

## Review

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# Gene amplification: mechanisms and involvement in cancer

**Abstract:** Gene amplification was recognized as a physiological process during the development of *Drosophila melanogaster*. Intriguingly, mammalian cells use this mechanism to overexpress particular genes for survival under stress, such as during exposure to cytotoxic drugs. One well-known example is the amplification of the dihydrofolate reductase gene observed in methotrexate-resistant cells. Four models have been proposed for the generation of amplifications: extrareplication and recombination, the breakage-fusion-bridge cycle, double rolling-circle replication, and replication fork stalling and template switching. Gene amplification is a typical genetic alteration in cancer, and historically many oncogenes have been identified in the amplified regions. In this regard, novel cancer-associated genes may remain to be identified in the amplified regions. Recent comprehensive approaches have further revealed that co-amplified genes also contribute to tumorigenesis in concert with known oncogenes in the same amplicons. Considering that cancer develops through the alteration of multiple genes, gene amplification is an effective acceleration machinery to promote tumorigenesis. Identification of cancer-associated genes could provide novel and effective therapeutic targets.

**Keywords:** amplicon; cancer; development; drug resistance; microarray.

**List of abbreviations:** Akt, v-akt murine thymoma viral oncogene homolog; AR, androgen receptor; BCL2, B-cell CLL/lymphoma 2; BFB, breakage-fusion-bridge; bHLHZ, basic-helix-loop-helix zipper; BIR, break-induced replication; BRAF, v-Raf murine sarcoma viral oncogene homolog B; BRCA2, breast cancer 2, early onset; BRD4, bromodomain-containing 4; CAD, carbamoyl-synthetase 2 aspartate transcarbamylase and dihydroorotase; CCND1, cyclin D1; CDK, cyclin-dependent kinase; CGH, comparative genomic hybridization; C-MYC, v-myc avian myelocytomatosis viral oncogene homolog; CNV, copy number variation; CRPC, castration-resistant prostate cancer;

DHFR, dihydrofolate reductase; DM, double minute chromosome; DRCR, double rolling-circle replication; DSB, double-stranded break; EGFR, epidermal growth factor receptor; EMSY, chromosome 11 open reading frame 30; ERBB2, v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2; ER $\alpha$ , estrogen receptor alpha; FISH, fluorescence *in situ* hybridization; FoSTeS, replication fork stalling and template switching; GRB7, growth factor receptor-bound protein 7; HR, homologous recombination; HSR, homogeneously staining region; MAPK, mitogen-activated protein kinase; MAX, MYC associated factor X; MDM2, mouse double minute 2 homolog; MEK, mitogen-activated protein kinase kinase 1; MTF, microphthalmia-associated transcription factor; MTX, methotrexate; NGS, next-generation sequencing; PAK1, P21-activated kinase 1; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PLP1, proteolipid protein 1; RAR $\alpha$ , retinoic acid receptor  $\alpha$ ; RAS, rat sarcoma viral oncogene homolog; RB, retinoblastoma; StAR, steroidogenic acute regulatory protein; STARD3, START domain containing 3; START, StAR-related lipid transfer; STAT3, signal transducer and activator of transcription 3; TKI, tyrosine kinase inhibitor; TOP2A, DNA topoisomerase 2- $\alpha$ .

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## Introduction

Gene amplification is defined as an increase in copy number of a restricted region of a chromosome arm (1). This amplified region is called an ‘amplicon’. Gene

amplification plays a crucial role in the normal developmental program, including in the amplification of chorion genes in *Drosophila melanogaster* ovaries (2) and amplification of rDNA in amphibian oocytes (3). Gene amplification in somatic cells was first observed by Breuer and Pavan (1955) during a morphological study of salivary gland chromosomes of *Rhynchosciara americana* larvae, a species of fly (4). The authors found that certain loci on salivary gland chromosomes swelled enormously, producing 'DNA puffs', and that the quantity of DNA in these loci increased compared with other loci, either simultaneously or after the swelling (5). In 1957, Rudkin and Corlette provided the first experimental evidence of gene amplification in *Rhynchosciara angelae* by measuring the quantity of absorbing material in a chromosome using microspectrophotometry (6). Large quantities of these developmental genes encoding rRNAs and structural proteins for eggshells and cocoons are necessary during development. In recent studies, two additional amplicons encoding a variety of proteins, including transporters and proteases, were identified in *Drosophila* follicle cells by a comparative genomic hybridization (CGH) array approach (7). In addition to its functions in physiological processes, gene amplification has attracted much attention for its involvement in cellular adaptation against cytotoxic drugs and, interestingly, in tumorigenesis (1, 8).

The first demonstration of gene amplification in cultured mammalian cells was the amplification of the dihydrofolate reductase (*DHFR*) gene in the AT3000 line of methotrexate (MTX)-resistant murine sarcoma 180 cells in 1978 (9). *DHFR* catalyzes the reduction of dihydrofolate to tetrahydrofolate, which is used in glycine, purine, and thymidylate synthesis. Methotrexate, a 4-amino analog of folic acid, inhibits *DHFR* activity and thus causes arrest of DNA replication and cytotoxicity (10). After exposure of cells to methotrexate, the surviving cells exhibited an increased copy number of the *DHFR* gene. This phenomenon was observed in several cell types, including Chinese hamster ovary cells as well as the AT3000 cell line. The occurrence of gene amplification in drug-resistant cells was observed not only with *DHFR* but also with the carbamoyl-synthetase 2 aspartate transcarbamylase and dihydroorotase (*CAD*) gene. Amplification of the *CAD* gene was observed in Syrian hamster cells resistant to *N*-phosphonacetyl-L-aspartate, an inhibitor of aspartate transcarbamylase (11).

The amplification mechanism of the *DHFR* gene in MTX-resistant cells has been frequently employed for recombinant protein production in mammalian cells (12, 13). Because the production of protein pharmaceuticals, including cytokines and humanized antibodies, requires

the use of cultured mammalian cells, a robust method for the production of large amounts of recombinant protein in mammalian cells is critically important. In the general method for mammalian cell-mediated production of recombinant proteins, the plasmid harboring the recombinant gene and a second plasmid containing a selection gene are first co-introduced into cells. The *DHFR* gene is the most frequently used selection gene. By adding MTX to the cells, the target gene co-amplifies gradually (13). Although almost all cells die by MTX treatment, the surviving cells that overproduce *DHFR* frequently contain several hundred to a few thousand copies of the recombinant gene (14). This method allows the production of cells that hyper-produce the target gene. Most *DHFR*-amplified cells produce up to 10- to 20-fold more recombinant protein than unamplified cells (12).

In 1976, Biedler and Spengler analyzed the amplified DNA in the MTX-resistant mammalian cell line and observed elongated chromosomal structures, which they named 'homogeneously staining regions' (HSRs) (15). Trypsin-Giemsa staining was used to visualize the differential banding pattern along the length of metaphase chromosomes, and staining showed that the long chromosome segments in drug-resistant Chinese hamster cells did not show any discrete 'bands' as did normal Chinese hamster cells (15). HSRs were thus easily recognizable.

Double minute chromosomes (DMs), which are small and often paired extrachromosomal elements, were also observed in MTX-resistant Chinese hamster cells (16). DMs vary in size among different cell lines and even within the same cells. They replicate in the cell cycle without centromeres (17). The *DHFR* gene tends to be amplified either on DM or in a HSR configuration, depending on the cell line, although the reason for such tendency remains unclear (17).

In this review, we first discuss the mechanisms of gene amplification and then focus on gene amplification in cancer. We will introduce typical oncogenes localized in amplicons and discuss their therapeutic potential.

## Mechanisms of generation of amplicons

As described above, gene amplification is detected in two forms: HSRs and DMs. DMs are circular extrachromosomal elements that replicate autonomously and lack a centromere and telomeres (18). Although gene amplification is often observed in cancer and other degenerative

disorders, the mechanisms of amplicon generation remain largely unknown. Four hypotheses have been proposed to explain the generation of amplicons: extrareplication and recombination, the breakage-fusion-bridge (BFB) cycle, double rolling-circle replication (DRCR), and replication fork stalling and template switching (FoSTeS).

## Extrareplication and recombination

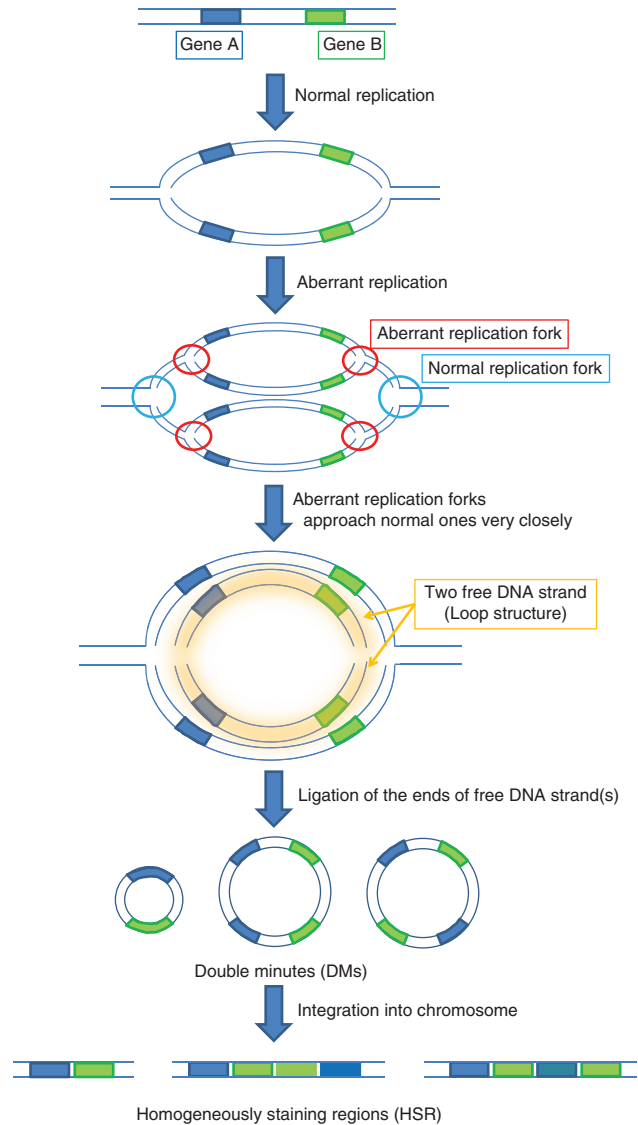
In 1972, Smith and Vinograd found that extrachromosomal circles were produced by transient treatment of cultured human cells with cycloheximide (19). Later, Woodcock and Cooper showed that transient inhibition of DNA synthesis by cycloheximide induced aberrant replication in human cultured cells, in which some DNA fragments were replicated more than once in normal replication (20). A previous study of chromosomes showed that all primary neuroblastoma cells had only DMs, although neuroblastoma cell lines cultured for a longer period of time had DMs and/or HSRs (21). These data imply that the primary product of amplification is DMs, and that HSRs are then produced by DMs. Furthermore, Osheim and Miller discovered multi-forked chromosomal structures, called ‘bubbles’, in the follicle cells of *Drosophila*, where the chorion gene was amplified (22). These studies propose a model of extrareplication and recombination shown in Figure 1.

## BFB cycle

The BFB cycle model (Figure 2) was proposed by McClintock using *Zea mays* in 1951 (23). She confirmed that BFB cycles occurred in chromosome 9 following X-ray treatment that induced a double-stranded break (DSB). BFB cycles could be induced by some clastogenic drugs in Chinese hamster fibroblast cells, and clones in which gene amplification occurred were resistant to these drugs (24). BFB cycles have also been observed in human cancer cells. For example, four osteosarcoma cell lines showed the existence of anaphase bridges, dicentric, chromosomal anomalies, and multipolar mitotic figures (25).

## DRCR

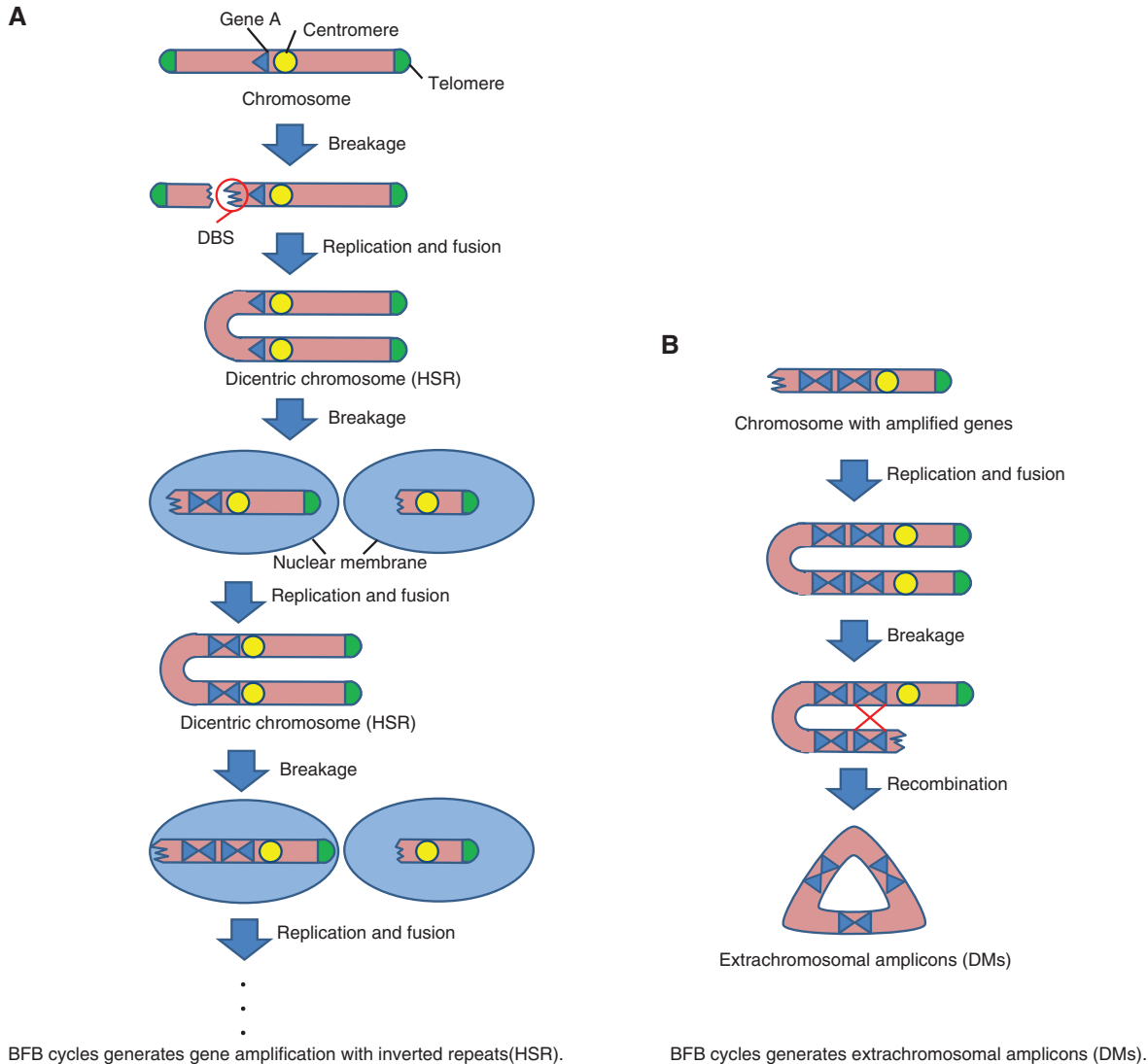
The DRCR system (Figure 3) was proposed by Futcher in 1986 for amplification of the yeast circular 2 micron plasmid (26), and later experimentally confirmed by



**Figure 1** Model of gene amplification generated by extrareplication and recombination (17, 37, 105).

This type of amplification is triggered by aberrant replication in a replication fork, resulting from a single additional initiation of replication within the replication fork. In this replication fork, there is a loop structure including the two free DNA strands (106). These strands can form three types of DMs by ligase. Some of these circular DNAs would be recombined into chromosomes, generating HSRs.

Volkert and Broach (27). Subsequently, a similar system was proposed for drug resistance gene amplification (28). In 2005, Watanabe and Horiuchi described a novel DRCR system of gene amplification based on break-induced replications (BIRs) in yeast (29). The authors used engineered yeast, in which a DSB occurs on a chromosome, and it yielded products similar to HSRs and DMs observed in higher eukaryotes. This system was recently improved for yeast and mammalian cells by using the cre-lox system



**Figure 2** The BFB cycle.

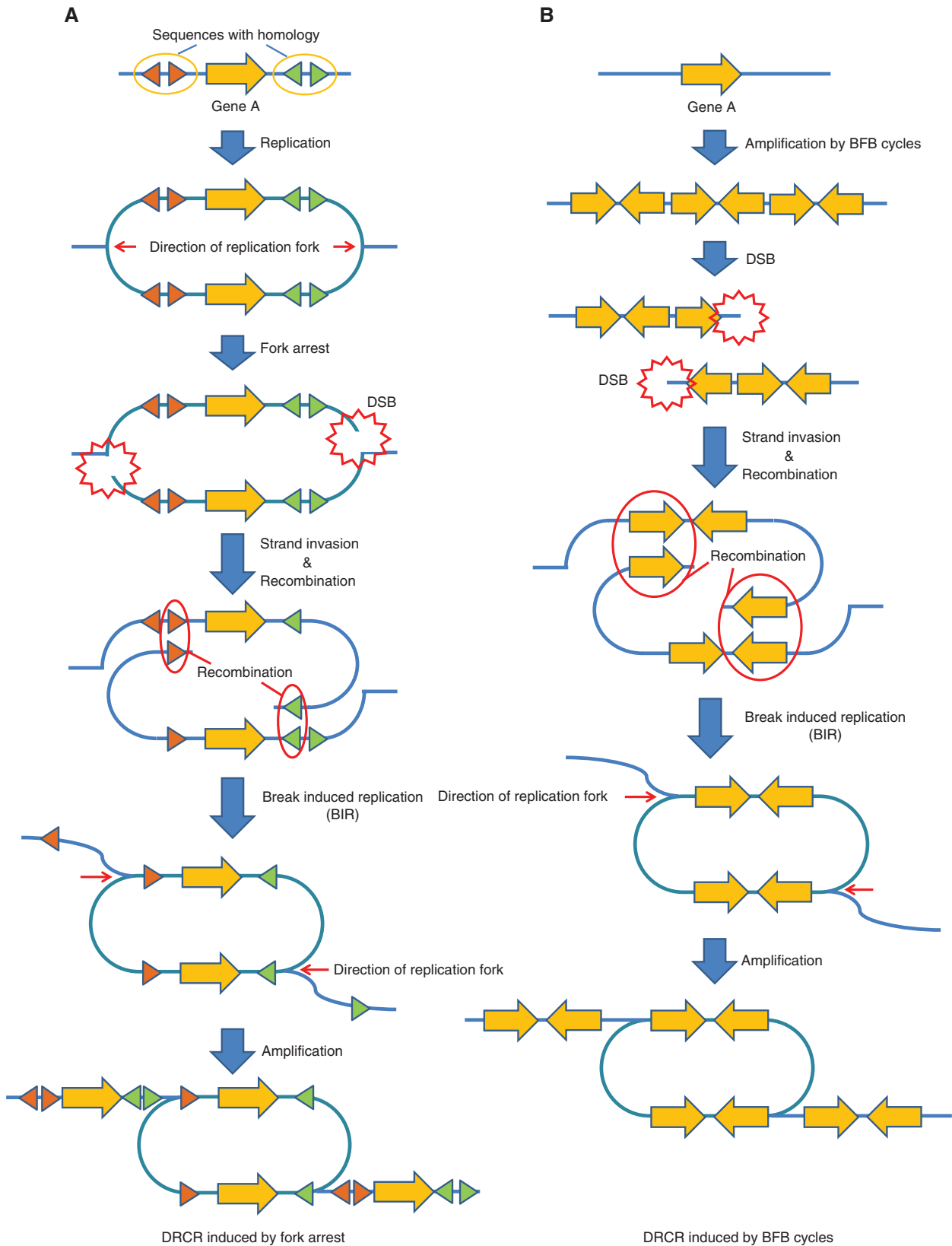
First, a DSB occurs in a chromosome, such that it loses a telomere (107). By replicating, sister chromatids that lack telomeres are produced. The two ends lacking telomeres fuse and form a dicentric chromosome. At anaphase, the two centromeres in the dicentric chromosome are pulled apart by kinetochore microtubules and form a chromatid bridge. This abnormal separation of the chromosome causes a random position on the chromatid bridge to be broken, and produces a new end lacking a telomere. This end fuses, forming a new dicentric chromosome and produces a new end without a telomere. Repeated cycles generate amplification with inverted repeats (HSR) (A) and extrachromosomal amplicons (DMs) (B) (108).

to induce DRCR (30). The DRCR system also yielded HSR/DM-type products similar to those of cancer cells. Although there is no evidence that DRCR naturally occurs in cancer cells, these findings imply a relationship between DRCR and gene amplification in tumor cells.

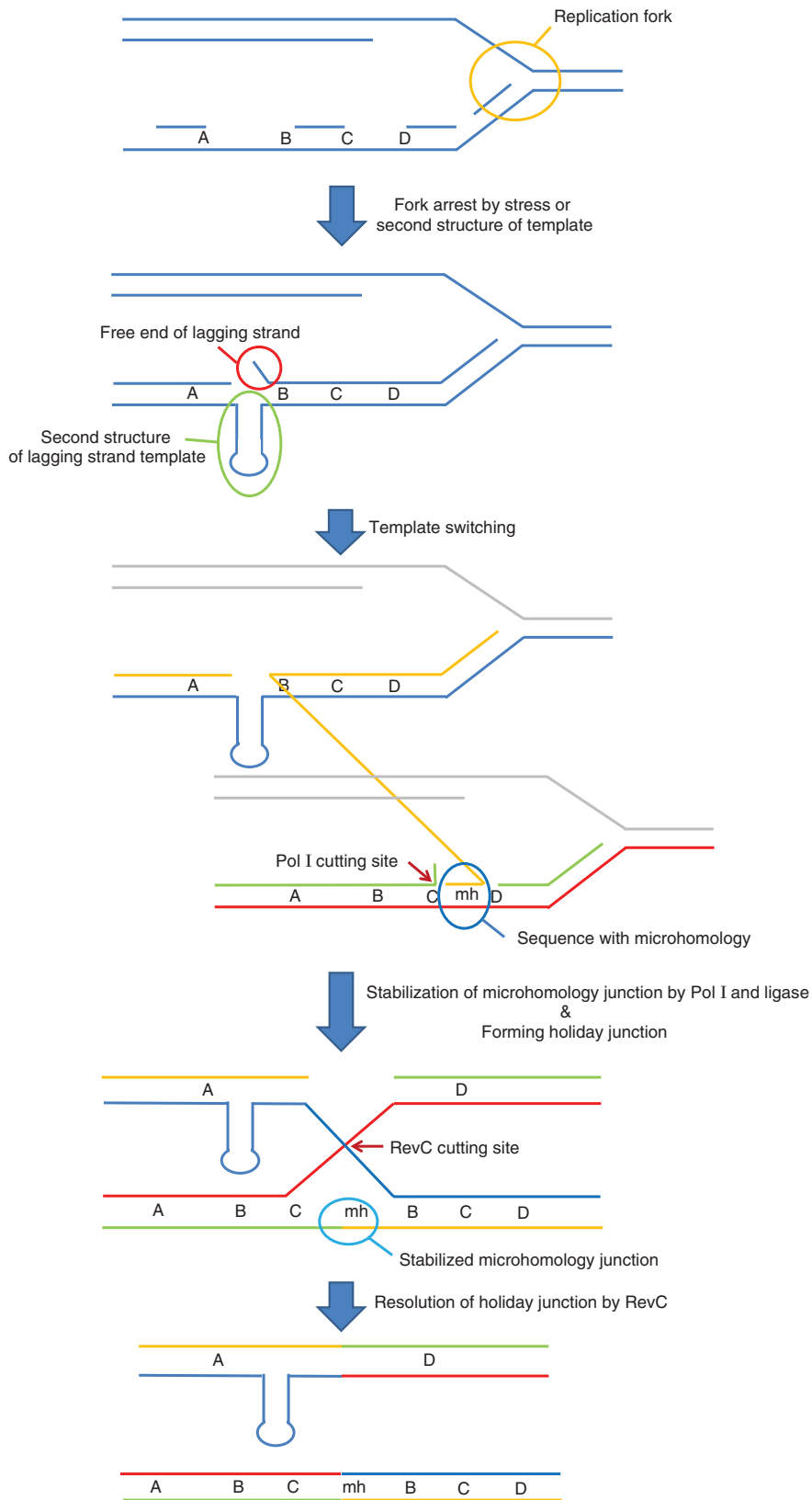
## FoSTeS

The FoSTeS model (Figure 4) was proposed by Slack, Thornton, Magner, Rosenberg, and Hastings in 2006

to explain gene amplification in *Escherichia coli* under stress (31). The authors discovered three pieces of data supporting FoSTeS. First, the junction sequences between amplified regions showed as little homology as 4–15 bp, indicating that homologous recombination (HR) does not occur, and were separated by 7–32 kb, which is too long for amplification to occur within a replication fork. Second, the 5' exonuclease domain of DNA polymerase I was essential for amplification, suggesting that the lagging strand is involved in this amplification. Third, the frequency of amplification was reduced by



**Figure 3** DRCR model of generation of amplicons proposed by Watanabe and Horiuchi (30). If the replication fork arrests (A) or BFB cycles occur (B) and subsequently cause a DSB on a chromosome, BIRs (109) between sequences with homology occur in both broken ends, and two replication forks chase each other, facilitating gene amplifications.



**Figure 4** FoSTeS.

When the replication fork stalls, a single-stranded lagging strand is exposed and anneals to the lagging strand template of another replication fork, forming a microhomology junction by polymerase I and ligase. Two products are generated by RuvC-mediated resolution of the Holliday junction.



overexpression of the Exo I 3' exonuclease, indicating that the 3' ends of DNA are important for amplification. A previous study suggested that FoSTeS may occur in Pelizaeus-Merzbacher disease patients, in which amplification of the proteolipid protein 1 (*PLP1*) gene was found (32). Furthermore, another study supported the FoSTeS model to explain genomic rearrangement and sequential complexities of two regions on the short arm of human chromosome 17 associated with several diseases: reciprocal Potocki-Lupski microduplication syndrome, Smith-Magenis microdeletion syndrome, reciprocal Charcot-Marie-Tooth disease type 1A, and duplication/hereditary neuropathies with liability to pressure palsies (33).

## Gene amplification in cancer

Gene amplification, or genomic DNA copy number aberration, is frequently observed in some solid tumors and has been thought to contribute to tumor evolution (1, 34–36). Chromosomal aberrations are found in DMs, HSRs, or interspersed regions throughout the genome. These occur in a wide variety of tumors and appear to be common in neoplastic cells and very rare in non-neoplastic cells (35, 36). Importantly, amplified oncogenes, such as *MYC*, *MYCN*, epidermal growth factor receptor (*EGFR*), and v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (*ERBB2*), have been found in amplicons (34, 37). *MYC* was the first oncogene that was proven to be amplified in a variety of tumor cells, including the neuroendocrine line derived from colon carcinoma, small-cell lung carcinoma, plasma cell leukemia, and murine osteocarcinoma (34, 37).

Gene amplification of oncogenes could be detected by several methods, including DNA-based techniques (PCR or Southern blot), molecular cytogenetic techniques (FISH, fluorescence *in situ* hybridization) with gene-specific probes, and comparative genomic hybridization (CGH) (38). PCR, Southern blot and FISH are mostly restricted to the analysis of known amplified genes (38). In contrast, CGH does not show this limitation. Genome-wide scanning of amplified chromosomal regions with CGH enables the detection of previously unknown amplified genes (38, 39), but it has limited sensitivity and resolution (39, 40). Therefore, amplification and copy number losses and gains can only be detected at limited resolution, in the 5–10 Mbase, and 2 Mbase levels, by standard CGH techniques if metaphase chromosomes are used as the target (39). To overcome this limitation, array-based CGH was

developed for both detection and mapping of amplified genes in 1998 (39, 41). The method of using well-defined genomic clones, such as bacterial artificial chromosome (BAC), P1 bacteriophage artificial chromosome (PAC), or cosmid clones is currently widely used (39, 41).

The use of next-generation sequencing (NGS), copy number variation (CNV), and structural variation observed in tumors has been recently extensively analyzed. NGS demonstrated high sensitivity and high-throughput abilities in terms of types and sizes of variants that can be detected (42, 43). Analysis of the cancer genome enables the identification of regions of significant CNVs and novel oncogene candidates as potential therapeutic targets (44, 45). Especially, NGS can be used to identify unknown fusion genes as diagnostic and therapeutic targets. In the following section, typical cancer-associated genes in the amplicons are described in detail.

## Oncogenes in the amplicons

One of the most investigated amplicons is localized on chromosome 17. In the 17q12-q21 amplicon, various cancer-associated genes are co-amplified with *ERBB2*, an important 'driver' oncogene for breast cancer. Here, we refer to the 17q12-q21 amplicon as the *ERBB2* amplicon. From the clinical perspective, *ERBB2* amplification occurs in 10–34% of breast cancer and this amplification is a significant predictor of relapse and survival in human breast cancer (46–48). In breast cancer, gene amplification of *ERBB2* is well correlated with its protein overexpression. Moreover, *ERBB2* amplification is observed also in gastric and esophageal cancers (49–51). When *ERBB2* is amplified, heterodimer formation between *ERBB2* and other *EGFR* family members on the cell surface is promoted. Formation of the heterodimer is considered to activate proliferation and survival signaling pathways, and thus contribute to oncogenesis (52). *ERBB2* amplification may cause the formation of not only heterodimers but also homodimers, and this may also contribute to oncogenesis. Thus, *ERBB2* has been a target of anticancer agents. Trastuzumab, a 95% humanized monoclonal antibody, is one of the well-known anticancer agents against *ERBB2* that targets the extracellular domain of the *ERBB2* receptor. This drug is effective in breast cancer patients with tumors exhibiting high expression or amplification of *ERBB2*.

Recent studies demonstrate that multiple overexpressed genes in amplicons, in addition to the typical driver oncogenes, also contribute to tumorigenesis. In

fact, several genes that are co-amplified with *ERBB2* are involved in tumorigenesis and progression. GRB7 (growth factor receptor-bound protein 7) is known to bind receptor tyrosine kinases and mediate signals to downstream effectors (53). Moderate expression of *ERBB2* alone did not generate transformed foci; however, co-expression of GRB7 together with *ERBB2* efficiently induced foci (54). Moreover, GRB7 was reported to bind *ERBB2* with high affinity (55). Together, these results support the oncogenic cooperation between *ERBB2* and GRB7.

Retinoic acid receptor alpha (*RAR $\alpha$* ) is also co-amplified with *ERBB2* in 23–32% of human *ERBB2*-positive breast cancers. The function of *RAR $\alpha$*  in *ERBB2*-positive tumors is somewhat controversial. One report shows that *RAR $\alpha$*  is associated with sensitivity to the antiproliferative/cytodifferentiating action of all-*trans* retinoic acid, which stabilizes the effect of lapatinib, a dual inhibitor of EGFR and *ERBB2* (56, 57).

Steroidogenic acute regulatory protein (StAR)-related lipid transfer domain containing 3 (*STARD3*), also known as metastatic lymph node 64 protein (*MLN64*), in the *ERBB2* amplicon is a protein that independently contributes to the malignant transformation of cancer. Via the StAR-related lipid transfer (*START*) domain, *STARD3* binds cholesterol and promotes steroidogenesis. Overexpression of *STARD3* thus promotes steroid hormone production in cancer cells, which may support the growth of hormone-responsive tumors (58, 59).

DNA topoisomerase 2-alpha (*TOP2A*) shows amplification independent of *ERBB2*, despite its vicinity to *ERBB2* on the chromosome, suggesting that the region at the *ERBB2* locus is composed of at least two distinct amplicons that harbor either *ERBB2* or *TOP2A* (49, 56, 60, 61). However, more cases with *TOP2A* aberrations were reported for tumors with *ERBB2* amplification than those without amplification (56). The *TOP2A* enzyme generates DNA breaks during cell division, and thus is essential for the cell cycle (62). *TOP2A* is targeted by cytotoxic agents such as anthracyclines, etoposide, and teniposide (52). In breast cancer, *TOP2A* amplification is a predictive marker for response to anthracyclines (63). Together these studies show that the significance of gene amplification is to simultaneously increase the amount of factors that are involved in tumorigenesis or progression either independently or cooperatively.

In addition to chromosome 17, amplicons also exist in other chromosomes (Figure 5) (64). Microphthalmia-associated transcription factor (*MITF*), located on 3p13, is a lineage-specific oncogene that is occasionally amplified in melanoma (65). *MITF* targets genes involved in cell-cycle arrest (*p21* and *INK4a*), cell proliferation (*TBX2* and

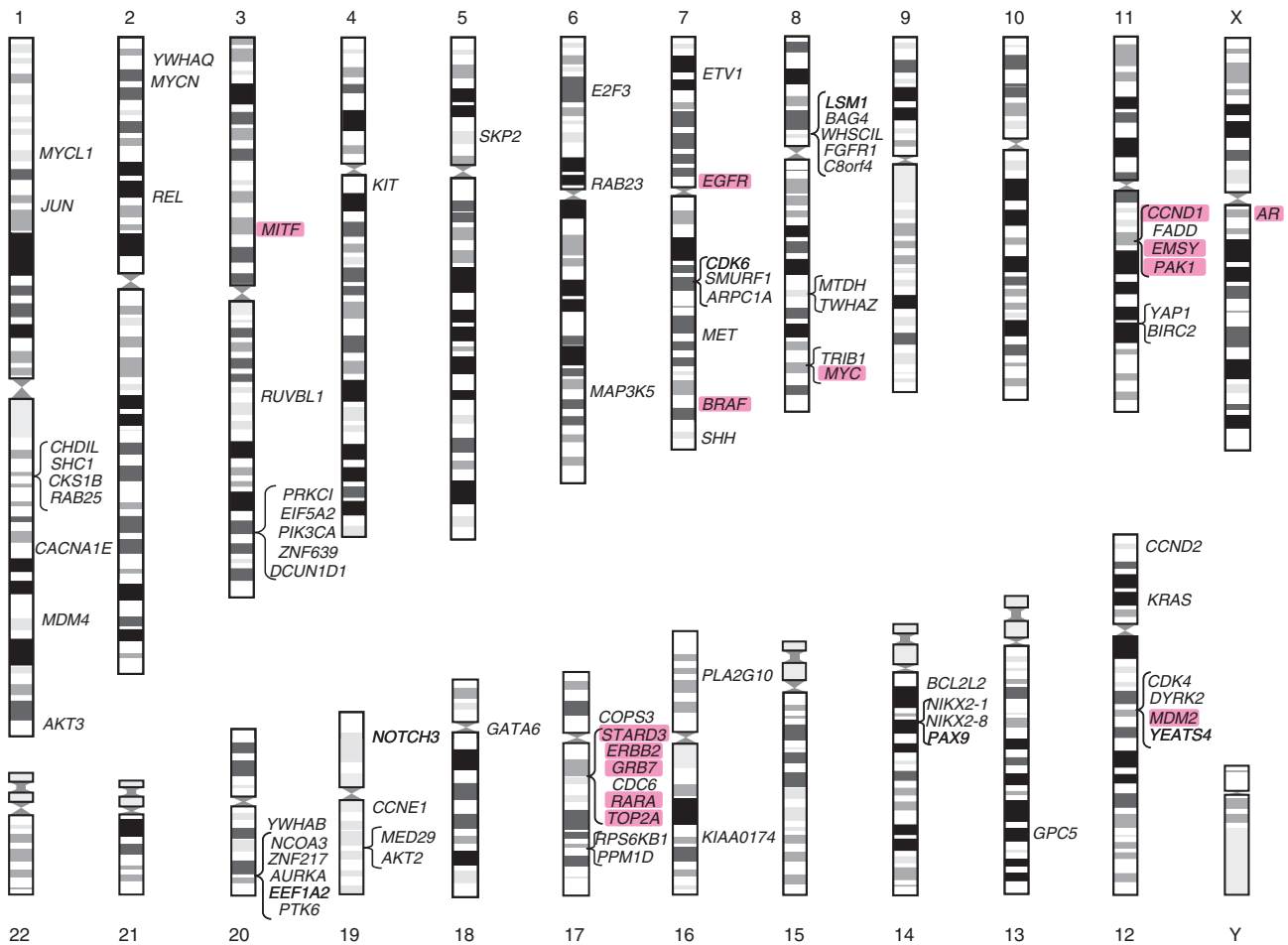
*CDK2*), and cell survival (B-cell CLL/lymphoma 2, *BCL2*) and differentiation (*TYR*, *TYRP1*, *DCT*, *MART1*, *AIM-1*, and *PMEL17*) (66). *MITF* is amplified in approximately 10–20% of primary melanoma cases, and the incidence is higher in metastatic melanoma. Furthermore, in metastatic melanoma, *MITF* amplification is related to a decrease of five years in survival (65). In addition, *MITF* can be used as an epithelioid melanoma marker, but not as a marker for rarer desmoplastic and spindle-cell melanoma variants (67, 68).

*EGFR* gene amplification in chromosome 7p12 causes many types of cancers, including malignant melanoma, breast cancer, colorectal cancer, and lung cancer (64, 69). *EGFR* is a member of the receptor tyrosine kinase ErbB family that is involved in cell proliferation, apoptosis resistance, survival, invasion, and migration. Ligand binding to *EGFR* induces a conformational change of the receptor and subsequent homo- or heterodimerization with other ErbB family proteins that result in the autophosphorylation of a tyrosine residue near the C-terminus. Downstream signaling pathways, such as RAS-RAF mitogen-activated protein kinase (MAPK) or PI3K-Akt pathways, are then activated via the phosphorylated tyrosine residues of *EGFR* (70). In glioblastoma multiforme, which accounts for the majority of malignant gliomas, *EGFR* overexpression is correlated with poor overall survival (71). *EGFR* amplification and overexpression are predictors of *EGFR*-targeted drugs.

One of the most successful *EGFR*-targeted therapies is the class of small molecule tyrosine kinase inhibitors (TKIs), which include gefitinib and erlotinib. TKIs block *EGFR*-mediated signal transduction by competing with ATP for binding to the tyrosine kinase domain of *EGFR* (72). In non-small-cell lung cancer, *EGFR* amplification is correlated with better response to gefitinib, which is more effective for patients carrying a somatic mutation in the *EGFR* gene (64). Conversely, some aberrations, like *MET* amplification, were reported to confer TKI resistance to *EGFR*-related cancer (73, 74). Therefore, combination therapies that target both *EGFR* and secondary aberrations would be more practical.

Another therapy is the *EGFR*-specific monoclonal antibody cetuximab. Unlike TKIs, cetuximab prevents *EGFR* signaling transduction by interfering with ligand binding, resulting in inhibition of *EGFR* dimerization and autophosphorylation. In addition, cetuximab reportedly induces *EGFR* internalization and destruction (72). Although cetuximab was proven to be efficient for *EGFR* in colorectal cancer, the presence of a *KRAS* mutation is significantly associated with poor response (75). Other studies demonstrate that v-Raf murine sarcoma viral





**Figure 5** Amplicons on various chromosomes. Genes mentioned in this review are highlighted.

oncogene homolog B (*BRAF*) mutation and *PIK3CA* mutation are also related to cetuximab resistance (76, 77). It may be necessary to examine not only *EGFR* amplification or mutation, but also the status of other drug resistance-related genes.

Mouse double minute 2 homolog (*MDM2*) gene amplification in chromosome 12q14 is found in at least 19 types of tumors, including sarcoma, esophageal carcinoma, and neuroblastoma (78). *MDM2* is a RING domain protein that plays an oncogenic role by inhibiting the p53 tumor suppressor. In most tumors, p53 is frequently inactivated by point mutation or deletion. However, even in cases in which *TP53* status is not aberrant, *MDM2* can abrogate the transcriptional activity of p53, which results in the increase of tumorigenic potential. In fact, transgenic mice that contain multiple copies of *Mdm2* transgene exhibit 100% incidence of tumorigenesis (79). Because cancers caused by *MDM2* amplification do not require the deregulation of p53,

*MDM2* amplification and *TP53* aberration tend to be mutually exclusive. Namely, *MDM2* amplification functions as a surrogate for loss or mutation of p53. Because of its critical role in tumor incidence, *MDM2* could be a therapeutic target. Through chemical library screening, Nutlin 3a was identified as a small-molecule *MDM2* antagonist (80). Nutlin 3a competes with p53 for binding to the *MDM2* N-terminus, and therefore disrupts the p53-*MDM2* interaction, which results in the recovery of wild-type p53. The Nutlin 3a derivative RG7112 is currently in clinical trials (81).

The cyclin D1 (*CCND1*) gene on chromosome 11q13 that encodes the cyclin D1 protein is amplified in various cancers, including breast, head and neck, lung, and bladder cancers (82). *CCND1* forms a complex with cyclin-dependent kinase (CDK) 4 or 6 and phosphorylates retinoblastoma (RB) protein, thereby initiating the G1/S cell-cycle transition (83). Furthermore, *CCND1* has a CDK-independent role as a transcriptional regulator. *CCND1*

interacts with various steroid hormone receptors and transcription factors, including estrogen receptor- $\alpha$  (ER $\alpha$ ), androgen receptor (AR), peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ), and signal transducer and activator of transcription 3 (STAT3) (84). In breast cancer, *CCND1* amplification is found in 15–20% of tumors (64, 84). Most *CCND1* amplifications are observed in ER-positive breast cancer and associated with poor prognosis and resistance to tamoxifen therapy (85).

*CCND1* amplification is frequently accompanied by co-amplification of the chromosome 11 open reading frame 30 (*EMSY*) gene, which is located at the same locus as *CCND1* 11q13. *EMSY* binds to the transactivation (TA) domain of breast cancer 2, early onset (BRCA2) and consequently inhibits its transcriptional activity (86). Therefore, *EMSY* amplification can be a substitute for loss or mutation of BRCA2 in breast cancer. Not only is *EMSY* amplification associated with poor prognosis, but patients with co-amplification of *EMSY* and *CCND1* also show worse outcomes than those with neither or one of the *CCND1* and *EMSY* genes amplified (85). P21 activated kinase 1 (*PAK1*), located at 11q13, also shows occasional co-amplification with *CCND1* (87). *PAK1* phosphorylates ER $\alpha$ , resulting in induction of upregulation of ER $\alpha$ -controlled proteins, like *CCND1* (88). Therefore, *PAK1* predicts for tamoxifen resistance.

Because *CCND1* amplification is one of the most common copy number alterations in human cancer (44), inhibiting *CCND1* function could be an attractive therapy. As the well-known role of *CCND1* is to induce the G1/S transition together with CDK4/6, CDK inhibitors that compete with ATP for binding to the active site of the kinase have been developed, and some have progressed to clinical trials (83). However, although such drugs exhibit beneficial effects to some extent, therapies focused on inhibiting CDK activity are incomplete, as *CCND1* has CDK-independent functions. To repress the entire negative response caused by *CCND1*, therapies that regulate *CCND1* at the protein level would be required.

The v-myc avian myelocytomatosis viral oncogene homolog (*C-MYC*) amplification in chromosome 8q24 is one of the most frequent copy number alterations in human cancer (44). *C-MYC* is a basic-helix-loop-helix-zipper (bHLHZ) transcription factor that activates various genes related to cell proliferation, apoptosis and inhibition of differentiation. *C-MYC* exerts its effect by forming a heterodimer with its partner bHLHZ protein MYC associated factor X (MAX), followed by binding specific DNA sequences called E-box sequences (89). In breast, colon, and lung cancers, *C-MYC* is proposed as the driver gene at the 8q24 amplicon (1). In addition,

patients carrying *C-MYC* amplification have a two-fold increase in risk of relapse and death (90). Because *C-MYC* induces apoptosis, a synergistic action with anti-apoptotic mechanisms, such as overexpression of BCL2 or BCL-X<sub>L</sub> or loss of the tumor suppressor p53, has been observed (89).

Owing to the broad range of *C-MYC* expression in human cancer, *C-MYC* has the potential to be a therapeutic target. Indeed, a study using the dominant-negative MYC mutant Omomyc in a transgenic mouse model demonstrated the feasibility of MYC-targeted therapy (91). Although developing a therapy that directly targets *C-MYC* is considered to be difficult, two studies have proven that the small-molecule bromodomain inhibitor JQ1 exhibits an anti-cancerous effect in multiple myeloma and acute myeloid leukemia, in which *C-MYC* is essential for tumor maintenance, both *in vitro* and *in vivo* (92, 93). Bromodomain-containing 4 (BRD4) binds to the acetylated lysine residue on histone proteins, resulting in transcriptional activation of cell-cycle-related genes. Bromodomain inhibition by JQ1 confers a repression of *C-MYC*-induced transcription and, surprisingly, MYC transcription itself. JQ1 has the potential to be a useful therapy for other cancers associated with *C-MYC*.

## Gene amplification and drug resistance

*AR* gene amplification in chromosome Xq11-13 is found in castration-resistant prostate cancers (CRPCs) (94). *AR* is a steroid hormone receptor that regulates cell growth and survival. Androgen binding causes a conformational change of *AR* that leads to *AR*-ligand complex translocation to the nucleus and subsequent gene transcription (95). Because all prostate cancers except for rare small cell carcinomas express *AR* at both the mRNA and protein levels, androgen ablation therapy has been the standard treatment (94). However, many prostate cancers are found to acquire resistance during the therapy, and such CRPCs have been reported to carry *AR* gene aberration. *AR* amplification is one of the genetic changes that occur in CRPCs. Approximately 80% of CRPCs exhibit increased *AR* gene copy numbers, and approximately 30% have high levels of amplification (94). *AR* amplification is correlated with increased expression, resulting in the increase of sensitivity to androgen (95). Interestingly, *AR* amplification was not observed before androgen ablation therapy in clinical tumors, suggesting that

AR amplifying cells that can proliferate under low levels of circulating androgen were selected during the therapy (96). Although CRPCs can respond to low androgen levels, their proliferation and survival are still androgen-dependent. Accordingly, combined therapy with gonadotropin-releasing hormone analogs and anti-androgens might be effective (97).

*BRAF* gene amplification in chromosome 7q34 also confers drug resistance. *BRAF* is a proto-oncogene that is mutated in several cancers, including melanomas and colorectal cancers (98). *BRAF* protein is a regulator kinase of the MAPK pathway. The most frequent mutation in *BRAF*, V600E, predicts for sensitivity to AZ628, an inhibitor of *BRAF* and to CI-1040 and its derivative PD0325901, both of which are inhibitors of mitogen-activated protein kinase kinase 1 (MEK), a kinase in the MAPK pathway downstream of *BRAF* (99, 100). Conversely, increased activity of CRAF which belongs to the same family as *BRAF* and a point mutation in MEK1 was identified as the mechanism that provides the resistance for AZ628 and the MEK inhibitor AZD6244 (selumetinib), respectively (101, 102). Recently, a colorectal cancer cell line model showed that V600E *BRAF* amplification also confers resistance to AZD6244 (103). *BRAF* amplification has been found in clinical colorectal cancer tumors. *BRAF* amplification is also found in 20% of malignant melanoma cell lines, although no significant correlation between copy-number increase and *BRAF* expression was observed (104). In colorectal cancer cell lines, V600E *BRAF* amplification increased the abundance of phosphorylated MEK and hindered the ability of MEK inhibitors (103). Interestingly, cells not treated with *BRAF* inhibitors included a small population of *BRAF* amplified cells (103). Therefore, clonal selection of cells carrying *BRAF* amplification might have occurred as a result of prolonged exposure to the drug. Because simultaneous inhibition of *BRAF* and MEK restores the sensitivity of *BRAF* inhibitor-resistant cells (103), a combined therapy has the potential to be an effective approach.

## Expert opinion and outlook

In this review, we have introduced the model of gene amplification and showed several examples of amplicons

in cancer. A considerable number of oncogenes have been identified in amplicons and their functions in cancer have been analyzed in detail, which emphasizes the significance of gene amplification during tumorigenesis. Furthermore, molecular targeted therapies such as trastuzumab have been developed to inhibit the functions of amplified oncogenes. Thus, it is reasonable to search for novel cancer-associated genes in amplicons if gene amplification accelerates the processes of tumorigenesis and progression. One typical approach is to knock down each gene to evaluate its function. We are also trying to express all the amplified genes simultaneously in a cell, however, novel experimental techniques need to be developed for this purpose. Even though several models to explain the mechanisms of gene amplification have been proposed so far, the complete mechanisms remain to be resolved. Understanding of the mechanisms may lead to the development of diagnostic markers as well as novel therapeutic approaches against gene amplification.

## Highlights

- Gene amplification was first discovered in the developmental program of *Drosophila melanogaster* and thereafter recognized in the response to cytotoxic drugs. Gene amplification is also observed in cancer and is one of the major causes of tumorigenesis.
- There are four models for the mechanisms of generation of amplicons: extrareplication and recombination, BFB cycle, DRCR and FoSTeS.
- Oncogenes are often observed in amplicons in cancer, and thus analyses have been conducted to identify novel oncogenes in amplicons. Several co-amplified genes also contribute to tumorigenesis in concert with oncogenes. Amplified oncogenes have the potential to serve as diagnostic and therapeutic targets.

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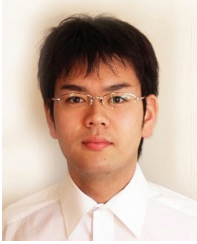
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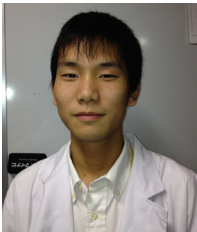
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