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Different Facets of Copy Number Changes: Permanent, Transient, and Adaptive

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Running Title: Facets of Copy Number Change

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Abstract

Chromosomal copy number changes are frequently associated with harmful consequences and thought of as an underlying mechanism for the development of diseases. However, changes in copy number are observed during development and occur during normal biological processes. In this review, we will highlight the causes and consequences of copy number changes in normal physiologic processes as well as cover the association with cancer and acquired drug resistance. We will discuss the permanent and transient nature of copy number gains and relate these observations to a new mechanism driving transient site-specific copy gains (TSSGs). Finally, we will discuss implications of TSSGs in generating intra-tumoral heterogeneity and tumor evolution and how TSSGs can influence therapeutic response in cancer.

Introduction

It was long been thought that the DNA sequences of healthy individuals were 99.9% identical to each other (1). However, genome-wide sequencing efforts in individuals from multiple ethnicities have revealed more variations in the genetic architecture than was previously appreciated (2-4).

These genomic alterations have been termed “structural variants”, which are further classified as being microscopic or sub-microscopic depending on the size of DNA involved (5). The microscopic variations have historically been identified through chromosome banding techniques (6) and comprise at least 500 kilobases (kb) of DNA (7). Examples of these variants are whole-chromosome gains or losses [referred to as aneuploidy; (7, 8)], translocations [change in location of a chromosomal segment; (9)], deletions [deletion of a DNA segment compared to the rest of the chromosome; (10)], duplications [a chromosomal segment that occurs as two or more copies per haploid genome; (11)] and inversions [reversal in orientation of a DNA segment compared to the rest of the chromosome; (12, 13)]. A schematic of structural variants resulting in copy number changes are shown in Figure 1. With the development of more sophisticated tools such as array-based comparative genomic hybridization [array CGH; (14-16)], smaller variants (sub-microscopic alterations) in the size range of 1kb-500kb can be detected (5). Genome sequencing has further revealed small insertions and deletions (INDELs) spanning from 1 to 10,000 base pairs across the human genome, which could cause considerable variability in the human population (17, 18).

The most common variant identified under sub-microscopic alterations is copy number variation (CNV). CNV is defined as a genomic segment of more than 1kb present at a variable copy number in comparison to a reference genome (19-22). The first studies

documenting the genome-wide presence of CNVs in normal human genome came from the work of Lee (23) and Wigler laboratories (24). These studies described more than 200 large-scale CNVs (LCVs; about 100kb or greater) in normal individuals. These studies also paved the way for the creation of Database of Genomic Variants (DGV) in 2004, which catalogues all the human CNVs and structural variations present in healthy individuals.

The sequencing efforts from the International HapMap Consortium (25) and 1000 Genomes project (26) have led to the identification and frequency determination of novel CNVs in the human genome. CNVs are now known to contribute to 4.8%-9.5% of the variability in the human genome (27, 28), which is more than what is accounted for by single nucleotide polymorphisms (SNPs; accounting for 0.1% of the variations) (29). Recently, the CNV map for the human genome was constructed (28), which documented all the small and large-scale CNVs present in normal healthy individuals. CNVs can either have no phenotypic consequences in individuals (4, 23, 24), or can lead to adaptive benefits that have been observed in a wide range of species (5).

One of the major challenges in the field is to distinguish benign CNVs (events that do not lead to phenotypic consequences) from pathogenic CNVs that underlie diseases (30). Pathogenic CNVs are often associated with deleterious consequences because of imbalance in gene dosage (31) and/or aberrant chromosomal structure (5, 7, 32, 33). Pathogenic CNVs have been associated with several disorders: obesity (34), diabetes (35), developmental disorders (36), psychiatric diseases (37) such as autism spectrum disorder (38), schizophrenia (39) and Alzheimer's disease (40, 41) and cancer (42-44). In this review, we will focus mainly on copy number alterations observed in cancer and their functional implications.

CNVs can either be present in the germline or can arise in phenotypically normal tissues and organs, which are then referred to as somatic CNVs (45, 46). Instead of being randomly present in the genome, CNVs are preferentially found to occur in regions that are rich in low copy repeats (segmental duplications) (47-50), heterochromatic areas (*e.g.*, telomeres and centromeres), replication origins and palindromic regions (28). There are several proposed mechanisms that underlie the generation of somatic CNVs: non-allelic homologous recombination (NAHR), non-homologous end joining (NHEJ), defects in DNA replication, DNA damage response and repair pathways. These mechanisms have been extensively discussed elsewhere, therefore we refer our readers to these reviews (32, 33, 51).

In this review, we explore the relationship between copy number changes and biological consequences, with a particular focus on development and tissue homeostasis under physiological as well as pathological conditions. This review will focus on these relationships especially in the context of cancer. We will further discuss a recently discovered process driving transient site-specific copy number gains (TSSGs) in cancer cells and its implications during adaptive responses such as stress and chemotherapeutic sensitivity.

Copy Number Changes In Development and Physiology

Chromosomal copy number changes and the associated gene amplifications and losses are observed during development in both lower and higher eukaryotes [reviewed in (7)]. The appearance of CNVs during normal biology suggests that copy number changes can have important functional consequences. A common hypothesis is that increased gene dosages during development provides an advantage during selective pressures and environmental conditions (7). Here, we will discuss examples from developmental

biology and their relationship to functional impact. We will also highlight the relationship between somatic CNVs and tissue homeostasis.

Several lower and higher eukaryotes use gene amplification to respond to cellular signals (Figure 2). Electron microscopy studies in the early 1970s demonstrated that ribosomal genes are amplified for the production of large amounts of ribosomes required during early embryogenesis (52) . Ribosomal DNA (rDNA) amplifications were observed during oocyte formation in amphibians *Xenopus leavis* (53-55), insects such as water beetles (56), molluscs (55) and in the macronuclear rDNA in *Paramecium* (57) and *Tetrahymena* (58). Thus, increase in ribosomal DNA synthesis to meet higher protein synthesis demands in different tissues, highlights gene amplification as a common principle in developmental biology.

Besides rDNA, specific chromosomal regions identified as “DNA puffs” are amplified and expressed to form structural proteins required for cocoon formation in the salivary gland of Sciarid flies (59, 60). Amplification of the DNA puffs occurs in response to the hormone ecdysone, which is required during larval development (60). Another example of gene amplification triggered by developmental signals can be observed during eggshell formation in *Drosophila* (61). Eggshells require amplification of chorion genes in the follicle cells of the ovary and these genes are expressed late in differentiation (61, 62). The amplifications of only specific chromosomal regions and genes and not the whole genome highlights the specific response that can occur across organisms. These examples suggest the ability of cellular cues to trigger these site-specific amplifications, which raises a question about what molecular mechanisms underpin this selective amplification across species.

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Examples of copy number variations are reported in various tissues in mammals. Using techniques such as SKY, FISH and single cell sequencing approaches, various groups have reported both small and large-scale changes in chromosomal copy number in mouse and human tissues, particularly in neurons, liver and skin fibroblasts (Figure 2). For example, approximately 33% of the neuroblasts in the embryonic mouse brain and 20% of neurons in the adult mouse cerebral cortex showed aneuploidy (63). The reduction in aneuploidy in the adult brain was hypothesized to be due to a neuroblast programmed cell death mechanism during brain development (64). Westra and colleagues also uncovered that 15-20% of neural progenitor cells in both mouse and human cerebellum exhibited aneuploidy (65) (Figure 2).

Additionally, high levels of sub-chromosomal CNVs (deletion and duplication events) were observed in the human frontal cortex neurons. Multiple copy number changes were noted within a small set of neurons, suggesting that CNVs might be restricted to either individual cells or specific neural lineages (66). These data suggest that the generation of copy number changes is an important process for achieving diversity in the neuronal populations during central nervous system development. However, this possibility has yet to be proven. It was reported that the transcripts arising from CNVs in the mouse brain are more tightly regulated when compared to other tissues such as lung, liver, heart, kidney and testis (67). It would be important to determine the rate of correlation between CNVs and expression changes in the human brain and whether there are underlying functional consequences of the affected transcripts in generating neural diversity and plasticity.

Somatic CNVs are also observed in mammalian hepatocytes and skin. A study by Duncan and colleagues suggests that approximately 50% of normal adult hepatocytes

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have changes in chromosomal numbers (gains or losses) such that genetically diverse sets of cells are present in the liver (68, 69). However, single cell next generation sequencing has reported a lower level of aneuploidy (<5%) in liver, skin and human neurons (70). The differences in the reported levels of aneuploidies could reflect the different types of assays employed to follow copy number changes (*i.e.*, FISH and SKY versus single cell sequencing, respectively).

The genetic variation resulting from the changes in copy number could be a mechanism employed during tissue development in order to achieve diversity in cell populations. Copy number variations may allow developing tissues to adapt to cellular and growth requirements during tissue expansion and organ development. Another advantage for the observed CNVs could be to adapt to encountered metabolic or toxic challenges, especially by hepatocytes (see discussion in the following section under Mammals). By identifying the regulatory features for regions undergoing CNV and the affected genes in different tissues, we would be able to understand tissue-specific gene expression and underlying diversity within tissues.

Copy Number Changes as an Adaptive Response

Many studies in bacteria, yeast and mammals have shown that copy number changes can arise as a consequence of selection, which may allow cells to exhibit an increased fitness and/or survival advantage. In this section, we discuss the relationship between different cellular conditions and the emergence of CNVs from different species (Figure 2).

Bacteria. Acquisition of antibiotic resistance can occur through the uptake of foreign DNA harboring resistance genes through the bacterial competence pathway (71). A

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recent study by Slager et al. demonstrated that different species of bacteria could increase the copy number of genes involved in the competence pathway (*com* genes) in response to antibiotics causing replication stress (72). These genes are located closer to the origin of replication (OriC) and their amplification occurs through multiple origin firing events at the OriC, which increases their copy number and transcription rates. In *Salmonella typhimurium*, gene amplification aids in the development of antibiotic resistance. Adaptation to the antibiotic cephalosporin occurred through amplification and increased gene dosage/expression of the β-lactamase gene [$bla_{TEM-1;}$ (73)]. The enzyme β-lactamase results in the hydrolysis of cephalosporin (74, 75), which results in reduced drug response.

These highlighted examples illustrate the impact selective pressure can have on DNA amplification and gene expression in bacteria (Figure 2). Additional examples have been observed and are discussed in a review by Sandegren et al. (76). Taken together, the existing data illustrate the relationship between input signals and changes at distinct regions of the bacterial genome. In the future, it will be interesting to know if this selection is based on fitness or the result of targeted DNA replication in prokaryotes.

Yeast. Similar to bacteria, yeast also exhibit changes in DNA content based on selective pressure. For example, gene rearrangements and copy number changes have been observed in *Candida albicans* when passaged through a murine host (77). It has been hypothesized that these changes in ploidy could generate genetic and phenotypic diversity required for adaptation in the new host environment. Consistent with these observations, CNV has been associated with anti-fungal drug resistance and adaptive benefits (78, 79). For example, fluconazole treatment in *C. albicans* results in the development of whole chromosome gains and aneuploidy (80). Upon CGH analyses for

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the copy number changes in 70 azole-resistant and -sensitive strains, Selmecki et al. found increased levels of aneuploidy in resistant strains (50%) compared to the sensitive ones [7.14%; (81, 82)]. Trisomies of chromosome 5, including a segmental aneuploidy consisting of an isochromosome (formed here by the attachment of two left arms of chromosome 5 around a single centromere) were also associated with azole-resistance. Gains of this isochromosome were associated with an increased expression of genes involved in drug resistance (82). Some of these genes encoded efflux pump proteins involved in resistance: an ATP-binding cassette transporter (ABC) and a multi-drug resistance transporter (83). Other genes were *ERG11* [a target of fluconazole; (84)] and *TAC1* [a transcription factor that upregulates ABC gene expression; (82)]. There is a need to identify other structural variations and affected genes conferring survival/adaptive advantage to antibiotics and whether these changes are conserved across other fungal species.

Consistent with gene amplification conferring a selective advantage, budding yeast exposed to nutrient deprivation exhibited gene amplifications that provided a cellular benefit (85). For example, glucose limitation in cultures resulted in the amplification of genes encoding glucose transporters (*HXT6* and *HXT7*), while sulfate-limitation resulted in the amplifications of *SUL1*, a gene that encodes for a high affinity sulfate transporter (Figure 2). The question remains as to whether these physiological input signals are able to drive selective DNA gains through a hardwired mechanism as observed in mammalian cells [discussed in section: Cancer and Transient Site-Specific Copy Gains (TSSGs)] or are the result of random selection. Resolving this issue could have a profound impact on our understanding of cellular fitness and antibiotics responses.

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Mammals. Mammals are no exception to selective pressures promoting copy number changes or copy number alterations impacting biological consequences. For example, copy number of the human salivary amylase gene *AMY1*, which aids in the hydrolysis of starch, is increased in populations that have a higher starch-content in their diets when compared to low-starch consuming populations (86). The increased copy number of *AMY1* also correlated with increased salivary amylase protein levels. This illustrates how diet induced selective pressures could be influencing copy number polymorphism in mammals. Other examples and the role of copy number polymorphism in human adaptation have been reviewed elsewhere (33, 87, 88). While these studies are correlative and suggest that environment impacts selection, they have yet to be shown as causal.

Increased or decreased copy number of certain genes can predispose an individual to diseases. For example, susceptibility of individuals to HIV/AIDS infection is increased in populations with a decreased copy number of the chemokine gene *CCL3L1*. This chemokine serves as a ligand for HIV co-receptor CCR5, which inhibits viral entry by binding to CCR5. However, HIV resistant individuals show duplications of the CCL3L1 locus (17q21.1) with increased CCL3L1 copies imparting resistance to HIV infections (89). Other examples of CNVs promoting susceptibility to diseases can be found in the case of psoriasis [associated with a copy number gain of *β-defensin* gene (90, 91)]; pancreatitis [copy number gain of *PRSS1; (92)]* and Crohn's disease [copy number loss of *HBD-2; (93)*], among others (20, 94). The question still remains as to whether there are mechanisms that would allow such changes to occur immediately in response to the stimuli in the population or whether this reflects some mutation that was selected for over time.

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Somatic mosaicism for CNVs within tissues can provide an adaptive response as well. CNVs within the liver can provide protection against tissue injury. Duncan et al. demonstrated in a chronic liver injury model that selective gene loss could provide resistance to liver injury (95). Deficiency of fumaryl acetoacetate hydrolase (*FAH*; an enzyme required in tyrosine catabolism) causes a build up of fatty acids and toxic metabolites that result in liver failure, which is known as Tyrosinemia. Conversely, deletion of the genes encoding enzymes that function upstream of *FAH* (e.g., homogentisic acid dioxygenase, HGD) is found to be protective for Tyrosinemia. Mice deficient for *FAH* and heterozygous for a mutation in *HGD* can generate healthy normal hepatocytes. These injury resistant, aneuploid hepatocytes (characterized by the loss of chromosome 16) are present in the liver and undergo expansion only when the liver is exposed to injury, demonstrating an adaptive response of cells to metabolic or toxic challenges.

Taken together, these few examples illustrate the CNVs present within populations and individual tissues and how these are associated with phenotypes. These data also emphasize the variation in the genome and how the environment and selective pressures can impact genetics. However, the question remains as to whether these genetic events occur after random selection or are the result of unidentified mechanisms that selectively alter the genetic landscape in response to external stimuli, and in turn, drive targeted *de novo* genetic changes.

Copy Number Alterations in Cancer and their Implications in Acquired Drug Resistance

Copy number alterations involving whole chromosomes and/or specific chromosomal segments are frequently observed in cancer (96, 97). Gains/amplifications of oncogenes

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and loss/deletion of tumor suppressor genes have been historically found to be major drivers of tumor development. For example, amplifications of *EGFR* in gliomas (98), *MYCN* in neuroblastoma (99), *MYC* in acute myeloid leukemia (100), *ERBB2* in breast (101), ovarian (102) and lung cancers (103). Similarly, loss/deletions in tumor suppressor genes such as *PTEN* (104), *TP53* (105) and *VHL* (106) are observed in a variety of tumors. The dependence of tumors on specific oncogenes for their proliferation and survival is referred to as oncogene addiction (107). By targeting these oncogenes, tumor cell growth becomes limiting or abrogated. For example, clinical success has been observed with the ERBB2 antibody Herceptin in the treatment of *ERBB2* amplified breast cancer (108), Crizotinib in the treatment of *MET* amplified non-small cell lung cancer (109), and the EGFR inhibitors Gefitinib and Erlotinib (these block the catalytic activity of EGFR) in lung cancer patients with *EGFR* mutations (110).

In addition to oncogene amplifications, copy number alterations of different chromosomal regions have been observed in cancer. A genome-wide analysis of copy number alterations in cancer has demonstrated a total of 76,000 gains and 55,000 losses across the 3,131 cancer samples analyzed (96). A typical tumor type was comprised of 17% amplifications and 16% deletions compared to less than 0.5% in normal samples (96). This data suggests that somatic copy number alterations are a frequent feature in cancer cells. Analyses across 17 tumor types demonstrated that 25% of the genome is affected by whole chromosome alterations and 10% of the genome by short chromosomal changes (focal events) in a typical tumor (96). Interestingly, the focally amplified regions often harbored known oncogenes (e.g., *MYC*, *CCND1*, *EGFR*, *NKX2-1* and *KRAS*), while the focally deleted genomic loci contained tumor suppressor genes (*TP53, CDKN2A/B and Rb1*). These observations suggest that the selective pressures associated with tumorigenesis might influence targeted amplification or deletion of

specific regions within tumor cells instead of occurring randomly, which would be reminiscent of the observations seen in bacteria and yeasts (Figure 2).

Focal amplifications can also harbor oncogenes or pro-survival genes that can influence drug response. For example, ~10% of cancers have a focal amplification of chromosome 1q21.2 that contains the anti-apoptotic gene *MCL1* (96). Another focally amplified anti-apoptotic gene that is observed in cancer is *BCL2L1* on chromosome 20q11.21 (96). Both of these genes are important for cell survival, hence their amplification within tumors could confer a distinct survival advantage. Consistent with this notion, Beroukhim et al. demonstrated that increased expression of these genes protected tumor cells from chemotherapy (96).

Chromosomal alterations in several distinct regions also influences pathogenesis in different tumor types. For example, in multiple myeloma (MM) disease progression is characterized partly by the focal amplifications of a proximal region of chromosome 1q (chr 1q). Several studies have identified a region of 10-15Mb corresponding to a chr 1q12-23 amplicon in MM. This region contains a large number of genes with amplifications or deregulated expression involved in myeloma pathogenesis, including *CKS1B* (111, 112), *MUC1* (113), *MCL1* (114), *PDZK1* (115), *IL-6R* (116), *BCL9* (117) and *UBE2Q1* (118). The amplification of a drug resistant oncogene *CKS1B* and the proximal chr 1q21 region has been reported in ~40% of newly diagnosed MM cases and in 70% of patients with tumor relapse (119, 120). The gains observed in *CKS1B* are in the range of one to three copies (111, 112). These focal amplifications are associated with poor prognosis and reduced response to cisplatin therapy (111). (Table I). Studies in cell culture further demonstrate that overexpression of *CKS1B* confers a reduced response to cancer chemotherapeutics (121). Similarly, amplification of the *PDZK1* gene

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within the chr 1q12-q22 region has been observed in primary cases of MM, and the overexpression of *PDZK1* in cells conferred resistance to melphalan, vincristine and cisplatin induced cell deaths (115) (Table I).

Gene amplifications are associated with drug resistance in several tumors (122-141) (Table I). For example, ovarian cancer patients with chr 1q12-21 amplification are more resistant to cisplatin treatment (142, 143). Amplifications of cyclin E1 (CCNE1) are present in 25% of high-grade serous ovarian cancer and are associated with poor survival and impart resistance to CDK2 inhibitors (144) (Table I). In the case of non-small cell lung cancer cells, an 11- to 13-fold higher copy number of chr 7q21.12 was detected by CGH in an acquired paclitaxel-resistant lung cancer model (NCI-H460/PTX250) compared with the parental cell line (NCI-H460). Most of the genes within this region were also highly expressed, including a multidrug transporter gene *MDR1/ABCB1* (131). These examples highlight how distinct regions in the genome are focally amplified and relate to altered patient outcome and cancer cell drug response. Whether, selective chromosomal alterations and gene amplifications in cells is a stochastic process or occurs in a directed manner in consequence to therapeutic pressure is yet to be determined.

DNA Amplification and Cancer Chemotherapeutic Resistance

Gene amplification serves as a biochemical basis for drug resistance in mammalian cells. This relationship to resistance was first documented in seminal work by Hakala (145-147) and Fischer (148) in the 1950s. They isolated highly resistant tumor cells under the presence of increasing concentrations of the drug methotrexate (MTX). MTX competitively inhibits the enzyme dihydrofolate reductase (DHFR), which catalyzes the conversion of dihydrofolate to active tetrahydrofolate, which is required for the *de novo*

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synthesis of thymidine. They found that the drug resistant cells had around 155 times the level of DHFR. They also found that the drug-resistant phenotype was unstable in murine sarcoma 180 cells, which coincided with the reduced DHFR enzymatic activity. Schimke's laboratory further characterized the mechanistic basis for the increased DHFR levels (149). It was shown that the cells developed resistance to MTX by overproduction of DHFR protein as a result of selective gene amplification (150). It was from the work of the Biedler and Spengler (151, 152) and Schimke laboratories in 1970s (150, 153) that showed the presence of cytogenetic structures associated with MTX-resistant cells. They found that the gene amplification accounts for the overproduction of DHFR in stable and unstable drug-resistant cells (Figure 3A).

Gene amplification forms two common structures: extrachromosomal double minutes (DM) and intra-chromosomal homogenously staining regions (HSRs). DMs were first observed in lung cancer cells in 1962 (154). They are defined as chromatin bodies that lack centromeres and telomeres that are not transmitted to 100% of daughter cells during mitosis (155) (Figure 1D). HSRs are chromosomal structures containing permanently integrated genes (Figure 1D). These were first described by Biedler and Spengler in 1976 (152) in drug-resistant cells. DHFR was found to reside on HSRs in highly methotrexate-resistant CHO cells (156) and murine leukemia cells (157). Kauffmann et al. further showed that the amplified *DHFR* genes were associated with DMs in unstable MTX-resistant cells (158).

A large body of work has contributed to our understanding of the generation of DMs and HSRs (159-162). For example, Storlazzi et al. investigated the structures of *MYCN* amplifications using eight neuroblastoma and two small cell carcinoma cell lines (162). The study provided evidence of generation of HSRs from DMs by an episome model

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wherein DNA segments are excised from a chromosome and then circularized and amplified to form DMs and chromosomally integrated to form HSRs. DMs are unstable and can be eliminated after drug treatment (163, 164), however HSRs are more stable (165) (Figure 1D and 3A). Amplified genes present on extrachromosomal DNA have been frequently observed in different tumor types (159, 166-168). The reversion of a malignant phenotype and cellular differentiation by the elimination of DMs has been shown extensively in a variety of tumors and cancer cell lines (167, 169, 170). Taken together, these observations demonstrate that transient gene amplifications can be an effective strategy for quick adaptation to selective pressures in tumor cells (Figure 3A).

In a recent study by Nathanson et al., another example of drug induced transient gene selection was demonstrated (Figure 3B). In this study, oncogenes maintained on extrachromosomal DNA were transiently gained/lost in response to drug treatment (171). Glioblastoma patients harbor a constitutively active oncogenic variant of epidermal growth factor receptor (EGFR-vIII) that is formed by the in-frame deletion of exon2-7 in the *EGFR* gene and found on extrachromosomal DNA (171, 172). The presence of EGFR-vIII makes tumor cells more sensitive to EGFR tyrosine kinase inhibitors (TKIs; (173)). The continued treatment with EGFR TKIs (e.g., Erlotinib) resulted in a loss of extrachromosomal EGFR-vIII, thus conferring resistance to the TKI. When the drug was withdrawn for a short period of time, there was an increase in EGFR-vIII on extrachromosomal DNA, and in turn, the cells were re-sensitized to Erlotinib treatment (Figure 3B). These data reiterate the reversibility of copy number gains and how transient copy number changes could impact chemotherapeutic response.

Furthermore, Nathanson and colleagues suggest that instead of a continuous therapeutic regimen, drug holiday during therapy might be a more effective mechanism

to restore the sensitivity of tumor cells to drugs. These studies raise the possibility that chemotherapy could result in the selection of cells with gene amplifications, which allow them to survive under this drug-induced stress (Figure 3). Therefore, understanding the mechanisms that result in transient or non-permanent amplifications of *DHFR*, *EGFR* and alike in cancer (Table I) will have a profound impact on how we view copy number control as well as how we identify novel biomarkers and therapeutic targets for treating drug resistant cancers.

Transient Site-Specific Copy Gains (TSSGs), Tumor Heterogeneity and Cancer Evolution

There are frequent gains/amplifications observed across cancer genomes, which are often thought to be permanent events (33, 160). However, a recent discovery from our laboratory (174, 175) suggests a possible mechanism for the observed intra-tumoral heterogeneity of copy number alterations observed in tumors. This recent discovery could also provide a molecular basis for the emergence of amplified drug resistant genes and enhanced cancer cell survival.

Chromatin modulation plays an important role in replication fidelity (176, 177). A recent study demonstrated that alterations in chromatin states could modulate copy number gains at distinct regions in the genome (175). KDM4A/JMJD2A demethylates tri-methylated histone H3 lysine 9 and 36 (H3K9/36me3) to a di-methylated state (K3K9/36 me2) (178-182). KDM4A overexpression promoted faster S-phase progression and altered replication timing at specific regions in the genome in a catalytically-dependent manner (175, 183). The faster S phase and regulation of replication timing was conserved from *C. elegans* to human cells and was the result of dysregulating specific HP1 members in the genome (HPL-2 in *C. elegans* and HP1γ in human cells) (183).

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Even though the S phase was faster in mammalian cells, the rate of cell proliferation was the same, which was consistent with the observed slowing into the G2/M phase. This delayed G2/M was not associated with major genome instability. However, KDM4A overexpression directly generated site-specific copy gains of regions affiliated with drug resistance (e.g., chr 1q21-22) by altering methylation states and heterochromatin association. KDM4A was enriched at these sites and promoted their re-replication. Furthermore, direct H3K9/36me3 interference promoted site-specific copy number gain events. This study demonstrated for the first time that an enzyme has the ability to directly regulate copy number gain at specific regions in the genome and that the chromatin/methylation states play an essential role in the process (175) (Figure 3C).

Since the copy number gained regions were not permanent and are only generated and present during S phase, they have been termed as transient site-specific copy gains (TSSGs) (174, 175, 184). Currently, we do not know the exact sizes of the re-replicated fragments and whether there are cellular checkpoints/machinery involved in their clearance. In fact, different cells in a population have differentially amplified regions and certain regions are mutually exclusive. Furthermore, the rate that these fragments are removed as cells move through S phases is different (174). It is important to determine the molecular features (*e.g.*, presence of repetitive elements, insulators, and other regulatory machinery) at and surrounding the re-replicated and copy gained regions. These molecular details will help establish whether unique sequence features or chromatin states have a predilection for re-replication and whether site-specific copy gains can be integrated in the genome.

Stabilization of KDM4A as a result of exposure to cellular triggers such as hypoxia also resulted in TSSGs in cell lines, tumors and normal primary cells (Figure 2; T cells) (174).

In fact, these copy number gains were found to be conserved at a syntenic region in zebrafish cells subjected to hypoxia. The return of cells to normoxia resulted in the reversion of copy number gains to the baseline levels (Figure 3C). Hence, generation of transient copy number gains could be an adaptive cellular response of cells to external stresses or stimuli. These data provide a mechanism for heterogeneity within a cell population even though the same genetic event occurred in the population.

The stabilization of KDM4A upon hypoxic exposure promoted copy number gains of the drug resistant oncogene *CKS1B (111, 112, 121, 185)*, which had a concomitant increase in transcripts (174). When cells were returned to normoxia, both copy number and transcripts of *CKS1B* returned to normal levels. Finally, we demonstrated that succinate [a natural inhibitor for KDM4 class of demethylases (186)] or chemical inhibition of KDM4A blocked the copy number gains upon hypoxic exposure. These data emphasize the impact that metabolites could have on copy number gain, but most importantly, identify a mechanism for blocking their generation (Figure 3C). Since drug resistant oncogenes are being increased, the inhibition of KDM4A may provide a novel mechanism for modulating TSSGs and provide a method for reducing 1q21 drug resistant associated cancers.

The fact that transient exposure to elevated KDM4A can promote copy number gain that is only present during S phase suggests that other mechanisms must be present to remove the TSSGs. Similar mechanisms may be involved in the removal of extrachomosomal *DHFR* and *EGFR* amplifications. The TSSG data support the notion that chromosomal regions with specific genes that confer a survival advantage are amplified to protect the cell. Selectively amplifying genes that confer distinct advantages either related to cell survival, metabolizing drugs, mounting responses to counteract drug

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sensitivity or features promoting tumorigenesis, could aid in the evolution/adaptation of cancer cells. The question remains as to whether the classical oncogenes (*e.g.*, *EGFR, MYC, ERBB2*, etc.; Table 1) are subjected to site-specific copy gains in tumors, and subsequent retention upon genetic, intrinsic or extrinsic exposure. Some extrinsic cues could be therapeutic and metabolic challenge, stress conditions (such as hypoxia, nutrient deprivation), vasculature and extracellular matrix plasticity. Future studies investigating their impact on TSSG and gene amplification will be critical.

Tumor Heterogeneity. Tumor heterogeneity presents a major diagnostic and therapeutic challenge in the treatment of cancer. Indeed, recent sequencing efforts with next generation sequencing helped in the tracing of clonal lineages in tumors (187, 188). Focal gains or losses of chromosomes can result in diversity within cells in a tumor population [intratumoral heterogeneity; (189)] as well as between tumors [intertumoral heterogeneity; (189)]. For example, next generation sequencing of five bladder tumors from patients with transitional cell carcinoma of the urinary bladder showed genomic rearrangements and mutational heterogeneity within tumors (188). Whole exome sequencing of samples from eighteen patients with chronic lymphocytic leukemia (CLL) revealed the emergence of sub-clones within selected population of cells treated with chemotherapy (190). These populations of cells might be more fit than the pre-treatment counterpart and could contribute to relapse after therapy. Thus, identifying the mutational landscape before and after chemotherapy could not only identify mechanisms of tumor relapse but also help to design effective therapeutic options for the elimination of dominant subclones arising after chemotherapeutic selection pressures.

Another mechanism contributing to intra-tumoral heterogeneity could be by the regulation of TSSGs from KDM4A levels, oxygen concentrations, cell division rates,

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metabolites and KDM4A inhibition. Cells could be cycling at different rates in a tumor population thereby affecting the rate at which re-replicated fragments are generated (Figure 3C). Differential levels of KDM4A expression, hypoxia levels or metabolic status in cells within a tumor population could also generate copy number gains at different rates thereby affecting heterogeneity. We hypothesize that the site-specific re-replication events could be one of the characteristics acquired in specific population of cells during subclonal divergence. Specific environmental, metabolic or therapeutic stress conditions can produce site-specific chromosomal alterations in the subclonal populations, which could either be transient, persisting only when the signal is there or could eventually become integrated elsewhere in the genome upon subsequent genetic/epigenetic changes. TSSGs within specific cell populations could either influence the emergence of the dominant sub-clone or it could go hand-in-hand with the germline mutations occurring during tumor evolution. Whether these events result in the emergence of the fittest clone that promotes survival and if these sets of "fit" cells clonally expand after a therapeutic challenge, is a hypothesis that needs to be investigated.

Conclusion

CNVs influence the ability of normal cells to respond to physiological triggers and can serve as an adaptive strategy for a variety of responses such as hypoxia, nutrient deprivation, toxic challenges or cell survival and proliferation. Alterations in copy number often lead to diseases such as cancer, where the tumor cells can also co-opt these aberrations as an adaptive response to amplify genes involved in chemotherapeutic resistance. It is important to determine whether the process of generating copy number alterations under normal physiological, developmental or pathological conditions are based on an active cell-directed and regulated mechanism or are the result of random aberrations that have occurred during cell division. Whether random or directed, it is

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important to understand that copy number changes are not always permanent. The recent discovery of a specific chromatin regulator controlling re-replication and site-specific copy number change suggests that copy number changes can be regulated and are reversible. These transient site-specific copy gains may generate intra-tumoral heterogeneity that could have important consequences in chemotherapeutic sensitivity and patient outcome. Hence, identifying regulators of CNVs and delineating processes affected by CNVs will be important therapeutically.

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FIGURE LEGENDS

Figure 1. Types of Copy Number Changes. (A) Representative examples of structural chromosomal alterations are shown with new sequence insertion (D), deletion of region AB, and duplication of sequence B (ABB). The reference chromosome is shown at the top. (B) Aneuploidy with whole chromosome gain (extra black chromosome) and loss (of black chromosome) is depicted with respect to a normal mitotic reference nucleus. (C) A part of chromosome (black) can be amplified or deleted (black) giving rise to segmental aneuploidy. This is demonstrated here involving rearrangement of only one chromosome. A more likely scenario is an unbalanced translocation, which is not shown in the figure. (D) Homogenously staining regions (HSR) and double minutes (DMs) are chromosomal structures that are generated as a consequence of gene amplification. HSRs are repeated units clustered at a single chromosomal locus (red) and DMs are unstable circular extrachromosomal DNA structures lacking a centromere or a telomere. In addition to these structures, amplicons can be present at a number of loci in the genome, which is not shown in the figure.

Figure 2. Copy number changes during normal development and physiology. Representative copy number changes are shown for organisms and specific tissues under different developmental and physiological conditions. Please refer to text for detailed description and corresponding references.

Figure 3. Permanent and Transient Adaptive Changes Under Different Cellular Conditions (A) Methotrexate treatment results in the amplification of DHFR gene (shown in red). DHFR can persist either as a stable structure such as an HSR or as an unstable DM that is lost upon subsequent cell division. (B) Continued treatment of glioblastoma cells with a tyrosine kinase inhibitor such as Erlotinib results in the loss of EGFR vIII positive extrachromosomal DNA (red) and its reemergence upon drug removal. (C) Hypoxia or overexpression of histone demethylase KDM4A results in site-specific genome amplification (red), which is generated every S phase. The amplification is reversible after KDM4 inhibitor treatment or with increased succinate dose. Manuscripts related to the data are discussed within the text.

Table I. Table representing a partial list of amplified genes that impact drug resistance. We apologize for not being able to cite or include all studies related to gene amplification and drug resistance.

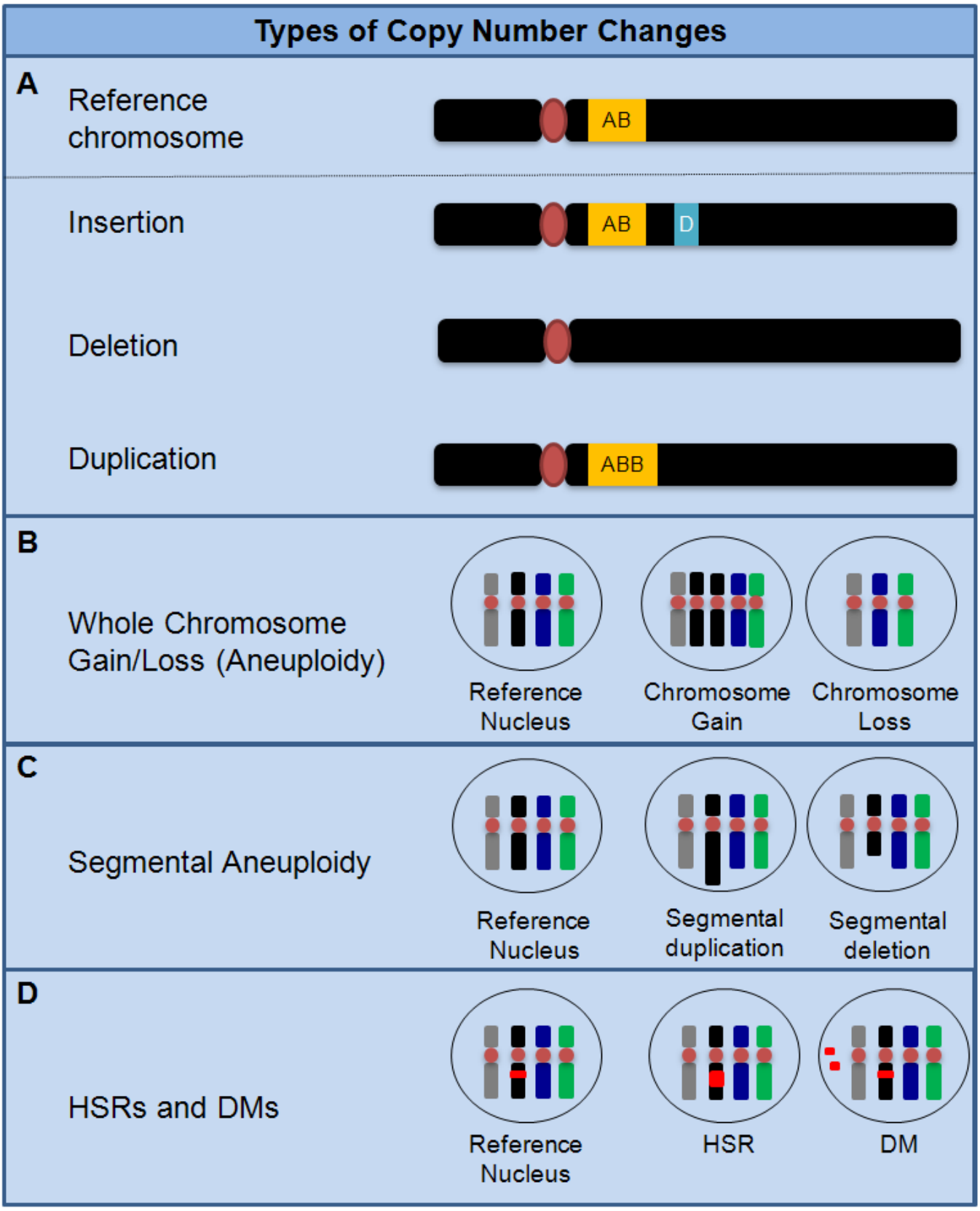


Figure 1. Mishra and Whetstine

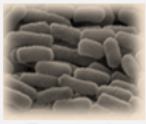
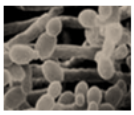
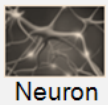
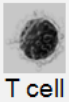
Organism	Condition	Consequence	References
 Bacteria	Replication stress in response to antibiotics	Gene Amplification (*e.g.*, upregulation of *com* genes, *β-lactamase* gene)	71-73
 Yeast	Resistance to Fluconazole in *C.albicans*	Aneuploidy (*e.g.*, Isochromosome formation)	81, 82
 Yeast	Nutrient Deprivation	Gene Amplification (*e.g.*, HXT6, HXT7, SUL1)	85
 Fly	a) Cocoon formation b) Egg shell formation	a) Salivary gland gene amplification b) Gene Amplification (*e.g.*, chorion genes)	60 62
 Frog	Oocyte formation	Ribosomal DNA amplification	55
 Mouse	Tissue Injury	Aneuploidy (*e.g.*, loss of chromosome 16)	69
 Human a)  Neuron b)  Hepatocyte c)  T cell	a) CNS development b) Liver cell proliferation c) Hypoxia	a) Aneuploidy and subchromosomal CNV b) Polyploidy c) Transient site-specific copy gains	63, 65, 66 69 174

Figure 2. Mishra and Whetstine

Molecular and Cellular Biology

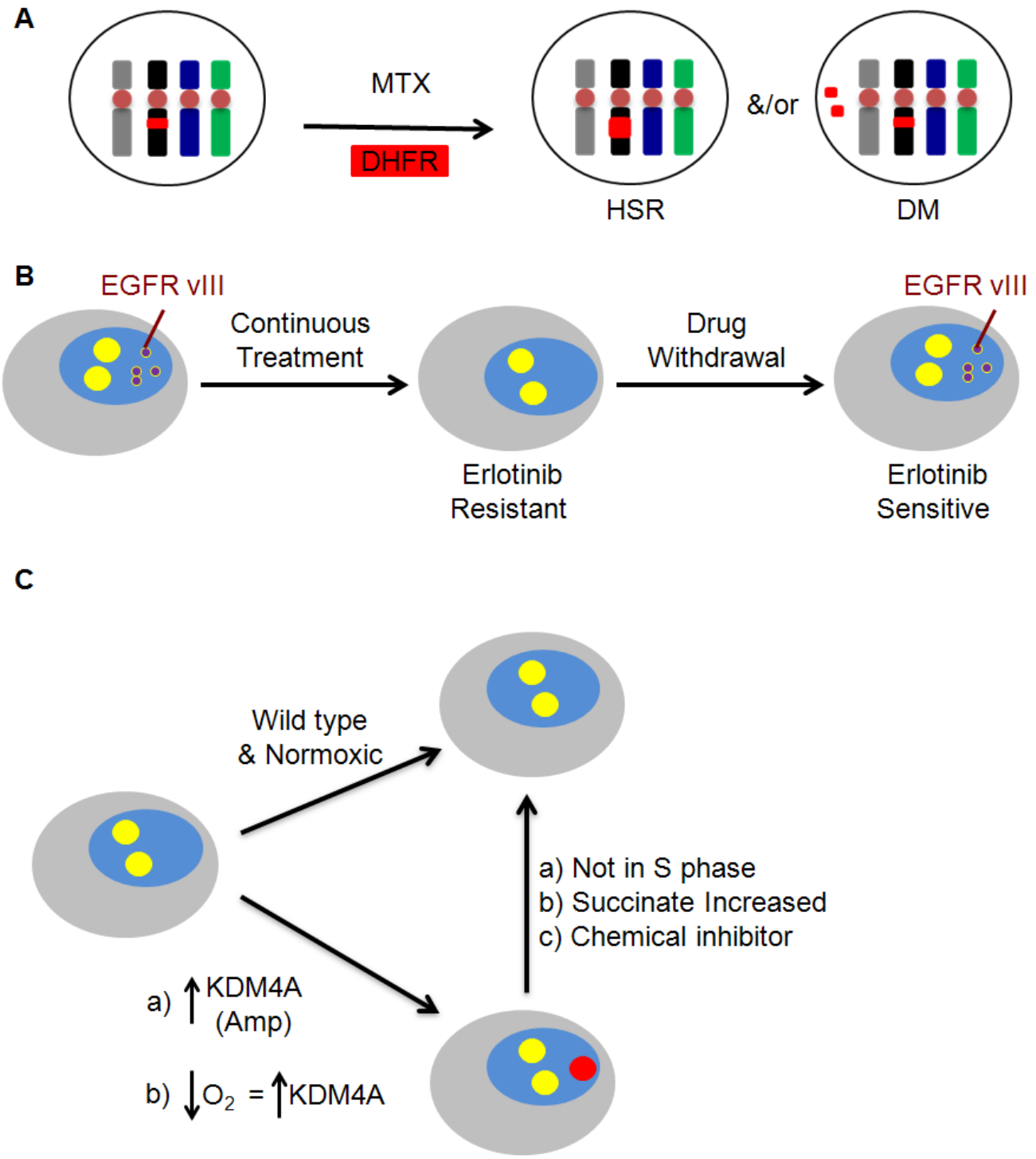


Figure 3. Mishra and Whetstine

Cancer Type	Therapeutic Agent	Genes Implicated in Resistance
Multiple Myeloma	Bortezomib, Cisplatin Melphalan, Cisplatin, Vincristine Dexamethasone	CKS1B (111, 121, 126) PDZK1 (115) FGFR3 (127)
Ovarian Cancer	Cisplatin, CDK2 inhibitors Paclitaxel	CCNE1 (128, 142) MDR1 (129, 130)
Lung Cancer	Gefitinib Paclitaxel Crizotinib	MET (123, 125) MDR1 (129, 130, 131) ALK, KIT (132)
Breast Cancer	Trastuzumab	MET (133), IQGAP1 (134)
Colorectal Cancer	Gefitinib 5-flurouracil	MET (124) TMYS (135)
CML	Imatinib	BCR-ABL (136)
Melanoma	Vemurafinib	BRAF (137, 138), BCL2A1 (139)
Leukemia	Methotrexate	DHFR (140, 141)