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Hypoxia-induced HIF1 α targets in melanocytes reveal a molecular profile associated with poor melanoma prognosis

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Summary

Hypoxia and HIF1 α signaling direct tissue-specific gene responses regulating tumor progression, invasion and metastasis. By integrating HIF1 α knockdown and hypoxia-induced gene expression changes, this study identifies a melanocyte-specific, HIF1 α -dependent/hypoxia-responsive gene expression signature. Integration of these gene expression changes with HIF1 α ChIP-Seq analysis identifies 81 HIF1 α direct target genes in melanocytes. The expression levels for ten of the HIF1 α direct targets – *GAPDH*, *PKM*, *PPAT*, *DARS*, *DTWD1*, *SEH1L*, *ZNF292*, *RLF*, *AGTRAP*, and *GPC6* – are significantly correlated with reduced time of Disease Free Status (DFS) in melanoma by logistic regression (P -value = 0.0013) and ROC curve analysis (AUC = 0.826, P -value < 0.0001). This HIF1 α -regulated profile defines a melanocyte-specific response under hypoxia, and demonstrates the role of HIF1 α as an invasive cell state gatekeeper in regulating cellular metabolism, chromatin and transcriptional regulation, vascularization and invasion.

Keywords

HIF1 α ; hypoxia; melanocyte; melanoma; metastasis

Introduction

The incidence of melanoma has increased over the past 20 years, and it has become clear that early diagnosis and excision of lesions is critical. Removal of early stage lesions correlates with a 5-year survival rate of over 98%, however, the 5-year survival rate drops dramatically to 62% and 18% for individuals who have progressed to regional lymph node or distal metastasis, respectively (SEER database, (Howlader et al., 2016)). Therefore, deciphering the underlying mechanisms that trigger primary lesions to metastasize has both prognostic and therapeutic importance.

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Current research has identified the most frequent driver mutations that cause melanoma progression, including BRAFV600E (~50%, (Bauer et al., 2011)), NRAS codons G12, G13 and Q61 (~20%, (Lee et al, 2011)), and NF1 loss of function mutations (~14%, (Cancer Genome Atlas Network, 2015)), all of which promote increased levels of MAPK signaling. However, the occurrence of these mutations alone does not correlate with clinical measures of outcome in melanoma patients, such as overall survival or disease free status (Rutkowski et al., 2014), suggesting these mutations alone provide an incomplete understanding of the complexity underlying melanoma metastasis. While one contributing factor may be the heterogeneity of additional genomic melanoma mutations (Cancer Genome Atlas Network, 2015; Hodis et al., 2012), notable heterogeneity of gene expression in localized tumor regions has been seen within individual lesions. This regional gene expression variation reflects altered protein and transcriptional regulation in response to changing nutrient and oxygen levels in the tumor microenvironment, thus modulating rates of energy production, stem cell renewal, cell proliferation, migration, differentiation and responsiveness to immune surveillance mechanisms.

Interestingly, the microenvironment for normal melanocytes located at the dermal-epidermal junction is slightly hypoxic. While dermal tissue has an oxygen partial pressure (pO_2) of 10%, the adjacent epidermis has a pO_2 that ranges from 10% to 0.5% (Evans et al., 2006). In melanomas, rapid cell growth within tumors leads to insufficient vascularization, resulting in localized regions of hypoxia that are associated with poor clinical outcomes (Bertout et al., 2008). Therefore, melanocytes and primary melanoma tumors that arise from skin melanocyte populations must respond to a microenvironment defined by a hypoxic gradient which is further exacerbated by reduced oxygen levels upon tumor growth (Bedogni and Powell, 2009).

Microenvironmental oxygen concentrations directly regulate the transcription factor hypoxia inducible factor one alpha subunit (HIF1 α , (Wang et al., 1995)). Under normoxia, HIF1 α is tightly regulated at the protein level, undergoing proteosomal degradation that is directed by von Hippel-Lindau protein (VHL) (Ivan et al., 2001; Jaakkola et al., 2001). At lower oxygen levels, HIF1 α is translocated to the nucleus, where it can heterodimerize with the constitutively expressed Aryl-Hydrocarbon-Receptor Nuclear Translocator (ARNT). HIF1 α -regulated genes control multiple cellular functions critical in cancer progression, including metabolism, cellular survival and proliferation, tumor invasiveness, vascularization, chromatin remodeling, and escape from adaptive immunity (Semenza, 2013; Wigerup et al., 2016), suggesting HIF1 α and its downstream pathways are important potential targets for therapeutic interventions.

In melanoma, a role for HIF1 α in promoting tumorigenesis is well substantiated. The BRAFV600E mutation results in increased HIF1 α expression and melanoma cell survival (Kumar et al., 2007). In addition, elevated HIF1 α expression levels correlate with a cellular phenotype switch from a proliferative to invasive phenotype, and this phenotypic plasticity is associated with melanoma drug resistance and poor prognosis (O'Connell et al., 2013; Widmer et al., 2013). Furthermore, B16F10 mouse melanoma cells exposed to hypoxia prior to injection in nude mice show increased tumor growth and metastatic potential (Cheli et al., 2012), and conversely loss of HIF1 α in a PTEN-deficient, BRAF-activated mouse model of

melanoma results in decreased tumor lymph node burden (Hanna et al., 2013). This mouse melanoma model confirms a crucial *in vivo* role for HIF1 α in regulating the proliferative to invasive cell changes that lead to regional and distal disease progression.

Previous analyses in other tissues have found that HIF1 α genome binding and HIF1 α -regulated genes are highly tissue-dependent (Benita et al., 2009; Chi et al., 2006; Denko et al., 2003; Widmer et al., 2013). Currently, there are limited genomic data that define the melanocyte transcriptional responses to HIF1 α and hypoxia. This study used ChIP-Seq and differential gene expression analyses to evaluate the genomic landscape that exists within melanocytes under hypoxia and in response to HIF1 α knockdown. The resulting data not only define HIF1 α genome occupancy and downstream targets in mammalian melanocytes, but also reveal a novel set of HIF1 α direct target genes that are significantly correlated with Disease Free Status in human primary melanomas, providing critical information for assessing melanoma progression.

Results

Hypoxia-responsive genes in melanocytes

Immortalized melanocytes under hypoxia showed increased migratory capacity and increased HIF1 α protein levels in comparison to immortalized melanocytes under normoxia (Figure 1A, B). Hypoxia-treated melanocytes also showed HIF1 α -dependent upregulation of known HIF1 α target genes (Figure 1C). As expected, *Hif1a* mRNA was not changed by hypoxia, consistent with the fact that protein stabilization regulates HIF1 α function (Figure 1C). Subsequent transcriptome analysis found that melanocytes are highly responsive to hypoxic conditions; 709 hypoxia-responsive genes were altered 1.5-fold under hypoxia, with 452 genes up-regulated and 257 genes down-regulated (Figure 1D, Table S1).

Consistent with hypoxia exposure activating HIF1 α in melanocytes, ~11% (51/452) of the hypoxia-responsive up-regulated genes were well-characterized HIF1 α targets, and the predicted canonical pathways identified by these genes included glycolysis, gluconeogenesis, axon guidance signaling, and HIF1 α signaling (Table S2). In addition to HIF1 α , the predicted transcriptional regulators included EPAS1/HIF2 α , MYC, VHL, and STAT4 (Figure 1D, Table S2). These transcription factors linked this up-regulated gene cohort of hypoxia-responsive genes in melanocytes to the anticipated HIF1 α /HIF2 α /ARNT pathway and its regulation by VHL, and also to pathways known to interact with HIF factors (Cancer Genome Atlas Network, 2015; Dang et al., 2008; Xu et al., 2005).

In contrast, the cellular functions of the 257 down-regulated hypoxia-responsive genes included multiple genes involved in pigmentation (*Mreg*, *Oca2*, *Rab27a* and *Slc24a5*), DNA replication (*Cdc6*, *Mcm2*, *Mcm3*, *Mcm4*, *Mcm5*, *Mcm6*, *Mcm7*, and *Rrm2*) and DNA repair (*Chaf1b*, *Exo1*, *Dna1*, *Lig1*, *Atrx*, *Rad54l*, *Fanca*, and *Pole*). Of the top predicted transcriptional regulators identified for the hypoxia down-regulated genes, which included TBX2, E2F family members, and the RB family of transcription factors (Figure 1D, Table S2), only RBL1 itself was also downregulated ~2-fold in this dataset. This highlights the potential for RBL1 to be an important factor in regulation of the down-regulated hypoxia expression profile. In total, these results suggest hypoxia triggers multiple melanocyte

cellular responses, characterized by activation of HIF1 α pathway genes and repression of Rb-E2F and TBX2 downstream pathway genes.

Previous studies found modest overlap between hypoxia gene expression datasets from different tissues, suggesting hypoxia causes cell-type-specific gene expression changes (Benita et al., 2009; Chi et al., 2006; Denko et al., 2003; Widmer et al., 2013). Direct comparison of eight hypoxia-annotated gene datasets from diverse tissues using Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) found that while these GSEA hypoxia-annotated datasets contained a total of 1184 genes that are differentially expressed in response to hypoxia, there was only a 2–28% average overlap between any two datasets. Similarly, the 709 melanocyte hypoxia-responsive gene set exhibited an average 28% overlap with the other eight hypoxia datasets (Table S3). Gene list comparisons for the eight GSEA hypoxia datasets and the hypoxia-responsive set (nine datasets total) revealed that no gene was consistently altered in all nine datasets, although a subset of genes was contained in at least seven of the nine datasets (*Bnip3*, *Adm*, *Bhlhe40*, *Bnip3l*, *Igfbp3*, *Mxi1*, *P4ha1*, *Slc2a3*, and *Vegf*). These results support a tissue-dependent hypoxia response, and suggest that tissue-specific cofactors and chromatin accessibility may direct many hypoxia-mediated gene expression changes.

HIF1 α -dependent genes in melanocytes

To assess the direct role of HIF1 α in regulating gene expression under hypoxia, melanocytes were subjected to siRNA-mediated HIF1 α knockdown (KD) followed by 24 hr hypoxia exposure. Under these conditions, multiple *Hif1 α* -directed siRNAs reduced HIF1 α protein and mRNA expression, as well as mRNA expression of the known HIF1 α target genes *Kdm3a* and *Gapdh* (Figure 1B, C). Transcriptome analysis using two independent *Hif1 α* -directed siRNAs identified 712 HIF1 α -dependent genes that were 1.5 fold differentially expressed in comparison to non-silencing controls in both siRNAs (Figure 1E, Table S4). Within this HIF1 α -dependent gene set, 362 genes were down-regulated by siHIF1 α KD, thus representing a HIF1 α -activated gene profile. These genes exhibited enrichment of both HIF1 α -responsive canonical pathways and predicted upstream regulators that were similar to those observed under hypoxia exposure (Table S5). A similar number of genes (350) was significantly up-regulated upon siHIF1 α KD, suggesting these genes are repressed by HIF1 α under hypoxia. Canonical pathways enriched for this HIF1 α -dependent repressed gene cohort included cell cycle control of cell replication, tRNA charging, and DNA damage response. In addition, these 350 HIF1 α -dependent repressed targets were predicted to have E2F4, ATF4, MYC, E2F6, and TBX2 as upstream regulators (Figure 1E, Table S5). Of these predicted transcriptional regulators, expression of ATF4 itself was increased 1.62-fold under HIF1 α KD (Table S4) and 17 genes were identified as ATF4 known target genes. Interestingly, 9/17 ATF4 target genes (*Slc7a5*, *Asns*, *Mthfd2*, *Psat1*, *Slc1a5*, *Shmt2*, *Trib3*, *Dit3* and *Cth*) were associated with MTORC1 signaling. MTORC1 pathways regulate both cell growth and metabolism, and MTORC1 pathway regulation is known to be repressed by hypoxia (Wouters and Koritzinsky, 2008). In summary, these HIF1 α -dependent genes define both activated and repressed gene targets, and also reveal that consistent pathways are regulated by hypoxia and HIF1 α in melanocytes.

HIF1 α -dependent/hypoxia-responsive melanocyte gene signature

To identify the subset of hypoxia-responsive genes in melanocytes that are under HIF1 α regulation, both the HIF1 α -dependent and hypoxia-responsive datasets were queried for genes consistently altered under both hypoxia and siHIF1 α KD conditions (Table S6). This analysis identified 251 genes that were comprised of two opposing cohorts: 185 genes that are both up-regulated under hypoxia and down-regulated in response to siHIF1 α KD, and have HIF1 α pathway members as the top predicted upstream regulators; and 66 genes that are both down-regulated under hypoxia and up-regulated in response to siHIF1 α KD. Contained within this melanocyte 251 gene signature were diverse cellular processes consistent with a hypoxia- and HIF1 α -mediated response (Table S7). Upregulated genes were enriched for known HIF1 α -activated hypoxia targets, many of which correspond to the enriched biological processes of glycolysis, MTORC1 signaling, epithelial to mesenchymal transition (EMT), heme metabolism, and angiogenesis. Processes enriched within the down-regulated gene signature included the G2M checkpoint of the cell cycle, MTORC1 signaling, unfolded protein response, xenobiotic metabolism and TGF β signaling. In total, this melanocyte-defined response to HIF1 α /hypoxia signaling captures a cell state switch from proliferative growth to a pro-migratory, slower cycling cell state that is known to be associated with hypoxia (O'Connell et al., 2013; Widmer et al., 2013).

HIF1 α genome-wide binding

To identify specific genomic regions defining HIF1 α binding in melanocytes under hypoxic conditions and facilitate the assessment of HIF1 α direct targets, Chromatin Immunoprecipitation (ChIP) was performed in melanocytes that had been subjected to 24 hr hypoxia conditions. Enrichment of HIF1 α binding at control loci was confirmed by qPCR, which showed HIF1 α binding at the proximal promoters of the known HIF1 α target genes *Kdm6b/Imjd3* and *Kdm3a*, both of which are involved in chromatin histone modifications (Figure 2A). Subsequent next-generation sequencing of two HIF1 α -ChIP bio-replicate samples (ChIP-Seq) identified 1773 HIF1 α chromatin binding regions/peaks genome-wide (Figure 2B, Table S8).

Assessment for the enrichment of known HIF1 α consensus binding within these 1773 HIF1 α -ChIP peak summit regions found enrichment of the motif ACGTG(A/C), which encompasses the HIF1 α :ARNT binding motif (A/G)CGTG (E-value = 1.2 e-18). The positions of the genome-wide HIF1 α -ChIP peaks were also assessed relative to annotated RefSeq genes. Of the 1773 HIF1 α binding peaks, 987 (56%) resided either within gene annotations or in the flanking 5kb upstream and downstream regions of RefSeq gene annotations, while 44% resided in distal intergenic regions (Figure 2C). Given the large number of HIF1 α binding regions distal to gene annotations, regions in close proximity to TSS were selected. A total of 537 HIF1 α -ChIP peaks located within $\pm$ 5kb of TSSs of individual genes were identified. These TSS peaks correlated with 591 Ensembl annotated genes (Figure 2B, Table S9). Pathway analysis for these 591 HIF1 α -ChIP-associated genes identified HIF1 α -regulated networks, as well as pathways reflective of a metabolic shift towards glycolysis (Figure 2D). These HIF1 α peaks and associated genes represent the first comprehensive inventory of putative HIF1 α binding locations associated with transcriptional targets in melanocytes under hypoxia.

81 HIF1 α direct target genes in melanocytes

Given that hypoxia can regulate HIF1 α -dependent as well as HIF1 α -independent pathways, and also that HIF1 α can have indirect, downstream effects, we sought to identify genes in the melanocyte-specific datasets that were putative direct targets for HIF1 α . HIF1 α direct targets were defined by their presence in both the 591 HIF1 α ChIP-associated gene dataset as well as the hypoxia-responsive and/or the HIF1 α -dependent gene sets. These criteria identified 81 HIF1 α direct targets: 30 genes changed only under hypoxia conditions, 15 genes altered only by siHIF1 α KD, and 36 genes consistently altered under both HIF1 α up-regulation by hypoxia and siHIF1 α KD (Figure 3A, Table S10). Most of these 81 HIF1 α direct targets (78%; 63/81) were activated by HIF1 α /hypoxia signaling, however 18/81 (22%) were consistent with repression by HIF1 α in melanocytes.

Of the 81 HIF1 α direct target genes, 51% (41/81) have been previously identified as known members of hypoxia-responsive pathways in other tissues (Table S10). These known HIF1 α targets were enriched for cellular functions including chromatin remodeling (*Kdm5b* and *Kdm6b/Imjd6*), mitochondrial proteins regulating autophagy (*Bnip3*, *Bnip3l*, and *Fam162a*), and a notable enrichment of proteins regulating glycolysis/gluconeogenesis (*Hk2*, *Gapdh*, *Pkm*, *Pgk1*, *Pfk1*, *Pdk1*, *Pgm2/PGM1*, *Pgam1*, *Pfkfb*, *Ldha*, and *Slc16a3*). The presence of these 41 known targets in the HIF1 α direct target gene set validates the HIF1 α -ChIP binding dataset, and also defines the specific gene family members utilized in melanocytes to regulate these known HIF1 α pathways. The remaining 49% (40/81) of the HIF1 α direct target genes were novel HIF1 α targets. Interestingly, cellular functions associated with the set of 40 novel HIF1 α direct target genes were also consistent with HIF1 α -regulated cellular mechanisms, including lipid and carbohydrate metabolism (*Unc13a*, *Aloxe3* and *Epm2a*), mitochondrial function (*Mpr154*), cell migration (*Tnfrsf9*), cell adhesion/repulsion (*Unc5a* and *Adgrl1*), and chromatin remodeling (*Suv420h1/KMT5B*) (Barderas et al., 2013; Gene Ontology Consortium, 2015; Yang et al., 2008).

One of the novel HIF1 α direct targets (*PRELID2*) and three previously known HIF1 α targets (*KDM3A*, *NAMPT*, and *SAP30*) were selected for secondary validation in 501mel melanoma cells under 48 hr siHIF1 α KD and 24 hr hypoxia conditions (Figure 3B). The mRNA expression of all 4 genes was significantly altered in response to siHIF1 α KD, consistent with these genes being differentially regulated by HIF1 α in both melanocytes and melanoma cells. Additional validation of HIF1 α protein binding at 21/40 of the novel genes was found in other ChIP datasets (Table S10, (Mimura et al., 2012; Mole et al., 2009; Salama et al., 2015)). Taken together, the identification of 40 novel HIF1 α direct target genes expands the repertoire of HIF1 α targets, justifying further investigation of HIF1 α in the regulation these genes both during melanocyte development and melanoma progression.

Enriched motifs localized at HIF1 α binding peak summits

The 81 HIF1 α direct target genes in melanocytes corresponded to 89 HIF1 α -associated ChIP-Seq peaks. These peaks were examined for overrepresented motifs at the HIF1 α -ChIP summit binding regions. For 63 of the 89 gene-associated peaks, the three most enriched motifs contained the well-known HIF1 α :ARNT consensus motif RCGTG (Figure 3C). However, similar to what has been observed in other tissues with different HIF1 α antibodies

(Mole et al., 2009; Tausendschön et al., 2015), not all HIF1 α target gene peaks contained an underlying RCGTG consensus motif. In addition, HIF1 α showed a broad enrichment pattern across an 80 bp window centered on the peak summit, rather than one central, discrete motif (Figure 3D). This pattern is similar to that described for E2F proteins, and suggests HIF1 α may associate with other DNA-protein complexes to regulate target gene expression (Bailey and Machanick, 2012).

Since the HIF1 α direct target binding profile suggested the presence of additional DNA-protein complexes, the analysis was expanded to include the full genome-spanning set of 1773 HIF1 α -ChIP peaks, to identify overrepresented, centrally located motifs near peak summits. Along with the broad, centrally located motif profile of the HIF1 α :ARNT consensus motif (P -value = 1.6 e-16), additional motif sequences with discrete region enrichment locations relative to the HIF1 α peak summit coordinates (Figure S1) were found. These profiles were as follows: (1) a single, centrally-located distribution profile for multiple motifs corresponding to members of the RFX family of proteins (P -values ranging from 1.1 e-46 to 1.2 e-27) (Figure S2); (2) motifs present on either side of the HIF1 α summit, associated with proteins NRF1 (P -value = 8.6 e-14), ZNF524 (P -value = 5.7 e-10), and GMBE2 (P -value = 1.6 e-7) in addition to the unassigned motif GGTTCTGA(A/T) (P -value = 3.5 e-21); and (3) two motifs distributed broadly across the area surrounding HIF1 α peak summits corresponding to XBP1 and KLF12 proteins (P -values = 5.5 e-7 and 5.5 e-7, respectively). Both the proximity of and distinct locations for these motifs relative to the peak summit suggest the potential for coordinated gene regulation between HIF1 α and these factors or family members, and possible complex or coordinated binding.

HIF1 α direct target genes correlate with Disease Free Status

Animal models have shown that HIF1 α is involved in melanoma tumor progression from the primary tumor to lymph node metastasis (Hanna et al., 2013). Therefore, the 81 HIF1 α direct target genes in melanocytes were examined for gene expression level differences in primary melanoma tumors that correlate with progression to metastatic disease. Clinical and gene expression data from the cutaneous melanoma provisional dataset of The Cancer Genome Atlas (TCGA) Research Network (<http://cancergenome.nih.gov/>, (Cancer Genome Atlas Network, 2015)) was queried for correlation of gene expression levels to the clinical metric Disease Free Status (DFS). DFS is a measure of tumor metastasis arising following resection of the primary tumor. As a control, the clinical parameters associated with this dataset were examined to confirm that worse prognosis correlates with increased tumor staging. Tumor staging is measured by standardized AJCC parameters, in which Stage I and Stage II describe localized tumor growth, and the transition from stage II to stage III is marked by the appearance of regional metastasis (Boland and Gershenwald, 2016). Similar to other clinical cohorts, the TCGA melanoma dataset demonstrated significant correlation of both DFS and Overall Survival (OS) with primary tumor progression from stage II to stage III (Figure S3).

First, the cohort of TCGA primary melanoma tumors was evaluated for correlation of DFS or OS with the expression profiles for the nine genes identified as differentially expressed in response to hypoxia in multiple tissues (*BNIP3*, *ADM*, *BHLHE40*, *BNIP3L*, *IGFBP3*,

MXII, *P4HA1*, *SLC2A3*, and *VEGF*). Logistic regression found no correlation between DFS or OS in primary tumors for either individual gene expression, or for expression of the panel of these nine genes together (data not shown).

Next, the 81 HIF1 α direct target genes in melanocytes were examined for correlation with clinical metrics in the TCGA primary melanoma tumors. Interestingly, expression levels for ten individual genes were significantly correlated with DFS in the primary tumors (Figure 4A–C). Logistic regression analysis for gene expression for the set of ten genes together also found that the combined profile significantly correlated with DFS in primary tumors (P -value <0.0013) and ROC analysis revealed an area under the curve (AUC) = 0.826 (P <0.0001, Figure 4D), consistent with the expression changes of these ten HIF1 α direct target genes discriminating for the time course of melanoma disease progression.

The set of ten DFS-associated, HIF1 α direct targets included three previously characterized HIF1 α target genes (*PKM*, *GAPDH*, and *ZNF292*) and seven novel genes (*AGTRAP*, *DARS*, *DTWD1*, *GPC6*, *PPAT*, *RLF*, and *SEH1L*). As both *DTWD1* and *SEH1L* loci had not been previously associated with HIF1 α -ChIP binding in other tissues, secondary validation of HIF1 α binding was performed in an additional melanocyte HIF1 α -ChIP biological replicate by qPCR. This confirmed significant enrichment of HIF1 α binding at the HIF1 α ChIP-Seq peak summit locations associated with *DTWD1* and *SEH1L* (Figure 5A).

A subset of the novel DFS-associated genes was assessed for HIF1 α -dependent expression changes in 501mel melanoma cells with siRNA-mediated HIF1 α KD (Figure 5B,C). Consistent with what was observed in melanocytes with HIF1 α KD, lower expression levels were observed for *DARS*, *RLF*, and *ZNF292* in 501mel cells with HIF1 α KD. However, out of the three genes tested that had higher expression upon siHIF1 α KD in melanocytes (*AGTRAP*, *DTWD1*, and *PPAT*), only *AGTRAP* exhibited elevated levels upon siHIF1 α KD in melanoma cells, consistent with HIF1 α repression. Conversely, *DTWD1* and *PPAT* were downregulated by siHIF1 α KD, thus responding as if they were activated targets in 501mel cells. Therefore, all novel targets tested were HIF1 α -dependent in both melanocytes and melanoma cells, with *AGTRAP* consistently demonstrating repression by HIF1 α signaling.

Hierarchical clustering analysis was performed for the ten DFS-associated, HIF1 α direct target genes across the entire 88 primary tumor dataset (Figure 6), to allow comparison of the expression of these ten genes within individual tumors. In addition, the distribution of the 88 tumors within the expression groups that were linked to shorter or longer DFS metrics for each of the 10 DFS-associated, HIF1 α direct target genes (shown in Figure 4) was determined by tallying the number of times each tumor was present within the high or low 25% expression groups for each gene (Figure 6, visualized by blue and red bars on right). Strikingly, tumor clustering for gene expression aligned well with the frequency scores correlated with shorter or longer DFS metrics. A group of tumors were frequently found within the DFS profiles corresponding to better prognosis (Figure 6, blue bars at right), and this tumor group was defined by consistent, lower expression levels in the seven HIF1 α target genes that showed lower expression correlated with longer DFS/better prognosis

(Figure 6, upper portion). Conversely, higher expression occurred for the remaining three genes (PKM, GAPDH and AGTRAP) within this same tumor cluster group.

In contrast, tumors more frequently associated with poor prognosis/shorter DFS (Figure 6, red bars at right) exhibited lower expression of PKM, AGTRAP and GAPDH, and heterogeneous up-regulation of the other seven genes, with notable tumor-to-tumor variation. This gene expression heterogeneity among tumors suggests a shorter time of DFS does not require simultaneous upregulation of all seven genes. In summary, this subset of seven HIF1 α direct target genes revealed a gene expression profile that is associated with better prognostic features in primary melanoma tumors when these genes exhibit low expression levels.

Discussion

While genomic mutations in driver genes that promote MAPK signaling pathways are fundamental to melanoma initiation, it is also clear that changes in the tumor microenvironment trigger dynamic changes in tumor gene expression, impacting metastasis progression and disease prognosis. As HIF1 α stabilization and activation occur in response to lowered oxygen concentration, HIF1 α is positioned to be both a key cellular sensor of tumor oxygen levels and a regulatory gatekeeper of metastatic cell state. The tissue-specific nature of HIF1 α genomic binding and target regulation requires a tissue-focused approach. However, melanoma tumors are inherently diverse and heterogeneous cell populations, therefore addressing HIF1 α and hypoxia responses in tumor tissue imposed technical challenges. Given that murine melanoma tumor animal models have found that hypoxia and HIF1 α play a key role in melanoma tumor progression (Cheli et al., 2012; Hanna et al., 2013), we used murine immortalized melanocyte cells to understand hypoxia- and HIF1 α -directed gene regulation occurring in melanocytes and to identify corresponding HIF1 α targets correlated with worse outcomes for melanoma disease progression. Our identification of HIF1 α direct targets using this mouse model system found that HIF1 α - and hypoxia-responsive pathways regulating melanoma tumor progression are conserved across species. Since new biomarkers defining cell states that predict clinical benefit are needed (Merlino et al., 2016), our analysis reinforces the utility of murine animal models in the identification of biomarkers at key stages of melanoma progression that are correlated with disease progression in humans.

Definitive classification of transcription factor direct and indirect targets requires correct assignment of regulatory loci to individual genes. Our conservative approach classified ~11% of the siHIF1 α -responsive genes as direct targets based on the location of HIF1 α binding in close proximity to TSSs, consistent with what has been observed in other tissues (Mimura et al., 2012; Mole et al., 2009; Salama et al., 2015). This may be an underestimate of HIF1 α direct targets, given that over 50% of the HIF1 α ChIP binding regions identified reside outside of gene structures and have the potential to regulate expression through long-range enhancers. While our analysis focused on HIF1 α direct target gene regulation and function, it also provided a foundation for understanding the complexity of hypoxic cell response through HIF1 α -independent and HIF1 α -indirect mechanisms. Prominent examples of both include RBL1 and ATF4, the predicted transcriptional regulators of hypoxia-

downregulated and siHIF1 α KD-upregulated gene subsets, respectively. Both RBL1 and ATF4 have significantly altered expression levels and are predicted to be upstream regulators of multiple genes with altered expression, however HIF1 α binding was not located near either gene, suggesting neither are under HIF1 α direct regulation. Our study identified multiple chromatin modifiers, DNA methylases and transcription factors that are direct targets of HIF1 α , and would themselves cause highly complex cellular responses to hypoxia that are independent and/or indirectly regulated by HIF1 α . Dissecting these downstream responses will require future studies.

Along with discovering both HIF1 α direct and indirect targets utilized within the melanocyte lineage, this study also revealed general trends for HIF1 α chromatin binding that are broadly applicable to HIF1 α binding across diverse tissue datasets. The melanocyte HIF1 α -ChIP data reveal cis-regulatory binding both with and without RCGTG-containing motifs, similar to what has been found in other tissues (Mole et al., 2009; Tausendschön et al., 2015). Even within the subset of melanocyte HIF1 α binding regions with RCGTG motifs, the consensus motif is distributed broadly across the HIF1 α -ChIP peak rather than at a single peak summit. Overall, these data suggest HIF1 α recruitment to DNA is not solely dependent upon on the RCGTG motif, and also suggest possible recruitment by factors other than the well-characterized cofactor ARNT. Consistent with this hypothesis, multiple motifs beyond RCGTG are enriched at HIF1 α binding loci, and these motifs show specific, defined locations relative to HIF1 α peak summits. One motif enriched at HIF1 α -ChIP peaks is for the transcription factor XBP1, which is of interest because HIF1 α -XBP1 complexes have been shown to occur at defined loci in breast cancer cells, where they drive gene expression leading to poor prognosis in triple negative breast cancer (Chen et al., 2014). Overall, these data highlight the need for future studies to determine more broadly how HIF1 α is recruited to gene regulatory loci, what the tissue-specific DNA binding components are that recruit HIF1 α to regulatory loci, and more specifically if XBP1-HIF1 α interactions also regulate gene expression correlated with prognostic outcomes in melanoma.

These studies present an unbiased, genome-wide approach to characterize the melanocyte lineage-specific response to hypoxia and HIF1 α activation, leading to the identification of 40 novel HIF1 α direct target genes. This is crucial information for deciphering HIF1 α 's role in melanoma progression, and again highlights that gene expression changes under hypoxia are cell specific, and thus generalizations cannot be made across cell types. Noteworthy among the novel targets is *AGTRAP*, a well-characterized inhibitor of angiotensin II type 1 receptor (AT1R, (Castrop, 2015; Cui et al., 2000; Gordan et al., 2007; Koshiji et al., 2004)). *AGTRAP* (along with *PKM* and *GAPDH*) displays higher gene expression in primary melanoma tumors with a better prognosis/longer DFS time, and overall the results in both melanocyte and melanoma cells suggest *AGTRAP* is a HIF1 α -repressed target. AT1R activation has been shown to promote vascularization in a mouse melanoma model (Egami et al., 2003), therefore, HIF1 α -mediated repression of *AGTRAP* would facilitate AT1R upregulation and is consistent with promotion of tumor vascularization. Consistent with upregulation of the AT1R signaling pathway occurring in melanocytes under hypoxic conditions, the AT1R downstream signaling targets *Cp* and *Amox1* are both up-regulated in the hypoxia-responsive/HIF1 α -dependent dataset.

In combination with the activation of signals to promote vascularization, a cell's ability to acquire invasive properties that promote migration away from the primary tumor is also a critical component of melanoma metastasis. Hypoxia-responsive melanocyte genes include 45 genes with GO annotation corresponding to cell motility. Within the smaller subset of 251 hypoxia-responsive and HIF1 α -dependent melanocyte genes, there were thirteen EMT-associated genes (*Cxcl12*, *Edil3*, *Fmod*, *Igf1bp3*, *Itga2*, *Loxl2*, *Plod1*, *Plod2*, *Spp*, *Postn*, *Serpine2*, *Spp1* and *Vegfa*), of which only two genes exhibit HIF1 α binding at the TSS (*Plod1* and *Plod2*). While this suggests that HIF1 α activation by hypoxia is a key regulator of multiple components of invasive cell state-associated gene signatures, it also indicates this complex regulation occurs through both HIF1 α direct and indirect mechanisms.

In addition to identifying a gene profile reflecting a HIF1 α -directed invasive response, this study discovered 10 HIF1 α direct target genes whose expression correlates with primary melanoma tumor progression to metastasis. Interestingly, this set of 10 genes spans multiple cellular functions, which may suggest this gene profile defines a genomic cell state rather than each gene in this profile having the individual capacity to drive invasiveness. These varied cell functions include the previously noted vascularization (*AGTRAP*) and invasion (*GPC6*), in addition to transcriptional regulation (*RLF* and *ZNF292*), cellular proliferation/cell division (*DTWD1* and *SEH1L*) and metabolism (*GAPDH*, *PKM*, *PPAT*, and *DARS*). Interestingly, RLF and ZNF292 are relatively uncharacterized proteins that demonstrate notable protein identity to each other, both within their zinc finger domains and uncharacterized C-terminal regions, suggesting related protein function and warranting further study of their downstream effects in light of their direct regulation by HIF1 α . Importantly, this DFS-associated gene cohort presents a novel diagnostic gene expression metric tightly linked to invasive tumor properties *in vivo*, placing HIF1 α in the position of regulatory gatekeeper of metastatic state in response to hypoxia.

In conclusion, this analysis defines the melanocyte lineage-specific HIF1 α response to hypoxia, identifies a unique HIF1 α -dependent/hypoxia-responsive gene expression signature as well as 81 HIF1 α direct targets utilized in melanocytes, and reveals 10 DFS-associated HIF1 α target genes which comprise a novel expression cohort profile linked with disease progression. These results also reveal how HIF1 α , as a sensor of cellular microenvironment, is uniquely positioned to coordinately regulate multiple cellular functions that correlate with the timing of primary to metastatic disease progression. Given the frequency with which acquired resistance is observed under current combined targeted and immune system-based therapeutic approaches in melanoma, additional targets and drug combinations are needed. The key regulatory position in which HIF1 α resides makes both HIF1 α and its downstream targets attractive molecules to screen for future novel therapeutic interventions.

Methods

Cell growth conditions

Analysis of hypoxia phenotype responses (migration, HIF1 α protein levels, known target expression changes), transcriptome analysis experiments under hypoxia and siHif1 α conditions, and HIF1 α genome-wide ChIP binding experiments used the immortalized mouse melanocyte cell line melan-Ink4a-Arf^{-/-}, obtained from Dr. Dorothy Bennett

(Sviderskaya et al., 2002). Cells were grown in RPMI media containing 200 pM Cholera Toxin and 200 nM 12-O-Tetradecanoylphorbol 13-acetate. Normoxic conditions were maintained in an ambient oxygen-supplemented incubator with 10% CO₂ at 37°C, providing ~21% oxygen. Hypoxia-treated cells were grown at 37°C in a hypoxic chamber supplied with 1% O₂, 5% CO₂, 94% N₂ for 24 hrs. 501mel cells, obtained from Dr. Yarden Samuels, were grown in RPMI with 10% serum and 2 mM glutamine.

Migration assays were performed using 24 well Biocoat 8 µM inserts (Fisher Scientific). Serum-containing media was used as a chemo-attractant below inserts. A total of 2.5×10^5 cells were plated in inserts using serum-free media and grown for 24 hrs. Three images for each of two bio-replicates/condition were counted.

HIF1α KD transfections in both human melanoma and mouse melanocyte cells were performed in triplicate using two, species-specific, independent siHIF1α double strand duplex RNAs and a non-silencing control siRNA NC-1 according to the Lipofectamine RNAimax protocol (Invitrogen) using 25pmol of oligo/6 well dish. HIF1α dsRNA duplex oligonucleotides (HIF1α Dsi RNA duplex oligos) were as follows: for murine *Hif1α*, si6 - MMC.RNAI.N0104431.12.6, (GGACGAUGAACAUCAAGUCAGCAAC) and si1 - MMC.RNAI.N0104431.12.1 (AGACAAUAGCUUCGCAGAAUGCUCU); for Human *HIF1α*, si9- HSC.RNAi.N001530.12.9 (GCACUCAUAUCAAGAAGUUGCAUUA), si5- HSC.RNAi.N001530.12.5 (ACCAUAUAGAGAUACACAAAGUCGG) and si4 - HSC.RNAi.N001530.12.4 (GAUGGAAGCACUAGACAAAGUUCAC) (Integrated DNA technologies). Transfections were grown under normoxic conditions for an initial 24 hrs, and then transferred to the hypoxic chamber for 24 hrs, thus providing 24 hr hypoxia/48 hr HIF1α KD conditions.

Transcriptome analysis and differential gene expression

RNA was isolated as previously described (Fufa et al., 2015). All experimental conditions were performed in 3X bioreplicates. Transcriptome analysis was performed using GeneChip® Mouse Gene 2.0 ST Arrays (Affymetrix). Gene lists with FDR of 0.05 and +/- 1.5-fold differential expression and hierarchical clustering analyses were obtained using Partek (Partek Incorporated). Analyses of differentially expressed genes, pathway enrichment, and upstream regulatory genes were obtained from Ingenuity Pathway Analysis Software (Qiagen). Quantitative changes in gene expression were assessed using StepOne (ThermoFisher). Statistical significance was calculated by t-tests or ANOVA with Bonferroni correction using GraphPad Prism. For each condition, cDNA was made using a high capacity cDNA reverse transcriptase kit (Applied Biosystems). Gene expression for each bioreplicate was tested in triplicate using the relative standard curve method. TAQMAN assays used were: (mouse) *Hif1α*, Mm00468869_m1; *Kdm3a*, Mm01182127_m1; *Gapdh*, 4352339E; *ACTB*, Mm00607939_s1; (human) *AGTRAP*, Hs01564425_m1; *DTWD1*, Hs00737889_m1; *ZNF292*, Hs00419514_m1; *RLF*, Hs00232629_m1; *PPAT*, Hs00601264_m1; *DARS*, Hs00154683_m1; and *GPC6*, Hs00170677_m1. Expression for *HIF1α*, *KDM3A*, *NAMPT*, *SAP30*, and *PRELID2* was assayed using SYBR® Select Master Mix (Thermo Fisher Scientific) with these primer sets: *HIF1α* (CCTGATGCTTTAACTTTGCT, AGTTTCTGTGTCGTTGCTG); *KDM3A*

(CCTGCAATAACGTACAAACC, TTGTTGATCTCCCAGAAGC); *NAMPT* (CCGACTCCTACAAGGTTACTC, TTTCACGGCATTCAAAGTAGG); *SAP30* (AGCTTCAGCAAGAGGATCC, GTAAAGATGCCTTGCGCTC); and *PRELID2* (GCTTTCTCCGAAAGTACCC, TCACCAGAGTGCTTAAGGA).

Gene Set Enrichment Analysis

The following eight hypoxia gene datasets that are annotated in GSEA (<http://software.broadinstitute.org/gsea/index.jsp>) were compared to the set of 709 melanocyte hypoxia-responsive genes: Subventricular zone (SVZ), QI_HYPOXIA; Astrocyte, MENSE_HYPOXIA_UP; Kidney, JIANG_HYPOXIA_NORMAL; Renal Carcinoma, JIANG, _HYPOXIA_CANCER; Neuroblastoma, FARDIN_HYPOXIA_11; Endothelial, MANALO_HYPOXIA_DN pooled with MANALO_HYPOXIA_UP; Winter review, WINTER_HYPOXIA_METAGENE; and Harris review, HARRIS_HYPOXIA. Pathway enrichment analysis of the HIF1 α -dependent/hypoxia-responsive 251 melanocyte gene signature was performed using GSEA hallmark gene sets (Subramanian et al., 2005), with an FDR q-value < 0.05.

Chromatin Immunoprecipitation and next generation sequencing

Approximately 2×10^7 cells were harvested, crosslinked at room temperature with fresh 1% formaldehyde for 9 minutes, and quenched with the addition of glycine to a final concentration of 125 mM for at least 15 minutes. Cells were then lysed on ice using 1 ml low-salt ChIP buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5% NP-40, 1.0% Triton X-100). Sonications were performed using Qsonica Q800R (Qsonica) with 70% and 10 sec on, 20 sec off cycles for 15 min to yield fragments of 500–100 bp. For ChIP-Seq analysis, immunoprecipitations were performed as previously described (Fufa et al., 2015) using 10 μ g/IP of HIF1 α antibody #AF1935 (R and D Systems) with normal goat IgG AB-108-c (negative control) IP performed in tandem. Illumina ChIP-Seq libraries were prepared as follows: ChIP-isolated DNA was size selected by 2% Agarose Nusieve gel, and a 200–500bp region was excised and purified using Qiaquick gel extraction kit. Adapter linker attachment was performed by PCR using 30ng of DNA and 2X Phusion master mix and primers, using conditions of 30 sec, 98°C, followed by 10 sec, 98°C, 30 sec, 65°C and 30 sec, 72°C with cycle number empirically determined, followed by a final 5 min, 72°C extension step. Adapter ligated PCR reactions were purified using Agencourt Ampure XP PCR Purification Beads per manufacturer's protocol. Statistical significance was calculated by t-tests using GraphPad Prism.

Two bioreplicates and input control DNA were sequenced for over 90 million unique Illumina reads. Quality control assessment was performed using FASTQC and GALAXY (Afgan et al., 2016). Reads were mapped to sequence build NCBI37/mm9 using BWA, and peak calling was performed using MACS1.4, using a cutoff of $P < 1.0 \times 10^{-5}$. The Genomic Regions Enrichment of Annotations Tools (GREAT) (McLean et al., 2010) was used to identify the 537 HIF1 α -ChIP peaks located within $\pm$ 5kb of TSSs, and the 591 genes correlated with these peaks. Enrichment of known HIF1 α consensus binding within the 1773 HIF1 α -ChIP peak summit regions was assessed using DREME (Machanick and Bailey, 2011). Assignment of peaks to genes and gene region-related structures were made

using summit peak coordinates. Overrepresented motifs present within HIF1 α peak summits using 400bp windows were identified using the MEME-ChIP Suite of tools (Machanick and Bailey, 2011).

ChIP validation qPCR

HIF1 α binding enrichment to genomic regions was verified by qPCR using SYBR® Select Master Mix and three technical replicates for each bioreplicate analyzed. Relative fold-change was determined by normalizing to percent input using the ddCt method. ChIP enrichment of genomic loci was assayed using the following primers: Negative control Chr6 (CCGAGGACCGCACCATTA, AAATAACGTCCACTAACATGAATAGCA), *Jmjd3* (ACACACGAGCAAGGAACGA, AAAACTCGCTCGGTCGTG), *Kdm3a* (TTCAGGCGTACGCAGTTAGA, TCAAAATGGCGGACCTAGAC), *Dtwd1* (AGGAAGCTGACGGTTCCTTT, GCATGCTCAGAGGGAGAATC), *Agtrap* (CGAACTCGGGAACAACTTC, GACGCCGTCTCCTAGCAA), and *Seh1l* (GTTTTTCGGAGGCGCACAC, AGCAGACGAAGCTGGGAGA).

Correlation with DFS and OS

Gene expression and clinical data for cutaneous melanoma primary tumors, a dataset generated by TCGA, were obtained from www.cbioportal.org. Tumors (88 for DFS and 103 for OS analysis) were first ranked using the expression of each hypoxia/HIF1 α target gene being queried. The top 25% of tumors (highest gene expressing tumors) and the bottom 25% of tumors (lowest gene expressing tumors) were plotted as a survival curve for DFS or OS. Significance between expression-defined, gene-specific cohorts was then assessed with respect to clinical outcome. Logistic regression analysis utilized expression data for the primary tumor expression data set and corresponding DFS and OS clinical data. FDR was calculated using Benjamini and Hochberg procedure.

Western blots

Whole cell lysates were harvested in Novex® Tris-Glycine SDS Sample Buffer (ThermoFisher Scientific) then sonicated and incubated at 95°C for 5 mins. Samples were separated on a 10% Tris-Glycine gel then transferred onto PVDF membrane via iBlot (ThermoFisher Scientific). Membranes were blocked for 1 hr in 5% non-fat milk, TBST followed by overnight incubation at 4°C in primary HIF1 α antibody (AF1935, R&D Systems) or monoclonal α -Tubulin antibody (SC-53646, Santa Cruz Biotechnology). Membranes were then washed in TBST and incubated with the appropriate secondary HRP-conjugated antibody (anti-goat for HIF1 α , anti-mouse for α -Tubulin; Jackson ImmunoResearch Laboratories). Protein band visualization was performed using Amersham ECL Prime Western Blotting Detection Reagent (RPN2232, GE Healthcare).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Hypoxia drives numerous gene expression changes that contribute to the progression of many types of cancer, including melanoma. The transcription factor HIF1 α is a critical regulator of the cellular hypoxia response, however the genes that respond to hypoxia and HIF1 α signaling are highly tissue-specific. We identified gene expression changes that occur under hypoxia and are directly regulated by HIF1 α in melanocytes. We found that expression levels for ten HIF1 α direct target genes significantly correlated with the clinical measurement of Disease Free Status in human primary melanoma tumors, thus discovering novel HIF1 α -regulated genes that could direct melanoma diagnosis and treatment.

Figure 1. Hypoxia and HIF1 α knockdown induce melanocyte target gene expression changes (A) The migratory ability of melan-Ink4a-Arf-/- cells is significantly increased by 24 hr hypoxia exposure (***, $P < 0.0001$). Representative images of migration wells for cells grown for 24 hr under normoxia (22% O₂) and hypoxia (1% O₂) are shown below the graph. (B) HIF1 α protein is stabilized by 24 hr hypoxia, and HIF1 α protein expression under hypoxia is eliminated by siHif1 α treatment. Molecular weights: HIF1 α =95 kDa, Tubulin = 50 kDa. (C) *Hif1 α* mRNA levels are unchanged under hypoxia, consistent with HIF1 α protein stabilization regulating signaling activity. *Hif1 α* mRNA levels are significantly reduced by multiple siRNAs (**, $P = 0.0015$). The mRNA expression levels of the known HIF1 α target genes *Kdm3a* and *Gapdh* are significantly increased by 24 hr hypoxia (*, $P < 0.05$), and are significantly downregulated with siHif1 α KD under hypoxia, returning to near-normoxic levels (*, $P < 0.05$). (D) 709 hypoxia-responsive genes were differentially expressed 1.5 fold under normoxia vs. hypoxia growth conditions. Ingenuity Pathway Analysis (IPA) predicted distinct upstream regulatory factors for upregulated and downregulated gene cohorts, listed at right. See Table S1 for the complete list of 709 hypoxia-responsive genes. (E) 712 HIF1 α -dependent genes were differentially expressed 1.5-fold in both siHif1 α KD RNAs as compared to non-silencing control. IPA predicted distinct upstream regulatory factors for upregulated and downregulated gene cohorts, listed at right. See Table S4 for the complete

list of 712 HIF1 α -dependent genes. Details of IPA analysis for predicted transcriptional regulators and downstream targets for the hypoxia-responsive genes and the HIF1 α -dependent genes are in Tables S2 and S5, respectively. Norm = normoxia; Hpx = hypoxia; Ctr = control si; si1 and si6 = independent HIF1 α -directed siRNA constructs.

Figure 2. HIF1α-ChIP in melanocyte cells under hypoxia

A) Validation of significant HIF1α binding enrichment at proximal promoters of the known HIF1α target genes *KDM6B* and *KDM3A* in melanocytes as compared to IgG negative controls and a negative control genomic region on Chr 6 (***, $P < 0.0001$). (B) Representative diagram showing that the HIF1α-ChIP bioreplicated peaks led to the identification of 591 genes with a HIF1α-ChIP peak located within ± 5 kb of their annotated TSSs. (C) Location of the 1773 replicated HIF1α-ChIP peaks with respect to RefSeq annotated gene structures. (D) Pathway enrichment analysis validates that the set of 591 genes with HIF1α-ChIP peaks located within ± 5 kb of TSSs are enriched for known HIF1α target genes. See Tables S8 and S9 for the complete lists of 1773 replicated HIF1α-ChIP peaks and 591 genes, respectively.

Figure 3. Identification of 81 HIF1 α direct target genes in melanocytes
A) Venn diagram illustrating the intersections of genes with HIF1 α -ChIP peaks (light orange), HIF1 α -dependent genes (purple), and hypoxia-responsive genes (red). (B) Quantitative gene expression for siHIF1 α KD in 501mel cells under 24 hr hypoxia for four of the 81 HIF1 α direct target genes verifies gene expression changes under siHIF1 α KD (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). HIF1 α is included as a positive control. (C) Centrimo motif analysis for the 89 peaks within +/- 5kb of TSSs for the 81 HIF1 α direct target genes finds that the top three enriched motifs (labeled 1, 2, and 3 in red, blue, and black, respectively) are consistent with the RCGTG HIF1 α :ARNT consensus. (D) Consensus motif profiles near HIF1 α -ChIP peak summits for the 89 peaks. Red, blue, and black line colors match the top 3 motifs shown in C. See Table S10 for the complete list of 81 HIF1 α direct target genes.

Figure 4. HIF1 α direct targets correlate with DFS in primary melanoma tumors

A–B) DFS plots (% Disease Free Status versus time in months) for primary melanoma tumor expression of the 10 HIF1 α direct target genes with significant P-value correlating gene expression with increased severity of DFS. A) *AGTRAP*, *PKM*, and *GAPDH* show reduced expression (25% lowest expression tumors, blue) correlating with poor prognosis/shorter DFS time. B) *DTWD1*, *SEH1L*, *ZNF292*, *DARS*, *GPC6*, *PPAT*, and *RLF* show increased expression (25% highest expression tumors, red) correlating with poor prognosis/shorter DFS time. C) Table of the 10 HIF1 α direct target genes with associated P-values and FDR values for individual correlation with DFS. D) ROC curve analysis for the set of DFS-associated HIF1 α direct target genes demonstrating the degree to which this set of 10 genes is predictive of DFS.

Figure 5. HIF1α direct target gene validation

A) Quantitative PCR verifies that HIF1α binding is significantly enriched at the proximal promoters of the novel HIF1α target loci *AGTRAP*, *DTWD1*, and *SEH1L* (***, $P < 0.0001$). B) Western blot shows that HIF1α is induced by hypoxia in 501mel melanoma cells, and this expression is reduced with multiple siHIF1α RNAs. N = normoxia; H = hypoxia; C = control siRNA. Molecular weights: HIF1α=95 kDa, Tubulin = 50 kDa. C) The DFS-associated, HIF1α direct target genes show significantly altered expression upon HIF1α KD in 501mel cells (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). No detectable expression was observed for *GPC6* in 501mel cells (data not shown). HIF1α and KDM3A were included as controls.

Figure 6. Hierarchical cluster analysis of primary melanoma tumors correlates with DFS prognosis

Hierarchical clustering of 88 primary tumors (rows) relative to expression levels of the ten DFS-associated, HIF1 α direct target genes (columns) is shown by the dendrograms and associated blue/gray/red blocks. Columns to the right of the cluster analysis show both a tally and a horizontal bar equal to the number of times each tumor was present in the highest or lowest 25% of tumors based upon rank gene expression of the 10 DFS-associated, HIF1 α direct target genes, as was used in the Figure 4 DFS plots. The horizontal blue bars

correspond to longer time of DFS/good prognosis, and the horizontal red bars correspond to shorter time of DFS/poor prognosis.

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