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Review Article

Mechanisms of aneuploidy induction by RAS and RAF oncogenes

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Abstract: Most cancers progress with the accumulation of genetic mutations with time and this is frequently associated with the acquisition of genomic instability in the form of whole chromosome changes, chromosomal rearrangements, gene amplifications or smaller changes at the nucleotide level. Whole chromosome instability (W-CIN), characterised by aneuploidy, is a major form of genomic instability observed in human cancers and several lines of evidence now support the argument that W-CIN is a promoter of tumourigenesis rather than being a passenger event. The primary mechanism proposed for evolution of CIN is abnormalities in mitosis/cytokinesis. However, mutations in genes directly involved in controlling mitosis/cytokinesis are rare in human cancers and so the mechanisms underpinning the evolution of CIN in cancers are not currently clear. On the other hand, mutations in *RAS* or *BRAF* are frequently found in human cancers, many of which demonstrate CIN, suggesting a possible link between deregulated signaling through the RAS/RAF/MEK/ERK pathway and CIN. In this review, we focus on a potential relationship between deregulated RAS/RAF signaling and CIN, and discuss possible mechanisms connecting the two.

Keywords: RAS, BRAF, CRAF, oncogenes, genome instability, aneuploidy, ERK, mouse models, cancer

1. Introduction

Genomic instability is a hallmark of cancer [1, 2], and chromosome instability (CIN) has been identified as a major form of genomic instability [3]. CIN is further categorized into either whole chromosome instability (W-CIN), characterized by numerical alterations of whole chromosomes, or structural chromosome instability (S-CIN), represented by chromosome rearrangements such as deletions, duplications and translocations [4]. While inappropriate DNA damage response/repair has been assumed to be the major cause of S-CIN, several different mechanisms have been proposed for W-CIN, including deregulated spindle assembly checkpoint (SAC) [5], alterations in kinetochore-microtubule (k-MT) dynamics [6], and defective chromosome cohesion [7], all of which facilitate, in principle, chromosome mis-segregation during mitosis.

The RAF/MEK/ERK MAP kinase pathway is one of the best-characterized signaling pathways downstream of the small GTPase RAS and comprises 3 RAF kinases (ARAF, BRAF, CRAF), two MEKs (MEK1 and MEK2) and two ERKs (ERK1 and ERK2) [8, 9]. In this pathway, RAF kinases phosphorylate MEK 1/2, which in turn phosphorylate ERK 1/2 to transduce the signal flow. Ligand-stimulation of several types of cell surface receptors induces conversion of 3 RAS homologues (HRAS, NRAS, KRAS) to active forms leading to recruitment of the RAFs to the plasma membrane and activation of the downstream ERK MAP kinase pathway. It is well established that the ERK pathway promotes cell proliferation through G1/S cell cycle progression [8], and mutations in RAS/RAF/MEK/ERK pathway components are found in various types of human cancers [9-12].

The fact that CIN and *RAS/RAF* mutations are a frequent occurrence in human cancers suggests a possible link between them. However, such a link has not yet been clearly demonstrated *in vivo*, especially in human cancers. Below we elucidate growing evidence for a role of these oncogenes in driving CIN in the evolution of cancers.

2. Summary of RAS/RAF/MEK/ERK pathway mutations in human cancer

Although the presence of *RAS* mutations in human cancer samples were initially documented nearly 30 years ago [9, 10], the advent of high throughput DNA sequencing projects in recent years has consolidated the fact that other components of the RAS/RAF/MEK/ERK are also mutational targets in human cancer. These sequencing efforts have identified *RAS* mutations in ~20-30% of human cancers, with *KRAS* mutations being far more common than either *HRAS* or *NRAS* mutations. *BRAF* is the next most commonly mutated gene in human cancers, at ~7-8% [11, 12]. Mutations in *CRAF, ARAF, MEK1 and ERK2* have been detected but are extremely rare while mutations affecting *MEK2* and *ERK1* have not been found so far. Although the vast majority of mutations are single point mutations, chromosomal translocations and rearrangements affecting *BRAF* or *CRAF* have been detected in some astrocytomas, melanomas, prostate and gastric cancer samples [13-16]. A summary of the current status of RAS/RAF/MEK/ERK pathway mutations in primary human cancer samples is provided in **Table 1**.

3. Signaling pathways downstream of RAS/RAF involved in mitosis

The ERK pathway is the best characterized signaling pathway downstream of the RAS/RAF cascade, promoting cell proliferation by facilitating G1/S cell cycle progression [8] or evoking premature cellular senescence when supra-physiological, robust activation is induced [17, 18]. In contrast to its well-established function in G1/S regulation, roles for the ERK pathway in mitosis are not entirely clear, especially in mammalian cells. The idea that ERK1/2 regulates mitotic progression comes primarily from studies on *Xenopus* egg extracts and oocytes and has been extensively reviewed elsewhere [19, 20]. In mammalian cells, although the activity of ERK1/2 has been reported to be enhanced during the G2/M transition [21, 22], the exact stage of the G2/M transition at which ERK1/2 is activated has been difficult to elucidate. A number of studies have shown that pharmacological and/or genetic inhibition of the ERK pathway delays the G2/M transition in mammalian cells [23-25]. However, long-term suppression of ERK1/2 activity in these studies makes it impossible to distinguish between direct effects of ERK1/2 and the ability of ERK1/2 to activate specific gene expression programmes required for progression through G2/M. A study utilizing live imaging and short-term MEK inhibition has demonstrated that direct ERK activity is dispensable for the G2/M transition, mitotic progression and spindle assembly in mammalian cells [26]. In contrast, depletion of ERK1/2 in human dermal keratinocytes, but not in fibroblasts, reportedly induced G2/M (or tetraploid G1) arrest through downregulation of cyclin B1 expression, suggesting that ERK-regulated gene expression programs, rather than direct ERK activity, could contribute to G2/M progression in a cell-type dependent manner [27]. Activated MEK1/2 and ERK1/2 have been reported to be detectable at centrosomes/spindle poles and kinetochores during mitosis, as assessed by immunofluorescence using phosphoantibodies [28-30]. However, there is little data to support functional roles at these locations and, furthermore, antibodies commonly used to detect phospho-MEK1/2 mitosis can cross-react with nucleophosmin, another nuclear phosphoprotein [31]. Another report has shown that ERK1/2 depletion and MEK inhibitor treatment failed to abrogate immunostaining of phosphorylated ERK at the mitotic apparatus [26], raising the possibility that the phosphorylated ERK in mitosis previously reported might be an immunological artifact. Overall, data indicating a role of the MEK/ERK pathway in G2/M progression needs to be carefully interpreted.

None of the above studies have addressed whether supra-physiological hyperactivation of the ERK pathway by oncogenic RAS/RAF affects mitotic progression. Indeed, when the ERK pathway is hyperactivated by ectopic expression of oncogenic RAS/RAF mutants, mitotic progression is reportedly perturbed [32-34]. In human melanoma cells, ectopic expression of ^{V600E}*BRAF*, the most common *BRAF* mutant detected in human cancers, was shown to promote aberrant activation of the SAC through stabilization of the mitotic checkpoint protein MPS1 in a MEK/ERK-dependent manner [32], and this led to chromosome mis-segregation and aneuploidization [33]. In contrast, ^{G12V}HRAS conditionally expressed in rat thyroid cells has been reported to attenuate the SAC activation in a MT-depolymerized condition, but this phenotype did not require MEK/ERK activity [34]. Thus, the biological consequence of hyperactivation of the ERK pathway in mitosis seems context-

Table 1. Summary of mutations of RAS/RAF/MEK/ERK pathway components detected in primary human cancer samples.

Cancer Type	Organ	% Common point mutation				Rare point mutation (# of cases)			Gene fusion (# of cases)	
		KRAS	NRAS	HRAS	BRAF	CRAF	ARAF	MEK1	BRAF	CRAF
Carcinoma	Oesophagus	3%	0%	0%	3%	ND	ND	ND	ND	ND
	Stomach	7%	3%	2%	1%	0%	0%	0%	AGTRAP-BRAF (1)	ND
	Colorectal	36%	4%	0%	10%	0%	G331C (1) T442T (1)	D67N (1)	ND	ND
	Pancreas	67%	0%	0%	2%	0%	0%	ND	ND	ND
	Liver	4%	2%	0%	0%	N115S (1)	ND	ND	ND	ND
	Biliary Tract	29%	2%	0%	10%	0%	ND	0%	ND	ND
	Thyroid	4%	7%	3%	44%	ND	ND	ND	AKAP9-BRAF (4)	ND
	Lung	16%	1%	0%	2%	A319S (1)	Q383H (1)	K57N (3) Y240Y (1)	ND	ND
	Skin	4%	4%	8%	0%	0%	ND	0%	ND	ND
	Kidney	1%	1%	0%	0%	0%	ND	0%	ND	ND
	Urinary Tract	4%	0%	8%	1%	0%	ND	0%	ND	ND
	Prostate	7%	1%	5%	4%	0%	0%	0%	SLC45A3 -BRAF (1)	ESRP1-CRAF (2)
	Breast	2%	2%	0%	1%	0%	0%	0%	ND	ND
	Ovary	11%	3%	0%	4%	S259A (1)	P561P (1)	0%	ND	ND
	Endo-metrium	15%	1%	0%	4%	ND	ND	ND	ND	ND
	Cervix	8%	2%	8%	1%	0%	ND	0%	ND	ND
Haemopoi-etic neo-plasms	Lymphoid (Non-HCL)	6%	10%	0%	1%	0%	0%	0%	ND	ND
	HCL	ND	0%	ND	100%	ND	0%	ND	ND	ND
	MM	4%	21%	0%	3%	0%	0%	0%	ND	ND
	Myeloid (Non-LCH)	4%	10%	0%	1%	0%	0%	0%	ND	ND
	LCH	ND	ND	ND	57%	ND	0%	ND	ND	ND
CNS Tumours		1%	1%	0%	3%	0%	0%	0%	KIAA1549-BRAF (130)*	SRGAP3-CRAF (2)*
Melanoma		3%	20%	1%	39%	0%	ND	K57N (1)	ND	ND

ND: Not Determined; HCL: hairy cell leukemia, MM: multiple myeloma, LCH: Langerhans cell histiocytosis All data was obtained from the Catalogue Of Somatic Mutations In Cancer (COSMIC: http://www.sanger.ac.uk/genetics/CGP/cosmic/) except for mutations in HCL [114], MM [115] and LCH [116] that were described in the corresponding references and mutations in MEK1 that were reported in references [117-120]. Only mutations detected in primary human cancer samples are included in the Table. A mutation in *ERK2* has been detected in only one ovarian cancer sample (A143A) but, for convenience, is not included in this table. *MEK2* and *ERK1* mutations have not been reported as yet. *For CNS tumours, BRAF translocations were detected in 46% of pilocytic astrocytoma and CRAF translocations in 4% of pilocytic astrocytoma

dependent. Another study utilizing HeLa cells depleted for the Raf Kinase Inhibitory Protein (RKIP) in which CRAF, but not BRAF, was activated, also demonstrated that ERK hyperactivation through CRAF attenuates the SAC function in a MT-stabilized condition by inhibiting the chromosome passenger protein Aurora B [35]. However, this study relied on using the MEK inhibitor PD098059 at 10μM, which has been shown by others to be insufficient to inhibit ERK activation in HeLa cells even at 100μM [22], as well as partial inhibition by dominant-negative MEK, to prove the contribution of ERK activation in this response. Further studies using more potent and specific inhibitors will be needed to confirm the significance of CRAF-induced ERK

hyperactivation in this context.

Whereas it is well established that BRAF signals primarily through the ERK pathway [36], CRAF has been reported to regulate other pathways independently of MEK/ERK activation [37]. These include ASK1-p38 MAP kinase, MST2-LATS-YAP1-p73, and ROKα-LIMK-COFILIN pathways, which may potentially contribute to mitotic progression through regulating the antephase checkpoint [38], mitotic cell death [39] and the SAC [40], and spindle positioning [41] and cytokinesis [42, 43] respectively. However, none of these pathways have been extensively investigated so far in the context of mitosis in the CRAF-deficient or -activated condition.

4. Aneuploidy and W-CIN

W-CIN is defined as a persistently high rate of losses/gains of whole chromosomes, and inevitably causes aneuploidy [3, 44]. However, single catastrophic events that transiently cause losses or gains of whole chromosomes can also result in aneuploidy without persistent instability, as seen in some types of congenital disorders. Even in cancers, chromosomally stable aneuploid tumors could develop through clonal evolution of aneuploid cells generated by such a single catastrophic event. Therefore, W-CIN must be evaluated by assessing the temporal dynamics of alterations in chromosome numbers or chromosome number variations at a single cell level, when only a single time point analysis is available. Single cell assays such as karyotyping and FISH are feasible in the latter case, but array CGH or DNA ploidy analysis using bulk populations on a single time point is not suitable for evaluating W-CIN, even if modal aneuploidy can be detected using these methodologies [44]. Of note, array CGH is a strong tool for detecting whole chromosome and segmental aneuploidy when clonally evolved, genetically homogenous cell populations are analyzed. Therefore, this method is useful for evaluating W-CIN when combined with single cell genomic DNA isolation or clonal subcultures.

Recent studies using yeast and mouse models with stable aneuploidy have demonstrated that aneuploidy itself has a negative impact on cell proliferation [45, 46]. This was also found to be the case in aneuploid daughter cells developed from chromosomally stable, near-diploid human cancer cells following transient pharmacological

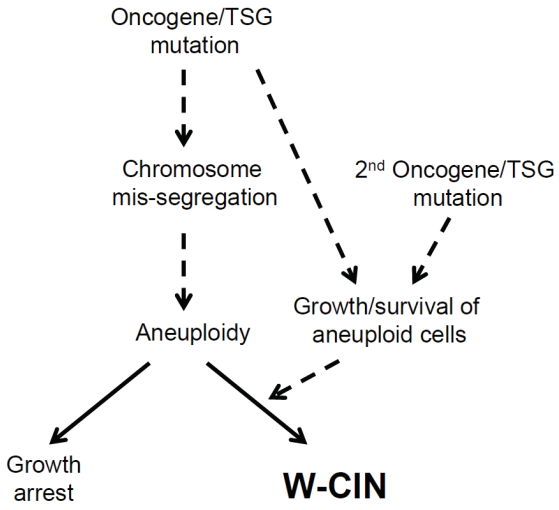

Figure 1. Evolution of Whole Chromosome Instability (W-CIN) in cancer. There are two distinct but intertwined characteristics that are required for evolution of CIN in cancer cells. The first is chromosomal heterogeneity that likely arises through chromosome mis-segregation and the second is compensatory mechanisms for avoiding aneuploidy-induced growth inhibition. These characteristics may be acquired either through mutation of a single oncogene/Tumour Suppressor Gene (TSG) or through sequential oncogene/TSG mutation.

perturbation of k-MT dynamics [47, 48]. In this case, proliferation of newly generated aneuploid cells was inhibited by p38 MAP kinase-mediated activation/stabilization of the tumor suppressor p53 [47]. Importantly, the cell line used in this study (HCT116) harbors the *^{G13D}KRAS* oncogenic mutation, indicating that signals mediated by ^{G13D}KRAS are not sufficient for circumventing this inhibitory effect. In contrast, further aneuploidization of CIN cancer cells did not induce growth inhibition [48], indicating that this growth-inhibitory machinery is abrogated in CIN cancer cells. Thus, W-CIN can be characterized not only by an increased rate of chromosome mis-segregation, but also by cancellation of aneuploidy-induced growth inhibition (**Figure 1**). It is currently unknown whether abrogation of the p38/p53 pathway can entirely explain the escape from the aneuploidy-induced growth inhibition in CIN cancers, or whether other mechanisms are involved in this process. Although pharmacological MEK inhibition in the presence of functional p38/p53 has been reported to have no impact on aneuploidy-induced growth inhibition [47], it has yet to be clarified as to

whether hyperactivation of the ERK pathway and/or MEK/ERK-independent CRAF effectors could be involved in escape from this growth-inhibitory machinery. Since the cell line used in this study harbors an oncogenic KRAS allele, it is important to elucidate whether oncogenic KRAS is still required for circumventing this growth inhibition when the p38/p53 pathway is disrupted.

5. The RAS/RAF cascade and W-CIN

5.1 Cell culture studies

Most of the early studies that evaluated the effect of oncogenic RAS on W-CIN were not ideal as they utilised indirect methodologies such as micronuclei formation [49-53]. However, a particularly elegant study by Woo and Poon utilised direct karyotyping combined with growth of cells under serum free conditions [54]. In this report, oncogenic ^{G12V}HRAS expressed in primary MEFs with a stable karyotype was shown to induce near-tetraploid aneuploidy within 24hrs of retroviral expression [54]. This phenotype satisfies the criteria of W-CIN, and was further enhanced when ^{G12V}HRAS was expressed in p53-deficient MEFs. p53 deficiency itself did not cause significant alterations of the MEF karyotype under the same conditions [54]. These findings fit the two-step theory for W-CIN establishment; the first step involving the induction of chromosome mis-segregation as promoted by oncogenic RAS in this situation, and the second step mediated by p53 deficiency supporting the growth of the resulting aneuploid cells (**Figure 1**).

Consistent with this assumption, several previous studies investigating mitotic phenotypes induced by ectopic expression of mutant RAS point to a potential role for oncogenic RAS in chromosome mis-segregation [49-52]. A recent study using an isogenic pair of human cancer cells with or without an endogenous oncogenic KRAS allele demonstrated that an endogenous level of oncogenic KRAS evokes chromosome mis-segregation with mitotic stress phenotypes [55]. Although it is largely unknown how oncogenic RAS induces aberrant mitosis, it is notable that this study demonstrated synthetic lethality between oncogenic KRAS and inactivation of PLK1 [55], suggesting that oncogenic RAS could deregulate pathways functionally compensated by PLK1. PLK1 is involved in almost all aspects of mitotic progression, regulating a wide variety of downstream substrates [56], some of which, such as CDC25C, are also reportedly regulated by the ERK pathway [57]. Detailed analysis of such common targets between PLK1 and the RAS/RAF/ERK pathway could in the future provide clues as to how oncogenic RAS deregulates mitotic progression.

Another possible mechanistic link between oncogenic RAS and W-CIN has emerged from studies investigating the role of the RB/E2F pathway in mitotic progression [58-61]. The tumor suppressor RB, which is classically mapped as downstream of the RAS/RAF/ERK pathway via G1 cyclins and CDKs, has been shown to transcriptionally suppress the key SAC protein MAD2 through regulation of the transcription factor E2F [58], and to control centromeric localization of condensin and cohesin that are important in maintaining centromeric structure and function [59, 60]. RB depletion by gene targeting or shRNA caused MAD2 overexpression [58] and centromeric dysfunction [60], resulting in aberrant mitosis with chromosome mis-segregation and aneuploidy as confirmed by karyotyping and FISH. Although G1 cyclin/CDK activation by the RAS/RAF/ERK pathway could promote phosphorylation of RB and E2F-induced transcriptional activation of MAD2, it is currently unclear whether RB phosphorylation by upstream signals affects its centromeric function [59, 60]. Further studies elucidating the effect of post-translational modification of RB on chromosome integrity will be needed to consolidate the significance of the RB pathway in connecting the RAS/RAF cascade and W-CIN.

In addition to oncogenic RAS, a recent study utilizing human melanoma cells and primary melanocytes ectopically expressing ^{V600E}BRAF revealed that the most common cancer-associated BRAF mutant induces not only mitotic abnormalities but aneuploidization during a relatively short period as confirmed by karyotyping and FISH [33]. Given that BRAF primarily signals via the ERK pathway [36], it is most likely that the observed aneuploidization is attributable to ^{V600E}BRAF-induced ERK hyperactivation, though a causative role of the ERK pathway in the aneuploid phenotype was not directly proven in this study. Two of the melanoma cell lines used in this study for ectopic ^{V600E}BRAF expression are also known to harbor the ^{Q61K}NRAS mutation, suggesting that the aneuploid phenotype might be a consequence of

the combinatorial effect of ^{V600E}BRAF and ^{Q61K}NRAS. In contrast, we have recently reported that primary splenic myeloid cells derived from Mx1-Cre;Braf$^{LSL\text{-}V600E/+}$ mice, in which ^{V600E}Braf is expressed from its endogenous allele in hematopoietic cells, maintain a relatively stable karyotype *in vivo* and for two weeks in culture [62]. Similarly, splenocytes from Mx1Cre;Kras$^{LSL\text{-}G12D/+}$ mice have also been reported to hold a stable diploid karyotype [63]. Thus, mouse hematopoietic cells could be relatively resistant to W-CIN induction by oncogenic RAS/RAF, compared to human melanocytes. It remains an open question as to what determines tissue type-specific sensitivity to W-CIN, if any. Protein expression levels of oncogenic RAS/RAF, downstream signal choice (PI3K, BRAF or CRAF) by oncogenic RAS, and compensatory mechanisms for aneuploidy-induced growth inhibition, may vary among tissue/tumor types and all of these could potentially affect development of the W-CIN phenotype.

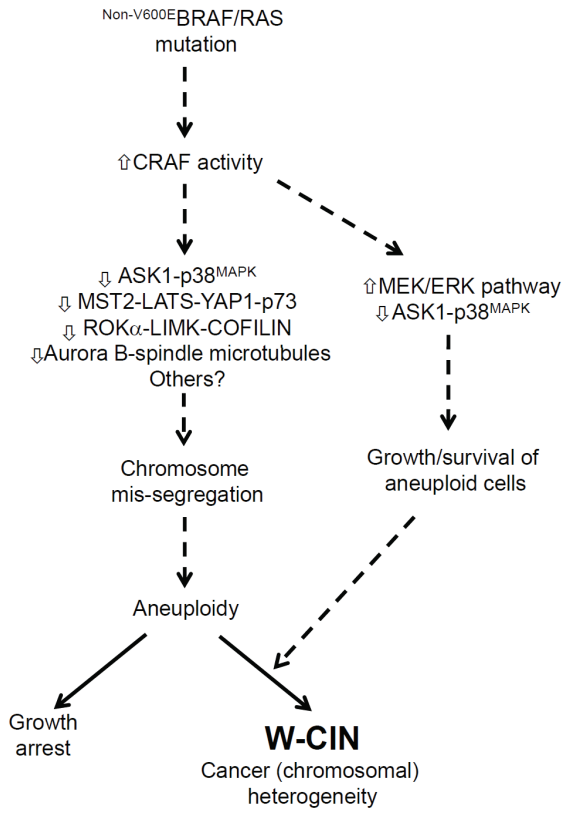

Figure 2. Role of CRAF in the evolution of Whole Chromosome Instability (W-CIN). Our investigations have provided evidence for a role of deregulated CRAF in the evolution of W-CIN [62, 87]. This finding came about through studies of impaired activity mutations of *BRAF* that are detected in human cancer and are involved in the cancer phenotype through their ability to heterodimerise and transactivate CRAF. It was found that deregulated CRAF was involved in driving the evolution of CIN downstream of mutant BRAF. However, the consequent deregulation of the MEK/ERK pathway was found not to be involved in the emergence of aneuploidy but, rather, was found to be required for the growth of aneuploid cells. This discovery fits the previously proposed dual model for the evolution of W-CIN (Figure 1). A number of MEK/ERK-independent effector pathways have been proposed previously for CRAF, as indicated, but which one(s) of these are involved in the two pathways leading to the evolution of W-CIN are not currently known. CRAF is a known effector of oncogenic RAS, and it will be important to assess in the future whether the emergence of W-CIN in RAS mutant tumours occurs through similar pathways. Our studies also ruled out a role for the most common BRAF mutant, ^{V600E}BRAF, as a single mutation in the evolution of W-CIN, which fits with the observation that ^{V600E}BRAF does not evidently signal through CRAF [86] and with the fact the ^{V600E}BRAF mutation is primarily linked with CIN-low CRCs [94]. However, ^{V600E}BRAF may function as a second oncogene to support growth/survival of aneuploid cells generated by the first oncogene/TSG in some types of cancers such as melanoma.

5.2 Mouse models

5.2.1 RAS models

Since the first transgenic mouse model for oncogenic RAS was reported in 1987, numerous different models utilizing exogenous transgene expression under tissue-specific promoters or knock-in mutations of endogenous loci have been developed [9]. A few studies of these mouse models have focused on CIN and most of these suggest that the role of oncogenic RAS in W-CIN *in vivo* is cooperative with other genetic events, such as p53 mutation [64] or MAD2 overexpression [65], as summarized in **Table 2**. However, it is still somewhat vague as to whether oncogenic RAS, as a sole genetic event, could affect chromosome integrity *in vivo*.

Using transgenic mouse models in which tissue-specific promoters drive oncogenic RAS, CIN and/or aneuploidy have been investigated in the resulting intestinal [66], lymphoid [67] and lung [65] tumors. When ^{G12V}KRAS was expressed under the control of the mouse villin promoter, more than 80% of aged mice developed intestinal tumors, ranging from adenomas with a moderate dysplasia to invasive adenocarcinomas [66]. However, none of these tumors exhibited aneuploidy when examined by flow

Table 2. RAS/RAF mouse models showing evidence of CIN. Although several RAS/RAF oncogenic mouse models have been reported in the past, the effects of oncogene expression on chromosome stability has only been examined in a few cases. The results of these are summarized in the table below.

Mouse strain	Description of model	CIN phenotype	Reference
RAS models - transgenic			
Villin-^{G12V}KRAS	Expression of ectopic ^{G12V}KRAS under the control of the villin promoter and examination of effects in intestinal epithelium	None observed. 4/6 tumours analysed showed a DNA diploid profile resembling that of normal mucosa.	[66]
Eμ-^{G12D}NRAS combined with p53 or Suv39h1 null alleles.	Expression of ectopic ^{G12D}NRAS under the control of the haemopoietic-specific promoter Eμ and examination of effects in lymphoid tissue.	Modest deviation from normal karyotype in Eμ-^{G12D}NRAS lymphomas on wild-type and *Suv39h1* null backgrounds. Tendency towards hyperdiploidy in Eμ-^{G12D}NRAS;*p53*-null lymphomas	[67]
Doxycycline-inducible CCSP-rtTA transactivator with rtTA-responsive Mad2 and/or ^{G12D}KRAS transgenes	Inducible expression of ectopic Mad2 and/or ^{G12D}KRAS under the control of the Clara cell secretory protein promoter and examination of effects in lung epithelium	Higher levels of aneuploidy in ^{G12D}KRAS lung (13%) than normal (2%) but a further three-fold increase in aneuploid cells (38%) in ^{G12D}KRAS plus Mad2-expressing lung. CGH analysis failed to detect any gross abnormalities.	[65]
RAS models – conditional knockins			
LSL-^{G12D}KRAS conditional knockin allele combined with Pdx1-Cre mouse, with or without the p53$^{R172H/+}$ allele	Expression of endogenous ^{G12D}KRAS in progenitor cells of the mouse pancreas.	Aberrant karyotypes in the vast majority of pancreatic ^{G12D}KRAS; p53$^{R172H/+}$ carcinoma cell lines but not from preinvasive ductal cells isolated from ^{G12D}KRAS pancreas. Abnormalities included W-CIN and S-CIN.	[64]
LSL-^{G12V}KRAS/β-geo combined with CMV-Cre mouse	Expression of endogenous ^{G12V}KRAS in MEFs	Abnormal karyotypes observed in late passage MEFs involving W-CIN and S-CIN without evidence of p53 or p19^{ARF} loss.	[70]
RAF models – conditional knockins			
LSL-^{V600E}BRAF combined with Mx1-Cre	Expression of endogenous ^{V600E}BRAF in haemopoietic cells and examination of effects in spleen	Stable karyotype in aberrantly growing ^{V600E}BRAF-expressing myeloid cells	[62, 81]
LSL-^{D594A}BRAF combined with CMV-Cre	Expression of endogenous kinase inactive ^{D594A}BRAF constitutively and examination of effects in spleen and MEFs	W-CIN observed in aberrantly growing ^{D594A}BRAF-expressing Cd11b+ myeloid cells and MEFs without evidence of p53 or p19^{ARF} loss.	[62]

cytometry, even in clonally-evolved advanced (invasive) adenocarcinomas [66]. Although flow cytometry is not sufficient for detecting cell-to-cell variability of chromosome numbers [44], these tumors also failed to display loss of heterozygosity (LOH) of the *Apc* locus. Since W-CIN has been suggested to promote tumorigenesis, at least in part through facilitating LOH of tumor suppressor loci [68], this study collectively implies that oncogenic KRAS in intestinal epithelial tumors may not induce W-CIN.

In contrast, T-cell lymphomas developed in Eμ-NRAS^{G12D} mice, in which mutant NRAS was

driven by the immunoglobulin heavy (μ)-chain enhancer, have been reported to exhibit aberrant karyotypes involving translocations between chromosomes 6 and 15, and gains of chromosomes 10 and 17, indicative of S-CIN and W-CIN respectively [67]. In this model, some tumors displayed modal hyperdiploidy with significant variability of chromosome numbers even in the limited number of metaphases analyzed. Interestingly, this phenotype was further worsened on a p53-deficient background, suggesting the possible synergy between oncogenic NRAS and p53 deficiency in W-CIN induction.

Transgenic mice expressing ectopic ^{G12D}KRAS specifically in alveolar epithelial cells in a chemically inducible manner have been developed [65] and, in this model, ^{G12D}KRAS expression in alveolar epithelium resulted in the development of lung adenocarcinomas. These tumors exhibited higher levels of aneuploidy as detected by FISH than wild-type or MAD2-overexpressing lung epithelium, indicating that oncogenic KRAS might promote W-CIN more drastically than the deregulated SAC function caused by exogenous MAD2 expression. Intriguingly, when both ^{G12D}KRAS and exogenous MAD2 expression were induced, a strong synergistic effect on aneuploidy induction was observed. In this context, it is currently unknown as to whether oncogenic KRAS enhanced chromosome mis-segregation, or compensated for the growth inhibition of aneuploid cells generated by perturbation of MAD2 expression, or both.

Controlling RAS transgene expression within physiologically relevant levels is quite challenging in mouse models using the exogenous promoters described above. To overcome this problem, conditional knock-in mouse models, in which oncogenic KRAS is expressed from the endogenous locus after Cre recombinase (Cre)-mediated removal of a lox-stop-lox (LSL) element were developed by two independent research groups [69, 70]. The first model was designed to express ^{G12D}KRAS (hereafter referred to as G12D), and has been used as a model for lung [69], colon [71, 72], hematopoietic [63, 73] and pancreatic [64, 74] tumorigenesis. Notably, primary pancreatic ductal adenocarcinoma cells arising in the G12D model with Pdx1Cre on a p53$^{R172H/+}$ background display a highly aberrant karyotype with characteristics of both W-CIN and S-CIN [64]. Again, the synergy between oncogenic KRAS and p53 deficiency regarding CIN induction was obvious. This was in contrast to preinvasive ductal cells with G12D alone, in which two thirds of metaphase spreads displayed a diploid karyotype in a similar way to normal (wild-type) pancreatic ductal cells, or the mice with p53 mutation alone which did not develop pancreatic tumors. However, in the future, detailed cytogenetic analyses of the preinvasive lesions and advanced tumors that arise in the context of G12D alone will be needed to elucidate any roles for ^{G12D}KRAS as a sole driver of CIN during pancreatic carcinogenesis. On the other hand, myeloproliferative neoplasms developed by crossing the G12D model to the Mx1Cre strain were reported to maintain a stable diploid karyotype [63]. Unfortunately, CIN phenotypes in colon and lung neoplasms arising in the G12D model have not been reported as yet.

The second knock-in KRAS model incorporated a bicistronic construct to express ^{G12V}KRAS as well as a marker gene β-geo (hereafter referred to as G12V/β-geo) [70]. Using this model, CIN was investigated in MEFs, in which ^{G12V}KRAS expression was induced by the CMVCre transgene. Although karyotype abnormalities were not evident in primary MEFs, tetraploidization and structural chromosome abnormalities were prominent at later passages, suggesting that *in vitro* culture stresses such as ROS production might contribute to the CIN phenotype. Since tetraploidization was commonly observed in both mutant and control MEFs at late passage, S-CIN rather than W-CIN seemed to be the predominant characteristic induced by ^{G12V}KRAS in this system. Surprisingly, in the G12V/β-geo model, no overt tumour formation was observed in adult epithelial tissues except for the lung when ^{G12V}KRAS expression was induced by CMVCre or 4OHT-induced CreER^{T} activation, making it difficult to follow the effects of ^{G12V}KRAS on CIN during *in vivo* carcinogenesis.

A number of possible explanations for the discrepancy between the G12V/β-geo and G12D models have been previously proposed [72]. We also believe that the different Cre expression/activation systems used in these two studies may be a critical factor in determining the distinct phenotypes. In the G12D model, tissue-specific but constitutively expressed (activated) Cre strains were used to induce solid tumors in

most cases, except for the induction of lung cancers (adenovirus-Cre) and skin papillomas (K14-CreER^{T}). In contrast, the CMVCre strain used in the G12V/β-geo model is thought to possess relatively weak recombinase activity, based on the fact that it has been reported to induce only mosaic recombination for some loxP flanked lesions [75] and its expression in primary MEFs does not evoke growth inhibition [62] or karyotype abnormalities [70]. Constitutively expressed Cre causes continuous genome damage [76-78] and this could, potentially, cooperate with oncogenic RAS in inducing carcinogenesis or, more specifically, the CIN phenotype. Overall, it would seem to be important to test if the CIN phenotypes observed in the various oncogenic RAS models can be reproduced in the absence of Cre or with weak, transient Cre activation.

5.2.2 RAF models

Within a decade since human cancer-associated *BRAF* mutations were first reported [11], transgenic mouse models for the most common ^{V600E}BRAF mutant using either tissue-specific, chemically-inducible promoters [79, 80] or conditional knock-in strategies involving the LSL element [81-83], have already been developed by several independent research groups including our own. However, expression of ^{V600E}BRAF in alveolar [82]/intestinal [84] epithelial cells or skin melanocytes [85] using knockin models consistently promotes oncogene-induced senescence (OIS) and thus a time window for analyzing CIN in these models is limited to an initial proliferation phase that occurs before the OIS program is engaged. As mentioned earlier, we have recently taken advantage of our conditional ^{V600E}BRAF knock-in mouse model intercrossed with the poly I:C-inducible Mx1Cre strain [62] to investigate the role of ^{V600E}BRAF in CIN. In this model, fetal myeloproliferative neoplasia spontaneously develops within a short time after birth even without poly I:C injection [81]. Cre-induced genome damage is minimized in this situation by the fact that poly I:C-induced Cre activation is avoided and, furthermore, the aberrantly growing myeloid cells in this strain do not exhibit obvious OIS responses. Interestingly, primary splenocytes derived from the Mx1Cre; Braf$^{V600E/+}$ mice maintain a stable diploid karyotype *in vivo* as well as following culture [62], indicating that hyperactivation of the BRAF/ERK pathway in the myeloid lineage does not compromise chromosome stability. Similar analyses for clinically-relevant cell/tissue types such as melanocytes, thyroid, and colon will be needed to clarify whether ^{V600E}BRAF is involved in W-CIN and, if so, whether tissue-specific differences exist.

Although the ^{V600E}BRAF mutant is detected in more than 90% of cancers with *BRAF* mutations, a number of other residues in BRAF are mutated at a lower frequency [86]. Aspartate 594 is the fourth most common BRAF residue mutated in human cancer and, interestingly, mutants of this residue are kinase inactive. The mechanisms by which these mutants contribute to tumour development has been an unresolved question. To investigate this, we have generated a conditional knock-in mouse model for ^{D594A}BRAF and recently reported its characterization [62, 87]. We have found that constitutive, heterozygous expression of this mutant in mice promotes W-CIN/aneuploidization of primary MEFs and splenic myeloid cells, even in the absence of the Cre recombinase [62]. This mutant was also found to heterodimerise with CRAF [87], inducing its hyperactivation and activation of the downstream MEK/ERK pathway. The W-CIN phenotype of cultured splenocytes was rescued by chemical inhibition using the RAF inhibitor sorafenib and by intercrossing the ^{D594A}*Braf* mice with kinase-inactive ^{D486A}*Craf* mice. However, MEK inhibition using the U0126 was unable to rescue W-CIN but inhibited the growth of aneuploid cells. Even though sorafenib suppressed the MEK/ERK pathway to some degree, it was found to promote splenocyte growth, presumably because it allows the retention of diploid cells that have a growth advantage. The fact that hyperactivation of the MEK/ERK pathway by itself is not sufficient to induce the emergence of W-CIN is consistent with analysis of mouse splenocytes expressing ^{V600E}BRAF mentioned above that retain a diploid karyotype. Taken together, these data suggest a model in which CRAF transactivation by ^{D594A}BRAF functions through MEK-independent pathways to promote W-CIN and through MEK-dependent pathways to promote the growth of aneuploid cells (**Figure 2**). As such this model fits the previously proposed scenario (**Figure 1**) whereby CIN arises through cooperation between aberrant signaling pathways that, on the one hand, promote chromosome mis-segregation and, on the other, promote the growth of the resulting aneuploid cells.

As mentioned above, CRAF has been suggested to activate a number of downstream signaling pathways in addition to the MEK/ERK pathway and an important next step will be to identify which of these CRAF effectors pathways is involved in chromosome mis-segregation and aneuploidy-induced growth. In preliminary investigations, Rok-α activity was found not to be altered in ^{D594A}BRAF-expressing cells synchronized at mitosis, suggesting the involvement of alternative pathways in the CIN response. In addition, it will be important to elucidate whether the same mechanisms operate in the context of other oncogenes acting upstream of CRAF, particularly RAS isoforms and Receptor Tyrosine Kinases (RTKs) that are mutated at a high frequency in human cancers. At least in human melanoma and mouse lung cancer, oncogenic RAS has been shown to be dependent on CRAF rather than BRAF [88, 89], raising the possibility that CRAF might be involved in W-CIN in these cancers.

5.3 Human colorectal cancer (CRC) studies

As mentioned earlier, the presence of aneuploid cells in human tumor samples does not necessarily mean W-CIN [44]. Because the evaluation of dynamic changes of karyotypes in culture is usually not feasible for primary human tumor samples, cell-to-cell variability of chromosome numbers as assessed by karyotyping or interphase FISH of primary tumour sections are the only realistic indicators of W-CIN. However, such examinations have not been routinely performed in clinical practice for solid cancers. Recently developed technologies including array CGH and SNP array are powerful tools for identifying aneuploidy, LOH and copy number variations when these abnormalities are homogenously distributed in the sample, but useless for evaluating on-going instability unless single cell or subclonal analysis is combined [44]. Thus, the major limitation in clinical studies for CIN is that the existence of aneuploidy has been used as a surrogate marker, but instability itself has not been directly measured. In this situation, it is important to validate the accuracy of static aneuploidy as a marker to estimate dynamic W-CIN, which might vary among tissues/tumor-types.

It has been well established that colon cancer lines with mismatch repair (MMR) deficiency maintain relatively stable, near-diploid karyotypes, but display microsatellite instability (MSI), another form of genomic instability [3]. On the other hand, MMR-proficient, microsatellite stable (MSS) colon cancer lines, which are usually aneuploid, have been reported to exhibit massively increased variability of chromosome number in clonal cultures [90], suggesting that static aneuploidy with the MSS phenotype in CRCs could faithfully reflect on-going W-CIN. Consistently, most near-diploid lines derived from colon cancers in the NCI-60 panel show minimal numerical heterogeneity in their karyotypes (which means they are chromosomally stable), whereas most aneuploid colon cancer lines in this panel display significant numerical heterogeneity (instability) [91]. In contrast, some ovarian, breast, and renal cancer lines display minimal numerical heterogeneity despite their aneuploid (hyper-diploid or near-triploid) karyotypes [91], suggesting that aneuploidy in these types of cancers does not specify W-CIN tumors. Thus, CRCs are ideal materials for correlation studies between *BRAF/KRAS* mutations and W-CIN (= aneuploidy = MSS) in humans.

KRAS mutations are detected in both MSI-high/CIN-negative and MSS/CIN+ CRCs, though the frequency is higher in MSS tumors [92-95]. Interestingly, while most mutations in MSS tumors (>80%) occur in codon 12 (G12) [92, 95], more than 50% of mutations in MSI-high tumors with hereditary MMR deficiency are in codon 13 (G13) [92], implying that there are potential differences in oncogenic functions between G12 and G13 mutants as well as in mutagenic mechanisms involved in the development of MSS/CIN+ and MSI-high/CIN-negative CRCs. *KRAS* mutations in MSS tumors are consistently detected throughout early-stage (Dukes' A) to advanced (Dukes' D) CRCs [93, 96], indicating that G12 mutations contribute to the establishment of early-stage MSS/CIN+ tumors [97, 98]. In MSI-high CRCs, however, the frequency of the mutations drastically increases during the progression from Dukes' A to Dukes' C [93], suggesting that the majority of *KRAS* mutations in CIN-negative tumors occur during a relatively late phase after MMR deficiency is established. These observations suggest a potential link between G12 mutations and CIN in the precancerous (adenoma) stage of MSS CRC development, whereas G13 mutations seem to facilitate later tumor progression without promoting CIN in MMR-deficient, MSI-high CRCs. Since more than two thirds of *KRAS*-mutated CRCs

are reported not to harbor p53 mutation [99], oncogenic *KRAS* might be linked to CIN in MSS CRCs independently of p53 mutations.

On the other hand, ^{V600E}*BRAF* mutation is strongly associated with the CpG island methylator phenotype (CIMP) [98, 100, 101], which often involves promoter hypermethylation of MMR genes causing sporadic MSI-high (CIMP-high/CIN-negative) CRCs [102]. In addition, some CRCs with the ^{V600E}*BRAF* mutation are characterized by the unique MSS/CIMP-high phenotype, which shares clinicopathological features with sporadic MSI-high CRCs, including a predominant proximal localization, serrated and mucinous morphology with poor differentiation, and near-diploid ploidy with little evidence of CIN [94]. Thus, in a similar way to the mouse studies described above, the ^{V600E}*BRAF* mutation is primarily linked to the CIN-low/negative phenotype, at least in CRCs. In contrast, the kinase impaired ^{D594}*BRAF* mutations, are reportedly associated with CRCs with the MSS/CIMP-low phenotype [103] and have distinct gene expression profiles [104] that are more similar to MSS/CIN+ CRCs with *KRAS* mutations than CIMP-high/CIN-negative, ^{V600E}*BRAF* tumors. These data suggest a potential link between non-V600E (especially D594) *BRAF* mutations and CIN in CRCs. However, it will be important to analyse larger cohorts of CRCs to consolidate this link as well as to investigate the underlying mechanisms, and particularly to identify whether CRAF plays a key role in the emergence of CIN in this context.

6. Future perspectives

6.1 Resolving the complexity of the RAS/RAF pathway

Despite significant progress over the last few decades in cancer biology related to *RAS and RAF* oncogenes, the mechanistic basis of how RAS/RAF signals contribute to chromosome segregation is yet largely unknown. The involvement of multiple *RAS/RAF* genes and multiple distinct *RAS/RAF* mutations makes research in this field extremely complicated [9]. Each of the three *RAS* genes is mutated in a different spectrum of human cancers (**Table 1**) and mutations affect a number of different residues within each [10]. It will ultimately be necessary to elucidate the roles for gene/mutation combinations in clinically relevant contexts and, to this end, studies using knock-in mouse models for *NRAS/HRAS* mutations at the codons 12 and 61 have just started [72]. Another complexity is that the three different RAF homologues can be activated to different extents by oncogenic RAS and, given the data shown above that hyperactive BRAF and CRAF may cause different types of genomic instability, it will be important to know which RAF homologue(s) is activated by oncogenic RAS in each tumour type. At least in human melanoma cells with *KRAS/NRAS* Q61 mutations and mouse lung cells with the ^{G12V}KRAS mutation, ERK activation relies on CRAF but not on BRAF [88, 89], indicating that oncogenic RAS preferentially activates CRAF in these contexts. This type of analysis will be needed in other cancers with *RAS* mutations.

Similarly, although the majority of oncogenic *BRAF* mutations occur at the V600 residue, *BRAF* point mutations at non-V600 residues [11, 12, 86] as well as gene rearrangements generating gene fusions that contain the BRAF kinase domain [13-16] have been described in various types of human cancers. Given data indicating that V600E and non-V600E (including D594) mutations may be involved in distinct types of genomic instability [62, 103], a role for each distinct *BRAF* mutation or rearranged BRAF fusion protein in MSI and W-CIN/S-CIN must be individually investigated in relevant cell/tissue types, such as melanoma and thyroid cancers for V600E [11, 12, 105], and lung cancers for non-V600E mutations [106, 107]. In addition, although there are only a few reported cases of *CRAF* mutations in human cancer, chromosomal translocations generating fusion transcripts containing the kinase domain of *CRAF* have also been found in prostate cancer and pilocytic astrocytoma as with *BRAF* (**Table 1**) [13, 14]. Examination of the role of CRAF in inducing CIN in these various cancers will be an important next step.

6.2 Aneuploidy/CIN-targeting therapeutics

With respect to therapeutic development, a comprehensive understanding of the functional linkage between the RAS/RAF pathway and W-CIN may provide new therapeutic options for treating CIN-positive cancers. As described above, PLK1 and some other mitotic proteins have been identified in genome-wide RNAi synthetic lethal screen for oncogenic *KRAS*, and cell lines mutated for *KRAS* are hypersensitive

to a PLK inhibitor and proteasome inhibitors [55]. Unfortunately, the cell lines (DLD-1 and HCT116) mainly used in this study were MMR-deficient, MSI-high/CIN-negative CRC cell lines, which leaves it an open question as to whether CIN cancers mutated for *KRAS* are also sensitive to PLK and/or proteasome inhibitors.

In CIN cancer cells, as indicated in **Figure 1**, there are two distinct but intertwined characteristics that might potentially be targeted by cancer-specific therapeutics. One is chromosomal (karyotypic) heterogeneity [108, 109] that likely drives clonal evolution of drug-resistant and/or metastatic subclones [110, 111], and the other is the compensative machinery against aneuploidy-induced growth inhibition [4, 112], which could be essential for the growth of CIN cancers but may be dispensable for normal diploid cells. A recent study using a conditional overexpressing ^{G12D}KRAS/MAD2 transgenic mouse model for lung cancer demonstrated that the enhanced CIN that occurred following co-overexpression of ^{G12D}KRAS and MAD2 increased tumour recurrence after abrogation of transgene expression [65]. It was argued that this increased relapse rate must be due to chromosomal heterogeneity established by pre-existing CIN before abrogation of the transgenes. This study provides a cautionary note to current therapeutic interventions as it suggests that chromosomal heterogeneity induced by W-CIN promotes the emergence of therapy-resistant subclones lacking the primary driver oncogenes. Thus, late intervention against ongoing W-CIN in already established cancers might be ineffective in preventing cancer recurrence following treatment.

On the other hand, it has been proposed that imbalances in protein subunit stoichiometry caused by aneuploidy could evoke proteotoxic stress [2, 112] that attenuates cell proliferation and/or induces apoptotic cell death (aneuploidy-induced growth inhibition). CIN cancer cells are assumed to develop mechanisms such as p38/p53 inactivation to evade such stress responses [4, 47], but are still more sensitive to further stress loading by proteotoxic compounds like AICAR and 17-AAG than normal diploid or near-diploid MSI cancer cells [113]. Thus, the identification of pathways controlling aneuploidy-induced proteotoxicity/growth inhibition in CIN cancers will be a vital step towards the development of aneuploidy/CIN-targeting therapeutics. In this context, it will continue to be of great interest to determine whether deregulation of the RAS/RAF cascade in CIN cancers contributes to the surpassing of aneuploidy-induced proteotoxicity/growth inhibition, and if so, how pharmacological modulation of the RAS/RAF pathway affects these processes.

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