate [252;253]. CSCs from a variety of tumors have been shown to be slow cycling and to exhibit an increased level of quiescence compared to the majority populations of cancer cells within certain tumors [254;255]. CSCs that survive chemotherapy, after an unspecified period of time, re-enter the cell cycle and re-establish the tumor. Chemotherapy and radiotherapy have been considered treatments of choice for the past half-century, often affording remarkable reductions in tumor burden. However, induction of a stem-like phenotype leads to the acquisition of resistance to both radio- and chemotherapy, a phenomenon that has been extensively documented in breast and ovarian cancers [256;257]. Today, we possess only a partial and incomplete understanding of the actual biochemical and cell-physiologic mechanisms underlying the intrinsic chemo- and radio-resistance of tumor cells that became insensitive after the acquisition of the stem state. In addition, the resistance to chemotherapy in normal stem cells has been attributed to high-level expression of anti-apoptotic proteins [258] and to ABC transporters that are capable of efflux of the Hoechst dye, creating the so called **"side population (SP)"** observed upon fluorescence-activated cell sorting [259-261]. As we demonstrated, uPAR Knock Out leads to the acquisition of resistant phenotype to drugs such as Vemurafenib and

Trametinib, a biological phenomenon that we connect with the slower proliferation rate of uPAR knock out cells, but also with their expression of ABCB1 protein. In addition, another characteristic of uPAR knock out cells can account for such chemo-resistance: with respect to uPAR expressing cells, our KO clones are characterized by an increased activation of ERK1/2 pathway, at the expense of PI3K/Akt. Thereby, probably because of such increase, these cells can better survive to drugs that selectively affect ERK pathway, which is the case of Vemurafenib (anti-$BRAF^{V600E}$) and Trametinib (anti-MEK) [243].

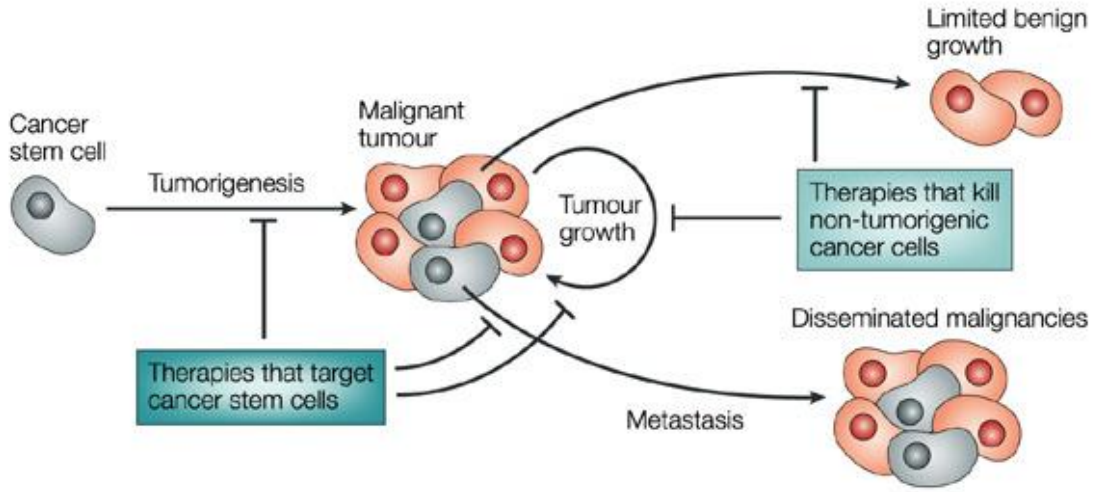


Figure 21 – CSCs chemoresistance and recurrence

Cancer stem cells (grey) self-renew and differentiate within tumours to form additional cancer stem cells as well as non-tumorigenic cancer cells (orange), which have limited proliferative potential. Therapies that kill, induce differentiation or prevent the metastasis of cancer stem cells represent potential cures. Therapies that kill primarily non-tumorigenic cancer cells can shrink tumours, but will not cure the patient because the cancer stem cells will regenerate the tumour. The intrinsic differences in tumorigenic potential among cancer cells might also explain why it is possible to detect disseminated solid cancer cells in patients that never develop metastatic disease. The identification and characterization of cancer stem cells should therefore also lead to diagnostic methods that can distinguish between disseminated tumorigenic and non-tumorigenic cells, as well as provide a better understanding of the mechanisms that regulate migration of cancer stem cells. [262]

Moreover, uPAR KO cells are also characterized by an enhanced resistance to apoptosis induced by Ultraviolet (UV) rays. Cells hit by UV are subjected to the formation of double strand breaks in DNA and the phosphorylated form of histone H2AX allows the DNA to be repaired. We showed that, in uPAR KO cells, not only pH2AX is less activated but also PARP shows lower activity in response to this kind of insult. Interestingly, by analyzing PARP signal in either wild-type or uPAR KO cells, we observed that its total expression is less in cells without uPAR expression. PARP is involved in DNA repair and in induction of programmed cell death. Thereby, its decreased expression could cause such increase in apoptotic threshold of uPAR KO cells. By the other hand,

we observed that A1 and A25 clones in standard condition are characterized by a basal apoptotic level, while uPAR expressing cells do not activate apoptotic program at all in normal condition. This is in accordance with the literature. Indeed, it has been demonstrated that downregulation of uPAR and cathepsin B decreased the Bcl-2/Bax ratio [222] and initiated partial extrinsic apoptotic cascade accompanied by the collapse of mitochondrial membrane potential in SNB19 glioma cells [263]. Moreover, Kin et al. [264] reported that downregulation of uPAR was associated with increased expression of the pro-apoptotic protein Bax in glioma cells. Taken together, these results suggest that uPAR downregulation could induced apoptosis by modulating the Bcl-2/Bax ratio accompanied by collapse of mitochondrial membrane potential. We also investigated the impact that uPAR deletion had on cancer metabolism. It is well known that during the Warburg effect, cancer cells generate ATP through glycolysis rather than oxidative phosphorylation, even under non-hypoxic conditions [265]. To confirm this, a recent study reported a metabolic switch to glycolysis that occurs upon generation of breast CSCs [266], in accordance with another one showing that the components of the mevalonate metabolic pathway are important in the generation of breast CSCs. Indeed, the inhibition of this pathway by using hydroxy-3-methylglutaryl CoA reductase blockers, resulted in loss of CSC specific properties [267]. These studies suggest that the alteration of the cell metabolism could be an essential step required for the entrance into the CSC state. In our study, despite we did not observe any significant variation in glucose uptake, in uPAR KO cells we noticed a strengthening in the glycolytic pathway at the expense of the oxidative-phosphorylation. In agreement with this, we observed an up-regulated expression of Lactate Dehydrogenase A (LDHA) and Monocarboxylate Transporter 1 (MCT-1). The first one catalyzes the inter-conversion of pyruvate and L-lactate with concomitant inter-conversion of NADH and NAD+ while the second one exports lactate to the external microenvironment [268;269]. This is a typical metabolic adaptation in tumor cells discovered in the 1920s by Warburg who showed that glycolysis, which normally increases under anaerobic conditions, was often enhanced in cancers in the presence of abundant oxygen, the so called aerobicglycolysis or “Warburg effect” [265;270-271].

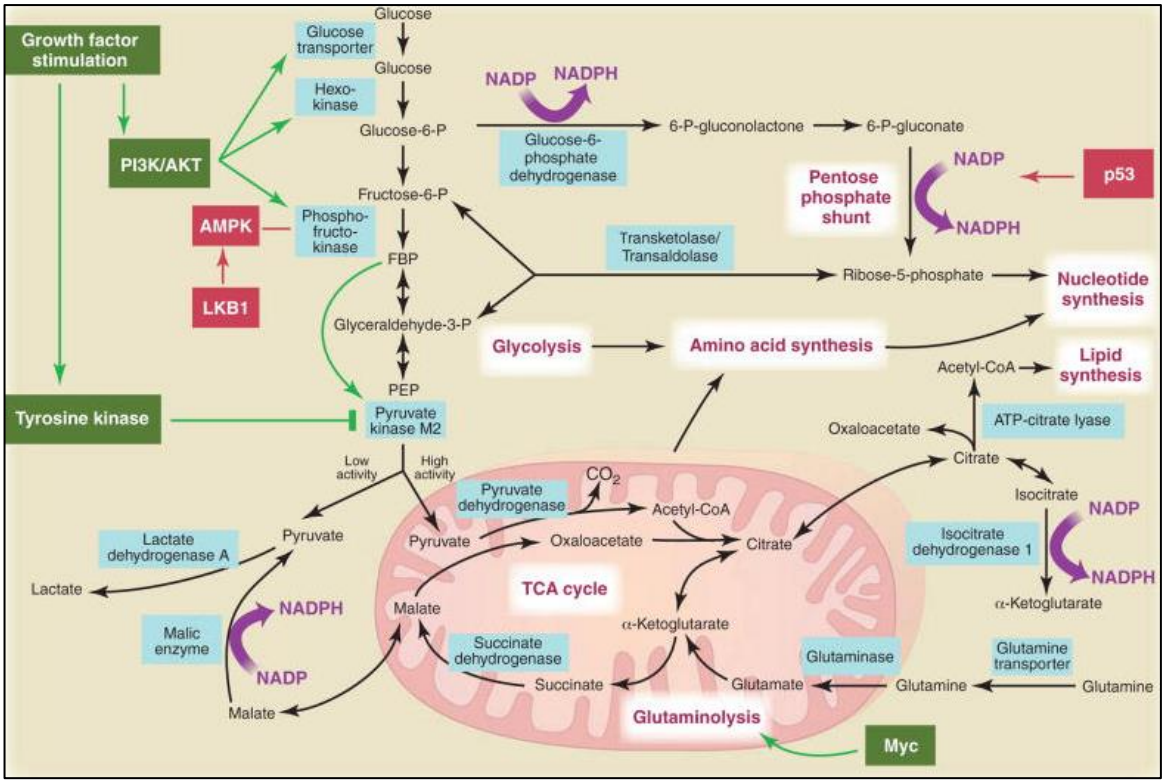


Figure 22 – The Warburg effect

The aerobic glycolysis mechanism at the base of tumor proliferation. [271]

This switch from oxidative phosphorylation to glycolysis, with its concomitant accumulation of lactate by-products in the tumor microenvironment, represents the best-known alteration of tumor cell metabolism [272]. It has been clearly proven that theconsequent increased acidification of the extracellular microenvironment drives tumor cell adaptive programs and may stimulates the evolution of the tumor niche [273-276]. Despite Glycolysis is a less efficient pathway to produce ATP, it is more rapid than mitochondrial oxidative phosphorylation. Another advantage of aerobic glycolysis for cancer cells is that generates less ROS than OxPhos metabolism, allowing cell adaptation to the intermittently hypoxic conditions prevalent in a poorly vascularized tumor. PKM2, which is up-regulated in uPAR KO cells, is commonly expressed in cancer cells and can be allosterically activated by fructose-1,6-bisphosphate [277-279] and drives the conversion of phosphoenolpyruvate to pyruvate, with concomitant phosphorylation of ADP to ATP. Cells can raise their intracellular NADPH by increasing flux through an alternative route of glucose metabolism called the pentose phosphate pathway (PPP), which is primarily anabolic, produces ribose sugars for nucleotide biosynthesis. In case of glycolytic precursors or intermediates accumulation, glucose-6-phosphate is oxidized by G6PD, catalyzing the generation of NADPH. The main substrate for the non-oxidative arm of the PPP is ribulose-5-phosphate, which can be generated either by the oxidative arm or by conversion from fructose-6-phosphate. Ribulose-5-

phosphate is then processed to ribose sugars that feed into nucleotide biosynthesis. In addition, intermediates generate precursors for amino acids, lipids, and hexosamine sugars via a collection of secondary biosynthetic pathways that support anabolic growth. G6PD work at low basal rate in non-transformed cells and it becomes strongly upregulated during tumorigenesis [280]. As we demonstrated, G6PD is much more expressed in uPAR KO cells meaning that it could be involved in the defense against oxidative stress and it can confer to the cells the substrates they need to proliferate. Regarding the glutamine pathway, uPAR KO cells have an upregulated expression of glutaminases (GLS) indicating that these cells, accordingly to the Warburg effect, exploit this mechanism to survive, proliferate and defend themselves from the oxidative stress. Indeed, once uptaken, glutamine is rapidly metabolized by GLS in glutamate that can be used directly for the synthesis of reduced glutathione by the enzyme glutathione cysteine ligase, sustaining the antioxidant response [281]. Alternatively, glutamine can be deaminated to produce α-ketoglutarate to fill levels of tricarboxylic acid cycle intermediates in a process named anapleurosis. Utilization of glutamine by transformed cells is commonly upregulated, consistent with the increased demand for energy, biosynthetic precursors, and redox substrates. The overall effect is the up-regulation of reduced glutathione synthesis to scavenge ROS, as well as the stimulation of the PPP to produce NADPH that can be used in turns to regenerate reduced glutathione. The Warburg effect is tremendously advantageous for the growth and the survival of many cancer cells. However, as we have demonstrated, when melanoma cells loss uPAR expression, while they acquire a glycolytic phenotype and stem features responsible for the acquisition of chemo-resistance and a higher apoptotic threshold, at the same time they become incapable to proliferate. This could be a two-edge sword because these cells, even if growing slowly, enrich the pool of CSCs causing in the long term the recurrence of the disease. As we know, in many solid tumors, there is an invasive front where the cells overexpress uPAR in order to transit from an epithelial phenotype to a mesenchymal one, to enhance their invasive and migratory ability and then intravasate into the bloodstream and metastasize in distant organs [282]. On the other hand, in the core of tumor mass there are cells expressing low levels of uPAR or even not expressing it at all. We speculate that these cells are able to regulate uPAR transcription in order to stop proliferating and became the bulk of cell resistant to chemotherapy. One of the mechanism used by these cells to abrogate uPAR expression could be through the DensityEnhanced Phosphatase-1 (DEP-1), which is a receptor-like tyrosine phosphatase. It regulates uPAR expression in confluent cells by inhibiting the VEGF-dependent activation of ERK1/2, leading to down-regulation of uPAR expression [283]. By contrast, overexpression of active ERK1 nullify the DEP-1 effect on uPAR. Therefore, these cells can regain uPAR expression in

case of need, and become again actively proliferating. We think that these cells, acquiring resistance to chemotherapy and stem traits, using this mechanism to hide uPAR expression, are the main responsible for the minimal residual disease. Being also a relatively small number of cells, we are actually incapable to monitor and then eradicate such cell population. Given our experience, we think that, regarding novel therapeutic strategy for melanoma treatment, it would be better, not to use gene therapies against uPAR but abrogate its function maintaining it physically present. For instance, using peptides like M25 [284-286], it would be helpful to uncouple uPAR from its partners in order to stop the uPAR-mediated signal transduction, and with standard chemotherapies reduce tumor growth and relapse.

Bibliography

1. Eden G, Archinti M, Furlan F, Ronan M and Degryse B The urokinase Receptor Interactome. Curr Pharma Des, 2011, 17, 1874-1889
2. Andreasen PA, Egelund R, Petersen HH The plasminogen activation system in tumor growth, invasion, and metastasis. Cell Mol Life Sci. 2000; 57: 25-40
3. Danø K, Behrendt N, Høyer-Hansen G, Johnsen M, Lund LR, Ploug M et al. Plasminogen activation and cancer. Thromb Haemost. 2005; 93: 676-681
4. Dass K, Ahmad A, Azmi AS, Sarkar SH, Sarkar FH Evolving role of uPA/uPAR system in human cancers. Cancer Treat Rev. 2008; 34: 122-136
5. Asuthkar S, Gondi CS, Nalla AK, Velpula KK, Gorantla B, Rao JS Urokinase-type plasminogen activator receptor (uPAR)-mediated regulation of WNT/β-catenin signalling is enhanced in irradiated medulloblastoma cells. J Biol Chem. 2012; 287: 20576-20589.
6. Ossowski, L., and J.A. Aguirre-Ghiso. 2000. Urokinase receptor and integrin partnership: coordination of signalling for cell adhesion, migration and growth. Curr. Opin. Cell Biol. 12:613–620
7. Blasi, F., and P. Carmeliet. 2002. uPAR: a versatile signalling orchestrator. Nat. Rev. Mol. Cell Biol.3:932–943
8. Andreasen, P.A., L. Kjøller, L. Christenson, and M.J. Duffy. 1997. The urokinase- type plasminogen activator system in cancer metastasis: a review. Int. J. Cancer. 72:1–22
9. Wei, Y., M. Lukashev, D.I. Simon, S.C. Bodary, S. Rosenberg, M.V. Doyle, and H.A. Chapman. 1996. Regulation of integrin function by the urokinase receptor. Science. 273:1551–1555.
10. Wei, Y., J.A. Eble, Z. Wang, J.A. Kreidberg, and H.A. Chapman. 2001. Urokinase receptors promote beta1 integrin function through interactions with integrin alpha3beta1. Mol. Biol. Cell. 12:2975–2986.
11. Margheri F, Papucci L, Schiavone N, D'Agostino R, Trigari S, Serratì S, Laurenzana A, Biagioni et al. Differential uPAR recruitment in caveolar-lipid rafts by GM1 and GM3 gangliosides regulates endothelial progenitor cells angiogenesis. J Cell Mol Med. 2015 Jan;19(1):113-23. Epub 2014 Oct 14.
12. Llinas P, Le Du MH, Gardsvoll H, et al. Crystal structure of the human urokinase plasminogen activator receptor bound to an antagonist peptide. EMBO J 2005; 24: 1655-63

13. Huang M, Mazar AP, Parry G, Hizagi AA, Kuo A, Cines DB. Crystallization of soluble urokinase receptor (suPAR) in complex with urokinase amino-terminal fragment (1-143). Acta Crystallogr D Biol Crystallogr 2005; 61: 697-700
14. Barinka C, Parry G, Callahan J, et al. Structural basis of interaction between urokinase-type plasminogen activator and its receptor. J Mol Biol 2006; 363: 482-95
15. Huai Q, Mazar AP, Kuo A, et al. Structure of human urokinase plasminogen activator in complex with its receptor. Science 2006; 311: 656-59
16. Look MP, van Putten WLJ, Duffy MJ, Harbeck N, Christensen IJ, Thomssen C, et al. Pooled analysis of prognostic impact of tumor biological factors uPA and PAI-1 in 8377 breast cancer patients. J Natl Cancer Inst. 2002, 94: 116-128.
17. Schmitt M, Mengele K, Napieralski R, Magdolen V, Reuning U, Gkazepis A, Sweep F, Brünner N, Foekens J, Harbeck N: Clinical utility of level-of-evidence-1 disease forecast cancer biomarkers uPA and its inhibitor PAI-1. Expert Rev Mol Diagn. 2010, 10: 1051-1067.
18. de Bock CE, Wang Y. Clinical significance of urokinase-type plasminogen activator receptor (uPAR) expression in cancer. Med Res Rev. 2004;24:13-39.
19. O'Halloran TV, Ahn R, Hankins P, Swindell E, Mazar AP. The many spaces of uPAR: delivery of theranostic agents and nanobins to multiple tumor compartments through a single target. Theranistics 2013;3:496-506.
20. Del Rosso M, Fibbi G, Pucci M, D'Alessio S, Del Rosso A, Magnelli L, Chiarugi V. Multiple pathways of cell invasion are regulated by multiple families of serine proteases. Clin Exp Metastasis. 2002;19:193-207.
21. Smith HW, Marshall CJ. Regulation of cell signalling by uPAR. Nat Rev Mol Cell Biol. 2010;11:23-36.
22. Behrendt N, List K, Andreasen PA, Dano K. The pro-urokinase plasminogen-activation system in the presence of serpin-type inhibitors and the urokinase receptor: rescue of activity through reciprocal pro-enzyme activation. Biochem J. 2003;371:277–287.
23. Vincenza Carriero M, Franco P, Vocca I, Alfano D, Longanesi-Cattani I, Bifulco K, Mancini A, Caputi M, Stoppelli MP. Structure, function and antagonists of urokinase-type plasminogen activator. Front Biosci. 2009;14:3782–3794
24. Binder BR, Mihaly J, Prager GW. uPAR-uPA-PAI-1 interactions and signalling: a vascular biologist's view. Thromb Haemost. 2007;97:336–342.
25. Ellis V, Behrendt N, Dano K. Plasminogen activation by receptorbound urokinase. A kinetic study with both cell-associated and isolated receptor. 1991;J Biol Chem 266:12752–12758.

26. Hajjar KA, Jacovina AT, Chacko J. An endothelial cell receptor for plasminogen/tissue plasminogen activator. I. Identity with annexin II. J Biol Chem. 1994;269:21191–21197. [PubMed]
27. Andronicos NM, Chen EI, Baik N, Bai H, Parmer CM, Kiosses WB, Kamps MP, Yates JR, III, Parmer RJ, Miles LA. Proteomics-based discovery of a novel, structurally unique, and developmentally regulated plasminogen receptor, Plg-RKT, a major regulator of cell surface plasminogen activation. Blood. 2010;115:1319–1330.
28. Quigley JP, Gold LI, Schwimmer R, Sullivan LM. Limited cleavage of cellular fibronectin by plasminogen activator purified from transformed cells. Proc Natl Acad Sci USA. 1987;84:2776–2780.
29. Goldfinger LE, Stack MS, Jones JC. Processing of laminin-5 and its functional consequences: role of plasmin and tissue-type plasminogen activator. J Cell Biol. 1998;141:255–265.
30. Pankov R, Yamada KM. Fibronectin at a glance. J Cell Sci. 2002;115:3861–3863.
31. Murphy G, Stanton H, Cowell S, Butler G, Knauper V, Atkinson S, Gavrilovic J. Mechanisms for pro matrix metalloproteinase activation. APMIS. 1999;107:38–44.
32. Ramos-DeSimone N, Hahn-Dantona E, Sipley J, Nagase H, French DL, Quigley JP. Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. J Biol Chem. 1999;274:13066–13076.
33. Waver AM, Hussaini IM, Mazar A, Henkin J, Gonias SL. Embryonic fibroblast that are genetically deficient in low density lipoprotein receptor-related protein demonstrate increased activity of the urokinase receptor system and accelerated migration on vitronectin. J Biol Chem 1997; 272: 14372-9
34. Nykjaer A, Petersen CM, Moller B, et al. Purified α2-macroglobulin receptor/LDL receptor-related protein bins urokinase plasminogen activator inhibition type-1 complex. Evidence that the α2-macroglobulin receptor mediates cellular degradation of urokinase receptor-bound complexes. J Biol Chem 1992; 267: 14543-6
35. Czekay RP, Aertgeerts K, Curriden SA, Loskutoff DJ. Plasminogen activator inhibitor-1 detaches cells from extracellular matrices by inactivating integrins. J Cell Biol 2003; 160: 781-91
36. Schvartz I, Seger D, Shaltiel S. Vitronectin. Int J Biochem Cell Biol 1999; 31: 539-44.
37. Wei Y, Waltz DA, Rao N, Drummond RJ, Rosenberg S, Chapman HA. Identification of the urokinase receptor as an adhesion receptor for vitronectin. J Biol Chem 1994; 269: 32380-8.

38. Ciambrone GJ, McKeown-Longo PJ. Vitronectin regulates the synthesis and localization of urokinase-type plasminogen activator in HT-1080 cells. J Biol Chem 1992; 267: 13617-22.
39. Gårdsvoll H, Ploug M. Mapping of the vitronectin-binding site on the urokinase receptor: involvement of a coherent receptor interface consisting of residues from both domain I and the flanking interdomain linker region. J Biol Chem 2007; 282: 13561-72.
40. Degryse B, Orlando S, Resnati M, Rabbani, SA, Blasi, F. Urokinase/urokinase receptor and vitronectin/αvβ3 integrin induce chemotaxis and cytoskeleton reorganization through different signalling pathways. Oncogene 2001; 20: 2032-43.
41. Sidenius N, Blasi F. Domain 1 of the urokinase receptor (uPAR) is required for uPAR-mediated cell binding to vitronectin. FEBS Lett 2000; 470: 40-6.
42. Muir E, Du JS, Fok-Seang J, et al. Increased axon growth through astrocyte cell lines transfected with urokinase. Glia 1998; 23: 24-34.
43. Deng G, Royle G, Wang S, Crain K, Loskutoff DJ. Structural and functional analysis of the plasminogen activator inhibitor-1 binding motif in the somatomedin B domain of vitronectin. J Biol Chem 1996; 271: 12716-23.
44. Stefansson S, Lawrence DA. The serpin PAI-1 inhibits cell migration by blocking integrin alpha V beta 3 binding to vitronectin. Nature 1996; 383: 441-3.
45. Minor KH, Peterson CB. Plasminogen activator inhibitor type 1 promotes the self-association of vitronectin into complexes exhibiting altered incorporation into the extracellular matrix. J Biol Chem 2002; 277: 10337-45.
46. Kamikubo Y, Neels JG, Degryse B. Vitronectin inhibits plasminogen activator inhibitor-1-induced signalling and chemotaxis by blocking plasminogen activator inhibitor-1 binding to the lowdensity lipoprotein receptor-related protein. Int J Biochem Cell Biol 2009; 41: 578-85.
47. Garg N, Goyal N, Strawn TL, et al. Plasminogen activator inhibitor-1 and vitronectin expression level and stoichiometry regulate vascular smooth muscle cell migration through physiological collagen matrices. J Thromb Haemost 2010; 8: 1847-54.
48. Zaidel-Bar R, Itzkovitz S, Ma'ayan A, Iyengar R, Geiger B. Functional atlas of the integrin adhesome. Nat Cell Biol 2007; 9: 858-67.
49. Tarui T, Mazar AP, Cines DB, Takada Y. Urokinase-type plasminogen activator receptor (CD87) is a ligand for integrins and mediates cell-cell interaction. J Biol Chem 2001; 276: 3983-90.
50. Wei Y, Czekay RP, Robillard L, et al. Regulation of α5β1 integrin conformation and function by urokinase receptor binding. J Cell Biol 2005; 168: 501-11.

51. Simon DI, Rao NK, Xu H, et al. Mac-1 (CD11b/CD18) and the urokinase receptor (CD87) form a functional unit on monocytic cells. Blood 1996; 88: 3185-94.
52. Yebra M, Parry GC, Strömblad S, et al. Requirement of receptorbound urokinase-type plasminogen activator for integrin αvβ5-directed cell migration. J Biol Chem. 1996 Nov 15;271(46):29393-9.
53. Chavakis T, May AE, Preissner KT, Kanse SM. Molecular mechanisms of zinc-dependent leukocyte adhesion involving the urokinase receptor and β2-integrins. Blood 1999; 93: 2976-83.
54. Silvestri I, Longanesi Cattani I, Franco P, et al. Engaged urokinase receptors enhance tumor breast cell migration and invasion by upregulating αvβ5 vitronectin receptor cell surface expression. Int J Cancer 2002; 102: 562-71.
55. Margheri F, Manetti M, Serratì S, et al. Domain 1 of the urokinase type plasminogen activator receptor is required for its morphologic and functional, β2 integrin-mediated connection with actin cytoskeleton in human microvascular endothelial cells: failure of association in systemic sclerosis endothelial cells. Arthritis Rheum 2006; 54: 3926-38.
56. Pluskota E, Soloviev DA, Plow EF. Convergence of the adhesive and fibrinolytic systems: recognition of urokinase by integrin αMβ2 as well as by the urokinase receptor regulates cell adhesion and migration. Blood 2003; 101: 1582-90.
57. Pluskota E, Soloviev DA, Bdeir K, Cines DB, Plow EF. Integrin αMβ2 orchestrates and accelerates plasminogen activation and fibrinolysis by neutrophils. J Biol Chem 2004; 279: 18063-72.
58. Demetriou MC, Pennington ME, Nagle RB, Cress AE. Extracellular α6 integrin cleavage by urokinase-type plasminogen activator in human prostate cancer. Exp Cell Res 2004; 294: 550-8.
59. Kwak SH, Mitra S, Bdeir K, et al. The kringle domain of urokinase-type plasminogen activator potentiates LPS-induced neutrophil activation through interaction with αvβ3 integrins. J Leukoc Biol 2005; 78: 937-45.
60. Franco P, Vocca I, Carriero MV, et al. Activation of urokinase receptor by a novel interaction between the connecting peptide region of urokinase and αvβ5 integrin. J Cell Sci 2006; 119: 3424-34.
61. Tarui T, Akakura N, Majumdar M, et al. Direct interaction of the kringle domain of urokinase-type plasminogen activator (uPA) and integrin αvβ3 induces signal transduction and enhances plasminogenactivation. Thromb Haemost 2006; 95: 524-34.

62. Degryse B, Fernandez-Recio J, Citro V, Blasi F, Cubellis MV. In silico docking of urokinase plasminogen activator and integrins. BMC Bioinformatics 2008
63. Degryse B, Resnati M, Rabbani SA, Villa A, Fazioli F, Blasi F. Src-dependence and pertussis-toxin sensitivity of urokinase receptor-dependent chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. Blood 1999; 94: 649-62.
64. Chapman HA, Wei Y, Simon DI, Waltz DA. Role of urokinase receptor and caveolin in regulation of integrin signalling. Thromb Haemost 1999; 82: 291-7.
65. Wei Y, Yang X, Liu Q, Wilkins JA, Chapman HA. A role for caveolin and the urokinase receptor in integrin-mediated adhesion and signalling. J Cell Biol 1999; 144: 1285-94.
66. Schwab W, Gavlik JM, Beichler T, et al. Expression of the urokinase-type plasminogen activator receptor in human articular chondrocytes: association with caveolin and β1-integrin. Histochem Cell Biol 2001; 115: 317-23.
67. Chintala SK, Mohanam S, Go Y, et al. Altered in vitro spreading and cytoskeletal organization in human glioma cells by downregulation of urokinase receptor. Mol Carcinog 1997; 20: 355-65.
68. Abu-Ali S, Sugiura T, Takahashi M, et al. Expression of the urokinase receptor regulates focal adhesion assembly and cell migration in adenoid cystic carcinoma cells. J Cell Physiol 2005; 203: 410-9.
69. Xue W, Mizukami I, Todd RF 3rd, Petty HR. Urokinase-type plasminogen activator receptors associate with β1 and β3 integrins of fibrosarcoma cells: dependence on extracellular matrix components. Cancer Res 1997; 57: 1682-9.
70. May AE, Neumann FJ, Schömig A, Preissner KT. VLA-4 α4β1 engagement defines a novel activation pathway for β2 integrin-dependent leukocyte adhesion involving the urokinase receptor. Blood 2000; 96: 506-13.
71. Barczyk M, Carracedo S, Gullberg D. Integrins. Cell Tissue Res. 2010 Jan;339(1): 269-80
72. Liu D, Aguirre Ghiso J, Estrada Y, Ossowski L. EGFR is a transducer of the urokinase receptor initiated signal that is required for in vivo growth of a human carcinoma. Cancer Cell 2002; 1: 445-57.
73. Kiyan J, Kiyan R, Haller H, Dumler I. Urokinase-induced signalling in human vascular smooth muscle cells is mediated by PDGFR- β. EMBO J 2005; 24: 1787-97.
74. Gallicchio MA, Kaun C, Wojta J, Binder B, Bach LA. Urokinase type plasminogen activator receptor is involved in insulin-like growth factor-induced migration of rhabdomyosarcoma cells in vitro. J Cell Physiol 2003; 197: 131-8.

75. Bauer TW, Fan F, Liu W, et al. Insulin-like growth factor-I-mediated migration and invasion of human colon carcinoma cells requires activation of c-Met and urokinase plasminogen activator receptor. Ann Surg 2005; 241: 748-56.
76. Bauer TW, Liu W, Fan F, et al. Targeting of urokinase plasminogen activator receptor in human pancreatic carcinoma cells inhibits c-Met- and insulin-like growth factor-I receptor-mediated migration and invasion and orthotopic tumor growth in mice. Cancer Res 2005; 65: 7775-81.
77. Guerrero J, Santibañez JF, González A, Martínez J. EGF receptor transactivation by urokinase receptor stimulus through a mechanism involving Src and matrix metalloproteinases. Exp Cell Res 2004; 292: 201-8.
78. LaRusch GA, Mahdi F, Shariat-Madar Z, et al. Factor XII stimulates ERK1/2 and Akt through uPAR, integrins, and the EGFR to initiate angiogenesis. Blood 2010; 115: 5111-20.
79. Jo M, Thomas KS, Marozkina N, et al. Dynamic assembly of the urokinase-type plasminogen activator signalling receptor complex determines the mitogenic activity of urokinase-type plasminogen activator. J Biol Chem 2005; 280: 17449-57.
80. Jo M, Thomas KS, O'Donnell DM, Gonias SL. Epidermal growth factor receptor-dependent and -independent cell-signalling pathways originating from the urokinase receptor. J Biol Chem 2003; 278: 1642-6.
81. Wang XQ, Sun P, Paller AS. Gangliosides inhibit urokinase-type plasminogen activator (uPA)-dependent squamous carcinoma cell migration by preventing uPA receptor/α5β1 integrin/epidermal growth factor receptor interactions. J Invest Dermatol 2005; 124: 839-48.
82. Nicholl SM, Roztocil E, Davies MG. Urokinase-induced smooth muscle cell responses require distinct signalling pathways: a role for the epidermal growth factor receptor. J Vasc Surg 2005; 41: 672-81.
83. Monaghan-Benson E, McKeown-Longo PJ. Urokinase-type plasminogen activator receptor regulates a novel pathway of fibronectin matrix assembly requiring Src-dependent transactivation of epidermal growth factor receptor. J Biol Chem 2006; 281: 9450-9.
84. Bakken AM, Protack CD, Roztocil E, Nicholl SM, Davies MG. Cell migration in response to the amino-terminal fragment of urokinase requires epidermal growth factor receptor activation through an ADAM-mediated mechanism. J Vasc Surg 2009; 49: 1296-303.
85. D'Alessio S, Gerasi L, Blasi F. uPAR-deficient mouse keratinocytes fail to produce EGFR-dependent laminin-5, affecting migration in vivo and in vitro. J Cell Sci 2008; 121: 3922-32.

86. Grimaldi G, Di Fiore P, Locatelli EK, Falco J, Blasi F. Modulation of urokinase plasminogen activator gene expression during the transition from quiescent to proliferative state in normal mouse cells. EMBO J 1986; 5: 855-61.
87. Boyd D. Examination of the effects of epidermal growth factor on the production of urokinase and the expression of the plasminogen activator receptor in a human colon cancer cell line. Cancer Res 1989; 49: 2427-32.
88. Amos S, Redpath GT, Dipierro CG, Carpenter JE, Hussaini IM. Epidermal growth factor receptor-mediated regulation of urokinase plasminogen activator expression and glioblastoma invasion via CSRC/MAPK/AP-1 signalling pathways. J Neuropathol Exp Neurol 2010; 69: 582-92.
89. Baek MK, Kim MH, Jang HJ, et al. EGF stimulates uPAR expression and cell invasiveness through ERK, AP-1, and NF-kappaB signalling in human gastric carcinoma cells. Oncol Rep 2008; 20: 1569-75.
90. Smith PC, Santibañez JF, Morales JP, Martinez J. Epidermal growth factor stimulates urokinase-type plasminogen activator expression in human gingival fibroblasts. Possible modulation by genistein and curcumin. J Periodontal Res 2004; 39: 380-7.
91. Sandberg T, Ehinger A, Casslén B. Paracrine stimulation of capillary endothelial cell migration by endometrial tissue involves epidermal growth factor and is mediated via up-regulation of the urokinase plasminogen activator receptor. J Clin Endocrinol Metab 2001; 86: 1724-30.
92. Adina Vultur, Meenhard Herlyn. SnapShot: Melanoma Cancer Cell Volume 23, Issue 5, 706, 13 May 2013
93. Haass NK, Smalley KS, Herlyn M. The role of altered cell-cell communication in melanoma progression. J Mol Histol. 2004 Mar; 35(3):309-18
94. Clark WH Jr, Elder DE, Guerry D 4th, Epstein MN, Greene MH, Van Horn M. A study of tumor progression: the precursor lesions of superficial spreading and nodular melanoma. Hum Pathol. 1984 Dec; 15(12):1147-65
95. Gaggioli C, Sahai E. Melanoma invasion - current knowledge and future directions. Pigment Cell Res. 2007 Jun;20(3):161-72
96. Chin L. The genetics of malignant melanoma: lessons from mouse and man. Nat Rev Cancer. 2003 Aug; 3(8):559-70
97. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R,

Jayatilake H, Gusterson BA, Cooper C, Shipley J,Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA. Mutations of the BRAF gene in human cancer. Nature. 2002 Jun 27; 417(6892):949-54

98. Gray-Schopfer VC, da Rocha Dias S, Marais R. The role of B-RAF in melanoma. Cancer Metastasis Rev. 2005 Jan; 24(1):165-83. Review. Erratum in: Cancer Metastasis Rev. 2005 Jun; 24(2):367

99. Chin L, Garraway LA, Fisher DE. Malignant melanoma: genetics and therapeutics in the genomic era. Genes Dev. 2006 Aug 15; 20(16):2149-82

100. Garnett MJ, Marais R. Guilty as charged: B-RAF is a human oncogene. Cancer Cell. 2004 Oct; 6(4):313-9

101. Wan PT, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, Jones CM, Marshall CJ, Springer CJ, Barford D, Marais R; Cancer Genome Project. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell. 2004 Mar 19; 116(6):855-67

102. Woods D, Cherwinski H, Venetsanakos E, Bhat A, Gysin S, Humbert M, Bray PF, Saylor VL, McMahon M. Induction of beta3-integrin gene expression by sustained activation of the Ras-regulated Raf-MEK-extracellular signal-regulated kinase signaling pathway. Mol Cell Biol. 2001 May; 21(9):3192-205

103. Huntington JT, Shields JM, Der CJ, Wyatt CA, Benbow U, Slingluff CL Jr, Brinckerhoff CE. Overexpression of collagenase 1 (MMP-1) is mediated by the ERK pathway in invasive melanoma cells: role of BRAF mutation and fibroblast growth factor signaling. J Biol Chem. 2004 Aug 6; 279(32):33168-76

104. Sumimoto H, Miyagishi M, Miyoshi H, Yamagata S, Shimizu A, Taira K, Kawakami Y. Inhibition of growth and invasive ability of melanoma by inactivation of mutated BRAF with lentivirus-mediated RNA interference. Oncogene. 2004 Aug12; 23(36):6031-9

105. Liang S, Sharma A, Peng HH, Robertson G, Dong C. Targeting mutant (V600E) B-Raf in melanoma interrupts immunoediting of leukocyte functions and melanoma extravasation. Cancer Res. 2007 Jun 15; 67(12):5814-20

106. Orgaz JL, Sanz-Moreno V. Emerging molecular targets in melanoma invasion and metastasis. Pigment Cell Melanoma Res. 2013 Jan; 26(1):39-57

107. Giorgio Parmiani. Melanoma Cancer Stem Cells: Markers and Functions. Cancers (Basel). 2016 Mar; 8(3): 34.

108. Fang D., Nguyen T.K., Lishear K., Finko R., Kulp A.N., Hotz S., Van Belle P.A., Xu X., Elder D.E., Herlyn M. A tumorigenic subpopulation with stem cell property in melanomas. Cancer Res. 2005;65:9228–9237.

109. Singh S., Clarke J.D., Terasaki M., Bonn V.E., Hawkins C., Squire J., Dirks P.B. Identification of a cance stem cells in human brain tumors. Cancer Res. 2003;63

110. Tirino V., Desiderio V., Paino F., De Rosa A. Human primary bone sarcoma contain CD133. Cancer Stem Cells. FASEB J. 2011;25:2022–2030.

111. Desiderio V., Papagerakis P., Tinino V., Zheng L., Matossian M., Prince M.E., Paino F., Mele L., Papaccio F., Montella R. Increased fucosylation has a pivotal role in invasive and metastatic properties of head and neck cancer stem cells. Oncotarget. 2014;6:71–84.

112. Eramo A., Lotti F., Sette G., Pilozzi E., Biffoni M., Di Virgilio A., Conticello C., Ruco L., Peschle C., de Maria P. Identification and expression of the tumorigenic lung cancer. Cell Death Differ. 2008;15:504–514.

113. Al-Hajj M., Wicha M.S., Benito-Hernandez A., Morrison S.J., Clarke M.F. Prospective identification of tumorigenic breast cancer cells. Proc. Natl. Acad. Sci. USA. 2003;100:3983–3988.

114. Quintana E., Schakleton M., Foster H.R., Fullen D.R., Sabel M.S., Johnson T.M., Morrison S.J. Phenotypic heterogeneity among tumorienic melanoma cells from patients that is reversibile and not hierarchically organized. Cancer Cell. 2010;18:510–523.

115. Perego M., Tortoreto M., Tragni G., Mariani L., Deho P., Carbone A., Santinami M., Patuzzo R., Della Mina P., Villa A., et al. Heterogeneous phenotype of human melanoma cells with in vitro and in vivo features of tumor-initiating cells. J. Invest. Dermatol. 2010

116. Held M.A., Curley D.P., Dankort D., McMahon M., Muthusamy V., Boserberg M.W. Characterization of melanoma cells capable of propagating tumors from a single cells. Cancer Res. 2010;70:388–397.

117. Singh, S. K. et al. Identification of human brain tumour-initiating cells. Nature 432, 396–401 (2004).

118. O'Brien, C. A., Pollett, A., Gallinger, S. & Dick, J. E. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 445, 106–110 (2007).

119. Ricci-Vitiani, L. et al. Identification and expansion of human colon-cancer-initiating cells. Nature 445, 111–115 (2007).

120. Li, C. et al. Identification of pancreatic cancer stem cells. Cancer Res. 67, 1030–1037 (2007).

121. Schatton, T. et al. Identification of cells initiating human melanomas. Nature 451, 345–349 (2008).

122. Yang, Z. F. et al. Significance of CD90+ cancer stem cells in human liver cancer. Cancer Cell 13, 153–166 (2008).

123. Bin-Bing S. Zhou, Haiying Zhang, Marc Damelin, Kenneth G. Geles, Justin C. Grindley& Peter B. DirksTumour-initiating cells: challenges and opportunities for anticancer drug discovery. Nature Reviews Drug Discovery 8, 806-823 (October 2009)

124. Gray-Schopfer V, Wellbrock C, Marais R. Melanoma biology and new targeted therapy. Nature. 2007 Feb 22;445(7130):851-7.

125. Wiedenheft, B., Sternberg, S.H. & Doudna, J.A. RNA-guided genetic silencing systems in bacteria and archaea. Nature 482, 331–338 (2012).

126. Fineran, P.C. & Charpentier, E. Memory of viral infections by CRISPR-Cas adaptive immune systems: acquisition of new information. Virology 434, 202–209 (2012).

127. Horvath, P. & Barrangou, R. CRISPR/Cas, the immune system of bacteria and archaea. Science 327, 167–170 (2010).

128. Barrangou, R. et al. CRISPR provides acquired resistance against viruses in prokaryotes. Science 315, 1709–1712 (2007).

129. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. Journal of Bacteriology. 169(12): 5429–33.

130. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell. 157 (6): 1262–78.doi:10.1016/j.cell.2014.05.010.

131. van Soolingen D, de Hass PE, Hermans PW, Groenen PM, van Embden JD. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of Mycobacterium tuberculosis. J Clin Microbiol. 31 (8): 1987–95.

132. Mojica FJ, Ferrer C, Juez G, Rodríguez-Valera F. Long stretches of short tandem repeats are present in the largest replicons of the Archaea Haloferax mediterranei and Haloferax volcanii and could be involved in replicon partitioning. Molecular Microbiology. 17: 85–93. doi:10.1111/j.1365-2958.1995.mmi_17010085.x.

133. Pourcel C, Salvignol G, Vergnaud G CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. Microbiology. 151 (Pt 3): 653–63.doi:10.1099/mic.0.27437-0.

134. Mojica FJ, Díez-Villaseñor C, García-Martínez J, Soria E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. Journal of Molecular Evolution. 60 (2): 174–82. doi:10.1007/s00239-004-0046-3.

135. Bolotin A, Quinquis B, Sorokin A, Ehrlich SD. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin". Microbiology. 151 (Pt 8): 2551–61. doi:10.1099/mic.0.28048-0.

136. Makarova KS, Grishin NV, Shabalina SA, Wolf YI, Koonin EV. A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action". Biology Direct. 1: 7. doi:10.1186/1745-6150-1-7.

137. Brouns SJ1, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, Dickman MJ, Makarova KS, Koonin EV, van der Oost J. Small CRISPR RNAs guide antiviral defense in prokaryotes. Science. 2008 Aug 15;321(5891):960-4. doi: 10.1126/science.1159689.

138. Marraffini LA. CRISPR-Cas immunity in prokaryotes. Nature. 526 (7571): 55–61. doi:10.1038/nature15386.

139. Garneau JE, Dupuis M, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P, Magadán AH, Moineau S. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. Nature. 468 (7320): 67–71.doi:10.1038/nature09523.

140. Deltcheva, E. et al. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 471, 602–607 (2011).

141. Jinek, M. et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337, 816–821 (2012).

142. Jeffry D Sander & J Keith Joung. CRISPR-Cas systems for editing, regulating and targeting genomes. Nature Biotechnology 32, 347–355 (2014) doi:10.1038/nbt.2842

143. Jiang, W., Bikard, D., Cox, D., Zhang, F. & Marraffini, L.A. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat. Biotechnol.* 31, 233–239 (2013).

144. Pattanayak, V. *et al*. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat. Biotechnol.* 31, 839–843 (2013).

145. Mali, P. *et al*. RNA-guided human genome engineering via Cas9. *Science* 339, 823–826(2013).

146. Cong, L. *et al*. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823 (2013).

147. Jinek, M. *et al*. RNA-programmed genome editing in human cells. *Elife* 2, e00471 (2013).

148. Cho, S.W., Kim, S., Kim, J.M. & Kim, J.S. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* 31, 230–232 (2013).

149. Hwang, W.Y. et al. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat. Biotechnol. 31, 227–229 (2013).

150. F Ann Ran, Patrick D Hsu, Jason Wright, Vineeta Agarwala, David A Scott & Feng Zhang. Genome engineering using the CRISPR-Cas9 system. Nature Protocols 8, 2281–2308 (2013)

151. Anders C, Niewoehner O, Duerst A, Jinek M. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. Nature. 2014 Sep 25;513(7519):569-73.

152. Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, Ishitani R, Zhang F, Nureki O. Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell. 2014 Feb 27;156(5):935-49.

153. Haft, D.H., Selengut, J., Mongodin, E.F. & Nelson, K.E. A guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. *PLoS Comput. Biol.* **1**, e60 (2005).

154. Gasiunas, G., Barrangou, R., Horvath, P. & Siksnys, V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proc. Natl. Acad. Sci. USA 109, E2579–E2586 (2012).

155. Sapranauskas, R. et al. The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli. Nucleic Acids Res. 39, 9275–9282 (2011).

156. Fu, Y., Sander, J.D., Reyon, D., Cascio, V.M. & Joung, J.K. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nat. Biotechnol.

157. Fu, Y. et al. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat. Biotechnol. 31, 822–826 (2013).

158. Hsu, P.D. et al. DNA targeting specificity of RNA-guided Cas9 nucleases. Nat. Biotechnol.31, 827–832 (2013).

159. Semenova, E. et al. Interference by clustered regularly interspaced short palindromic repeat (CRISPR) RNA is governed by a seed sequence. Proc. Natl. Acad. Sci. USA 108, 10098–10103 (2011).

160. Mali, P., et al. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nat. Biotechnol. 31, 833–838 (2013).

161. Cradick, T.J., Fine, E.J., Antico, C.J. & Bao, G. CRISPR/Cas9 systems targeting beta-globin and CCR5 genes have substantial off-target activity. Nucleic Acids Res. 41, 9584–9592 (2013).

162. Ding, Q. et al. Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs. Cell Stem Cell 12, 393–394

163. Ran, F.A. et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 154, 1380–1389

164. Cho, S.W. et al. Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. Genome Res. 24, 132–141

165. Kuzminov, A. Single-strand interruptions in replicating chromosomes cause double-strand breaks. Proc. Natl. Acad. Sci. USA 98, 8241–8246

166. Cortes-Ledesma, F. & Aguilera, A. Double-strand breaks arising by replication through a nick are repaired by cohesin-dependent sister-chromatid exchange. EMBO Rep. 7, 919–926

167. Wang, T., Wei, J.J., Sabatini, D.M. & Lander, E.S. Genetic screens in human cells using the CRISPR-Cas9 system. Science 343, 80–84

168. Shalem, O. et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. Science343, 84–87

169. Koike-Yusa, H., Li, Y., Tan, E.P., Velasco-Herrera, M.D.C. & Yusa, K. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. Nat. Biotechnol. 32, 267–273

170. Li, D. et al. Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. Nat. Biotechnol. 31, 681–683

171. Yu, Z. et al. Highly efficient genome modifications mediated by CRISPR/Cas9 in Drosophila. Genetics 195, 289–291

172. Bassett, A.R., Tibbit, C., Ponting, C.P. & Liu, J.L. Highly efficient targeted mutagenesis of Drosophila with the CRISPR/Cas9 system. Cell Reports 4, 220–228

173. Friedland, A.E. et al. Heritable genome editing in C. elegans via a CRISPR-Cas9 system. Nat. Methods 10, 741–743

174. Gratz, S.J. et al. Genome engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease. Genetics 194, 1029–1035

175. Lo, T.W. et al. Precise and heritable genome editing in evolutionarily diverse nematodes using TALENs and CRISPR/Cas9 to engineer insertions and deletions. Genetics 195, 331–348

176. Waaijers, S. et al. CRISPR/Cas9-targeted mutagenesis in Caenorhabditis elegans. Genetics195, 1187–1191

177. Tzur, Y.B. et al. Heritable custom genomic modifications in Caenorhabditis elegans via a CRISPR-Cas9 system. Genetics 195, 1181–1185

178. Katic, I. & Grosshans, H. Targeted heritable mutation and gene conversion by Cas9-CRISPR in Caenorhabditis elegans. Genetics 195, 1173–1176

179. Chiu, H., Schwartz, H.T., Antoshechkin, I. & Sternberg, P.W. Transgene-free genome editing in Caenorhabditis elegans using CRISPR-Cas. Genetics 195, 1167–1171

180. Li, J.F. et al. Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nat. Biotechnol. 31, 688–691

181. Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J.D. & Kamoun, S. Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. Nat. Biotechnol. 31, 691–693

182. Shan, Q. et al. Targeted genome modification of crop plants using a CRISPR-Cas system. Nat. Biotechnol. 31, 686–688

183. Xie, K. & Yang, Y. RNA-guided genome editing in plants using a CRISPR-Cas system. Mol. Plant 6, 1975–1983

184. Jiang, W. et al. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. Nucleic Acids Res. 41, e188

185. Urnov, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S. & Gregory, P.D. Genome editing with engineered zinc finger nucleases. Nat. Rev. Genet. 11, 636–646

186. Bikard, D. et al. Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. Nucleic Acids Res. 41, 7429–7437

187. Qi, L.S. et al. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152, 1173–1183

188. Maeder, M.L. et al. CRISPR RNA-guided activation of endogenous human genes. Nat. Methods 10, 977–979

189. Perez-Pinera, P. et al. RNA-guided gene activation by CRISPR-Cas9-based transcription factors. Nat. Methods 10, 973–976

190. Konermann, S. et al. Optical control of mammalian endogenous transcription and epigenetic states. Nature 500, 472–476

191. Cheng, A.W. et al. Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. Cell Res. 23, 1163–1171

192. Gilbert, L.A. et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell 154, 442–451

193. Chen, B. et al. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. Cell 155, 1479–1491

194. Esvelt, K.M. et al. Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. Nat. Methods 10, 1116–1121

195. Hou, Z. et al. Efficient genome engineering in human pluripotent stem cells using Cas9 from Neisseria meningitidis. Proc. Natl. Acad. Sci. USA 110, 15644–15649

196. Benjamin Beck and Cédric Blanpain. Unravelling cancer stem cell potential. Nature Reviews Cancer 13, 727–738 (2013)

197. Shaheenah Dawood, Laura Austin, and Massimo CristofanilliCancer Stem Cells: Implications for Cancer Therapy. Oncology (Williston Park). 2014 Dec;28(12):1101-7, 1110

198. Antonija Kreso and John E. DickEvolution of the Cancer Stem Cell Model. Stem Cell. 2014 Mar 6;14(3):275-91.

199. Mazar AP, Ahn RW, O'Halloran TV. Development of novel therapeutics targeting the urokinase plasminogen activator receptor (uPAR) and their translation toward the clinic. Current pharmaceutical design. 2011; 17: 1970-1978

200. Margheri F, Luciani C, Taddei ML, Giannoni E, Laurenzana A, Biagioni A, Chillà A, Chiarugi P, Fibbi G, Del Rosso M. The receptor for urokinase-plasminogen activator (uPAR) controls plasticity of cancer cell movement in mesenchymal and amoeboid migration style. Oncotarget. 2014;5:1538-53.

201. Laurenzana A, Biagioni A, D'Alessio S, Bianchini F, Chillà A, Margheri F, Luciani C, Mazzanti B, Pimpinelli N, Torre E, Danese S, Calorini L, Del Rosso M, Fibbi G. Melanoma cell therapy: Endothelial progenitor cells as shuttle of the MMP12 uPAR-degrading enzyme. Oncotarget. 2014;5:3711-27.

202. Laurenzana A, Biagioni A, Bianchini F, Peppicelli S, Chillà A, Margheri F, Luciani C, Pimpinelli N, Del Rosso M, Calorini L, Fibbi G. Inhibition of uPAR-TGFβ crosstalk blocks MSC-dependent EMT in melanoma cells. J Mol Med (Berl). 2015 Jul;93(7):783-94.

203. Reya T, Morrison SJ, Clarke MF, Weissman IL.Stem cells, cancer, and cancer stem cells. Nature 2001;414:105–11

204. Hendrix M, Seftor EA, Meltzer PS, et al. The stem cell plasticity of aggressive melanoma tumor cells. In: S Sell. Stem cells handbook. Totowa (NJ): Humana Press, Inc.; 2003. p. 297–306.

205. Reed JA, Finnerty B, Albino AP. Divergent cellular differentiation pathways during the invasive stage of cutaneous malignant melanoma progression. Am J Pathol 1999;155:549–55.

206. Fang D, Hallman J, Sangha N, et al. Expression of microtubule-associated protein 2 in benign and malignant melanocytes: implications for differentiation and progression of cutaneous melanoma. Am J Pathol 2001; 158:2107–15.

207. Maria Vinci, Sharon Gowan, Frances Boxall, Lisa Patterson, Miriam Zimmermann, William Court, Cara Lomas, Marta Mendiola, David Hardisson and Suzanne A EcclesAdvances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. BMC Biology201210:29

208. Sreelatha Gopinath, Rama Rao Malla, Christopher S. Gondi, Kiranmai Alapati, Daniel Fassett, Jeffrey D. Klopfenstein et al. Co-Depletion of Cathepsin B and uPAR Induces G0/G1 Arrest in Glioma via FOXO3a Mediated p27Kip1 Upregulation PLoS One. 2010 Jul 22;5(7):e11668.

209. Jones SM, Kazlauskas A. Growth-factor-dependent mitogenesis requires two distinct phases of signalling. Nat Cell Biol 3: 165-172, 2001

210. Lui VW, Grandis JREGFR-mediated cell cycle regulation. Anticancer Res. 2002 Jan-Feb;22(1A):1-11.

211. Tiffany E. Taylor, Frank B. Furnari and Webster K. CaveneeTargeting EGFR for Treatment of Glioblastoma: Molecular Basis to Overcome ResistanceCurr Cancer Drug Targets. 2012 Mar; 12(3): 197–209.

212. Harding J, Burtness B.Cetuximab: an epidermal growth factor receptor chemeric human-murine monoclonal antibody.Drugs Today (Barc). 2005 Feb;41(2):107-27.

213. James A. Ramirez, Joan Guitart, M. Sambasiva Rao, Leslie K. DiazCyclin D1 expression in melanocytic lesions of the skin. Annals of Diagnostic Pathology Volume 9, Issue 4, August 2005, 185–188

214. Ren S, Rollins BJ. Cyclin C/cdk3 promotes Rb-dependent G0 exit. Cell. 2004;117:239–51.

215. Rickert P, Seghezzi W, Shanahan F, Cho H, Lees E. Cyclin C/CDK8 is a novel CTD kinase associated with RNA polymerase II. Oncogene. 1996;12:2631–40.

216. Solmi R, Lauriola M, Francesconi M, Martini D, Voltattorni M, Ceccarelli C et al. Displayed correlation between gene expression profiles and submicroscopic alterations in response to cetuximab, gefitinib and EGF in human colon cancer cell lines. BMC Cancer. 2008 Aug 8;8:227.

217. Goran Hamid Mohammad, S. W. M. Olde Damink, Massimo Malago, Dipok Kumar Dhar and Stephen P. Pereira Pyruvate Kinase M2 and Lactate Dehydrogenase A Are Overexpressed in Pancreatic Cancer and Correlate with Poor Outcome PLoS One. 2016; 11(3): e0151635. Published online 2016 Mar 18.

218. Bolzoni M, Chiu M, Accardi F, Vescovini R, Airoldi , Storti P et al.Dependence on glutamine uptake and glutamine addiction characterize myeloma cells: a new attractive target. Blood. 2016 Aug 4;128(5):667-79.

219. Isei Tanida, Takashi Ueno, Eiki Kominami LC3 and Autophagy Autophagosome and Phagosome Volume 445 of the series Methods in Molecular Biology™ pp 77-88

220. Katayama K, Noguchi K, and Sugimoto Y Regulations of P-Glycoprotein/ABCB1/MDR1 in Human Cancer Cells. New Journal of Science Volume 2014 (2014), Article ID 476974

221. Landreville S, Agapova OA, Kneass ZT, Salesse C, Harbour JW. ABCB1 identifies a subpopulation of uveal melanoma cells with high metastatic propensity. Pigment Cell Melanoma Res. 2011 Jun;24(3):430-7.

222. Ramarao Malla,Sreelatha Gopinath,Kiranmai Alapati,Christopher S. Gondi, Meena Gujrati,Dzung H. Dinh,Sanjeeva Mohanam,Jasti S. RaoDownregulation of uPAR and Cathepsin B Induces Apoptosis via Regulation of Bcl-2 and Bax and Inhibition of the PI3K/Akt Pathway in Gliomas. Plos One October 29, 2010

223. Steelman LS, Chappell WH, Abrams SL, Kempf RC, Long J, Laidler P, Mijatovic S, Maksimovic-Ivanic D, Stivala F, Mazzarino MC, Donia M, Fagone P, Malaponte G, Nicoletti F, Libra M, Milella M, Tafuri A, Bonati A, Bäsecke J, Cocco L, Evangelisti C, Martelli AM, Montalto G, Cervello M, McCubrey JA.Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. Aging (Albany NY). 2011 Mar;3(3):192-222.

224. Diwakar R Pattabiraman and Robert A. Weinberg Tackling the cancer stem cells – what challenges do they pose? Nat Rev Drug Discov. 2014 Jul; 13(7): 497–512.

225. Ghiaur G, Gerber J, Jones RJ. Concise review: Cancer stem cells and minimal residual disease. Stem Cells. 2012 Jan;30(1)

226. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.Cell. 2006 Aug 25;126(4):663-76. Epub 2006 Aug 10.

227. Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S.The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells.Cell. 2003 May 30;113(5):631-42.

228. Guilai Shi and Ying Jin. Role of Oct4 in maintaining and regaining stem cell pluripotency. Stem Cell Research & Therapy20101:39

229. Kyung Hee Noh, Bo Wook Kim, Kwon-Ho Song, Hanbyoul Cho, Young-Ho Lee, Jin Hee Kim, Joon-Yong Chung, Jae-Hoon Kim, Stephen M. Hewitt, Seung-Yong Seong,Chih-Ping Mao, T.-C. Wu and Tae Woo Kim. Nanog signaling in cancer promotes stem-like phenotype and immune evasion. J Clin Invest. 2012 Nov 1; 122(11): 4077–4093.

230. Weiren Luo, Siyi Li, Bailu Peng, Yanfen Ye, Xubin Deng and Kaitai Yao. Embryonic Stem Cells Markers SOX2, OCT4 and Nanog Expression and Their Correlations with Epithelial-Mesenchymal Transition in Nasopharyngeal Carcinoma. PLoS One. 2013; 8(2): e56324.

231. Del Rosso M, Fibbi G, Pucci M, D'Alessio S, Del Rosso A, Magnelli L, Chiarugi V Multiple pathways of cell invasion are regulated by multiple families of serine proteases. Clin Exp Metastasis 19:193–207PubMedCrossRefGoogle Scholar

232. Fibbi G, Caldini R, Chevanne M, Pucci M, Schiavone N, Morbidelli L, Parenti A, Granger HJ, Del Rosso M, Ziche M Urokinase-dependent angiogenesis in vitro and diacylglycerol production are blocked by antisense oligonucleotides against the urokinase receptor. Lab Invest 78:1109-1119

233. Fibbi G, Ziche M, Morbidelli L, Magnelli L, Del Rosso M Interaction of urokinase with specific receptors stimulates mobilization of bovine adrenal capillary endothelial cells. Exp Cell Res 179:385–395

234. D'Alessio S, Margheri F, Pucci M, Del Rosso A, Monia BP, Bologna M, Leonetti C, Scarsella M, Zupi G, Fibbi G et al Antisense oligodeoxynucleotides for urokinase-plasminogen activator receptor have anti-invasive and anti-proliferative effects in vitro and inhibit spontaneous metastases of human melanoma in mice. Int J Cancer 110(1):125–133

235. Rao JS, Gondi C, Chetty C, Chittivelu S, Joseph PA, Lakka SS Inhibition of invasion, angiogenesis, tumor growth, and metastasis by adenovirus-mediated transfer of

antisense uPAR and MMP-9 in non-small cell lung cancer cells. Mol Cancer Ther 4:1399–1408

236. Jo M, Lester RD, Montel V, Eastman B, Takimoto S, Gonias SL Reversibility of epithelial-mesenchymal transition (EMT) induced in breast cancer cells by activation of urokinase receptor-dependent cell signaling. J Biol Chem 284:22825–22833

237. Lester RD, Jo M, Montel V, Takimoto S, Gonias SL uPAR induces epithelial-mesenchymal transition in hypoxic breast cancer cells. J Cell Biol 178:425–436

238. Serratì S, Margheri F, Pucci M, Cantelmo AR, Cammarota R, Dotor J, Borràs-Cuesta F, Fibbi G, Albini A, Del Rosso M TGFbeta1 antagonistic peptides inhibit TGFbeta1-dependent angiogenesis. Biochem Pharmacol 77:813–825

239. Santibanez JF Transforming growth factor-Beta and urokinase-type plasminogen activator: dangerous partners in tumorigenesis-implications in skin cancer. Dermatol 597927

240. Margheri F, Schiavone N, Papucci L, Magnelli L, Serratì S, Chillà A, Laurenzana A, Bianchini F, Calorini L, Torre E et al (2012) GDF5 regulates TGFß-dependent angiogenesis in breast carcinoma MCF-7 cells: in vitro and in vivo control by anti-TGFß peptides. PLoS One 7:e50342

241. Ulisse S, Baldini E, Sorrenti S, D'Armiento M. The urokinase plasminogen activator system: a target for anti-cancer therapy. Current cancer drug targets. 2009;9:32–71.

242. Boonstra MC, Verspaget HW, Ganesh S, Kubben FJ, Vahrmeijer AL, van de Velde CJ, Kuppen PJ, Quax PH, Sier CF. Clinical applications of the urokinase receptor (uPAR) for cancer patients. Current pharmaceutical design. 2011;17:1890–1910

243. Creighton CJ, Li X, Landis M, et al. Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. Proc Natl Acad Sci USA. 2009;106:13820–13825.

244. Tehranchi R, Woll PS, Anderson K, et al. Persistent malignant stem cells in del(5q) myelodysplasia in remission. N Engl J Med. 2010;363:1025–1037.

245. Singh SK, Clarke ID, Terasaki M, et al. Identification of a cancer stem cell in human brain tumors. Cancer Res. 2003;63:5821–5828.

246. Nordling CO. A new theory on cancer-inducing mechanism. Br J Cancer. 1953;7:68–72.

247. Ashley DJ. The two “hit” and multiple “hit” theories of carcinogenesis. Br J Cancer. 1969;23:313–328.

248. Knudson AG., Jr Mutation, cancer: Statistical study of retinoblastoma. Proc Natl Acad Sci USA. 1971;68:820–823.

249. Nowell PC. The clonal evolution of tumor cell populations. Science. 1976;194:23–28.

250. Mazzieri R, Masiero L, Zanetta L et al. Control of type IV collagenase activity by components of the urokinase-plasmin system: a regulatory mechanism with cell-bound reactants. EMBO J 1997; 16(9): 2319–32.

251. Ferrara N. Role of vascular endothelial growth factor in the regulation of angiogenesis. Kidney Int 1999; 56: 794–814

252. Nathan Moore and Stephen Lyle. Quiescent, Slow-Cycling Stem Cell Populations in Cancer: A Review of the Evidence and Discussion of SignificanceHindawi Publishing Corporation Journal of Oncology Volume 2011, Article ID 396076, 11

253. Anjomshoaa A, et al. Slow proliferation as a biological feature of colorectal cancer metastasis. Br J Cancer. 2009;101:822–828.

254. Roesch A, et al. A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. Cell. 2010;141:583–594.

255. Pece S, et al. Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. Cell. 2010;140:62–73.

256. Gupta PB, et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell. 2009;138:645–659.

257. Kurrey NK, et al. Snail and slug mediate radioresistance and chemoresistance by antagonizing p53-mediated apoptosis and acquiring a stem-like phenotype in ovarian cancer cells. Stem Cells. 2009;27:2059–2068.

258. Feuerhake F, Sigg W, Hofter EA, Dimpfl T, Welsch U. Immunohistochemical analysis of Bcl-2 and Bax expression in relation to cell turnover and epithelial differentiation markers in the non-lactating human mammary gland epithelium. Cell Tissue Res. 2000;299:47–58.

259. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med. 1996;183:1797–1806.

260. Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci U S A. 2004;101:781–786.

261. Zhou S, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med. 2001;7:1028–1034.

262. Ricardo Pardal, Michael F. Clarke & Sean J. Morrison Applying the principles of stem-cell biology to cancer. Nature Reviews Cancer 3, 895-902 (December 2003)

263. Gondi CS, Kandhukuri N, Kondraganti S, Gujrati M, Olivero WC, et al. (2006) RNA interference-mediated simultaneous down-regulation of urokinase-type plasminogen activator receptor and cathepsin B induces caspase-8-mediated apoptosis in SNB19 human glioma cells. Mol Cancer Ther 5: 3197–3208.

264. Kin Y, Chintala SK, Go Y, Sawaya R, Mohanam S, et al. (2000) A novel role for the urokinase-type plasminogen activator receptor in apoptosis of malignant gliomas. Int J Oncol 17: 61–65.

265. Warburg O, Wind F, Negelein E. The Metabolism of Tumors in the Body. J Gen Physiol. 1927;8:519–530.

266. Dong C, et al. Loss of FBP1 by Snail-mediated repression provides metabolic advantages in basal-like breast cancer. Cancer Cell. 2013;23:316–331.

267. Ginestier C, et al. Mevalonate metabolism regulates Basal breast cancer stem cells and is a potential therapeutic target. Stem Cells. 2012;30:1327–1337.

268. Fantin VR, St-Pierre J, Leder P. 2006. Attenuation of LDH-Aexpression uncovers a link between glycolysis, mitochondrialphysiology, and tumor maintenance. Cancer Cell 9: 425–434.

269. Le A, Cooper CR, Gouw AM, Dinavahi R, Maitra A, Deck LM,Royer RE, Vander Jagt DL, Semenza GL, Dang CV. 2010.Inhibition of lactate dehydrogenase A induces oxidative stressand inhibits tumor progression. Proc Natl Acad Sci 107:2037–2042

270. Warburg O. 1956. On respiratory impairment in cancer cells. Science 124: 269

271. Vander Heiden MG, Cantley LC, Thompson CB. 2009. Understandingthe Warburg effect: The metabolic requirements of cell proliferation. Science 324: 1029–1033.

272. Gillies RJ, Robey I, Gatenby RA. 2008. Causes and consequencesof increased glucose metabolism of cancers. J Nucl Med 49(Suppl 2): 24S–42S.

273. Warburg O. 1956. On the origin of cancer cells. Science 123:309–314.

274. Gatenby RA, Gillies RJ. 2004. Why do cancers have high aerobicglycolysis? Nat Rev Cancer 4: 891–899.

275. Peppicelli S, Bianchini F, Calorini L. Extracellular acidity, a "reappreciated" trait of tumor environment driving malignancy: perspectives in diagnosis and therapy.Cancer Metastasis Rev. 2014 Sep;33(2-3):823-32.

276. Peppicelli S, Bianchini F, Torre E, Calorini L. Contribution of acidic melanoma cells undergoing epithelial-to-mesenchymal transition to aggressiveness of non-acidic melanoma cells.Clin Exp Metastasis. 2014 Apr;31(4):423-33. doi: 10.1007/s10585-014-9637-6. Epub 2014 Jan 28.

277. Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A,Gerszten RE,Wei R, Fleming MD, Schreiber SL, Cantley LC.2008a. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature 452:230–233.

278. Christofk HR, Vander Heiden MG, Wu N, Asara JM, CantleyLC. 2008b. Pyruvate kinase M2 is a phosphotyrosine-bindingprotein. Nature 452: 181–186.

279. Vander Heiden MG, Locasale JW, Swanson KD, Sharfi H,Heffron GJ, Amador-Noguez D, Christofk HR, Wagner G,Rabinowitz JD, Asara JM, et al. 2010. Evidence for an alternativeglycolytic pathway in rapidly proliferating cells. Science329: 1492–1499.

280. Kuo W, Lin J, Tang TK.Human glucose-6-phosphate dehydrogenase (G6PD) gene transforms NIH 3T3 cells and induces tumors in nude mice.Int J Cancer. 2000 Mar 15;85(6):857-64.

281. Vaughn AE, Deshmukh M. 2008. Glucose metabolism inhibitsapoptosis in neurons and cancer cells by redox inactivation of cytochrome c. Nat Cell Biol 10: 1477–1483.

282. Alpízar-Alpízar W, Christensen IJ, Santoni-Rugiu E, Skarstein A, Ovrebo K, Illemann M, Laerum OD.Urokinase plasminogen activator receptor on invasive cancer cells: a prognostic factor in distal gastric adenocarcinoma.Int J Cancer. 2012 Aug 15;131(4):E329-36. Epub 2011 Oct 20.

283. Patrick M. Brunner, Patricia C. Heier, Judit Mihaly-Bison, Ute Priglinger, Bernd R. Binder and Gerald W. Prager. Density enhanced phosphatase-1 down-regulates urokinase receptor surface expression in confluent endothelial cells. Blood 2011 117:4154-4161

284. Rosemary Bass, Vincent Ellis. Regulation of urokinase receptor function and pericellular proteolysis by the integrin α5β1Thrombosis and Haemostasis, 101 (5). pp. 954-962.

285. Simon DI, Wei Y, Zhang L, Rao NK, Xu H, Chen Z, Liu Q, Rosenberg S, Chapman HA.Identification of a urokinase receptor-integrin interaction site. Promiscuous regulator of integrin function.J Biol Chem. 2000 Apr 7;275(14):10228-34.

286. Ying Wei, Johannes A. Eble, Zemin Wang, Jordan A. Kreidberg, and Harold A. Chapman. Urokinase Receptors Promote β1 Integrin Function through Interactions with Integrin α3β1. Mol Biol Cell. 2001 Oct; 12(10): 2975–2986.