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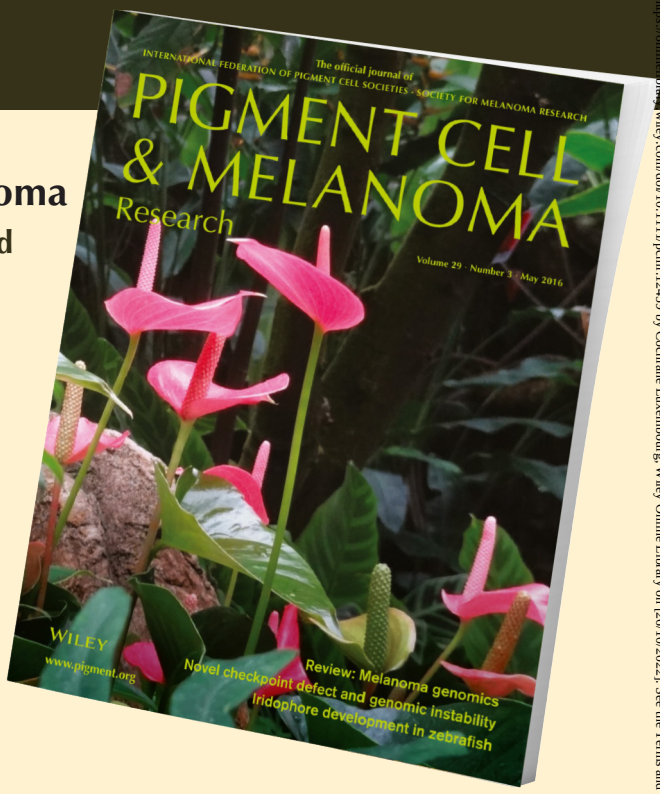

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DOI: 10.1111/pcmr.12459
Volume 29, Issue 3, Pages 266–283

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KEYWORDS exome sequencing/genomics/melanoma/mutation/ultraviolet radiation/driver gene

PUBLICATION DATA Received 30 November 2015, revised and accepted for publication 25 January 2016, published online 1 February 2016

doi: 10.1111/pcmr.12459

Summary

Somatic mutation analysis of melanoma has been performed at the single gene level extensively over the past several decades. This has provided considerable insight into the critical pathways controlling melanoma initiation and progression. During the last 5 yr, next-generation sequencing (NGS) has enabled even more comprehensive mutational screening at the level of multigene panels, exomes and genomes. These studies have uncovered many new and unexpected players in melanoma development. The recent landmark study from The Cancer Genome Atlas (TCGA) consortium describing the genomic architecture of 333 cutaneous melanomas provides the largest and broadest analysis to date on the somatic aberrations underlying melanoma genesis. It thus seems timely to review the mutational landscape of melanoma and highlight the key genes and cellular pathways that appear to drive this cancer.

Scope of review

As genomewide approaches are unbiased ways to identify cancer 'driver' genes, we summarize in this article only those findings from whole-genome sequencing (WGS) and whole-exome sequencing (WES) studies of cutaneous melanomas originating from non-glabrous skin. The review is limited in its scope to this subtype since at this time, very few samples of acral, mucosal, and uveal melanoma have been subjected to WGS or WES (Furney et al., 2012, 2013a,b; Harbour et al., 2010; Hodis et al., 2012; Krauthammer et al., 2012, 2015; Martin et al., 2013; Turajlic et al., 2012) and in-depth analysis of each of these melanoma subtypes will warrant a separate review in due course. This review is also restricted to treatment naïve samples and attempts not to include tumors from patients who have received general or molecularly targeted chemotherapies. Thus, for example, we do not discuss WGS/WES studies directed at identifying mechanisms of BRAF inhibitor resistance, a topic that similarly deserves its own review in the near future. Lastly, we conclude this review with a discussion on the intersection of the somatic mutation landscape and germline genetics that confers susceptibility to melanoma.

The cutaneous melanoma genome: a high mutation rate and a signature of UVR exposure

The advent of NGS technologies enabled tremendous opportunities to gain insight into the genetics of cancer in a systematic and unbiased approach. The first report describing a catalog of somatic mutation events in cancer was from a commercially available melanoma cell line that was published in 2010 (Pleasance et al., 2010). This WGS study sequenced COLO-829, an immortal cell line established from a pretreatment metastasis of malignant melanoma of a 43-yr-old male, and its matched normal lymphoblastoid cell line. This approach of sequencing of both tumor and matching normal tissue enabled a comprehensive analysis of the somatic genetics driving the growth of an individual cancer, and in this case, also revealed a clear signature of DNA damage caused by ultraviolet radiation (UVR).

WGS revealed a total of 32 325 single-base and 510 double-base substitutions, the majority of which were C>T/G>A or CC>TT/GG>AA transitions, respectively. This mutational spectrum was consistent with signatures associated with UVR exposure (Pfeifer et al., 2005), a known environmental risk factor for melanoma. When

neighboring sequence context of these C>T substitutions was considered, 92% of mutations occurred at the 3′ base of a pyrimidine dinucleotide, another key characteristic of UVR-induced lesions (Pfeifer et al., 2005), further substantiating the role of UVR to the mutational processes in this cell line.

The correlation of a UVR mutation signature in cutaneous melanoma genomes became further substantiated as increasing NGS studies began exploring larger cohorts of melanomas (Hodis et al., 2012; Krauthammer et al., 2012). In particular, it was apparent that this association was a feature common to sun-exposed cutaneous melanomas, while sun-shielded melanomas (such as acral, mucosal, and uveal melanomas) exhibited a lower mutation burden. This contrast explained some of the known differences in the etiology of these melanoma subtypes and is briefly explored in a later section of this review (Body site differences in driver mutations).

When WES and WGS datasets across a variety of cancers were compared it became apparent that melanoma exhibited a much higher somatic mutation burden relative to other cancers, with a median of >10 mutations/Mb (Alexandrov et al., 2013; Lawrence et al., 2013). This is considerably high considering the average mutation rate in the least frequently mutated cancer, acute myeloid leukemia, is 0.37 mutations/Mb (Lawrence et al., 2013). A high mutation rate in melanoma was replicated by TCGA, with a mean of 16.8 mutations/Mb (Watson et al., 2015). In that study, 76% of primary (44 of 85) and 84% (221 of 262) of metastatic melanomas exhibited a signature consistent with UVR exposure. However, it should be noted that the mutation rate in melanoma varies substantially by body site, such that sun-shielded cutaneous melanomas have markedly lower mutation burdens than those melanomas arising on chronically sun-exposed body sites (Krauthammer et al., 2012).

The high mutation rate observed in melanoma creates a major challenge for oncologists and researchers in distinguishing *bona fide* driver events from the background noise of passenger mutations (Lawrence et al., 2013). Although it was expected that sequencing larger sample sets would increase the ability to detect driver genes, paradoxically a number of NGS studies appeared to show the opposite due to limitations of the mathematical modeling used at the time (Lawrence et al., 2013). It quickly became apparent that use of these early approaches, relying on the principle that significantly mutated genes would accumulate more mutations than expected by chance, was leading to bloated gene lists that contained spurious and false-positive driver events. Detailed bioinformatics showed that extensive mutation heterogeneity existed across different regions of cancer genomes, such that relative accumulated mutation rates differed between genes expressed at high or low levels, or genes replicated early or late during mitosis (Lawrence et al., 2013). As such, it became apparent that future large-scale NGS studies aiming to identify the key driver genes in melanoma would require both increasing the sample cohort size and utilizing a range of sophisticated bioinformatic analyses designed to account for the underlying mutational heterogeneity in cancer (explained in more detail in the section Driver genes in TCGA melanoma study).

Coding mutations in melanoma driver genes

As the very first exploratory studies of melanoma using WGS applied to single samples (Pleasance et al., 2010; Roychowdhury et al., 2011), there have been a growing number of NGS studies that have reported new, and often known, mutated genes that appear to drive development of a subset of these tumors. The vast majority of studies have been limited to the use of WES, and to date, the number of cutaneous melanomas analyzed by WGS is extremely small, thus highlighting this type of in-depth whole-genome assessment as an unmet need. The earliest WES studies were also restricted in the number of samples sequenced (range 7–14), although with time sample numbers increased and there are now several studies that report on >100 tumors (Table 1). The first melanoma WES study assessed 14 tumors and focused on genes with recurrent mutations or high mutation load (Wei et al., 2011). Half of the tumor cohort had BRAF V600E mutations and nine other genes (*TRRAP, DCC, FCRL1, ZNF831, CPT1A, LRRN3, NOS1, PLCH1,* and *SLC17A5*) had recurrent mutations in two samples. A prevalence screen of these hotspot mutations was conducted in an additional 153 melanomas, which led to the identification of one additional mutation in *DCC, FCRL1,* and *ZNF831*, as well as four mutations occurring at the same position in *TRRAP*. The latter substitution, giving rise to a S722F change in the encoded protein, part of a large multimeric complex with histone acetyltranseferase activity, proved to be an activating mutation. Mutant TRRAP transformed NIH3T3 cells more effectively than wild-type TRRAP and knockdown of TRRAP in cells carrying a mutant allele resulted in apoptosis. Little attention has been given to TRRAP in melanoma since this initial report, which is surprising given that its overall mutation prevalence across several large melanoma WES studies, including TCGA, is 12% (Table 1). In the search for genes with more mutations than expected by chance, Wei et al. (2011) identified *GRIN2A* mutations in 6 of 14 melanomas subjected to WES and a further 11 of 153 in the prevalence screen. Mutations were distributed along the length of the gene and included several nonsense mutations, thus indicating a likely tumor suppressor role for GRIN2A, an ionotropic glutamate receptor, in melanoma. The overall prevalence of *GRIN2A* mutations across the TCGA, Broad, and Yale datasets is almost 22% (Table 1).

Through WES analysis of eight melanoma cell lines Stark et al. (2011) confirmed frequent mutations of

Table 1. Summary of WGS and WES studies of melanoma and comparison of key gene mutation frequencies. The mutation frequency data were extracted from cBioPortal. Melanoma WGS/WES studies were selected using the following criteria: (i) studies had at least three samples of cutaneous (non-acral) melanoma, and (ii) somatic mutations were detected by comparing matched normal samples. All relevant studies are listed in chronological order of publication

Publication	Number of CMM Patients§	Sample type	Study type (WES or WGS)	Novel gene(s) highlighted¶	Mutation prevalence in discovery paper (%)	cBioPortal mutation frequency in TCGA/Broad/Yale datasets				Comments on discovery paper
						TCGA SKCM (N = 345)	Broad (N = 121)	Yale (N = 91)	Total (N = 557)	
Wei, X. *et al*. 2011	14	metastases	WES	*GRIN2A*	42.9%	23.5%	24.8%	11.0%	21.7%	Frequency only for TRRAP p.Ser722Phe given in discovery paper
				TRRAP	4.0%	11.9%	14.0%	9.9%	12.0%	
Stark, M. S. *et al*. 2011	8	cell lines	WES	*MAP3K5*	9.0%	6.4%	7.4%	4.4%	6.3%	
				MAP3K9	15.0%	8.4%	5.8%	5.5%	7.4%	
Nikolaev, S. I. *et al.* 2011	7	cell lines	WES	*MAP2K1*	6.3%	5.2%	5.0%	0.0%	4.3%	
				MAP2K2	1.6%	2.0%	3.3%	0.0%	2.0%	
Berger, M. F. *et al.* 2012	23	metastases	WGS	*PREX2*	44.0%	22.0%	28.9%	17.6%	22.8%	Study also included 2 acral melanomas
Hodis, E. *et al.* 2012	121	15 primary tumors, 30 metastases and 76 short-term cultures derived from metastases	WES	*RAC1*	5.0%	6.1%	5.0%	7.7%	6.1%	
				PPP6C	9.1%	7.5%	9.1%	8.8%	8.1%	
				ARID2	9.1%	13.3%	9.1%	11.0%	12.0%	
				STK19	4.1%	4.1%	4.1%	0.0%	3.4%	
				SNX31	6.6%	3.2%	6.6%	1.1%	3.6%	
				TACC1	6.6%	2.6%	6.6%	1.1%	3.2%	
Krauthammer, M. *et al. 2012*	73	metastases and short-term cultures derived from metastases	WES	*RAC1*	9.8%	6.1%	5.0%	7.7%	6.1%	Study comprised 147 samples, with 99 having matched DNA. Of the latter, 14 were acral melanomas, 7 were mucosal melanomas, 5 were uveal melanomas and 73 were cutaneous melanomas.
				PPP6C	13.1%	7.5%	9.1%	8.8%	8.1%	
				ARID2	18.0%	13.3%	9.1%	11.0%	12.0%	
				DCC	34.4%	20.3%	22.3%	25.3%	21.5%	
				PTPRK	19.7%	11.3%	9.9%	14.3%	11.5%	
Mar, V. J. *et al* .2013	34	primary tumors	WES	*NOTCH1*	5.9%	3.8%	3.3%	0.0%	3.1%	
				KIT	8.8%	4.3%	3.3%	4.4%	4.1%	
				NF1	17.7%	13.3%	13.2%	14.3%	13.5%	
Gartner, J. J. *et al*. 2013	29	metastases	11 WGS & 18 WES	*BCL2L12*	3.4%	2.6%	0.0%	3.3%	2.2%	A synonymous mutation reported in discovery paper but only non-synonymous mutations listed in cBioPortal
Aydin, I. T. *et al*. 2014	8	metastases	WES	*FBXW7*	8.1%	3.5%	3.3%	3.3%	3.4%	
Lu, C. *et al*. 2015	17	primary tumors	5 WGS & 12 WES	N/A	N/A	N/A	N/A	N/A	N/A	Study was of childhood & adolescent melanomas and included 'conventional melanomas', Spitzoid melanomas and melanomas arising in congenital naevi. One patient also had an acral melanoma. WGS was carried out on fresh frozen specimens whereas WES was done on FFPE specimens
Wong, S. Q. *et al*. 2015	20	cell lines	WES	*RQCD1*	4.0%	2.9%	2.5%	3.3%	2.9%	RQCD1 mutation was found in 3 of 20 cell lines (15%), then prevalence of 4% derived from analysis of 715 primary melanomas
Ding, L. *et al*. 2015	13	metastases	WGS	*EPHA3*	25.0%	9.9%	13.2%	7.7%	10.2%	15 metastases were from 13 patients
TCGA. 2015	331	266 metastases and 67 primary tumors	WES	*BRAF*	52.0%	51.3%	62.8%	37.4%	51.5%	From the list included here, *IDH1* and *DDX3X* are the only novel driver genes identified in the TCGA paper. which also listed another 28 significantly mutated genes by the MutSig method (Q<0.01), but not including previously highlighted genes such as *PREX2*, *GRIN2A*, and *BCL2L12*. The other 'known' melanoma driver genes are listed here in order to provide their mutation frequencies across the three largest melanoma WGS/WES studies
				NRAS	28.0%	27.8%	25.6%	20.9%	26.2%	
				CDKN2A	13.0%	13.3%	19.0%	1.1%	12.6%	
				TP53	5.0%	15.1%	19.0%	12.1%	15.4%	
				ARID2	14.0%	13.3%	9.1%	11.0%	12.0%	
				IDH1	5.7%	5.8%	2.5%	5.5%	5.0%	
				PPP6C	7.0%	7.5%	9.1%	8.8%	8.1%	
				PTEN	8.5%	8.7%	11.6%	3.3%	8.4%	
				DDX3X	6.3%	6.1%	5.0%	0.0%	4.8%	
				RAC1	6.3%	6.1%	5.0%	7.7%	6.1%	
				MAP2K1	5.1%	5.2%	5.0%	0.0%	4.3%	
				NF1	4.0%	13.3%	13.2%	14.3%	13.5%	
				RB1	3.8%	3.5%	5.0%	0.0%	3.2%	
Krauthammer, M. *et al.* 2015	117	20 primary tumors, 61 metastases, 8 short-term cultures derived from primary tumors, 28 short-term cultures derived from metastases	WES	*RASA2*	4.2%	4.6%	4.1%	2.2%	4.1%	Study reports on 213 samples but 109 were also in the previous Krauthammer et al., publication, therefore the mutation frequencies are not completely independent. Matched normal DNA was available for 133 samples, 16 of which were from unknown melanoma histotype
				PTPN11	3.8%	3.2%	0.8%	2.2%	2.5%	
				SOS1	6.6%	2.9%	4.1%	7.7%	3.9%	
				RAF1	2.3%	3.2%	1.7%	1.1%	2.5%	
				SPRED1	2.3%	1.4%	0.8%	1.1%	1.3%	
Shain, A. H. *et al.* 2015	20	primary tumors	low coverage WGS & high coverage WES	*NFKBIE*	15.0%	0.3%	0.8%	0.0%	0.4%	All samples are desmoplastic melanomas and *NFKBIE* mutations occur in the gene promoter
				CBL	11.0%	4.3%	5.0%	5.5%	4.7%	
				CDH2	19.0%	4.1%	5.0%	5.5%	4.5%	
				FSIP1	13.0%	3.5%	5.0%	3.3%	3.8%	
				ARID1A	22.0%	4.6%	11.6%	7.7%	6.6%	
				PAK3	19.0%	3.2%	3.3%	2.2%	3.1%	
				IL36A	3.0%	3.2%	2.5%	0.0%	2.5%	
Arafeh, R. *et al.* 2015	501	see comments	36 WGS & 465 WES	*SETD2*	5.4%	5.2%	5.0%	5.5%	5.2%	Data are collected from various sources including TCGA, Broad, YALE and others
				RASA2	5.4%	4.6%	4.1%	2.2%	4.1%	

[a]Number of CMM patients reflects only those with cutaneous melanoma on non-glabrous skin (i.e. excluding acral) with matched normal DNA for comparison.

[b]Novel gene highlighted in discovery paper (i.e. not reported to be substantially mutated in melanoma before) applies to all studies except TCGA, which serves as a reference study to highlight other commonly identified driver genes.

TRRAP and *GRIN2A* in melanoma and identified mutations in two MAP3-kinases other than BRAF, MAP3K5, and MAP3K9. A prevalence screen in an additional 77 melanoma cell lines found these were mutated in 9 and 15% samples, respectively. The mutations were spread over the length of the two genes, included a nonsense mutation and showed loss of heterozygosity in some samples, implying they were inactivating, a notion that was supported by structural modeling, in vitro kinase assays and reduced MEK/ERK and JNK phosphorylation with some of the mutant isoforms. *MAP3K5* and *MAP3K9* are mutated in 6-7% of melanomas (Table 1).

Remarkably, given their overall rarity (2–4%, Table 1), Nikolaev et al. (2011) identified mutations in *MAP2K1* and *MAP2K2*, encoding MEK1 and MEK2, respectively, in WES analysis of only seven melanoma cell lines. Both mutations occurred in samples with non-V600E BRAF mutations (although with other potentially activating mutations: K601E in one cell line and G593S and L597R on different alleles in the other) and led to constitutive

activation of ERK, as well as resistance to MEK inhibitors. A prevalence screen in another 127 melanomas from 121 patients found one additional MAP2K1 E203K mutation and also identified four samples with MAP2K1 P204S substitutions, as well as one sample with each of MAP2K1 F53L, MAP2K1 N382H, and MAP2K2 S154F mutations. In the extended series of tumors, it was apparent that the *MAP2K1* and *MAP2K2* mutations showed no correlation with activating hotspot mutations in either *BRAF* or *NRAS*, indicating they are insufficient to lead to full oncogenic transformation of melanoma.

The only high-coverage WGS study of multiple melanoma samples carried out to date identified *PREX2* mutations, encoding a negative regulator of PTEN, in 10 of 23 cutaneous (non-acral) tumors (Berger et al., 2012). *PREX2* mutations occurred along the entire gene and included frequent nonsense mutations, implicating it as a tumor suppressor. However, paradoxically, overexpression of some mutant PREX2 isoforms (including several truncated versions) in immortalized melanocytes led to increased tumorigenesis compared to wild-type *PREX2*-expressing melanocytes when injected into nude mice. Although, across other large WES melanoma datasets, the mutation frequency of *PREX2* is consistently high (overall ~23%), it was not flagged by TCGA (Watson et al., 2015) as a significantly mutated gene (SMG) (Figure 1), and it is not normally expressed in the pigment cell lineage. Thus, its relevance to melanoma genesis is uncertain.

The earliest WES study to analyze a substantial number of melanomas included 121 tumors and introduced a statistical approach to identify SMGs based on predicted functional impact of the mutations as well as 'local' mutation rate (i.e. relative abundance of mutations between exons and introns/UTRs of the same gene) (Hodis et al., 2012). This approach identified 6 novel melanoma driver genes: *RAC1, PPP6C, STK19, SNX31, TACC1,* and *ARID2*) (Table 1), the first 3 of which harbored recurrent mutations, the other 3 having more widely distributed mutations, suggesting they are likely loss of function. None of these genes had previously been recognized as being significantly mutated in melanoma. RAC1 is a small G-protein member of the Rac subfamily of Rho GTPases that regulate cellular events including activation of protein kinases. Expression of the P29S hotspot mutant, but not its wild-type counterpart, in normal murine melanocytes enhances ERK phosphorylation (Krauthammer et al., 2012), and confers resistance to RAF inhibition in melanoma cells (Watson et al., 2014). The high loss-of-function mutation burden in *ARID2*, encoding a member of the SWI/SNF chromatin remodeling complex, pointed to it being a new and significant tumor suppressor gene in melanoma. Notably, truncating mutations in *ARID2* were found in isolated samples in all 3 previous melanoma WES studies (Nikolaev et al., 2011; Stark et al., 2011; Wei et al., 2011) and its overall mutation frequency in melanoma is 12% (Table 1). Other known melanoma genes that were found to have a statistically high mutation burden by Hodis et al. (2012) included: *BRAF, NRAS, TP53, PTEN, CDKN2A,* and *MAP2K1*.

Simultaneous to the previous publication, Krauthammer et al. (2012) also identified the RAC P29S hotspot mutation as well as frequent mutations (including recurrent) in *PPP6C*, through WES analysis of 147 melanomas of diverse histological subtypes (99 having matched normal DNA for comparison and 73 of these being cutaneous melanomas). This study highlighted the following genes as having statistically higher numbers of nonsense mutations than expected by chance: *ARID2, DCC, TP53, NF1, ZNF560, FAM58A,* and *ME1*, thus adding more support to earlier studies implicating *ARID2* (Hodis et al., 2012) and *DCC* (Wei et al., 2011) as novel melanoma suppressors, and also substantiating the role of *NF1* as a key melanoma driver. Krauthammer et al. (2012) found that the *RAC1* mutation occurred

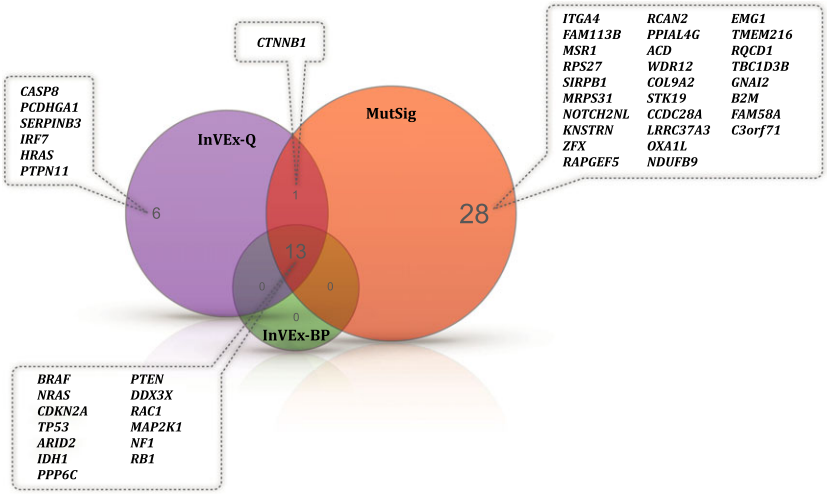

Figure 1. Significantly mutated genes (SMGs) found in TCGA melanoma study. Three methods were used to identify SMGs in TCGA melanoma data: (i) MutSig: 42 SMGs identified by MutSig with the criteria Q < 0.1 and RSEM <1 in at least 70% samples. (ii) InVEx-Q: 20 SMGs identified by InVEx with either functional mutation burden or loss-of-function test (Q < 0.1). (iii) InVEx-BP: 20 SMGs identified by InVEx with either functional mutation burden or loss-of-function tests (Bonferroni P < 0.05).

significantly more frequently in chronically sun-exposed melanomas of males; however, this observation did not hold true in a study of 814 primary melanomas (Mar et al., 2014). Ectopic expression of the RAC1 P29S isoform in mouse melanocytes led to increased proliferation and migration relative to cells transfected with wild-type RAC1 (Krauthammer et al., 2012). The *RAC1* and *PPP6C* genes are mutated in 6 and 8% of melanomas overall (Table 1).

The first study to focus solely on primary melanomas conducted WES analysis of 34 cutaneous tumors and found mutations in the well-known drivers *BRAF, NRAS, HRAS, KIT, RAC1, NF1, TP53,* and *CDKN2A* (Mar et al., 2013). The authors also highlighted *NOTCH1* mutations that occurred in two *BRAF* wild-type samples. Although *NOTCH1* mutations were absent from the Yale dataset, the overall mutation frequency across a large number of melanomas subjected to WES/WGS is ~3% (Table 1).

Prior to publication of TCGA melanoma data, several other small WES studies (n = 8–29 samples) reported on additional potential driver genes, which warrant corroboration in larger studies and functional assessment to determine their roles in melanoma development. These include the following: *FBXW7* (Aydin et al., 2014), *RQCD1* (Wong et al., 2015), and *EPHA3* (Ding et al., 2014), which are mutated in ~3–10% of melanomas (Table 1).

Driver genes in TCGA melanoma study

Identifying which somatic mutations are likely to be 'drivers' in pathogenesis and elucidating how mutated genes affect the biology of a given tumor are fundamental challenges in cancer genomic studies (Watson et al., 2013). In particular, when compared to other cancers, cutaneous melanomas have the highest mutation burden due to UVR exposure, thus necessitating stringent approaches to distinguish driver versus passenger mutations (Lawrence et al., 2013). Recently, TCGA published a seminal melanoma genomics study describing the landscape of somatic alterations in cutaneous melanomas through DNA, RNA, and protein-based analysis of 333 primary and/or metastatic melanomas from 331 patients (Watson et al., 2015). Two methods (MutSigCV and InVEx) were used to identify SMGs in the WES data. The algorithm MutSigCV was developed to correct for variations in mutation burden by taking into account patient-specific mutation frequency and spectrum as well as incorporating mRNA expression and gene-specific DNA replication time (Lawrence et al., 2013). InVEx uses a more stringent permutation-based approach to compare mutation rates in introns versus exons and ascertain genes with a somatic mutation distribution showing evidence of positive selection for non-silent mutations (Hodis et al., 2012). Three mutation burden scores are calculated by InVEx: functional mutation burden, synonymous mutation burden, and loss-of-function (LoF) mutation burden. The InVEx method is particularly applicable to cancers with high mutation load, such as melanoma. TCGA melanoma analysis using InVEx was strict when parameters were set to reduce the false-positive rate, and only 13 core genes (*BRAF, NRAS, TP53, NF1, CDKN2A, ARID2, PTEN, PPP6C, RAC1, IDH1, DDX3X, MAP2K1, and RB1*) were identified with Bonferroni's corrected $P < 0.05$, but extended to 20 genes using $Q < 0.1$ as a stringency cutoff. An additional 28 genes were identified by MutSigCV ($Q < 0.1$ and RSEM > 1 in 70% samples), including promoter mutation in *NDUFB9* (Poulos et al., 2015), 5′ UTR mutation in *RPS27* (Dutton-Regester et al., 2014), and synonymous mutation in *TMEM216* (Guan et al., 2015) (Table 1). While the method to identify SMGs in TCGA study through intersection of MutSigCV and InVEx may have generated the top significant driver genes in melanoma, it also left out some well-known melanoma driver genes identified by other studies, for example *PREX2, GRIN2A, ERBB4, ADAMTS18, BCL2L12, SOX10, MITF, and KIT*, which were ranked far down the list in TCGA paper (Table 1). For example, *PREX2* had a high mutation frequency among all TCGA, Broad, and Yale melanoma samples but ranked 193th by MutSig, with P-value 0.002 and *q*-value 0.197. Surprisingly, it was ranked 6890th by InVEx functional mutation burden (P-value = 1 and *q*-value = 1) and ranked 4461th by InVEx LoF (P-value = 1, and *q*-value = 1). In addition, identifying SMGs in melanoma is highly susceptible to discovery cohort bias, for example, there are very limited *CDKN2A, ITGA4, and FAM113B* mutations in the Yale dataset compared to TCGA and Broad datasets. Thus, the driver mutations highlighted in any individual study are somewhat limited in their ability to fully inform on the mechanisms of melanoma development. This limitation is primarily due to insufficient sample size; if all cohorts comprised thousands of tumors, then mutation frequencies for each gene should be very similar between studies, assuming no intrinsic differences between tumor cohorts, for example histological subtype, UVR exposure, and body site.

Post-TCGA

Subsequent to the TCGA report there have been three other melanoma WES studies published (Arafeh et al., 2015; Krauthammer et al., 2015; Shain et al., 2015a). Two of these identified *RASA2* as a key driver gene in melanoma, particularly in tumors carrying *NF1* mutations (Arafeh et al., 2015; Krauthammer et al., 2015). This finding shows cooperativity between these genes in regulating melanoma development. Similarly, *NF1* mutations were found to occur significantly more frequently with other RASopathy genes, for example *SOS1, PTPN11, RAF1,* and *SPRED1* (Krauthammer et al., 2015). *RASA2* mutations are distributed along the length of the gene, although some are recurrent, but all appear to be loss-of-function mutations, indicating it is a new

melanoma tumor suppressor, with defects in ~4% of melanomas overall (Table 1). *RASA2* encodes a GTPase-activating protein for RAS (i.e. a RasGAP) and mutations result in RAS activation, leading to increased proliferation and migration of melanoma cells and poorer overall survival of stage III melanoma patients (Arafeh et al., 2015).

The most recent melanoma WES study (Shain et al., 2015a) focused on cutaneous melanomas with desmoplastic features. Analysis of 20 tumors found they lacked the most common *BRAF* and *NRAS* hotspot mutations but carried a variety of other mutations which would result in the activation of the MAPK and PI3K pathways, for example, in *NF1, MAP2K1, MAP3K1, PIK3CA, RAC1, EGFR, MET, ERBB2, CBL, SOS2*, and *PTPN11*. Of note, the latter three are RASopathy genes, hence providing further support for this family of genes being important regulators of melanoma genesis. The most striking finding in the desmoplastic melanomas was the high frequency of recurrent promoter mutations in *NFKBIE*, which are discussed in more detail in the next section of this review.

Promoter mutations

With the advent of new analytical approaches, together with larger numbers of tumors being subjected to WGS, the increasing role of mutations in gene regulatory regions, particularly gene promoters, has been appreciated. For example, a reanalysis of the WGS data from Berger et al. (2012), concentrating on non-coding mutations, identified two recurrent mutations at positions chr5:1295228 and chr5:1295250 in the *TERT* promoter in 17 of 19 melanomas (Huang et al., 2013). Focused sequencing of a further 51 melanomas gave a combined mutation prevalence of 71% (50 of 70 samples) and confirmed that the mutations occurred in a mutually exclusive fashion, implying redundancy in their functional consequence. The mutations were predicted to create new binding sites (core motif GGAA) for the ETS family of transcription factors and were shown by luciferase reporter assays to result in 2- to 4-fold increased expression of *TERT* compared to the wild-type promoter. Recently, Bell et al. (2015) showed that GABP is the specific ETS transcription factor that binds to the mutated motifs in the *TERT* promoter, a finding since replicated in melanoma (Makowski et al., 2015). Furthermore, mutant *TERT* promoters exhibit H3K4me2/3 marks of active chromatin, recruiting GABP transcription factors, while the wild-type allele retains marks of epigenetic silencing, indicating that mutation results in mono-allelic expression of *TERT* (Stern et al., 2015). Both of the most common mutations in *TERT* are C>T transitions (minus strand), characteristic of UVR-induced mutagenesis. Further support for this notion comes from a simultaneous report of both germline and somatic *TERT* promoter mutations in melanomas, in which two additional recurrent tandem GG>AA (CC>TT) somatic mutations generating ETS binding sites were reported at positions chr5:1295228/1295229 and chr5:1295242/1295243 (Horn et al., 2013). That study reported *TERT* promoter mutations in 25 of 77 (33%) primary melanomas and 45 of 53 (85%) metastases, indicating that the mutations likely occur late in tumor progression. This hypothesis was supported by the lack of any of the above mutations in 25 melanocytic nevi analyzed (Horn et al., 2013). However, a recent sequencing study of 37 primary melanomas and adjacent precursor regions that included a histopathological spectrum of lesions, revealed a high frequency of *TERT* mutations (>70%) in 'intermediate lesions' and melanomas in situ and low frequency in benign nevi (Shain et al., 2015b). These findings suggest that *TERT* mutations are selected for early in tumorigenesis once lesions progress beyond benign status; however, these findings need to be replicated in larger sample sets. In melanoma, *TERT* promoter mutations co-occur with *BRAF* mutations, as well as *BRAF* plus *CDKN2A* mutations, more frequently than expected by chance (Horn et al., 2013), implying a degree of cooperativity.

An independent analysis of some of the same melanoma WGS data as above, but in the context of a pan-cancer analysis of 863 tumors of diverse types, identified the chr5:1295228 and chr5:1295250 *TERT* promoter mutations in 10 of 17 melanomas and showed that the mutations resulted in increased *TERT* expression (Weinhold et al., 2014). That study also identified *SDHD* promoter mutations, which occurred only in melanoma and no other cancer type. The recurrent mutations, at positions chr11:111957523 and ch11:111957541 and chr11:111957544, were all C>T substitutions leading to loss of putative ETS binding sites, which, using other TCGA-derived melanoma expression and WES data, were shown to be associated with lower levels of *SDHD* expression. Notably, melanoma patients with *SDHD*-mutated tumors had significantly worse overall survival than patients without such mutations.

A similar study that used TCGA-derived WGS data from 505 tumors of 14 different types, including melanoma, showed that the recurrent *TERT* promoter mutations resulted in increased *TERT* expression, although the latter was highly dependent on tumor type, with melanoma *not* being one of the cancers showing the strongest association (Fredriksson et al., 2014). This observation accords with the TCGA melanoma WES study in which only the chr5:1295228 mutation was associated with a difference in the level of *TERT* expression. Fredriksson et al. (2014) reported a nominally significant ($P = 0.047$) association between *BRAF* and *TERT* promoter mutations in melanoma.

Fredriksson et al. (2014) also identified *DPH3* promoter mutations in WGS data from 6 of 38 (16%) melanomas. These C>T substitutions occurred 25, 26, and 29 base-pairs upstream of the transcription start site of the gene (genomic positions: chr3:16306504, chr3:16306505, and

chr3:16306508). Analysis of an extended series of melanomas gave a *DPH3* promoter mutation prevalence of 35 of 263 (13%). These mutations do not create or abolish obvious transcription factor binding sites, and they are not associated with significant differences in *DPH3* expression (Fredriksson et al., 2014), thus their significance is unknown. However, they have been shown to significantly co-occur with *NF1* mutations (Fredriksson et al., 2014), thus suggesting a functional role. This study alludes to the difficulties with interpreting the functional consequences of promoter mutations more generally and emphasizes the need for these to be followed up mechanistically.

Most recently, Bastian and colleagues (Shain et al., 2015a) identified a recurrent mutation (chr6: 44233400) in the promoter of *NFKBIE* in 9 of 20 (45%) desmoplastic melanomas subjected to low-coverage WGS and high-coverage WES. Additional mutations in this gene were found in another six samples. Analysis of an extended series of melanomas showed that such mutations were also present in non-desmoplastic melanomas. In all cases, the *NFKBIE* promoter mutations occurred in tumors lacking the common *BRAF* V600E and *NRAS* Q61R or *NRAS* Q61K hotspot mutations, indicating that they provided a compensatory growth stimulating signal to that off the MAPK and PI3K pathways. *NFKBIE* encodes IκBε, an inhibitor of NF-κB signaling, which acts by sequestering NF-κB in the cytoplasm. Mutations in the promoter of *NFKBIE* result in constitutive loss of NF-κB translocalization to the nucleus and therefore presumed loss of functional activation of this pathway. The open question is whether the *NFKBIE* promoter mutations are oncogenic or tumor suppressive. The recurrent hotspot mutations and occasional amplifications of the locus seen in some desmoplastic melanomas argues for an oncogenic role, however, the finding of several melanomas with biallelic mutations or loss of heterozygosity at the locus, suggests a tumor suppressor role (Shain et al., 2015a). Mechanistic studies are needed to distinguish between these possibilities.

5′UTR mutations

Compared to promoter sequences, mutations occurring in 5′UTRs in melanoma have been explored to a lesser extent. The 5′UTR, extending from the start of mRNA transcription to the base prior to the initiation start codon (AUG), plays a key role in the translational regulation of the transcript and can contain a variety of motifs that are responsible for these functions. Early reports of 5′UTR variations in melanoma were limited to the description of the role of germline mutations in some key melanoma genes. Targeted sequencing of 5′UTR regions of *CDKN2A*, a gene that is mutated in 30–40% of melanoma families, revealed a small number of kindreds with a G to T transition 34 basepairs upstream of the initiation codon (Liu et al., 1999). This variant, and others, has been shown to result in preferential expression (compared to wild-type sequence) or result in reduced mRNA translation efficiency (Bisio et al., 2010; Liu et al., 1999). In addition, another 5′UTR mutation that was first described in human melanomas was identified in *TP53* (C119T), suggesting that this tumor suppressor may be translationally regulated in a similar manner (Khan et al., 2013).

In regard to somatic 5′UTR mutation events in melanoma, only a limited number of genes have been reported and are currently 'descriptive' in regard to their mechanistic role in tumorigenesis. One of these genes, *RPS27*, was first described after a systematic analysis of recurrent 'hotspot' mutations collated from WES datasets (Dutton-Regester et al., 2014). In the subset of novel recurrent mutations (64 of 178) that were independently validated in a cohort of 489 melanomas, 4 of the top 10 most frequent hotspots were within 5′UTRs (*RPS27*, *RBM22*, *CHCHD2*, and *UMPS*). Remarkably, *RPS27* was mutated at a frequency of 9.6% (47 of 489), ranking it as the gene with the fourth most frequent recurrent mutations in melanoma (ranked order: *TERT*, *BRAF*, *NRAS*, *RPS27*, *RAC1*). The *RPS27* mutation resulted in a C to T transition at the first base of the major translational start site in the 5′UTR (Dutton-Regester et al., 2014). This site is part of a 5′ terminal oligopyrimidine tract (5′TOP), a feature common to the family of ribosomal proteins and which is a motif required for the translational control of the protein in a growth-dependent manner. The mutation does not abolish the 5′TOP, but rather extends it by an additional base (CTTTCC to CTTTTCC). Although the functional significance of this mutation has yet to be determined, its high frequency of mutation strongly suggests an oncogenic role in melanoma.

Other NGS studies of melanomas have also described a number of 5′UTR mutations. TCGA reported on two recurrent 5′UTR mutations, the first of these being the previously described mutation in *RPS27* (27 of 331, 8.1%) (Watson et al., 2015). The other recurrent mutation occurred in *MRPS31* (17 of 331, 5.1%), a ribosomal protein similar to *RPS27*, but which has not been previously linked to cancer.

The independent analysis of pan-TCGA data by Weinhold et al. (2014) also identified a number of recurrent 5′UTR mutations in melanoma. In their analysis, mutations with the highest incidence occurred in *PES1* (5 of 17, 29%), *DHX16* (4 of 17, 23%), and *DERL1*, *TBC1D31*, *LRRC4C,* and *EEF1A1* (each 3 of 17, 17%). *PES1*, *DERL1,* and *EEF1A1* have previously been implicated in tumorigenesis; however, it should be noted that these mutations were not validated by secondary screening and no functional analyses have been performed to assess their biological relevance.

Although the landscape of 5′UTR mutations has begun to be described in melanoma, a number of challenges remain for the field. Firstly, the biological function of 5′UTR mutations can be hard to assess, which means distinguishing driver versus passenger mutation events

will remain a difficulty. Secondly, the current analyses from the large genetic datasets are heavily represented by tumors that have been sequenced by coding region or exome-based approaches. As such, these samples may have poor sequencing coverage around the outlying genic edges of the probes or baits used to generate the exome libraries. This is of concern for assessment of variants in 5′UTR and promoter regions, because even if 'captured' they may have reduced sequencing depth. Indeed, this effect was shown to result in a number of false-negative calls for the *RPS27* mutation identified in WES data, which was only corrected for during independent secondary validation screening (Dutton-Regester et al., 2014). Based on this observation, it is possible that a number of 5′UTR mutations are currently under-reported or have yet to be identified. As larger numbers of studies begin to use WGS approaches, the latter-described limitation should be overcome.

Synonymous mutations

Synonymous or 'silent' mutations are variations in gene coding sequences that do not result in a change in the amino acid encoded by the affected codon. To date, the role of synonymous mutations in tumorigenesis has largely been ignored. This is not surprising due to the difficulties associated with distinguishing driver mutations among the background passenger mutation rate (of which synonymous mutations are a large contributor) and subsequent challenges associated with assessing their biological function. Despite this, some synonymous mutations have been shown to have a direct impact on gene function, including gene splicing, transcript stability and translation, protein folding, and miRNA-based regulation in a variety of non-neoplastic diseases (Gotea et al., 2015).

The first study to link a synonymous mutation to the process of tumorigenesis was from a WGS and WES study of 29 melanomas (Gartner et al., 2013). An analysis of recurrent mutation events within this dataset revealed 16 synonymous mutations occurring in at least two samples. Secondary screening in 169 additional melanomas revealed recurrent synonymous mutations occurring at a significantly elevated rate in *OR4C3* and *BCL2L12*. The *BCL2L12* mutation (C51T, F17F) was the most frequent of the two, occurring in approximately 4% of melanomas (12 of 285). Interestingly, it was shown experimentally that the F17F mutation did not affect normal protein function, rather it led to the accumulation of BCL2L12. This effect was due to the overexpression of the mutant transcript compared to the wild type due to disruption of a miRNA binding site (hsa-miR-671) putatively responsible for the steady-state control of the protein. It was also shown that accumulation of mutant protein led to increased resistance of p53-dependent apoptosis, suggesting a prosurvival role for BCL2L12 in melanoma.

Although the TCGA did not specifically report on the status of silent mutations in melanoma, an independent analysis using the same dataset identified *TMEM216*, *CRB1,* and *CDKN2A* as genes significantly enriched for synonymous mutations (Guan et al., 2015). *TMEM216*, ranked the most significant using a multifilter approach, was mutated in a highly conserved region in a splice site of exon 3 (138T>G, G46G splice) and occurred in approximately 3% of patients (8 of 276). Mutations in *TMEM216* had a non-UVR-induced mutation profile and occurred in a mutually exclusive pattern with other members of the MAPK pathway including *NRAS* and *RAC1* (there were melanomas with dual *TMEM216* and *BRAF* mutations; however, co-occurrence is not significant). Furthermore, this same mutation was observed in 3 of 289 patients with low grade glioma, possibly indicating this variant has significance in tumors of neural crest lineage. It was suggested that the splice site mutation could act in a tumor suppressor fashion and be involved in the *RHOA*/*GNA12* pathway.

As increasingly reports begin to look at synonymous mutations as driver events in cancer (Supek et al., 2014), it is clear that more detailed analyses and alternative approaches to determine the biological significance of these mutations will be required. Furthermore, as there are now significant catalogs of large-scale sequencing cancer datasets available, closer examination of the role of synonymous mutations in tumorigenesis is warranted.

Fusions and structural aberrations

Transcript fusions and structural variations involving chromosomal rearrangements represent a unique class of mutation that can disrupt normal gene activity or create novel oncogenic fusion proteins. Oncogenic fusion events have historically been associated with hematological malignancies; comparatively, the identification of similar events in solid cancers has largely been limited to reports from the last decade as a result of NGS analyses (Kumar-Sinha et al., 2008). Regarding melanocytic tumors, only a small subset appear to have oncogenic fusions, but these usually involve members of the MAPK pathway, including *BRAF* and *RAF1* (Botton et al., 2013; Dessars et al., 2007; Hutchinson et al., 2013; Menzies et al., 2015; Palanisamy et al., 2010). One of the earliest reports of a fusion event involving *BRAF* in melanocytic neoplasms, was a case report in two large congenital nevi with chromosomal translocations of *BRAF* that resulted in the removal of the auto-inhibitory N-terminal regulatory domain with the kinase domain (Dessars et al., 2007). It was noted that due to the relative rareness of *BRAF* oncogenic mutations in congenital melanocytic nevi and non-cutaneous melanoma, that a translocation event may represent an alternative and unique molecular oncogenic mechanism to activate the MAPK pathway.

Further exploring this idea, Palanisamy et al. (2010) used paired-end transcriptome sequencing in *ETS* rear-

rangement negative prostate cancers and identified fusion events involving *BRAF* and *RAF1*. After screening a larger set of tumors, it was found that although rare (1–2%), recurrent rearrangements involving *BRAF* and *RAF1* occurred in prostate cancers, gastric cancers, and melanomas (Palanisamy et al., 2010). In their dataset, *BRAF* and *RAF1* each exhibited a fusion event in 1 of 131 melanomas. Of note, tumors exhibiting *BRAF*/*RAF1* fusions lacked oncogenic point mutations in *BRAF*, further suggesting that these events may act in a mutually exclusive fashion. Overexpression of these fusions in cell lines resulted in increased rates of cell proliferation, activation of the MAPK pathway, and increased sensitivity to RAF and MEK inhibitors.

Expanding on these findings, an analysis of 848 comparative genomic hybridization profiles of melanocytic tumors revealed 10 tumors with DNA copy number changes indicative of putative *BRAF* fusion events (Botton et al., 2013). Closer inspection revealed six tumors with the *BRAF* kinase domain fused in-frame with six different N-terminal partners, and similar to the Palanisamy study, none of these exhibited mutations in the common melanoma oncogenes *BRAF*, *NRAS*, *KIT,* and *GNAQ*. This mutual exclusivity was also replicated in another study that explored the occurrence of *BRAF* fusions in 'pan-negative' melanomas, or melanomas without mutations in *BRAF*, *NRAS*, *KIT*, *GNAQ,* and *GNA11* (Hutchinson et al., 2013). In this set of tumors, 2 of 24 (8%) contained *BRAF* fusions, each with different N-terminal partners.

Some of these studies have alluded to the clinical relevance of *BRAF* fusions in melanoma, with multiple lines of evidence to suggest possible efficacy of MEK inhibitors to treat this subset of tumors. This includes in vitro experiments showing that *BRAF* fusions result in activation of the MAPK pathway, increased proliferation and invasiveness, and higher sensitivity to MEK inhibition (Botton et al., 2013; Hutchinson et al., 2013; Palanisamy et al., 2010). In addition, a cell line derived from a *BRAF* fusion patient showed response to Sorafenib in vitro (but not Vemurafenib), and concurrently, a clinical response with Sorafenib was observed in the patient (Botton et al., 2013). Lastly, a case report involving prospective targeted therapy in two heavily pretreated melanoma patients with *BRAF* kinase fusions showed evidence of clinical activity with the MEK inhibitor trametinib (Menzies et al., 2015). These results warrant further efforts exploring the clinical activity of MEK inhibitors in the, albeit small, subset of patients with *BRAF* fusions.

Outside of fusions involving *BRAF,* recent large-scale DNA and RNA sequencing analyses have begun to identify recurrent fusions in other kinases. Comprehensive targeted sequencing of 140 spitzoid melanocytic neoplasms revealed recurrent fusions in *BRAF* (n = 7, 5%), as well as a number of other kinase genes including *ROS1* (n = 24, 17%), *NTRK1* (n = 23, 16%), *ALK* (n = 14, 10%), and *RET* (n = 4, 3%) (Wiesner et al., 2014). All fusions were mutually exclusive and were found throughout the entire biological spectrum of neoplasms including Spitz nevi, atypical Spitz tumors and spitzoid melanomas, further suggesting that these are *bona fide* early pathogenic events. Similar to previously reported *BRAF* fusions, 5′ partner genes, while varying significantly, all contributed coiled-coil domains to the fusion proteins, suggesting a similar mechanistic function resulting in dimerization and auto-activation of the respective kinases. From a clinical perspective, *ALK*, *ROS,* and *RET* fusions in other cancers have been successfully treated with FDA-approved kinase inhibitors, although these drugs have yet to be assessed in spitzoid neoplasms.

Fusions involving the kinase gene *MET* have also been identified in melanoma, although again predominately occurring in tumors with spitzoid histopathology (Yeh et al., 2015). This study identified six melanocytic tumors with *MET* fusions, each with different N-terminal partners. All samples with fusions lacked known melanoma oncogene mutations. Functional analysis revealed that two of the fusions (*TRIM4-MET* and *ZKSCAN1-MET*) resulted in constitutive activation of the phosphoinositol-3 kinase (PI3K) and phospholipase C gamma-1 (PLCγ1) pathways and that the FDA-approved MET inhibitor cabozantinib could block increased pathway activity.

The use of RNA-seq has also been used to identify gene fusions in melanoma (Berger et al., 2010). In one of the earliest studies to adopt this technology to cancer genomics, paired-end RNA-seq was used to sequence 10 patient-derived melanoma short-term cultures and cell lines. Using an integrative genomic analysis, 11 novel-expressed gene fusions and 12 novel chimeric read-through transcripts were identified. Although none of the genes involved in the fusions events were recurrent, a number of interesting genes were identified, including *RB1*, a well-characterized tumor suppressor gene in melanoma. Other fusions involving known cancer-related genes included *PARP1*, *RECK1*, *CCT3,* and *GNA12*. Interestingly, one of the read-through transcripts, CDK2-RAB5B, was found to be recurrent in 4 of the 10 RNA-seq samples, resulting in premature truncation of the kinase domain and possibly affecting transcript stability by changes in the 3′UTR.

Lastly, as part of TCGA report, an integrative analysis of copy number, RNA-seq, and WGS data identified 224 candidate fusion drivers in 333 melanomas (Watson et al., 2015). Previously reported, fusion events involving *BRAF* and *RAF1* were identified, as well as a number of novel recurrent fusion genes, including *AKT3* (n = 4), *MITF* (n = 3), and *HMGA2* (n = 3). Interestingly, there was a significant enrichment of fusions and complex structural variations in so-called triple wild-type melanomas (i.e. those harboring no mutations in *BRAF*, *NRAS*, or *NF1*) that lacked a UVR mutation signature. This observation provides further evidence to support the view that

the above events represent a unique mutational process to generate oncogenic drivers in this subset of tumors. However, it should be noted that the vast majority of genes involved in fusions identified by TCGA are non-recurrent. As such, it remains a significant challenge to determine whether those fusions represent driver or passenger mutations. Using an orthogonal genetic approach, a pan-cancer analysis of TCGA datasets identified a *WASF2-FGR* fusion in one melanoma that also occurred in two other cancers (Stransky et al., 2014). However, it is clear that genetic analyses alone are insufficient and that robust experimental validation will be required to conclusively determine mechanistically the biological role of fusion events in melanoma.

Body site differences in driver mutations

A decade ago, several landmark genetic studies provided compelling evidence that melanomas arising on chronically sun-damaged (CSD) skin differ from those arising on skin without chronic sun damage (non-CSD) in terms of site of primary tumor, age of onset, speed of development, and clinical/histological presentation and that such tumors likely represent genetically distinct subtypes. The discovery of frequent *BRAF* mutations in melanomas (Davies et al., 2002) and melanocytic nevi (Pollock et al., 2003) provided the initial opportunity to search for genetic differences between tumors arising on skin with or without evidence of chronic sun damage (based on the presence or absence of severe solar elastosis in hematoxylin and eosin (H&E) stained sections), as well as tumors from non-UVR-exposed body sites (glabrous skin, mucosa). *BRAF* mutations were common in non-CSD tumors but rare in those arising in CSD skin, or alternatively in non-UVR exposed body sites (Maldonado et al., 2003) This approach was further extended to explore differences in DNA copy number across the genome, as well as *BRAF* and *NRAS* mutation status (Curtin et al., 2005). In addition to the previously reported distribution of *BRAF* mutations, distinct patterns of chromosomal loss or gain were observed for the subtypes. Focal gains of the *CCND1* locus, loss of chromosome arm 3q, and chromosome 22 gains were found to preferentially occur in the CSD subgroup, while chromosome arm 10q loss was found to be more frequent in the non-CSD subgroup; no significant difference was found in the frequency of *NRAS* mutation. Further supporting the suggestion that CSD and non-CSD tumors are genetically distinct, focal amplifications of *KIT* were subsequently observed in CSD, acral, and mucosal melanomas, but not non-CSD melanomas. Sequencing of *KIT* identified oncogenic mutations in this gene in some samples, but amplifications or mutations of *KIT* were not found in non-CSD tumors (Curtin et al., 2006a,b). These data provided compelling evidence that genetic heterogeneity in melanomas is at least partially attributable to the presence of distinct genetic subgroups associated with different patterns of sun exposure.

Large-scale WGS and WES studies may provide an opportunity to both replicate these findings and identify and catalog additional genetic lesions associated with specific patterns of sun exposure if histological assessment of sun damage at the site of the primary tumor is recorded. Mar et al. (2013) performed exome sequencing on a small set of melanomas (n = 34) for which the degree of solar elastosis adjacent to the primary tumors was assessed. Overall, the mutation rate in melanomas arising on CSD skin was approximately five-fold higher (21 mutations/Mb versus 3.8 mutations/Mb) than in those arising on skin without severe sun damage, with CSD tumors also harboring a higher proportion of tandem CC>TT mutations. Consistent with previous reports, *BRAF* V600E mutations were more common in non-CSD tumors, while *KIT* mutations were identified solely in CSD tumors. Additionally, consistent with a previous report that conducted focused sequencing of *BRAF*, *BRAF* V600K mutations tended to be found in CSD tumors (Menzies et al., 2012). Still, the sample size in this study was too small to derive further meaningful associations between the degree of solar elastosis and other specific gene mutations, and data from histological assessment of sun damage at the site of the primary tumor have been lacking for most of the larger tumor sequencing datasets. For example, due to lack of available material on many cases, the degree of solar elastosis of the incident primary melanoma could not be evaluated in a sufficient number of TCGA cases to yield meaningful results. An imperfect surrogate for UVR exposure that can be evaluated in most datasets is body site of primary tumor and is available for a subset of the TCGA melanomas (Watson et al., 2015). The associated mutation data (Table 2) are largely consistent with previously reported differences in CSD and non-CSD tumors. Specifically, *BRAF* mutations tend to be found more commonly in tumors arising on the trunk (low/intermittent UVR), versus either the extremities or head/neck, whereas *NRAS* mutations occur more often on body sites more typically associated with chronic UVR exposure (e.g. extremities, head, and neck). Mutations or focal amplifications of *KIT* largely occur in head/neck tumors. Similarly, amplifications of *CDK4* and *CCND1* trend toward higher frequencies in tumors from the head/neck and extremities.

An alternative approach to separate tumors into subgroups with potentially different UVR exposure patterns is assessment of the proportion of mutations within a tumor that could be ascribed to UVR mutagenesis, for example C>T or CC>TT mutations, or perhaps more precisely in the context of a mutational signature based on context of flanking nucleotides (Alexandrov et al., 2013). Analysis by TCGA using the former approach, specifically the criteria proposed by Brash (2015) (> 60% C>T transitions or >5% CC>TT) to classify samples as

Table 2. Differences in gene mutation/amplification/deletion frequency by body site of primary tumor in 278 TCGA melanomas with complete data

Gene (Mut/CNV)	Extremities (110)	Gene alteration frequency: Trunk (92)	Head and neck (18)	Others and unknown (58)	Fisher's exact test *P*-value: Extremities versus trunk	Extremities versus head and neck	Trunk versuss head and neck
BRAF : MUT	45%	62%	39%	47%	0.02	0.80	0.12
TP53 : MUT	15%	13%	39%	19%	0.84	0.02	0.01
NRAS : MUT	36%	25%	33%	29%	0.09	1.00	0.56
PPP6C : MUT	6%	7%	22%	5%	1.00	0.05	0.06
NF1 : MUT	14%	12%	17%	14%	0.83	0.72	0.70
DDX3X : MUT	5%	5%	22%	7%	1.00	0.03	0.04
RAC1 : MUT	5%	4%	17%	12%	0.76	0.11	0.08
ARID2 : MUT	15%	9%	17%	12%	0.20	1.00	0.38
IDH1 : MUT	5%	5%	6%	9%	1.00	1.00	1.00
RB1 : MUT	3%	5%	0%	2%	0.47	1.00	0.59
CDKN2A : MUT	16%	12%	17%	17%	0.42	1.00	0.70
MAP2K1 : MUT	5%	4%	6%	7%	1.00	1.00	1.00
PTEN : MUT	8%	9%	0%	10%	1.00	0.36	0.35
KIT : MUT	3%	3%	17%	3%	1.00	0.04	0.05
CDK4 : MUT	3%	2%	6%	2%	1.00	0.46	0.42
MITF : AMP	4%	7%	6%	12%	0.52	0.54	1.00
MDM2 : AMP	4%	2%	11%	7%	0.69	0.20	0.12
KIT : AMP	2%	1%	11%	9%	1.00	0.09	0.07
CCND1 : AMP	7%	1%	6%	9%	0.04	1.00	0.30
CDK4 : AMP	6%	1%	6%	5%	0.07	1.00	0.30
CDKN2A : HOMDEL	34%	28%	28%	29%	0.45	0.79	1.00

The data were extracted from cBioPortal and samples with clinical data but without mutation/CNV data were removed. Fisher's exact test was used to determine statistical significance. P-values < 0.05 are highlighted pink.

associated with high-UVR, revealed no distinct difference between samples harboring either *BRAF*, *NRAS*, or *NF1* mutant samples in terms of proportion of high- or low-UVR samples; the largest proportion of low-UVR samples occurred in triple wild-type samples (Watson et al., 2015). In contrast, a small sequencing study focused on pediatric melanoma found that most samples, particularly conventional melanomas (non-spitzoid, not associated with a congenital nevus) harbored a high number of somatic mutations, and in each sample, over 80% of mutations were consistent with a UVR damage signature (Lu et al., 2015). Nearly, all of these conventional melanomas, whether occurring on the trunk, extremities, or head/neck, harbored *BRAF* V600E mutations (13 of 15, 87%; one of the two lacking the *BRAF* V600E mutation was an acral melanoma), as well as hotspot *TERT* promoter mutations (12 of 13, 92%), a pattern consistent with adult non-CSD melanomas.

Alignment of susceptibility and somatically mutated genes

As one might have expected, there is some overlap between the genes and pathways altered somatically and those with germline mutations conferring susceptibility to melanoma. The most salient of these are briefly summarized below.

Telomere maintenance pathway

There is an emerging body of evidence suggesting that telomere dysfunction not only plays a key role in melanoma progression, but also risk of developing melanoma. To date, rare, high-penetrance mutations to multiple genes involved in telomere maintenance have been identified in multiple-case melanoma families, including mutations to the *TERT* promoter (Harland et al., 2015; Horn et al., 2013), as well as to the protein-coding sequence of the shelterin complex members *POT1* (Robles-Espinoza et al., 2014; Shi et al., 2014), *ACD* (Aoude et al., 2014), and *TERF2IP* (Aoude et al., 2014). While thus far mutations to these telomere-related genes have been found in only a very small fraction of high-density melanoma families, genomewide association studies (GWAS) have provided evidence for a much broader role for telomere maintenance genes in conferring risk of developing melanoma in the general population. GWAS-identified telomere-related melanoma risk loci include several previously implicated as being associated with telomere length, including the following: *TERT-CLTPM1L* (Barrett et al., 2015; Gibbs et al., 2015; Law et al., 2012; Nan et al., 2011; Rafnar et al., 2009; Stacey et al., 2009), *TERC*, *RTEL1*, and *OBFC1* (Law et al., 2015). The somatic *TERT* promoter hotspot mutations discussed earlier (Horn et al., 2013; Huang et al., 2013) appear to account for the majority of driver

aberrations in telomere maintenance genes in melanoma (found in 64% of melanomas assayed by TCGA) and are preferentially found in tumors with either *BRAF*, *RAS,* or *NF1* mutations (frequency of 75, 70 and 80%, respectively, versus 10% of triple wild-type tumors) (Watson et al., 2015). Amplification of the *TERT* locus is a less common event, preferentially found in (~15% of) the triple wild-type melanomas (Watson et al., 2015). Individually, other telomere-related genes implicated in melanoma risk appear to be altered infrequently. The shelterin complex member *ACD*, while listed among the top 83 recurrently mutated genes in the TCGA dataset (MutSig $Q < 0.10$), is nonetheless somatically altered in only ~3% of tumors, while *POT1, TERF2IP*, *RTEL1,* and *OBFC1* are each mutated at insufficiently high frequencies to support clear roles as driver melanoma genes (2.5, 1, 2.5 and 1%, respectively). Thus, while the majority of melanomas harbor somatic aberrations to the telomere maintenance pathway, most mutations occur within a single gene, *TERT*.

CDKN2A/CDK4/CCND1/RB1 pathway

The role of mutations in members of the p16/CDK4/cyclin D1/Rb pathway in melanoma development, both somatically and in the germline, has been well established. The identification of pathogenic, high-penetrance *CDKN2A* mutations in melanoma families resulting in the loss of the p16 protein (Hussussian et al., 1994; Kamb et al., 1994a) quickly followed on from the discovery of recurrent deletions and somatic mutations in melanomas as well as other cancers over 20 yr ago (Kamb et al., 1994b; Nobori et al., 1994). Germline *CDKN2A* mutations have since been found in ~35–40% of multiple-case melanoma families (Goldstein et al., 2006). Similarly, the discovery of hotspot mutations in the p16-binding domain of *CDK4* in melanoma families (Zuo et al., 1996) followed the discovery of similar mutations in melanoma tumors (Wölfel et al., 1995). These germline *CDK4* mutations occur in families at a much lower (2–3%) frequency (Goldstein et al., 2006). Further, patients with bilateral retinoblastoma, who almost universally have germline inactivating mutations to the retinoblastoma 1 tumor suppressor (*RB1*), have up to an 80-fold increased risk of developing melanoma (Draper et al., 1986; Fletcher et al., 2004), underscoring the importance of this pathway melanoma predisposition. Finally, melanoma GWAS have provided evidence that this pathway may influence risk beyond high-density melanoma families and extend to the general population, with genomewide significant risk loci identified in the vicinity of both *CDKN2A* and *CCND1* (Barrett et al., 2011; Bishop et al., 2009; Falchi et al., 2009; Law et al., 2015). Somatically, the importance of this pathway in melanoma is likewise clear and reinforced by recent genomic studies. More than half (56%) of TCGA melanomas (with both copy number and mutation data available) harbor aberrations in at least one pathway member. *CDKN2A* is mutated or deleted in 44% of TCGA melanomas (13% of samples harbor mutations), a frequency similar to that observed in most other WES and WGS datasets (Table 1). This figure, however, may nonetheless overestimate the prevalence of *CDKN2A* aberrations in thinner, less advanced tumors; data from Shain and colleagues suggest that *CDKN2A* mutations are less frequent in early lesions, with homozygous loss occurring predominantly in invasive tumors (Shain et al., 2015b). *CDK4* (7% overall, 3% mutated, 4% amplified), *CCND1* (7% overall, primarily amplifications), and *RB1* (5% overall, primarily point mutations and homozygous deletions) are each aberrant in a much smaller proportion of TCGA tumors (Watson et al., 2015).

TP53/p14^{ARF}/MDM2 pathway

While most *CDKN2A* mutations, both germline and somatic, affect either p16 alone or alternatively both p16 and p14^{ARF} proteins, 2–3% of melanoma families have been found to have mutations altering the alternative first exon of *CDKN2A* encoding only p14^{ARF} (Harland et al., 2005; Hewitt et al., 2002; Randerson-Moor et al., 2001) suggesting that p14^{ARF} itself plays a role in melanoma susceptibility and implicating the p14^{ARF}/*TP53* pathway in melanoma biology. Consistent with this notion, 57% of TCGA melanomas harbor somatic alterations in *CDKN2A*, *TP53*, or *MDM2*. In addition to the previously mentioned *CDKN2A* deletions or mutations (44% of TCGA tumors), *TP53* is mutated in 15% of TCGA melanomas, a frequency consistent not only with data from other WGS and WES datasets (Table 1) (Berger et al., 2012; Hodis et al., 2012; Krauthammer et al., 2012, 2015; Watson et al., 2015), but also a large meta-analysis of *TP53* mutations in melanoma compiled from multiple focused studies (Hocker and Tsao, 2007). Much as the case with *CDKN2A*, however, *TP53* mutations appear to occur later during tumor development, suggesting that early lesions may harbor such aberrations at a lesser frequency (Shain et al., 2015b). Reinforcing data from previous studies implicating DNA copy number gain at the *MDM2* locus (Jönsson et al., 2007; Muthusamy et al., 2006; Stark and Hayward, 2007) focal amplifications of *MDM2* are observed in about 5% of TCGA melanomas (Watson et al., 2015) and are accompanied by co-amplification of the *CDK4* locus in approximately half of these tumors, suggesting that at least in some tumors co-amplification of *CDK4* and *MDM2* may substitute for loss of p16 and p14^{ARF}.

Pigmentation pathways

There is considerable epidemiological data establishing a connection between light skin color and melanoma risk. Among a number of genes known to mediate pigmentation, the melanocortin-1 receptor (*MC1R*) is well established as a medium-penetrance locus for melanoma susceptibility (Bishop et al., 2009; Raimondi et al., 2008). Beyond *MC1R*, GWAS have further highlighted multiple melanoma risk loci that also strongly contribute

to pigmentation phenotypes at a level of genomewide significance, including the following: *ASIP* (Brown et al., 2008; Gudbjartsson et al., 2008), *TYR* (Bishop et al., 2009), *SLC45A2* (Barrett et al., 2011), and *OCA2* (Amos et al., 2011; Law et al., 2015). Additionally, although not identified through GWAS, *MITF,* encoding a key melanocyte transcription factor and regulator of pigmentation, has also been identified as a medium-penetrance melanoma predisposition gene (Bertolotto et al., 2011; Yokoyama et al., 2011). Somatically, alteration of pigmentation genes is relatively infrequent. Collectively, *TYR*, *TYRP1*, *DCT*, *SLC24A5*, *SLC45A2*, and *PMEL* are mutated in 21% of TCGA melanomas. While some such variants are clearly inactivating, including likely homozygous deletions in *TYR*, *TYRP1*, *DCT*, and *SLC24A5*, the significance of other variants, which include a mix of amplifications and point mutations, is less clear. On the other hand, both *SOX10*, which regulates *MITF* expression, as well as *MITF* itself, both appear to be altered somatically with significant frequency. Garraway et al. (2005) initially reported amplification of the *MITF* locus in approximately 10% of primary melanomas, with a higher observed frequency in metastases (21%). A subsequent study noted somatic protein-coding mutations of both *MITF* (8% of primary tumors, 12% of metastases) and *SOX10* (9% of primary tumors, 6% of metastases) in addition to *MITF* amplifications (in 10% of metastases), with more than 20% of tumors having aberrations in at least one of these two genes (Cronin et al., 2009). Data from TCGA melanoma project (from which both copy-number and sequencing data are available) similarly show copy number aberrations and mutations in both genes, albeit with somewhat lower frequencies. Collectively, either *MITF* (amplified in 6.5%, mutated in 2%) or *SOX10* (amplified in 2%, mutated in 4%) is somatically altered in about 15% of TCGA melanomas (Watson et al., 2015).

MAPK and PI3K/AKT pathways

While it is evident that the MAPK and PI3K/AKT pathways are altered and activated in the majority of melanomas, a major role in susceptibility has yet to emerge. Although deleterious germline mutations in RAS/MAPK pathway members are sometimes found, they are largely associated with a class of developmental disorders not associated with increased melanoma risk (RASopathies, reviewed in Tidyman and Rauen, 2009), perhaps reflecting the essential role of the this pathway in regulation of cell growth, differentiation, cell cycle, and senescence. Rare, germline mutations in the *PTEN* tumor suppressor, on the other hand, lead to Cowden Syndrome, an autosomal-dominant disorder associated with increased lifetime risk of multiple cancers, including melanoma (Tan et al., 2012). However, there is little evidence of a significant role for common germline variants within or near genes in these two pathways mediating melanoma risk. While germline variants in *BRAF* have been previously reported to be associated with risk of melanoma in candidate gene studies, including reports of an association in men (Meyer et al., 2003), as well as a reported weak association in an Australian case–control study (James et al., 2005), GWAS have not provided evidence of a significant role for germline variation in *BRAF*, or any other core genes directly involved in MAPK or PI3K signaling (Law et al., 2015).

Somatically, *BRAF*, *NRAS*, and *NF1* collectively represent the most significantly mutated MAPK pathway genes; overall, 86% of TCGA melanomas harbor a mutation in at least one of these three genes. The respective mutation frequencies for each of these genes (52, 28 and 14%) largely align well with those observed in other WGS and WES datasets (Table 1) (Berger et al., 2012; Hodis et al., 2012; Krauthammer et al., 2012, 2015; Watson et al., 2015). Hotspot mutations in other Ras genes, while observed in melanoma, appear to be exceptionally rare (1% of samples mutated each for *HRAS* and *KRAS*). Beyond these genes, TCGA data have also provided some further support for previously identified driver mutations in downstream MAPK effectors. *MAP2K1* and *MAP2K2* mutations, originally identified from a small WES sequencing study (Nikolaev et al., 2012; Stark et al., 2011), appear to be mutated with frequencies consistent with the original report (5, 2%, respectively, Table 1), with *MAP2K1* listed among the core set of TCGA SMGs. Mutations in *MAP3K5* and *MAP3K9*, identified in a similar study (Stark et al., 2011) are found at a somewhat lesser frequency (6 and 8%, respectively) than originally reported.

NRAS and *PTEN* represent the most commonly mutated PI3K pathway members in melanoma. Somatic alterations to *PTEN* are collectively found in 14% of TCGA melanoma tumors, with 6% of these likely homozygous deletions and the remainder mutations. While this frequency aligns well with estimates from previous reports, data from Shain and colleagues suggest *PTEN* mutations tend to occur predominantly in invasive melanomas and thus may be less frequent in early lesions (Shain et al., 2015b). At the same time, this figure is likely to underestimate the proportion of advanced melanomas with functional PTEN loss, given both the higher frequency with which allelic loss at the *PTEN* locus (Goel et al., 2006; Mikhail et al., 2005; Pollock et al., 2002; Tsao et al., 1998) as well as loss of PTEN expression are observed in melanomas (Goel et al., 2006; Lin et al., 2008). Consistent with previous reports (Curtin et al., 2006a; Davies et al., 2008; Omholt et al., 2006), alterations in other prominent pathway members downstream of Ras, including *PIK3CA* (altered in 5% of samples overall) and *AKT3* (altered in 8% of samples overall, most of which are copy-number gains), occur at relatively low frequencies. As a whole, somatic alterations within this geneset are found in 46% of TCGA melanomas. Somatic events and or altered gene expression of additional downstream pathway members may further contribute to PI3K/AKT pathway activation across melanomas. Most

are found to be mutated relatively infrequently, consistent with previous reports (Shull et al., 2012), with the PTEN-regulating protein PREX2 a notable exception. *PREX2* gene mutations were observed in approximately 27% of TCGA melanomas, a somewhat higher frequency than the 14% mutation rate originally reported (Berger et al., 2012; Watson et al., 2015).

While there is considerable evidence that multiple RTKs upstream of these pathways play significant roles in melanoma biology, relatively few show frequent somatic genetic alterations (Prickett et al., 2009). Among those that are frequently altered include multiple previously reported genes including *KIT*, mutated or amplified each in 4% of TCGA melanomas, respectively, as well as *PDGFRA*, located approximately 350 kb from and frequently co-amplified with *KIT* (Curtin et al., 2005, 2006b). Consistent with previous reports of *ERBB4* and *FLT1* mutations spread out across their respective protein-coding regions in almost 20 and 10% of melanomas, respectively (Prickett et al., 2009), *ERBB4* and *FLT1* mutations are found in 16 and 12% of TCGA melanomas (Watson et al., 2015).

Conclusions and future directions

NGS technology has allowed an unprecedented and in-depth analysis of somatic genetic events of cutaneous melanoma. Satisfactorily, the large-cohort sequencing efforts have consistently reported key drivers and SMGs in melanoma, including both known (*BRAF*, *NRAS*, *TP53*, *CDKN2A,* and *PTEN*) and newly identified genes (*NF1*, *RAC1*, *PPP6C*, *IDH1,* and *ARID2*) (Hodis et al., 2012; Krauthammer et al., 2015; Watson et al., 2015). Despite this, it is likely that a number of candidate cancer genes are yet to be identified as the current datasets have not yet reached saturation for the detection of SMGs (Lawrence et al., 2014). Using an average background mutation rate of 13 mutations/Mb (as approximately predicted for melanoma), even the largest NGS compilation analysis of 501 melanomas could only predict with complete power genes that are mutated in 10% or more of patients (Arafeh et al., 2015). Furthermore, based on a similar background mutation frequency, it has been predicted it will take 5300 samples to reach 100% power to create a comprehensive catalog of candidate cancer genes that are mutated in $\leq$2% of melanomas (Lawrence et al., 2014).

Adding to the difficulty of reaching enough samples to detect SMGs in melanoma is the fact that all non-cutaneous melanoma subtypes are currently under-represented in existing NGS datasets. Although it appears that acral, uveal, and mucosal melanomas have a lower background mutation rate, access to the number of samples required for sufficient power (while significantly smaller than that required of cutaneous melanoma) will remain challenging, as these subtypes present clinically at low frequency.

As such, there remains the need to continue large-scale sequencing efforts, both of cutaneous and non-cutaneous melanomas, in order to comprehensively catalog the somatic mutation landscape of melanoma.

There are also different types of mutation events that are yet to be explored in detail and analyses of these may reveal novel candidate driver genes. As mentioned previously in this review, areas of the non-coding genome warrant closer examination as it is likely that mutations in 5′UTR and promoter regions may be currently underrepresented due to the limitations of WES. In addition, the role of synonymous mutations in melanoma genesis is still in its infancy, and comprehensive maps of fusion events and structural variations have yet to be extensively compiled. Other areas that may point to new SMGs include events occurring within the 3′UTR such as disruption of miRNA binding sites that can modulate downstream pathway activity (Forloni et al., 2014), mutations affecting distal enhancers or resulting in super-enhancer hijacking (Northcott et al., 2014), mutations within transcription factor binding sites (Katainen et al., 2015), or the expression of novel transcript isoforms (Wiesner et al., 2015).

Further exploitation of the sequencing data currently generated may also reveal new SMGs and additional insights into melanoma biology. One area that has yet to be extensively addressed is the role of germline genetics influencing the somatic mutation landscape. Although it is routine practice to sequence matched normal DNA in order to ascertain somatic mutation events in cancer sequencing studies, this germline sequence data is currently underutilized. Thorough examination of this data may reveal unexpected insights into possible synergistic roles between germline variants and somatic mutation events in cancer.

Lastly, and although outside the scope of this review, it is important to note the role of somatic and germline genetics influencing the clinical management and treatment of melanoma patients. Further exploration of data linking clinical presentation, genetic sequencing, therapeutic regimen, and overall response in retrospective and prospective studies will be useful in identifying new diagnostic and prognostic biomarkers. High somatic mutation burden in melanoma has already been linked to favorable outcomes of immune-based therapeutic approaches using CTLA-4 antibodies (Snyder et al., 2014; Van Allen et al., 2015). Such analyses of genomic correlates and exceptional patient responders will be useful in guiding improved therapeutic options and could lead to new insights into the biological pathways governing melanoma genesis.

Looking to the future, establishing multi-integrated datasets combing WES, WGS, and RNA-Seq information on large cohorts of cutaneous and non-cutaneous melanomas from various stages of tumorigenesis, in conjunction with rigorous functional validation of candidate driver genes, will help establish a comprehensive

catalog of somatic mutation events which will be informative for guiding the clinical management of melanoma patients.

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