Hoek et al. 2006

Model system	Experiment	Result	Conclusion
GSE4845 (Based on BRAF/NRAS mutations)	ANOVA selection and multiple-testing correction (Benjamini et al., 2001) to look for gene expression changes correlating with activating mutations of BRAF or NRAS	No genes with expression changes upon activating mutations for either	No statistically significant correlation between mutations and expression levels. It is likely the correlations were found in cells within the same cohort.
GSE4845	Unsupervised hierarchical clustering multiple testing controlled statistical methods to select probe sets with cohort-specific expression patterns	3 cohorts for melanoma cells with 223 common genes with cohort specific exression	3 possible cell states
GSE4845	Self organised map (SOM) analysis to check which genes are coregulated and RT PCR + Western Blot (MITF/INHBA)to confirm relative expression levels	that 105 were tightly linked to one of two distinct motifs	Motif 1 shows downregulation of 51 co-regulated genes in cohort C compared with those in cohorts A and B. Motif 2 shows upregulation of 54 co-regulated genes in cohorts B and C compared with those in cohort A (Motif 1 - melanocytic, motif 2 - TGF-β regulated, extra cellular regulators)
GSE4845	Student t- test and p-value for 134 reference genes shown to be involved in metastatic potential	54 significant in Zurich set, with 20 from 223 common genes	Different cohorts and expression are reflections of differences in metastatic potential.
GSE4845 Zurich	*in vitro* motility with time-lapse photography and TGF - β treatment	a cleared field repopulated within 24 h with melanomas from cohorts A and C, while cohort B melanomas were appreciably slower. Cohort A detaches and migrates while B and C migrate along substrate. Cohort C was less affected by TGF-β. Cohort A grew two-fold faster	Supports the metastatic potential theory
GSE4845 Zurich cohorts B and C	Immunohistochemistry for Wnt/β-catenin and MITF	B shows positive correlation in levels of two proteins and C has low levels of both proteins	In cohort C Wnt signalling is deactivated

- Bloethner et al., 2005 and Pavey et al., 2004 identified BRAF and NRAF mutants to have differential gene expression. Surprisingly, the genelists are completely different!
- Proliferative and weakly metastatic cells maintain a neural crest-like transcriptional signature through Wnt signalling. However, the induction of TGFb-like signalling, possibly through microenvironmental changes brought about by hypoxia or inflammation, drives the expression of factors inhibitory to Wnt signalling and resulting in cells which are less proliferative but have high metastatic potential
- Thus two transcriptional proogrammes underlie the molecular differences between the metastatic potential

Hoek et al., 2008

Model system	Experiment	Result	Conclusion
Tumour cell lines	Check if the two states pertain to different MITF levels using imunohistochemistry	in proliferative signature cell lines, 93% of cells were positive for nuclear staining for Mitf whereas invasive signature cell lines showed no positivity	Proliferative show high levels and invasive show low levels of MITF
Tumour cell lines	Check if the two states pertain to different Ki67 levels using imunohistochemistry	94% of proliferative signature cells and 45% of invasive signature cells had positively stained nuclei. No variation in mRNA levels	Proliferative show high levels and invasive show low levels of MITF. Ki67 antigen is a suitable marker for identifying regions undergoing differential rates of proliferation.
Tumour cell lines	MITF siRNA	Mitf depletion from proliferative signature melanoma cells made them less susceptible to TGF-β mediated growth inhibition	Mitf mediates the growth-inhibitory effect of TGF-β
Tumour cell lines	TGF-β treatment	Mitf mRNA and protein levels decrease with TGF-β	TGF-β–mediated growth inhibition may be effected by reduction of MITF expression
Proliferative and invasive cells in athymic nude mice	Seeded *in vivo* for tumour formation	tumor volume exceeded 100 mm3 for proliferative at 14 +/- 3 days and for invasive in 59 +/- 11 days	Time period for growth was wide spread in invasive, than proliferative.Unlike proliferative signature–seeded initiation, invasive signature-seeded initiation may be dependent on microenvironmental variation.
Tumours from proliferative and invasive seeding	Immunohistochemistry staining of tumours for MITF and Ki67	Additionally, we found that Mitf-stained nuclei tended to concentrate within the peripheral margins of the tumors. Ki67 was also indistinguishable.Mitf-positive nuclei were also enriched for Ki67-positive	Tumors seeded with invasive or proliferative signature cell lines were not distinguishable.

- Ki67 antigen is not detected in G0, and it may be an absolute requirement for cell proliferation, the authors conclude that invasive signature cells spend more time in G0 (quiescence).
- Increasing Mitf expression in melanoma has been shown to be a proliferative factor and involved in Cdk2 production and activity
- Melanoma cells proximal to the interface between host tissues and the tumor are actively undergoing increased rates of proliferation.
- *in vitro* proliferation rates for invasive signature cells serially passaged over the same time frame as conducted for the xenograft experiments showed no change in proliferation rates, thus not due to contamination.
- *in vitro* studies in which poorly aggressive cells, grown on three-dimensional matrices preconditioned by aggressive lines, showed up-regulation of extracellular matrix modifying genes and increased invasive ability (Seftor EA et al., 2006)
- This location of proliferative phenotype melanoma cells at the tumor periphery directly contradicts the long-held assumption that these cells represent the tumor invasive front.
- Hypothesis - the thickness of the primary tumor may, rather than serve to bring its peripheral cells closer to vascular egress, determine the extent to which cells deeper within it experience microenvironmental change, which (as we contend) drives the switch to a more invasive phenotype.
- The caveat - that much of our hypothesis rests on expression signatures obtained in vitro and thus may not fully recapitulate the in vivo biology of melanoma

Verfaille et al., 2015

Model system	Experiment	Result	Conclusion
GEO datasets ref	Unsupervised hierarchical clustering and SOM	3 clusters - proliferative, invasive and immune- related signature. Significant overlap with Hoek et al. 2006	immune signature possibly due to infilterating immune cells/lymphocytes.
TCGA cohort	exome re-sequencing analysis	No specific enrichments for mutations in BRAF, NRAS or any of the other well-established melanoma driver genes	The two states are driven by transcriptional reprogramming rather than genetic mutations
Immune cell samples	Non-negative matrix factorization and GSEA	fall into invasive and proliferative phenotypes	Might represent a layer underlying melanoma phenotypes
SKMEL-5 (n=1) + Gembarska, A. et al.(freshly isolated human melanoma samples, n=10)	Short passage followed by transcriptome and chcromatin landscape analysis	Identified a new gene signature for each state, consisting of 772 and 643 genes for the proliferative and invasive phenotypes with 100% upreglation of Hoek signatures	The two expression profiles we identified likely arise through gene regulation by cis-regulatory modules and no correlation to mutations
Same eleven samples	CHiP- seq and open chromatin profiling (FAIRE-seq17) to identify *cis*-regulatory elements against two important histone modifications representing activated (H3K27ac) and repressed (H3K27me3) chromatin marks	H3K27ac and FAIRE-seq tracks indicate an active and open SOX10 promoter, respectively, in the nine proliferative samples with high SOX10 expression. The SOX10 promoter lacks activating marks, but carries repressing H3K27me3 marks in the two invasive cultures	Thousands of regulatory genes show difference in the two states ussing SOM analysis
Same eleven samples and TCGA (tumour biopsies)	Analysis of (differential) H3K27ac, FAIRE and H3K27me3 peaks around the transcription start site (TSS) of their nearest differentially expressed genes	TSS of invasive genes shows strong regulatory activity in the invasive samples, but low activity in the proliferative samples, while the repressive H3K27me3 mark shows the opposite and vice versa	Confirms that thespecific chromatin marks are robust predictors of the transcriptional activity in a specific cellular state.
Same eleven samples and TCGA (tumour biopsies) - proliferative cells	Publicly available CHiP-seq data to identify TFs that bind to the regulatory regions identified for proliferative states	SOX10 most active with 2437 of these regions and one of the highest scoring targets lies upstream of SOX10 (using CHiP-seq track) and SOX10 target region is present upstream of MITF. The second most enriched motif is an E-box motif (MITF binds E-box motifs), so correlation verified using CHiP seq. One of the binding sites includes upstream region of SOX10	SOX10 plays a major role and is autoregulated. MITF/SOX10 feedback or autoregulation is possible. They are the major TF regulators of the proliferative states

- No genetic mutation to account for metastasis has been identified, supporting the possibility of epigenetic/ chromatin regulation for the same.
- When comparing enhancers active in the proliferative transcriptional state to 110 different sets of tissue-specific enhancers identified by the expression of enhancer-RNA, melanocyte is identified as the cell type with the highest overlap of enhancers. In contrast, the invasive melanoma enhancers overlap most strongly with enhancers specifically active in skin fibroblasts, which are known to harbour a mesenchymal regulatory programme
- 7q34 duplication was found to be enriched in the invasive samples which contains BRAF gene
- overexpression of BRAF was recently shown to drive a rapid and reversible switch in a specific subset of invasive-related TFs

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Same eleven samples and TCGA (tumour biopsies) - invasive cells	Publicly available CHiP-seq data to identify TFs that bind to the regulatory regions identified for invasive states	AP-1 most enhanced and CHiP-seq track ranked FOSL2 first (AP-1 complex protein) . TEAD factors were second most enhanced with strong enrichment in TEAD CHiP	TEAD and AP1 are the major TF regulators for invasive state.
GEO datasets (?)	Checked for coexpression of regulators and target genes identified followed by perturbation analysis for SOX10, FRA1 and JUN. GSEA was used to analyse target gene predictions	Overlap was observed between target gene sets and experimental	Predictions validated! High overlap between AP-1 and TEAD genes was also observed, possibly due to cooperativity.
Same eleven samples and TCGA (tumour biopsies)	focused on one particularly relevant target gene of the invasive network, SOX9 and performed Circularized Chromosome Conformation Capture sequencing (4C-seq)	31 significantly active regulatory regions were identified—clustered into eight subregions—within 1.4Mb around SOX9 and positively correlated with SOX9 promoter activation. SOX9 positive cells (invasive?) showed high level of upstream sequence activation for SOX9 while SOX9 negative region showed no interaction between distal activation site and enhancer.	multiple distal enhancers can interact with a single promoter, and that (long-range) chromatin interactions can differ between melanoma cellular states.
Same eleven samples	KD of all four TEADs and assayed for invasion	Predicted target genes (SOX9, SERPINE, EHPA2) downregulated, significant decrease in invasive properties	TEADs contribute to the establishment of the invasive transcriptional cell state and its associated cellular phenotype
CCLE	Correlation analysis	Positive correlation between TEADs target gene signature and BRAF inhibitor response (in BRAF mutants) and MEK inhibitor response	TEAD-mediated transcription is one of the determinants that contribute to the increased resistance of the invasive melanoma cells to MAPK pathway inhibition
Short term cultures	IC_{50}determination for BRAF and MEK inhibitors	Invasive cultures are significantly more resistant, KD of all four TEADs significantly resensitizes cells	TEADs transcriptional response aids drug resistance in invasive cells in melanoma

- the observed architecture of loops, of SOX9 long distance interaction, differs strongly between invasive and proliferative samples, elegantly explaining the differential expression of this target gene.

Tsoi et al., 2018

Model system	Experiment	Result	Conclusion
GSE80829	Unsupervised hierarchical clustering	4 clusters with no gain in cluster stability with increasing clusters and distinct groups in PCA by clusters	4 clusters of cells possibly exist
ESC differentiating into melanocyte	PCA of the differentiation stage gene expression profiles and projection of melanoma along this PCA space	4 clusters separated out in space	Distinct differentiation profiles of 4 clusters
4 clusters of cells	Enrichment analysis of differentiation- related GO processes	C1 was enriched for undifferentiated (invasive phenotype genes like cell adhesion,migration, immune repose, MITF low), C2 for invasive/immune phenotype with neural crest cell like markers, C3 for transitory (neural crest markers and pigmented-associated), C4 for melanocytic markers.	Validation
4 clusters of cells	RTK levels and enrichment	C1 and C2 had MITF low, AXL high, elevated SMAD3. Undifferentiated has lower levels of neural crest cell markers and higher levels of SOX9,EGFR which occurs due to SOX10 loss. C3 and C4 have higher levels of MITF and low AXL. C4 showed higher differentiation with more enrichment for MITF and its target genes.	Validation
4 clusters of cells	Submap to Hoek cohorts	Informative mapping relations identified for all clusters. C1 and C2 are cohort C, C3 cohort B and C4 cohort A,	Subclassification of Hoek's cohorts
Same	Observed differentiation trajectory position score for each sample using a "center of mass" when treated with MAPKi	In BRAF mutants, cells shift from initial differentiated to dedifferentiated phenotype. NRAS mutants/alterate splicing do not show this trajectory! However the alternate splicing mutation shows gain of MAPK without change in differentiation state	Dedifferentiation and MAPK reactivation are not mutually exclusive and dedifferentiation can be a transient response of adaptive resistance
CCLE and SKCM	Validation in cell lines and tumor biopsies	Strong concordance (R=0.87) between maximum expressed value of genes in cell lines and tumour, residual disparity from non tumour cells. Upon removal of these cells, tumour replicates trajectory upon PCA.	Cell lines retain tumour like properties

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TCGA and GDSC	Methylation of promoters for differentiation affiliated genes	Promoter methylation beta values are inversely correlated with expression for AXL, MITF, SOX10, SMAD3 and CTNNB1 and genome methylation replicated the arc pattern upon PCA	Consistency in tumour and cell lines and epigenetic/ chromatin regulation as a key factor
CCLE and GDSC	hierarchical clustering to identify drug clusters with a similar mechanism of action followed by correlation beteen dedifferentiation and sensitivity	Dedifferentiation increased sensitivity to ferroptosis drugs	Potential drug target

- The dedifferentiation state can be stabilized, such as through loss of SOX10 by epigenetic reprogramming
- Greater expression of dedifferentiation signatures within the relapse group compared to the control both in the tumors and in tumor-derived cell lines suggesting that immune cell dependent dedifferentiation is not a factor

Rambow et al., 2018

Model system	Experiment	Result	Conclusion
PDX injected in mice	mice were exposed to the dabrafenib- trametinib (DT) combination	All treated lesions rapidly shrunk (phase 1) to reach an impalpable size (phase 2) within 15 days. Continuous treatment invariably led to development of resistance (phase 3)	Presence of MRD
Above derived resistant tumour cells	DNA sequencing	dentified de novo mutations in the MEK1 and NRAS genes and BRAF amplification and splicing mutants in several lesions	similar to aquired resistance to MAPKi
MEL006 dsRed-positive cells from drug-naive (T0) and MRD (phase 2)	genomic DNA of individual cells to massively parallel sequencing	heterogeneous alterations in chromosomes 2 and 7.However, the subclonal distribution at T0 and MRD did not differ significantly. Analysis of a larger number of cells by DNA fluorescence in situ hybridization analysis confirmed this finding	MRD is established through a non-mutational adaptive process and that PDXs are well-suited for studying the mechanisms of drug tolerance in vivo.
Resistant cells	Bulk RNA sequencing	increased pigmentation due to MITF activity and localisation, was seen in a fraction of the drug-tolerant melanoma cells.there was also a large fraction of cells that became completely devoid of any MITF staining at phase 2. Uniform MITF levels in drug naive	adaptive response to MAPK-inhibition is not uniform and distinct populations can arise in vivo.
Cells isolated at differnt time points	scRNA seq and cells were distributed along pseudotemporally ordering paths	contrasting levels of MITF activity at phase 2, Four distinct melanoma transcriptional states were detected, transition occurs from “proliferative” melanoma cells to MITFhigh “pigmented” cells (differentiation lineage) or to MITFlow/negative cells, which either adopted the SOX10low “invasive” or SOX10high NCSC	drug exposure first promotes a transient transition from a “proliferative” to a “starved”-like state from which cells then make the decision to move along a differentiation trajectory to become “pigmented” or a dedifferentiation path and to either become NCSCs or invasive.