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Squamous cell cancers: a unified perspective on biology and genetics

G. Paolo Dotto[1*] and Anil K. Rustgi[2*]

[1]Department of Biochemistry, University of Lausanne, Epalinges, CH-1066, Switzerland and Cutaneous Biology Research Center, Massachusetts General Hospital, Charlestown, MA 02129, USA; Email: paolo.dotto@unil.ch

[2]Division of Gastroenterology, Departments of Medicine and Genetics, Abramson Cancer Center, University of Pennsylvania Perelman School of Medicine, PA. 19104; Email: anil2@mail.med.upenn.edu

*Co-Corresponding authors

SUMMARY

Squamous cell carcinomas (SCCs) represent the most frequent human solid tumors and a major cause of cancer mortality. These highly heterogeneous tumors arise from closely interconnected epithelial cell populations with intrinsic self-renewal potential inversely related to the stratified differentiation program. SCCs can also originate from simple or pseudo-stratified epithelia through activation of quiescent cells and/or a switch in cell fate determination. Here, we focus on specific determinants implicated in development of this disease by recent large-scale genomic, genetic and epigenetic studies and complementary functional analysis. This evidence indicates that SCCs from various body sites, while clinically treated as separate entities, have common determinants, pointing to a unified perspective of the disease and potential new avenues for prevention and treatment.

INTRODUCTION

Squamous cell carcinomas (SCCs) are, individually and in aggregate, among the most common forms of human cancer (Cancer Facts and Figures 2015, American Cancer Society) (Figure 1). Development of these tumors is linked closely to genomic perturbations, genetic mutations and/or altered expression of key molecules involved in various stages of squamous cell lineage commitment and/or terminal differentiation. Alterations of underlying stromal cells also play an important role in development of these tumors and recent evidence indicates that they may even be a primary determinant, besides promoting escape from immune surveillance and resistance to chemotherapy (reviewed in (Dotto, 2014; Junttila and de Sauvage, 2013)).

Stratified epithelial cells giving rise to SCCs are found across the entire surface of the external tegument as well as the nasal cavity, oropharynx, esophagus, and anogenital region. In addition, squamous metaplasia occurs in the respiratory tree and urinary tract as a reactive response to a variety of noxious conditions (e.g. cigarette-smoking) (Ishizumi et al., 2010). Induction of squamous differentiation in this context could have a protective role, increasing tissue resilience and, as discussed below, cell survival. However, if metaplasia persists, there is an enhanced opportunity for dysplasia and cancer risk. Interestingly, an inverse process of intestinal metaplasia, i.e. formation of an intestinal-resembling epithelium at the distal esophagus and gastro-esophageal junction as a result of acid and bile reflux and chronic inflammation, is associated

with an elevated risk of adenocarcinoma rather than squamous carcinoma (Rustgi and El-Serag, 2015). Although rare, SCC can be also found in atypical tissues, such as the thyroid (Tunio et al., 2012), prostate (Malik et al., 2011) and breast (Grabowski et al., 2009); however, the origin of these tumors has not yet been identified.

A distinguishing feature of SCCs is their high degree of cellular heterogeneity, with cell populations at various stages of differentiation, which are able to reverse lineage commitment to proliferative stages as well as enter into quiescent, slow-cycling growth phases. These features make them particularly difficult to target with monotherapeutic approaches (Elkabets et al., 2015).

Differentiation therapy has been proposed to exhaust cancer-initiating cell populations by forcing them into terminal differentiation (Brooks et al., 2014; Cruz and Matushansky, 2012). However, countering the promise of this approach is the risk that it could rather enhance cell survival and resistance to chemotherapeutic agents and contribute to pro-oncogenic immune evasion (Cruz and Matushansky, 2012; Facompre et al., 2012). Exploring the crosstalk of pathways at the intersection of squamous cell renewal, terminal differentiation, stromal alterations and immune surveillance is thus of major importance for future preventive and therapeutic approaches against SCC.

The classification of SCCs commonly follows the anatomical divisions of clinical medicine where, for example, head and neck SCCs are treated separately from those of the anogenital region. However, it is becoming increasingly clear that SCCs share important properties, as revealed by a

commonality of genomic, genetic and epigenetic alterations and a similar impact of the underlying mesenchyme. This opens up new and exciting avenues for a unified perspective on the biology of SCCs and translation into common and novel therapeutic approaches to these widespread and poorly controlled cancers.

ETIOLOGY OF SCCs

The basement membrane marks the histological delineation between stratified epithelia and underlying mesenchymal compartment. The basal layer comprises relatively round, proliferative cells that have a high nuclear/cytoplasmic ratio. This ratio reverses with each successive layer, where cells flatten out into the non-dividing terminally differentiated "squames" after which squamous epithelium is named. Tight regulation of this proliferation-differentiation gradient is needed to maintain the barrier function and is disrupted by stress in the form of infection, carcinogens, drugs and radiation. Key carcinogenic insults leading to SCC development differ in relation to the protective function of the epithelia from which they originate, but others are shared (Hussain et al., 2001) (Table 1).

- Ultraviolet light: Ultraviolet A and B radiation (UVA and UVB) are irrefutable carcinogens to human skin (Armstrong and Kricker, 2001; Narayanan et al., 2010). UVB (280-315 nm) causes direct DNA damage, with a typical, but not exclusive, signature of C→T substitutions, resulting from covalent cyclobutane dimer formation between adjacent pyrimidine rings. UVA (315-400 nm) exposure

is also mutagenic through more indirect mechanisms involving production of reactive oxygen species (ROS) (Bachelor and Bowden, 2004) and 8-oxo-guanine (8-oxo-7, 8-dihydro-7′-deoxyguanosine) formation, which results in G-T transversions during replication. UVA causes additional multiple effects, including chromosomal aberrations and widespread alterations in cellular proteins and lipids (Ridley et al., 2009; Wischermann et al., 2008). Besides cancer cells of origin, the pro-tumorigenic effects of UV exposure are likely due to its impact on the surrounding tissue environment, with induction of inflammation and immune suppression (Narayanan et al., 2010; Ridley et al., 2009).

Cutaneous SCCs (CSCCs) are typically indolent tumors, rarely yielding metastasis (<5%), late in the course of the disease. Nevertheless, the massive incidence of CSCCs as the second most frequent type of human malignancy worldwide makes this cancer type a major health concern, accounting for 20% of all skin cancer-related deaths. Additionally, multiple and recurrent CSCCs are a major cause of death for the many organ transplant recipient patients under treatment with calcineurin inhibitors for immunosuppression (Euvrard et al., 1995). The specifically increased risk of CSCCs as opposed to other SCCs in internal organs points to a synergy between carcinogenic UV light exposure and calcineurin inhibition that deserves further exploration (Dotto, 2011; Dziunycz et al., 2014).

- Cigarette-Smoking: Lung SCC (LSCC) is classed as a non-small-cell lung

cancer: a group particularly resistant to chemotherapy, with 97% of cases attributable to smoking (Herbst et al., 2008). A strong association has also been found between smoking and tumors classed anatomically as “head and neck” SCC (HNSCC). HNSCCs are the sixth most common malignancy worldwide (Leemans et al., 2011) and can be divided both histologically (Woolgar and Triantafyllou, 2011) and on the basis of global transcriptional analysis with etiology specific profiles (Chung et al., 2006; Chung et al., 2004; Martin et al., 2014). Smoking is also a critical risk factor in esophageal SCC (ESCC) (Rustgi, NEJM 2014).

Since the 1950’s, studies have identified over 7000 chemicals in cigarettes, at least 40 of which have known carcinogenic properties (DeMarini, 2004). Despite this diversity, smoking has a relatively predictable mutagenic signature, preferentially acting on guanine base pairs and creating G→T substitutions (Alexandrov et al., 2013). Importantly, many polyaromatic hydrocarbons and cigarette smoke components are inactive and require metabolic activation for causing genotoxic damage. Surface epithelial tissues are commonly replete with antigen-internalizing and -presenting dendritic cells. Independently of their role in the immune system, these cells have been shown to play an essential role in activation of the hydrocarbon 7,12-dimethylbenz[**a**]anthracene (DMBA) in a skin mouse model of squamous carcinogenesis (Modi et al., 2012). Carcinogenic conversion of smoke components into mutagenic agents can also occur by liver detoxification enzymes.

- Alcohol: Similarly to many smoking related carcinogens, ethanol is only carcinogenic as a first-pass metabolite, resulting in the formation of acetyaldehdye-DNA adducts, (Brooks and Zakhari, 2014). While readily demonstrated in a variety of assays systems, the mutagenic impact of ethanol in patients is more debatable, the situation being complicated by the low acute toxicity of this substance and the resulting excessive doses that are usually employed for testing (Phillips and Jenkinson, 2001). Like smoking, the pro-carcinogenic consequences of alcohol exposure likely involve widespread tissue alterations and chronic inflammation (Franke et al., 2005). As a systemic carcinogen, it is unsurprisingly linked to a wide range of cancers and there is synergy between alcohol and cigarette-smoking in the pathogenesis of various types of SCC, specifically HNSCC (Herbst et al., 2008) and ESCC (Rustgi and El-Serag, 2015).

ESCC has a particularly poor prognosis, with 5-year survival rates rarely exceeding 20% (Figure 1). ESCC stands as the 8th most commonly diagnosed cancer in the world and the 6th leading cause of cancer-related mortality (Rustgi and El-Serag, 2015). ESCC is especially frequent in males (>4:1), and has especially high incidence in north central China (in 25% or more of adults above age 35), central Asia, the eastern part of the African continent and parts of South America (Taylor et al., 2013). This geographic bias has been linked to differences in various dietary and lifestyle habits as well as genetic polymorphisms, rather than alcohol/tobacco consumption (Taylor et al., 2013).

- Infectious agents: Pathogens create pro-oncogenic environments in two major ways: 1) expression of pathogen-derived oncogenes and/or inactivation of host tumor suppressor genes; 2) chronic inflammation and reduced immunosurveillance.

The first mechanism of infectious oncogenesis is exemplified by human papilloma viruses (HPVs), which produce the E6 and E7 oncoproteins upon integration into the genome of the host keratinocytes (Doorbar et al., 2015; Egawa et al., 2015). The cell cycle is then perturbed by functional inactivation of the key tumor suppressor proteins p53 and p105-Rb by E6 and E7, respectively. Through this mechanism, HPV is held responsible for a staggering 96% of cervical SCC (CvSCC) (Doorbar, 2006) and has gained recognition as an important cause of HNSCC (Leemans et al., 2011). A significant association with ESCC has also been reported (Ludmir et al., 2015).

Generally, HPV-positive HNSCC has a better clinical prognosis than its HPV-negative counterpart. The reasons for this remain to be understood but may be due to their different genetic causes, where HPV-related cancers tend to retain wild type *TP53*, while HPV-negative tumors are very frequently associated with pro-oncogenic gain-of-function mutations in *TP53* and other genes (Cancer Genome Atlas, 2015). Most likely, disparities in tumor behavior are also linked to different patient populations, where HPV-positive cancers develop in younger generally healthy individuals, while the HPV-negative form affects older people with a history of alcohol and/or tobacco abuse (which are in turn associated with

poor health-seeking behavior) (Leemans et al., 2011).

Epstein-Barr virus (EBV) is another DNA tumor virus, of the herpes family, associated with nasopharyngeal carcinoma (NPC), which is endemic in certain regions of China, specifically the southwestern area. It is not clear why EBV infection is specifically associated with NPC. A possibility is that the nasopharynx is rich in lymphocytes, which can act as a reservoir for viral spread to neighboring epithelial cells. NPC is distinguished into two histological subtypes: keratinizing (WHO1) and non-keratinizing squamous cell carcinoma (WHO2/3). The latter, which accounts for 80% of cases, is universally associated with latent EBV infection, exclusively in tumor cells and not surrounding lymphoid infiltrates (Shah and Young, 2009). Moreover, EBV infection has been associated with ESCC (Jenkins et al., 1996).

EBV-induced oncogenesis results from a multistep process involving a number of genetic and epigenetic (methylation) changes in the host genome (including *CDKN2A* inactivation and *CCNDD1* amplification) coupled with persistent EBV infection. Several viral nuclear proteins are involved in establishment of latency (EBNA2, EBNA3A, 3B, 3C, and leader protein or LP), which control transcription of viral and host genes via interactions with the key effector of canonical Notch signaling, RBPJκ (Raab-Traub, 2012). In established NPC, these viral proteins are not detected, and two other EBV latent genes with well demonstrated transforming properties are instead expressed (Kempkes and Robertson, 2015). One codes for LMP1 (Latent membrane protein 1), which functions as an activated TNF family member and can activate multiple

pathways, including MAPK/JNK, PI3K/AKT and NF-κB. The other EBV transforming gene codes for LMP2A (latent membrane protein 2A), a 6 trans-membrane domains protein with a long cytoplasmic C-terminus tail and adaptor /scaffold function, which in epithelial cells can activate the PI3K/AKT and β-CATENIN signaling pathways. In addition to these protein-coding genes, recent evidence indicates that latency-associated EBV miRNAs are also implicated in NPC development (Cai et al., 2015).

The trematode *Schistosoma haematobium* (*S. haematobium*) is an exemplary case of parasite-induced inflammation and cancer, specifically bladder SCC (BSCC) (Odegaard and Hsieh, 2014). Following *S. haematobium* infection of the urinary tract, primary tumors usually develop in the bladder wall, within the fibrotic granuloma tissue that forms around the parasitic eggs (Odegaard and Hsieh, 2014). Chronic inflammation is thought to cause metaplasia of the transitional urothelium into squamous tissue before an accumulation of oncogenic mutations that culminate in uncontrolled cell growth. Although much information exists on the oncogenic effects of the inflammatory host reaction to *S. haematobium,* it is important to note that the parasites are also directly carcinogenic (Botelho et al., 2011). In fact, parasitic sterols secreted by the *S. haematobium* egg sac are metabolized by P450 enzymes, generating catechol-estrogens that readily undergo oxidation and conversion into highly reactive quinine and quinone metabolites (Botelho et al., 2011). These compounds are able to react directly with DNA or indirectly, via generation of ROS. Besides the strong association with *S. haematobium* infection, BSCC development is linked

to other agents (e.g. cigarette-smoking, aniline dyes) that result in the concentration of carcinogens in the urine.

GENETIC LANDSCAPE OF SCC DEVELOPMENT

In concert with amplification and deletion of specific chromosomal regions, mutational analysis of selected candidate genes has been recently supplanted by whole exome sequencing of many tumor types coupled with expression profiles. Together with mutations in protein encoding sequences, more recent whole genome analysis has highlighted the existence of mutations in gene regulatory regions and the post-transcriptional potential of epigenetic alterations and non-coding RNAs (Mathelier et al., 2015). In this context, activation of transposable elements and transposition can be also involved (Helman et al., 2014; Tubio et al., 2014).

An important finding is that a large fraction of gene mutations found in tumors are already present in normal tissues (Martincorena et al., 2015) (Tomasetti et al., 2013), so that accumulation and combination of these mutations may be more important than their individual occurrence (Tomasetti and Vogelstein, 2015). As an alternative to clonal selection, a “Big Bang” mode of cancer development is also possible, whereby clinically detectable tumors result from expansion of a single cell population with genetically dominant alterations intermingled with “private” (i.e. non dominant) changes occurring within non-selected but still co-expanded sub-clones (Sottoriva et al., 2015). This would explain the high level of inter-tumor heterogeneity and the indication that “some

tumors appear born to be bad" from the beginning (Sottoriva et al., 2015). The fact that different cancer driver mutations of the same genes, like *TP53*, *HRAS* or *NOTCH1*, can be found in separate parts of the same SCCs (South et al., 2014) points to possible convergent selection. However, the presence of clones with multiple mutations of these genes also in normal epithelium suggests that may have a more passenger than driver function and/or act in concert with other determinants of carcinogenesis. Most notable among these are changes in the surrounding stromal tissue, which, as we recently reviewed (Dotto, 2014), can occur at various levels and play a primary role not only in progression of the disease but also initiation.

For mutational analysis of tumors, significance of the observed frequencies needs to be adjusted on the basis of levels of gene mutations in normal matching tissues (Gonzalez-Perez et al., 2013; Lawrence et al., 2013; Schroeder et al., 2014). This is of special importance for SCCs, given their high accumulative gene mutation frequency. For example, in studies of LSCCs (Hammerman et al., 2012) and CvSCCs (Ojesina et al., 2014) researchers found a total of 8.1 and 4.2 mutations per megabase, respectively. A 5-15-fold higher mutation rate was found in CSCC (>50-60 mutations per megabase, with a median of 1200 mutations per tumor) to be compared with a 10-fold lower mutation rate in normal sun-exposed skin (Pickering et al., 2014; South et al., 2014), in concert with a high rate of gene mutations, chromosomal instability is another feature of SCC. In fact, one study in HNSCC estimated that copy number alterations (CNAs) affecting discrete chromosomal regions and polyploidy

account for up to 70% of tumors (Pickering et al., 2013). In another, focused on CSCC and associated precursor lesions, UV hotspot mutations in the kinetochore *KNSTRN* gene were reported to cause disruption of chromatid cohesion and aneuploidy (Lee et al., 2014).

Integrated analysis of genomic, epigenetic and gene expression alterations point to significant similarities between SCCs from various body sites, such as HNSCC, LSCC, ESCC and CSCC (Hoadley et al., 2014; Pickering et al., 2013; Pickering et al., 2014) (Table 2). However, mutations of certain genes appear to be more frequent or potentially specific to a given SCC. For example, AJUBA inactivating mutations are found in HNSCC, CSCC and ESCC, but not in LSCC and CvSCC (Table 2). A complex interplay between environmental risk factors, host genetic predisposition and tumor cell genomics/genetics/epigenetics may nurture certain mutations preferentially. It is important to note, however, that reported presence and frequencies of gene alterations can vary even within studies of the same SCC type, due to confounding geographic/ethnic differences of patient populations, stages and grades of tumors, depth of nucleotide sequencing analysis, number of tumor samples, and degree of normal/tumor cell admixture in the studied samples.

The broad spectrum of gene alterations identified in SCCs may be grouped into two categories: one, with a likely cancer driver function in a variety of cancer types, and the other, affecting genes with a preferential or selective role in SCCs in a mutation gene network centered around squamous cell fate decisions and/or the squamous terminal differentiation program. By analysis of

large data sets of tumors, specific gene mutations that are mutually exclusive with others can be identified, even if statistical significance of negative associations is limited to very few cases (Figures 2 and 3). Given the many altered genes, by either deletions/amplifications or point mutations, only a representative number will be discussed on the basis on this logic, referring to a more complete list of frequently mutated genes and/or other alterations in Table 2.

CELL CYCLE REGULATORY GENES

- *TP53* and *CDKN2A/RB1* genes: *TP53* mutations are the most frequently identified somatic mutations in SCCs from all body sites (Table 2). Missense "hot spots" mutations are very common, which result in dominant negative and/or gain-of-function properties through three possible mechanisms (Freed-Pastor and Prives, 2012; Muller and Vousden, 2014). The first relates to the tetramer complex formation of p53 and its ability to interact, in either wild type or mutated forms, with the two other family members, p63 and p73, affecting their function. The second involves modulation of gene expression by mutant p53 mediated by its association with other transcriptional factors. The third results from altered DNA binding specificity of mutant p53. As a result, a whole range of deregulated genes has been identified with pro-survival, pro-invasive and pro-tumorigenic functions (Freed-Pastor 2012; Muller 2014). The different impact on tumorigenesis of missense versus loss of function *TP53* mutations is best appreciated in mouse models, with "knock-in" missense mutations resulting in a

shift towards epithelial-derived cancers (see, for instance, (Lang et al., 2004; Olive et al., 2004)).

Suppression of p105-Rb activity by loss-of-function mutations of the CDK inhibitor *CDKN2A* is also very common in SCCs, while mutations of the *RB1* gene itself have been found less frequently except in ESCC (Song et al., 2014). Interestingly, putative cancer driver mutations in many genes including *TP53* are already frequent in normal photo-exposed skin, with the notable exception *CDKN2A* mutations, suggesting that these may be a critical trigger of cancer development (Martincorena et al., 2015).

As mentioned, the incidence of *TP53* and *CDKN2A/RB1* mutations is much reduced in HNSCCs and CvSCCs linked with HPV infection. Here, expression of viral E6 and E7 has been shown to inhibit the p53 and p105-Rb proteins, thus rendering direct genetic mutation dispensable (Cancer Genome Atlas, 2015; Ojesina et al., 2014).

- *CCDN1* and *MYC*: The genes coding for Cyclin D1 and c-Myc are also commonly amplified in SCCs (Figures 2 and 3) and amplification of these genes, like *TP53* and *CDKN2A* mutations, are frequent in HPV (-) HNSCCs but rare or absent in their HPV (+) counterparts (Cancer Genome Atlas, 2015). *FBXW7* codes for a component of the SCF ubiquitin E3 ligase complex involved in degradation of a number of key cell regulatory molecules including c-Myc, cyclin E and Notch1 (Welcker and Clurman, 2008). Loss-of-function mutations in *FBXW7* are especially frequent in CvSCC (Ojesina et al., 2014), but occur also in

SCCs from other body sites (Table 2). An important target of FBXW7 in HNSCCs is the anti-apoptotic Mcl-1 protein, which has been implicated in increased cancer cell survival and as a possible therapeutic target (He et al., 2013).

TYROSINE KINASE RECEPTORS

- Epidermal growth factor receptor (EGFR): SCCs are also linked to frequent amplification and, in some cases, mutations of tyrosine kinase receptor genes (Figures 2 and 3; Table 2). The frequent amplification of *EGFR* and closely related *ERBB2* can contribute to elevated receptor activity in HNSCCs and ESCCs (Li et al., 2014). Interestingly, in a recent comprehensive study of HNSCCs, *EGFR* amplification was found in 15% of the (HPV-) tumors but in none of their (HPV+) counterparts (Cancer Genome Atlas, 2015). As EGFR is a redundant receptor to multiple ligands, it is particularly attractive target for chemotherapy by either small molecule inhibitors (Robinson and Sandler, 2013) or blocking antibodies (Pirker, 2015). Favorable response to small molecule inhibitors is observed in the case of activating *EGFR* mutations, such as frequently occurring in lung adenocarcinomas but not squamous carcinomas. By contrast, some beneficial effects are elicited by treatment with anti-EGFR antibodies even in the absence of *EGFR* mutations, through mechanisms that are still poorly understood (Pirker, 2015). Resistance to antibody treatment can occur through a number of mechanisms, including AKT activation, suggesting that combinatorial therapies with PI3K inhibitors have conceptual merit (Brand et al., 2011; Iida et al., 2013). Another pathway of resistance to anti-EGFR therapy

is via increased activity of another tyrosine kinase receptor, c-MET (Burtness et al., 2013). C-MET is engaged by the hepatocyte growth factor (HGF) ligand, and its phosphorylation triggers a variety of signaling pathways, similarly to EGFR. While *MET* may be amplified in small subsets of HNSCC, ESCC and LSCC, mutations are not prevalent. Regardless, dual anti-EGFR and c-MET therapy holds promise for therapeutic intervention (Burtness et al., 2013; Liao et al., 2012).

- Fibroblast growth factor receptors (FGFRs): The *FGFR1* and, to a lesser extent, *FGFR2* and *FGFR3* are also frequently amplified in SCC from various body sites and, in HNSCCs, amplification of these genes occurs mostly in tumors without *EGFR*, *CCND1* or *MYC* amplification (Figure 2). As is the case for these other genes, even *FGFR1,2* gene amplification occurs selectively in (HPV-) tumors (Cancer Genome Atlas, 2015). Unlike *EGFR*, at least in LSCCs, amplification of *FGFR* genes can be accompanied by activating mutations mapping to the extracellular or intracellular regions of the receptors (Liao et al., 2012), making these molecules possible therapeutic targets (Liao et al., 2013; Liao et al., 2012). Importantly, *FGFR1, FGFR2 and FGFR3* gene fusions have also been identified in various cancer types, including HNSCCs and LSCCs (Wang et al., 2014; Wu et al., 2013). The resulting protein products have heterogeneous oligomerization domains fused to an intact FGFR tyrosine kinase region and exhibit elevated susceptibility to pharmacologic inhibition both in vitro and *in vivo*, offering a window of therapeutic opportunity (Dienstmann et al.,

2014).

While *FGFR* gene activation is mostly viewed as pro-oncogenic, activating *FGFR3* mutations were the most frequent genetic alteration found in expanding clones of keratinocytes in photo-aged but otherwise normal human skin (Martincorena et al., 2015). This raises the possibility that activation of this receptor may confer upon these cells a selective advantage without conferring a tumorigenic phenotype (Martincorena et al., 2015). Consistent with this view, activating *FGFR3* mutations are also found in >40% of seborrheic keratosis, very common keratinocyte-derived tumors of the skin that do not, or very rarely, progress into malignancy (Hafner et al., 2006; Logie et al., 2005). Furthermore, functional FGFR3 activation in these cells can trigger differentiation, in parallel with increased proliferation (Mandinova et al., 2009).

RAS/MAPK AND PI3K SIGNALING

- RAS: Downstream of tyrosine kinases, small RAS GTPases provide a key signaling node notoriously hard to target, even if better understanding of their membrane association is opening new perspectives (Cox et al., 2015). Mutations of *HRAS* are found with variable frequencies in SCCs from various body sites, a likely reflection of the fact that activation of the pathway at other levels, such as tyrosine kinase receptors, can also occur. In this context, it is interesting to note that, in a comprehensive study of HNSCC, *HRAS* mutations were selectively found in HPV-negative tumors (Cancer Genome Atlas, 2015) and that no *HRAS* mutations were detected in a previous study of cervical cancer, the other major

SCC type associated with HPV infection (Ojesina et al., 2014). This may be explained by the fact that, besides E6 and E7, expression of the HPV E5 protein has been connected previously with transformation through activation of surface TK receptors (DiMaio and Mattoon, 2001).

An elevated incidence of *HRAS* mutations (>20%), with a lower frequency of *KRAS* and *NRAS* mutations, was reported in two genomic studies of cutaneous SCCs, which may reflect the fact that overall gene mutation frequencies in these tumors are substantially higher than in SCCs of internal organs (Pickering et al., 2014; South et al., 2014). Importantly, an even greater frequency of *HRAS* mutations (>40%) was found in the cutaneous SCCs and keratoacanthomas that develop in melanoma patients treated with the B-RAF inhibitor vemurafenib (South et al., 2014; Su et al., 2012). The underlying reasons remain to be fully elucidated but could involve paradoxical activation of MAPK signaling and accelerated growth of *HRAS* harboring lesions (Arnault et al., 2012; Su et al., 2012) and/or more complex mechanisms that can be counteracted by combined treatment with B-RAF and MEK inhibitors (Robert et al., 2015) and/or inhibitors of COX-2 (Escuin-Ordinas et al., 2014).

- **PI3K**: As in many other tumor types, the PI3K/AKT signaling pathway is frequently affected by gene amplification and/or mutations, consistent with its key role in cell survival. The 3q26/28 chromosomal region, encompassing *PIK3CA*, as well as the *TP63* and *SOX2* cell lineage genes discussed below, is frequently amplified in various SCCs (Hoadley et al., 2014) (Figures 2 and 3). Activating

mutations of the *PIK3CA* gene are also a frequent finding in various SCCs (Figures 2 and 3; Table 2), with loss of *PTEN* as an alternative possible mechanism for deregulated AKT signaling and consequently increased cell survival (Liao et al., 2012). A dual role of specific AKT isoforms in squamous differentiation is also to be noted with possibly important therapeutic implications (Naeem et al., 2015; Okano et al., 2000; Saoncella et al., 2014).

GENES INVOLVED IN SQUAMOUS CELL FATE DETERMINATION

- *TP63*: The basal cell compartment in most squamous tissues is believed to harbor stem cells or progenitor cells. Such cells are characterized by elevated expression of *TP63*, a member of the *TP53* gene family (Crum and McKeon, 2010). *TP63* codes for two main isoforms TAp63 and ΔNp63 (lacking an N-terminal transactivation domain), each of which gains additional diversity through alternative splicing (α, β, γ, δ, ε sub-isoforms). This gene is critical for epithelial development in mice and humans (Crum and McKeon, 2010). The transition from the simple epithelium to the stratified epithelium occurs at different developmental times around mid-gestation. In the developing skin, where this process has been studied in great detail, *TP63* plays a key role in the maintenance of stem cell populations and/or the transition from simple to stratified and glandular epithelia (Crum and McKeon, 2010). In this context, it has been implicated in the switch from a horizontal to vertical plane of epithelial cell division that accompanies stratification (Knoblich, 2010; Lechler and Fuchs, 2005).

TP63 has also been shown to play a key role in the balance between epithelial/keratinocyte proliferation in antagonism with p53 and Notch signaling (Dotto, 2009). However, the role of p63 in SCC has been the subject of debate, with divergent conclusions. Aggregate evidence suggests that p63 plays a positive tumor promoting function in the initial stages, but suppressive at later stages (Missero and Antonini, 2014). This protein is used as a diagnostic marker of squamous versus adenocarcinoma forms of lung and esophageal cancer (Crum and McKeon, 2010), and it is very frequently overexpressed in SCCs of various body sites. In fact, amplification of the *TP63* locus is a strikingly common occurrence in this kind of tumors, cervical carcinoma included, while *TP63* amplification is rarely found in other tumor types with the exception of uterine CS (Figures 2 and 3). *TP63* and *SOX2* have adjacent chromosomal localization and are frequently co-amplified with cooperative effects on control of diverse genomic loci (Watanabe et al., 2014).

TP63 missense mutations of uncertain significance have been found only in a small minority of SCCs (specifically HNSCCs), while they are a frequent occurrence in melanomas, consistent with a proposed role of *TP63* in malignant progression of this tumor type (Matin et al., 2013). A direct p63 target of likely relevance in the context of SCC development is *FGFR2*, with increased FGFR signaling promoting cancer development (Ferone et al., 2012; Ramsey et al., 2013). In other settings, specifically breast cancer, loss of p63 function has been associated with increased invasion and metastatic spread through a number of possible mechanisms (Adorno et al., 2009; Hu et al., 2008; Piccolo et al., 2013).

TP63 is also likely to play an important role in early tissue alterations preceding cancer development. In fact, its inappropriate and increased expression has been linked to squamous metaplasia in both tracheal and esophageal epithelium (Daniely et al., 2004).

- *SOX2*: *SOX2* codes for a member of the Sox family of transcription factors with a key role in pluripotency of embryonic stem cells and reprogramming of cell fate, stem cell potential and cancer (Weina and Utikal, 2014). It is also essential to embryonic development of the esophagus and its separation from the trachea and the emergence of a stratified squamous epithelium (Que et al., 2007). In the lung, *SOX2* appears to be selectively involved in cancer development along the squamous lineage, as amplifications of the gene occur at a staggering frequency (>50%) in LSCCs (Bass et al., 2009), while, in adenocarcinoma, another cell lineage determinant gene, *NKX2-1*, is amplified instead (Weir et al., 2007). *SOX2* amplification is also critical in ESCC and LSCC (Bass et al., 2009) and is frequent in HNSCC (Schrock et al., 2014) (Figures 2 and 3). *TP63*, *SOX2* and *PIK3CA* reside in the 3q chromosomal region and co-amplification of these genes together with *FGFR1* has been recently reported in LSCCs, pointing to a possibly important level of cross-activation (Toschi et al., 2014).

In LSCCs, *SOX2* and *PRKCI* are also frequently co-amplified, with phosphorylation of SOX2 by protein kinase C iota, the *PRKCI* gene product, enhancing Hedgehog ligand production and consequently increased cancer stem cell potential (Justilien et al., 2014). In skin SCCs, the *SOX2* gene is rarely

amplified, but *SOX2* appears to be selectively expressed in cancer stem cell populations and be required for skin SCC development, with *TP63* as one of its direct transcriptional targets (Boumahdi et al., 2014). SOX2 and p63 have also been found to physically interact and converge on a large number of common gene targets with pro-oncogenic potential like *ETV4*, in ESCC and LSCC cell lines (Watanabe et al., 2014). The activity of SOX2 extends to control of *NOTCH1* and *NOTCH2* expression, with interplay between the two cell regulatory networks playing a possibly important role in determining cells of origin and subtype of KRAS-induced lung tumors (Xu et al., 2014b).

- NRF2: The interplay between production of reactive oxygen species (ROS) and metabolism plays an important role in the balance between stem cell renewal and commitment to differentiation (Bigarella et al., 2014). The NRF2 transcription factor is a key regulator of enzymes involved in the protective response against ROS and in compound metabolism (Schafer and Werner, 2015). Activating mutations of *NFE2L2*, coding for NRF2, are a frequent event in SCCs of various types, mutually exclusive with putative loss-of-function mutations of the NRF2-inactivating *KEAP1* gene (Liao et al., 2012).

Recent evidence indicates that self-renewal of basal epithelial stem cells of the large airways is controlled by dynamic variations in ROS levels through NRF2-dependent activation of Notch signaling (Paul et al., 2014) with possibly important implications for cancer development. More directly, in the skin, NRF2 function has been linked to heterogeneity of SCC stem cell populations, with

association and stabilization of NRF2 by $p21^{CDKN1A}$ resulting in enhanced ROS protection and resistance to chemotherapeutic agents (Oshimori et al., 2015). Such mechanism may also contribute to the enhanced resistance to these agents in keratinocytes with NOTCH1 activation in which *CDKN1A* expression is induced (Mandinova et al., 2008).

Reflecting this complexity of biological functions, pharmacological NRF2 activation exerts significant chemopreventive effects in both experimental and clinical settings (Schafer and Werner, 2015), while NRF2 inhibitory compounds have promising therapeutic potential for diminishing survival of tumor cells (Liao et al., 2012).

SQUAMOUS DIFFERENTIATION NETWORK

- NOTCH: Notch signalling is a key developmental pathway and form of direct cell-cell communication that is used to synchronize behaviour of closely connected cells (Kopan and Ilagan, 2009). Of the major developmental signalling pathways, Notch is the only one with an established direct role in the switch between proliferation and differentiation of keratinocytes (Dotto, 2008; Lefort and Dotto, 2004). It is also involved in maintenance of normal skin structure and function, through control of the permeability barrier function (Demehri et al., 2009a; Demehri et al., 2009b; Dumortier et al., 2010). This pathway appears to play an equally important regulatory role in the oral (Papagerakis et al., 2014),

esophageal (Croagh et al., 2014) and bronchial epithelia (Garcia Campelo et al., 2011).

Of the four family members expressed in mammalian cells, *NOTCH1* plays an especially important role in promoting keratinocyte differentiation and tumor suppression (Dotto, 2008). This gene is a direct target of p53 in keratinocytes and its down-modulation in keratinocyte-derived tumors and SCCs – with resulting defects in differentiation - can be explained by mutation of *TP53* (Lefort et al., 2007; Yugawa et al., 2007), down-modulation of *TP53* expression by increased EGFR activation (Kolev et al., 2008) or pharmacological inhibition of calcineurin activity via increased ATF3 expression (Wu et al., 2010). In cervical carcinoma cells, *NOTCH1* down-regulation is required for sustained HPV E6 expression and consequently compromised p53 function (de Wilde et al., 2008; Talora et al., 2005; Talora et al., 2002). In this context, however, a positive role of the Notch pathway in enhancing cancer stem potential has also been proposed (Bajaj et al., 2011). This may be amenable to substantially different roles of this pathway in distinct cell populations of the same lineage and/or a pro-survival function of Notch shared across cell types (Dotto, 2008). In ESCC cells, compromised *NOTCH1* expression, resulting from concomitant loss of *TP53* and *KLF5* transcription, was linked to malignant progression (Yang et al., 2011), and in primary esophageal keratinocytes Notch activation induces senescence through a $p16^{INK4a}$-dependent mechanism (Kagawa et al., 2014).

Together with reduced expression, inactivating mutations of *NOTCH1* have been found with elevated frequency in HNSCC, LSCC, ESCC and CSCC

(Figures 2-4; Table 2), consistent with a role in tumor suppression. However, as discussed in the context of CvSCCs, this may be an over-simplification, consistent with the identification of activating *NOTCH1* mutations in a subset of HNSCCs (Sun et al., 2014). Transcriptome analysis of tumors for signs of Notch activity is difficult to interpret. In fact, "canonical" Notch targets of the Hes/Hey families function as transcriptional repressors of their own expression (Iso et al., 2003), so their observed up- or down-regulation can be variously interpreted as Notch activation or suppression. More importantly, SCCs are highly heterogeneous tumors and augmentation or suppression of Notch activity needs to be interpreted in the context of specific cell types (growing versus differentiating tumor cells, intermingled stromal cells of various types, infiltrating inflammatory cells).

Like *NOTCH1*, *NOTCH2* and *NOTCH3* are also frequently mutated in SCCs, with both missense substitutions and nonsense and frameshift alterations (Table 2, Figure 4). While *NOTCH2* does not play an essential role in keratinocyte differentiation and tumor suppression, combined loss of *NOTCH1* and *NOTCH2* has more significant consequences than loss of *NOTCH1* alone (Pan et al., 2004), pointing to a complementary function of the two receptors. Loss of *NOTCH3*, individually or in combination with *NOTCH1* and/or *NOTCH2*, results in no phenotype in mouse skin (Pan et al., 2004). The specific role that this receptor may play in human stratified epithelia and cancer has only started to be addressed, with the finding that loss of *NOTCH3* in the esophageal epithelium disrupts normal stratification and differentiation (Ohashi et al., 2010).

- Fat1: A distinguishing feature of squamous epithelia is their tight cell-cell junction organization and packing, with polarization along the basal-apical axis but also along the main body axis. Mutations in genes encoding classical adherens junctions and desmosomal proteins, like *DSG1-4*, occur in SCCs of various types but with relatively low frequencies. By contrast, frequently mutated is *FAT1* (Figures 2 and 3; Table 2), belonging to the cadherin superfamily, whose ortholog in Drosophila plays a well demonstrated tumor suppressing function as well as a key role in planar cell polarity (Sadeqzadeh et al., 2014). In mammals, four *FAT* family members have been identified, of which *FAT4* is the most closely related to the Drosophila gene, while the others, *FAT1* included, exert both synergistic and antagonistic functions (Saburi et al., 2012). Recent studies, focused mostly on *FAT1*, point to a complex interplay with other major pathways like ß-catenin and HIPPO signalling (Sadeqzadeh et al., 2014). However, this field is still in its infancy and the role that these molecules, as well as other planar cell polarity components, play in squamous cell differentiation and cancer remains to be elucidated.

EPIGENETIC REGULATORS

Epigenetic regulators, specifically enzymes involved in DNA methylation and histone modification, are attractive drug targets, as epigenetic alterations are potentially reversible and can critically regulate the balance between cancer stem cell renewal and commitment to differentiation (Campbell and Tummino, 2014).

The overall mutation incidence of this family of genes in SCCs is >50%. Frequently mutated genes in SCCs from various body sites include *EZH2*, *EP300, MLL2*, *MLL3*, *NSD1*, *MED1, DDX3*, and *SYNE1* (Table 2). Earlier studies showed that p300 (coded by *EP300)* is essential for cell cycle withdrawal of terminally differentiating keratinocytes (Missero et al., 1995), with subsequent work implicating p300 in a large variety of transcriptional regulatory mechanisms involved in this process (Wang et al., 2013). Another epigenetic regulator specifically implicated in squamous differentiation is EZH2, a key component of the Polycomb repressive complex 2 with histone methyltransferase activity and serves as a drug target (McCabe and Creasy, 2014). *EZH2*, which is frequently mutated in cancer (Yamaguchi and Hung, 2014), controls proliferative potential of self-renewing keratinocyte populations by repressing the *INK4A-INK4B* locus and preventing the recruitment of AP1 transcriptional factors to terminal differentiation marker genes (Ezhkova et al., 2009). Sustained EZH2 activity is required for survival of keratinocytes cancer stem cell populations (Adhikary et al., 2015), and in both skin and lung bronchial epithelium increased *EZH2* expression has been associated with malignant SCC progression (Behrens et al., 2013; Xie et al., 2014). EZH2 is also a mediator of the negative effects that increased levels of HOTAIR have on E-cadherin expression in HNSCC cells, with consequently enhanced malignant progression (Wu et al., 2015).

The mixed lineage leukemia (*MLL*) genes (*MLL2* and *MLL3*: *KMT2D* and *KMT2C*, respectively) encode H3K4 methyltransferases. *MLL* translocations result in MLL fusion proteins that play a causative role in acute myelogenous

leukemia (Krivtsov and Armstrong, 2007). Truncating or missense mutations in these genes have also been described in a variety of tumors, including most SCCs, especially CvSCC (Figures 2 and 3; Table 2). The specific consequences of these mutations in SCC development remain to be established. *MED1*, coding for a component of the transcriptional coactivator complex Mediator (MED) with an essential role in keratinocyte differentiation (Oda et al., 2012), is also frequently mutated in SCCs. The impact of mutations in this and other chromatin modifying genes in SCC development remains an interesting topic for future studies.

Besides mutations of these genes, additional evidence points to a key role of epigenetic modifications in SCC development. Super enhancer and transcriptional profiling of stem cell populations isolated from keratinocyte-derived SCCs revealed that the Ets2 transcription factor is a key regulator of epigenetic changes associated with malignant behavior, acting upstream of other transcription factors like Elk3 and AP1 family members and in possible antagonism with polycomb silencing (Yang et al., 2015) . While *ETS2* and related family members are rarely mutated in SCCs, these transcription factors are under positive RAS/MAPK control, providing a possible link between activation of this pathway and chromatin alterations.

FUTURE DIRECTIONS: A UNIFIED APPROACH TO RESEARCH, DETECTION, PREVENTION AND THERAPY

SCCs have been anatomically separated for convenient clinical management, which is not without historical merit. However, as discussed in this review, their etiologies and molecular properties point to a landscape with sundry similarities. As depicted in Figure 5, an essential commonality that distinguishes SCCs from all other cancer types are genetic alterations of specific determinants of squamous differentiation, most notably *NOTCH, TP63* and *SOX2* genes, and their interplay with general regulators of the cancer process such as p53 as well as cyclin D1, the latter downstream of EGFR and RAS activation. Furthermore, growth/differentiation of epithelial cells is tightly linked to cell adhesion, and altered expression and/or mutations in cell adhesion genes like *CDH1*, *CTNND1 and DSG1-3* (coding for E-cadherin, p120-catenin and desmogleins, respectively) and integrin receptor genes play a critical role in various aspects of SCC behavior (Janes and Watt, 2006; Jeanes et al., 2008).

SCCs viewed in this unified fashion may merit standardized approaches to research efforts in prevention, diagnosis, prognosis and therapy. For instance, inducers of terminal cell differentiation could be beneficial to exhaust cancer stem cell populations harnessed by a common type of molecular mechanisms. Conversely, as squamous differentiation results in increased cell survival, inhibitors of the differentiation process when this is activated could be beneficial in combination with conventional pro-apoptotic chemotherapeutic agents or drugs against recently identified targets (such as PI3K and AKT inhibitors). Consideration of common properties across SCCs as they relate to epigenetics, genomics, genetics and transcriptomes may serve as a foundation for individual

and combinatorial therapeutics, as well as understanding the mechanistic basis of treatment resistance.

A unified view of SCCs also prompts attentive consideration of reported differences in response to specific treatments depending on body sites. Some of these differences may indeed reflect specific frequencies of gene mutations, various cells of origin and/or intermediate steps, such as squamous dysplasia in the lung. However, other reported differences may be the result of independent clinical trials being carried out for various SCCs, different criteria, and patients' staging. A case of relevance is the response of patients with advanced LSCC to second-line treatment with erlotinib (a reversible EGFR tyrosine kinase inhibitor) and, with greater efficacy, afatinib (an irreversible ErbB family blocker) (Soria et al., 2015). While advanced stage HNSCC was initially reported not to respond to erlotinib, this perspective has changed (Gross et al., 2014), and a favorable response of HNSCC patients to afatinib has now been observed (Machiels et al., 2015)**.**

A unified view of SCC is also likely to extend to two emerging and interconnected areas of general importance for cancer management, one involving stromal alterations and the other the immune system. Several lines of evidence indicate that changes in stromal tissue can play a primary role not only in progression of the disease but also initiation (Dotto, 2014). Convergent pathways are involved, including Notch/CSL (Hu et al., 2012) (Procopio et al., 2015), TGF-ß (Bhowmick et al., 2004) (Goudie et al., 2011) and SHH/Gli signaling (Junttila and de Sauvage, 2013). Collectively, stromal alterations in

these and other pathways make an attractive target for preventive and therapeutic approaches against SCC development. (Dotto, 2014; Junttila and de Sauvage, 2013).

Neoadjuvant chemotherapy and radiation are mainstays of early stages of SCC treatment. Adjuvant chemotherapy is also often pursued after surgery. Newer and rapidly emerging therapeutics are kinase inhibitors and immunotherapy, the latter of which involves immune checkpoint inhibitors (Sharma and Allison, 2015). The number of somatic genetic mutations in cancers may be critical in exploiting the balance between immune surveillance and immunosuppression, and hence, in immunotherapy. SCCs in various body sites are among the cancer types with the highest percentage of somatic genetic mutations (Alexandrov et al., 2013), and mutations of *HLA* genes in these tumors suggest a possible stratification of patients for their predicted response to immunomodulatory agents (Liao et al., 2012). An important concept is that genetic alterations of tumors can impact the immune microenvironment, opening some novel windows of opportunity for treatment around STK11 (LKB1)/AMPK signaling (Xu et al., 2014a). Additionally, immature myeloid cells have been demonstrated to be important in ESCC and represent a population of cells for therapeutic targeting (Karakasheva et al., 2015; Waldron et al., 2013).

Overall, by viewing SCCs at various body sites as a cohesive theme, new insights can be gained. However, more consistency is needed between studies of various types, standardizing major confounders such as geographic/ethnic differences of patient populations, stages and grades of tumors, degree of

normal/tumor cell admixture, intrinsic tumor cell heterogeneity and the contribution of stromal components.

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LEGENDS

Table 1. Environmental risk factors for SCC

Table 2. Common genetic mutations by function in SCC

Figure 1. Incidence and mortality for major SCC types

* Non-melanoma skin cancer (NMSC) comprises SCC and basal cell cancer and incidence is challenging to estimate as these cases are not required to be reported to US or worldwide cancer registries. A study in 2006 noted 3.5 million NMSC cases among 2.2 million people in the United States.

** Non-small cell lung cancers (NSCLC) comprise about 85% of all lung cancers, and are divided further into SCC, adenocarcinoma and large cell cancer. Thus, lung SCCs represent a significant component of the NSCLCs.

*** Head/neck cancers are almost exclusively SCC. They are classified further as follows: oral cavity (including tongue and mouth), oropharynx (including tonsil and oropharynx) and other HNC (including larynx and poorly-specified tumors of the lip/oral cavity/pharynx)

****In the US there is a higher proportion of esophageal adenocarcinomas than SCC, this proportion is reversed for worldwide statistics.

Statistics calculated from: Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. GLOBOCAN 2012

v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr, accessed on 27/09/2015.

Figure 2. Pattern of frequently altered genes (>5% frequency) in HNSCC. The results shown here are based upon data generated by the cBioPortal for Cancer Genomics (http://www.cbioportal.org/index.do) (Cerami et al., 2012; Gao et al., 2013), and represent the genes relevant to this review with >5% alteration frequency. The dataset included all tumor samples with sequencing and CNA data (n=279). Mutually exclusive alterations were found for 94 gene pairs, only 2 of which statistically significant: *TP53* - *EP300* (p = 0.01) and *KMT2D* – *NFE2L2* (p = 0.038).

Figure 3. Pattern of frequently altered genes (>5% frequency) in LSCC. The results shown here are based upon data generated by the cBioPortal for Cancer Genomics (http://www.cbioportal.org/index.do) (Cerami et al., 2012; Gao et al., 2013), and represent the genes relevant to this review with >5% alteration frequency. The dataset included all tumor samples with sequencing and CNA data (n=178). Mutually exclusive alterations were found for 90 gene pairs, only 1 of which statistically significant: *TP53* – *KMT2C* (p = 0.017).

Figure 4. Specific pattern and mapping of Notch1, Notch2 and Notch3 mutations in HNSCCs. The results shown here are based upon data generated by the cBioPortal for Cancer Genomics (http://www.cbioportal.org/index.do) (Cerami et al., 2012; Gao et al., 2013).

Figure 5 Model of potential interactions of epigenetic and genetic alterations in SCC development.

Schematic of potential interactions amongst epigenetic and genetic alterations that may contribute, directly and indirectly, to SCC initiation and progression. Proteins with commonly accepted tumor promoting and suppressing functions are highlighted in red and blue respectively, while a protein involved in epigenetic regulation, p300, is highlighted in green.

Figure 1

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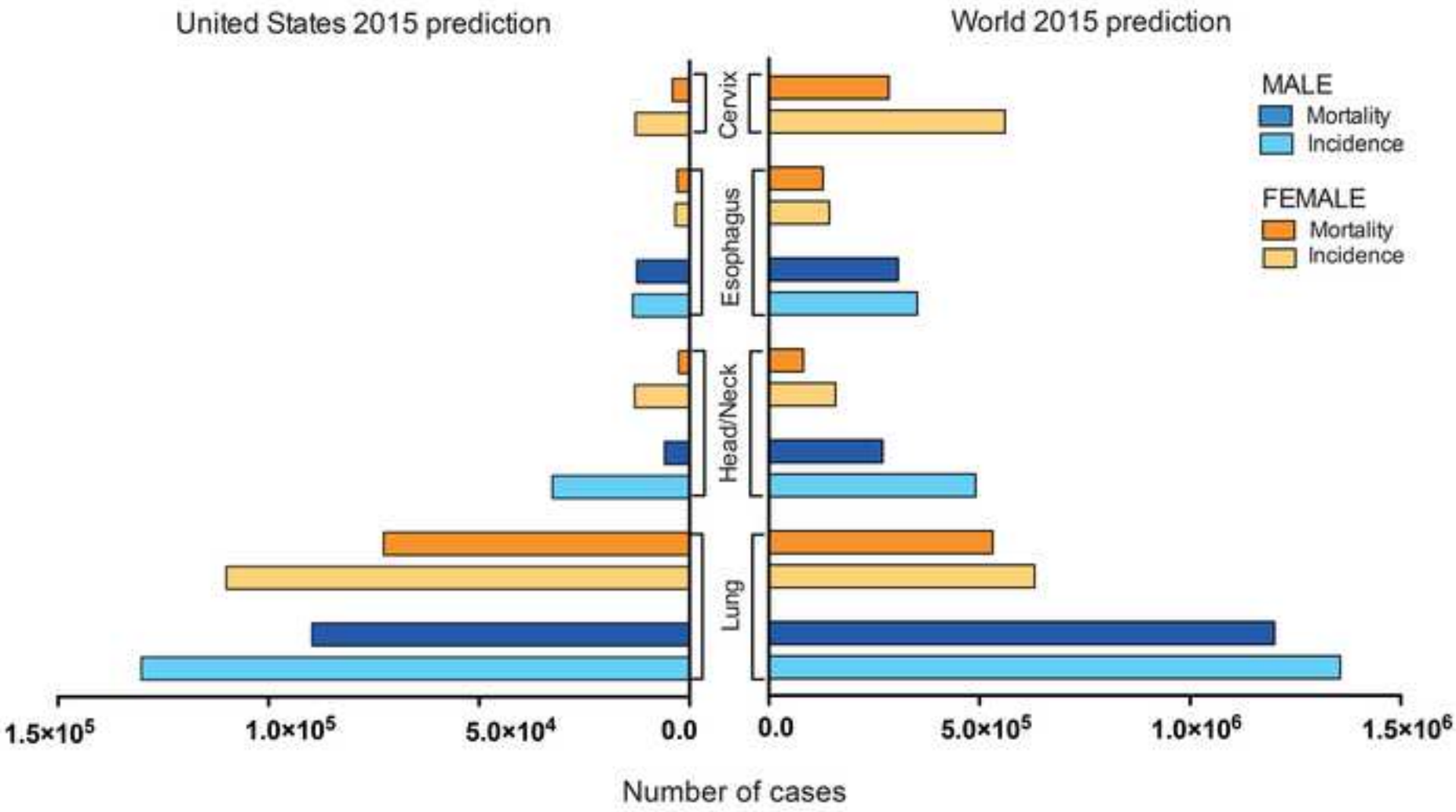

Figure 2

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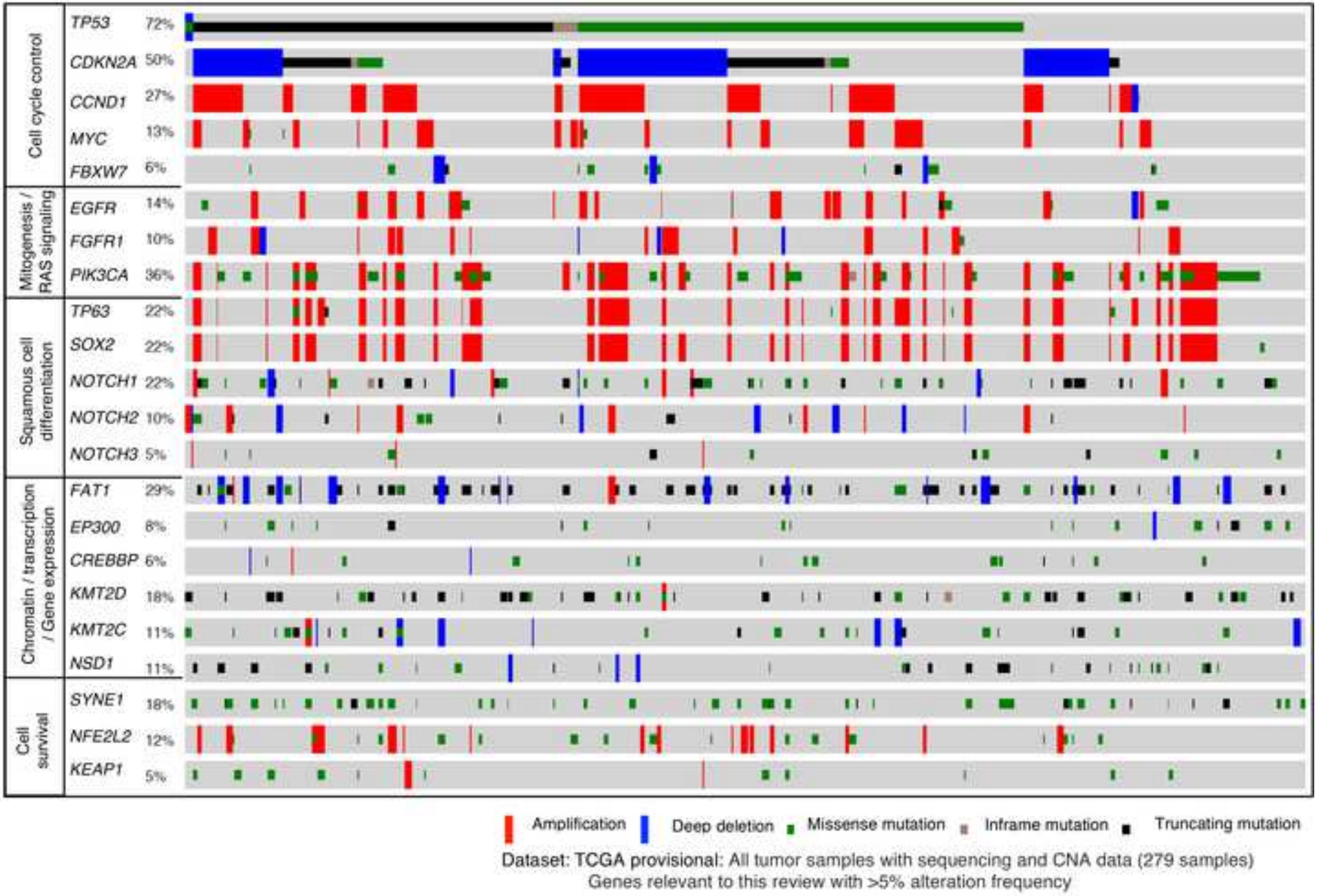

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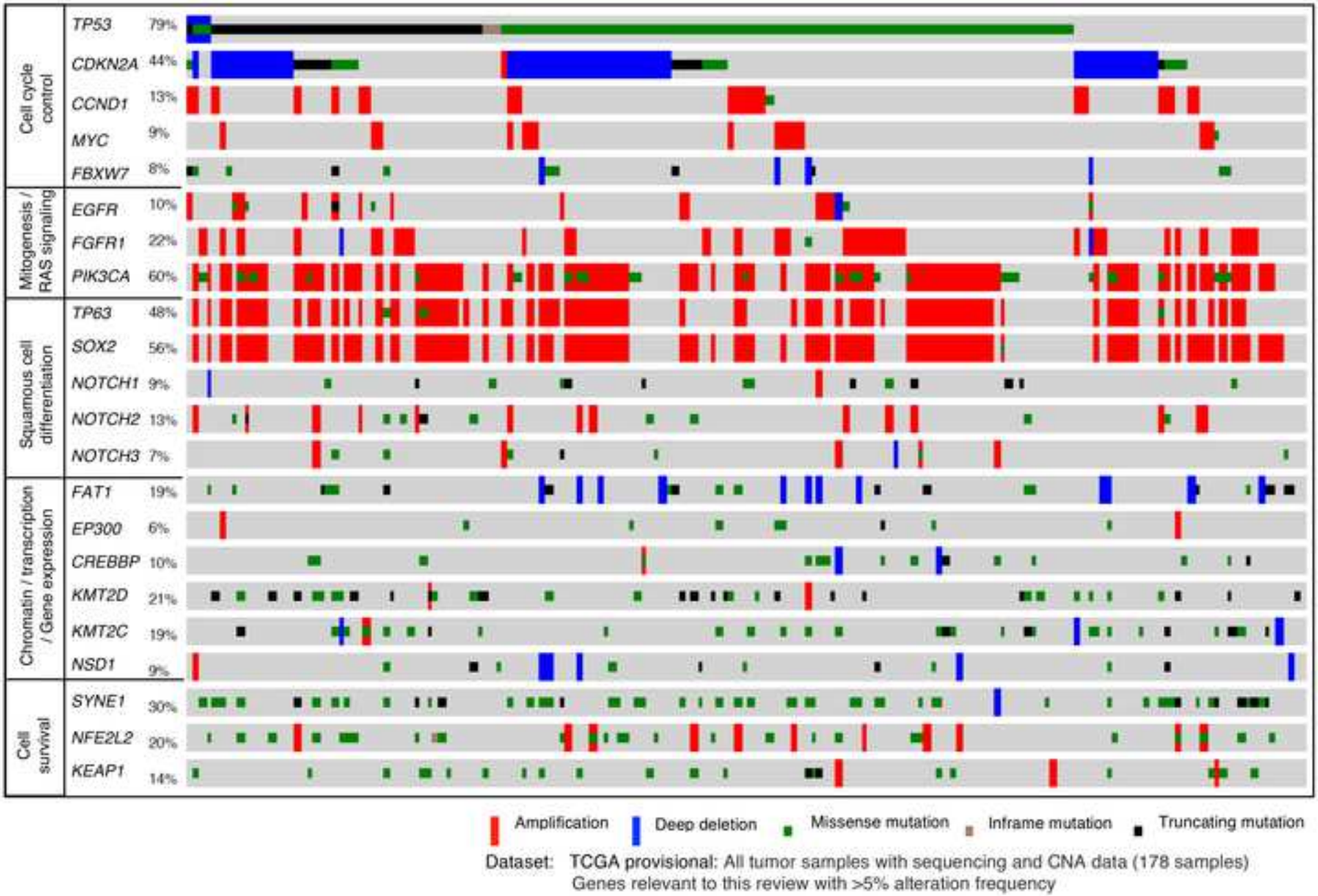

Figure 4

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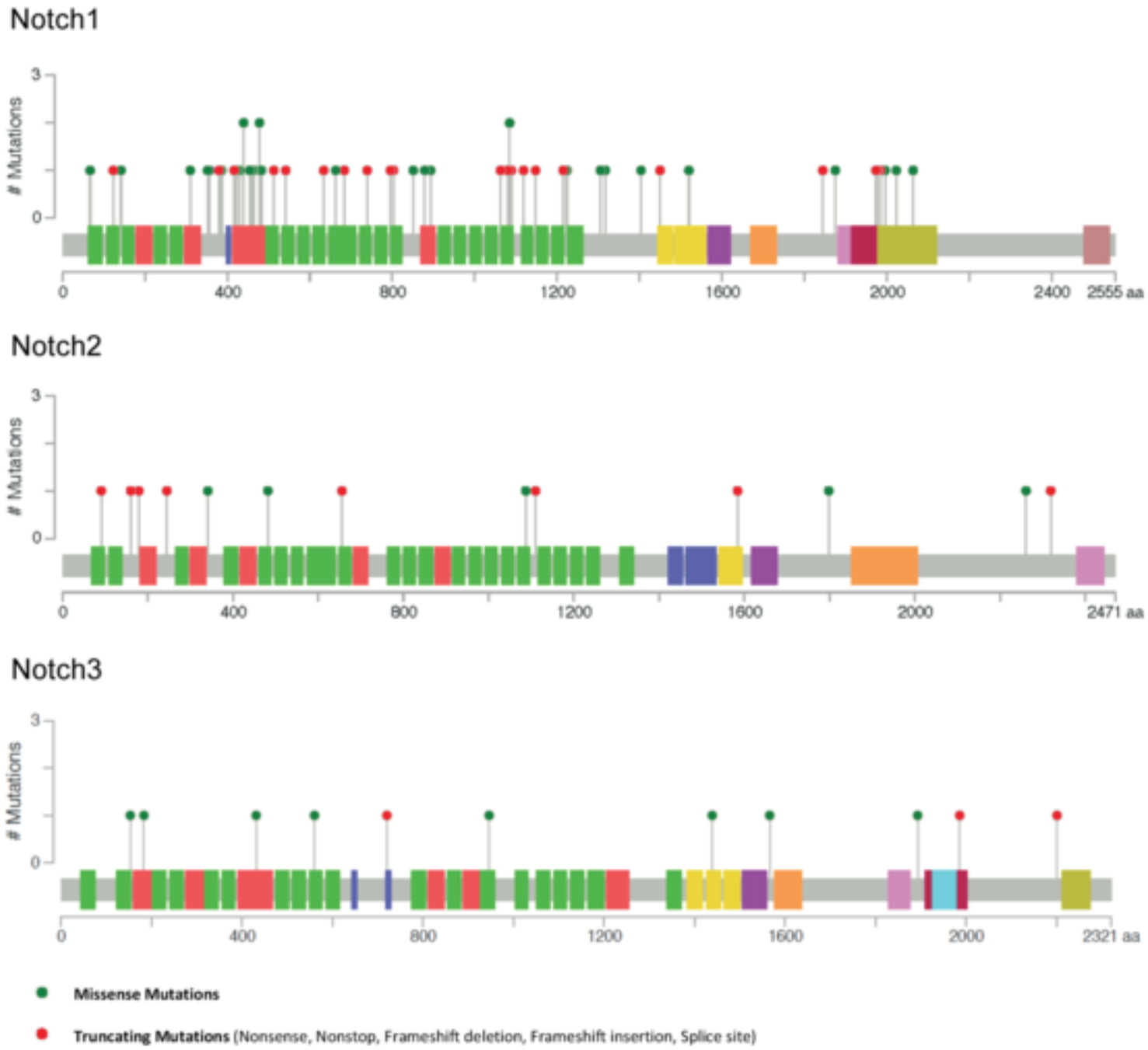

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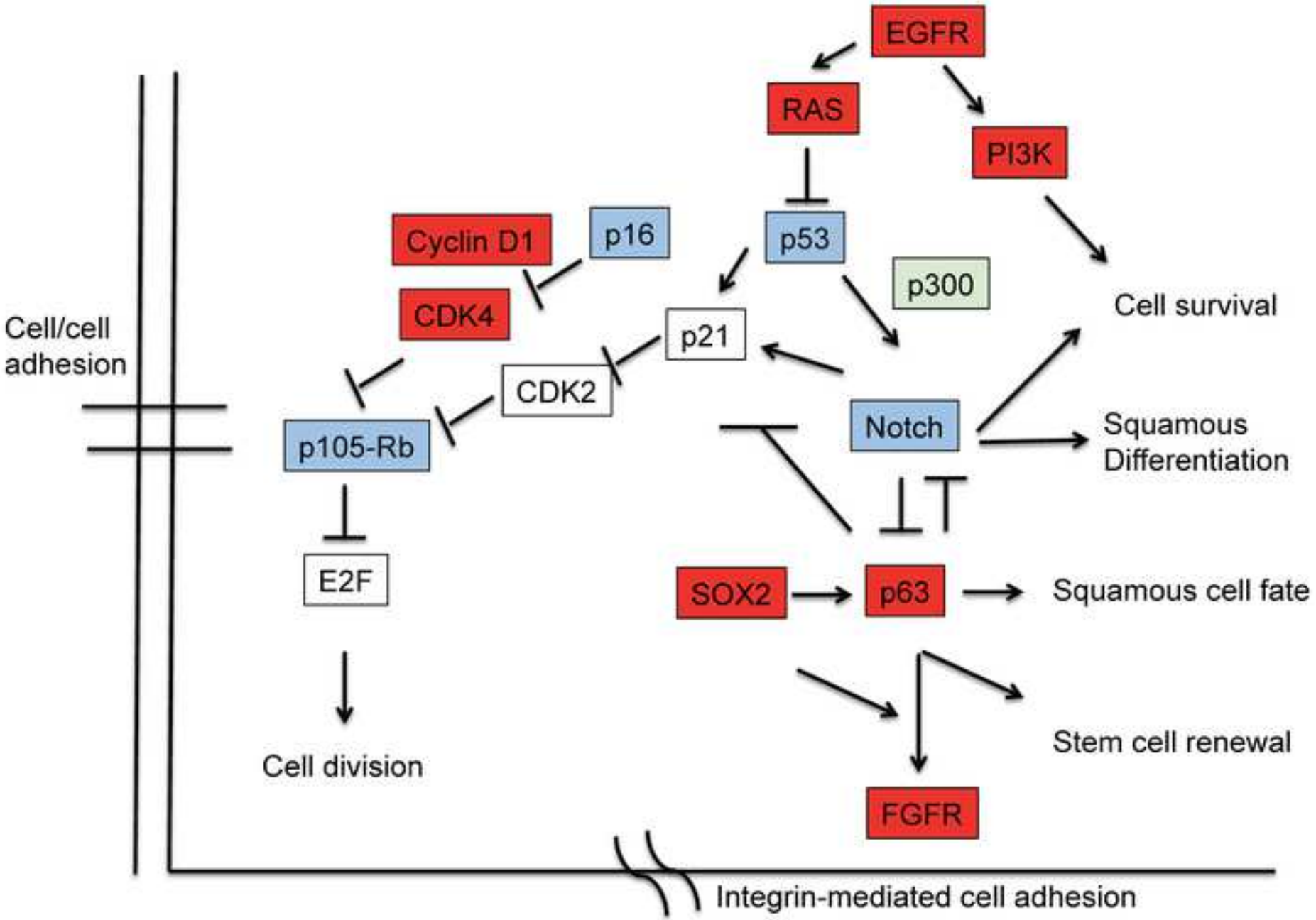

Table 1

Skin	**UV exposure** □ Sun sensitivity (low melanin) □ A history of excessive sun exposure □ Use of tanning beds **Immunosuppressive therapies** □ Calcineurin inhibitors **A history of skin cancer** □ Recurrence rate **Infection** □ HPV
Lung	**Cigarette** □ History of smoking (quantity, type and duration) □ Exposure to second hand smoke **Radon gas** **Asbestos** (especially among smokers) **Metals** □ Arsenic, cadmium, chromium **Organic solvents** **Radiation** **Air pollution** (e.g. diesel exhaust) **Miscellaneous**: □ Rubber manufacturing, paving, roofing, painting, and chimney sweeping **Infection** □ *Mycobacterium tuberculosis*
Head/Neck and Esophageal	**Cigarette** □ History of smoking (quantity, type and duration) **Alcohol** □ History of drinking (quantity and duration) **Nutritional deficiencies** □ Vitamins, minerals **Infection** □ HPV □ EBV
Cervical	**Infection** □ HPV **Miscellaneous**: Exacerbated by immunosuppressed state, cigarette-smoking, high number of childbirths, long-term oral contraceptive use

Table 2

Genes	Relative frequency of mutation (%)							
	CSCC[1]	**HNSCC[2]** (HPV mixed)	**HNSCC[2]** (HPV-)	**HNSCC[2]** (HPV+)	**CvSCC[3]**	**ESCC[4]**	**LSCC[5]**	**Mixed SCC[6]**
Cell cycle control								
TP53	61-95	72	47-100	3-5	5	82-93	72-91	65
RB1		5	1-4	5-6	4	7-9	4-15	6
CDKN2A	33-44	49	9-86			4-20	3-44	24
CCND1		26	22-45	3		33	12	16
MYC		13	6-14	3			5	6
FBXW7		6	5-20	4-5	15-16	3-5	4-6	7
Mitogenesis / RAS signaling								
EGFR		13	15	6-12		6	4-8	8
FGFR1		10	10			1	7-17	4
FGFR2		1	2			1	3	
FGFR3		3	2	11-14			2-4	
DDR2[7]		3	3-6	6			3	
HRAS	16-23	3	3-9	6-9			3	7
KRAS	13-14	1		6-10	4		3	6
NRAS	5	3					1	
AJUBA[7]	18	7	7			2-7		
MAPK1		3			8		2	
PIK3CA	10	35	5-34	22-56	14-20	5-9	9-48	29
PTEN		3	5-12	6-20		1	8-11	7
BRAF	18	3	1				5	
Squamous cell differentiation								
TP63		22	8-19	28	4		29	
SOX2		21	5	15			42	18
NOTCH1	59-73	21	15	6-17		9-13	8-15	12
NOTCH2	51-63	10	8-9		20	4	8	
NOTCH3		5	3		4	2-6	6	
FAT1	44	29	14-32	3		4-11	19	
Chromatin / transcription / gene expression control								
EZH2		2	3	18		1	3	
EP300		7			16	3-10	4	
KMT2D (MLL2)[7]	69	17	10-18	10-18		4-19	20-24	
KMT2C (MLL3)[7]	39	10		10		3-6	17	
NSD1[7]		11	10			2	8	
MED1		3	5		4	1	4	
DDX3[7]		2	4		4		1	
SYNE1[7]		17	10-22	9		7-10	29	
Cell survival								
NFE2L2		12	1-14	4		5-10	15-18	
KEAP1		4	5			3	12-16	
CUL3[7]		5	2			1	7	
CASP8[7]	23	11	10-11	3	12	1	1-4	

1) Pickering et al., 2013; South et al., 2014; Wang et al., 2011 **2)** Agrawal et al., 2011; Cancer Genome Atlas, 2015; Lechner et al., 2013; Pickering et al., 2013; Seiwert et al., 2015; Stransky et al., 2011; Hedberg et al., 2016 **3)** Muller et al., 2015; Ojesina et al., 2014); **4)** Gao et al., 2014; Lin et al., 2014; Song et al., 2014; Zhang et al., 2015 **5)** Hammerman et al., 2012; Hoadley et al., 2014; Kim et al., 2014 **6)** Mixed SCC: 30% HNSCC, 25% LSCC, 10% CSCC, 10% CvSCC; Schwaederle et al., 2015**. 7)** Genes not described in the text: *DDR2,* Discoidin Domain Receptor Tyrosine Kinase 2; *AJUBA,* Ajuba LIM protein; *KMT2D (MLL2),* Lysine (K)-Specific Methyltransferase 2D; *KMT2C (MLL3),* Lysine (K)-Specific Methyltransferase 2C; *NSD1,* Nuclear Receptor Binding SET Domain Protein 1; *DDX3,* DEAD (Asp-Glu-Ala-Asp) Box Helicase 3, X-Linked; *SYNE1,* Spectrin Repeat Containing, Nuclear Envelope 1; *CUL3,* Cullin 3; *CASP8,* Caspase 8, Apoptosis-Related Cysteine Peptidase.