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(54) Title: INHIBITION OF A lncRNA FOR TREATMENT OF MELANOMA

(57) Abstract: The present application relates to the field of cancer, particularly the field of melanoma. It was found that a particular long non-coding RNA (lncRNA) is specifically upregulated in melanoma (but not other tumor) cells as compared to melanocytes. Inhibition of this lncRNA in melanoma cells leads to induction of apoptosis and is a novel therapeutic strategy in the treatment of melanoma.

Inhibition of a lncRNA for treatment of melanoma

Field of the invention

The present application relates to the field of cancer, particularly the field of melanoma. It was found that a particular long non-coding RNA (lncRNA) is specifically upregulated in melanoma (but not other tumor) cells as compared to melanocytes. Inhibition of this lncRNA in melanoma cells leads to induction of apoptosis and is a novel therapeutic strategy in the treatment of melanoma.

Background

Cutaneous malignant melanoma is the leading cause of skin cancer related deaths. Its incidence is on the increase worldwide faster than any other cancer, with 5-year survival rates for patients with distant metastatic disease being less than 20%. Improvement of clinical outcomes for this aggressive, chemo- and radio resistant, disease remains a major clinical challenge. Significant progress in our understanding of the etiologies and genetic underpinnings of melanoma has nevertheless been made. These advances have recently led to promising results in trials of targeted therapies for this disease. The Ras/Raf/MEK/ERK pathway has been identified as the main regulator of cell proliferation in melanoma, with ERK being hyperactivated in up to 90% of human melanomas. Activating NRAS mutations are a common route to activating this pathway; mutations affecting codon 61 being the most prevalent (NRASQ61K). BRAF, one of the three human RAF genes, is also frequently mutated in melanomas, with the most common mutation being a glutamic acid for valine substitution at position 600 (V600E). BRAFV600E stimulates constitutive ERK signaling, leading to melanocyte hyper-proliferation. Early clinical experience with the novel class I RAF-selective inhibitor, PLX4032, demonstrated an unprecedented 80% anti-tumor response rate among patients with BRAFV600E-positive melanomas; unfortunately, patients acquire drug resistance within a few months of an initial response and combination therapies with MEK inhibitors are currently being investigated.
p53 pathway inactivation, which mainly arises as a consequence of inactivating mutations or allelic loss of the p53 gene itself, is the most common molecular defect in human cancers. Intriguingly, the p53 locus is intact in over 95% of melanoma cases, raising questions as to the pathogenic relevance of p53 in the etiology of melanoma tumor formation. At the same time, there is an increasing body of evidence supporting a relevant role for p53 in melanoma development. Loss of p53 cooperates with melanocyte-specific overexpression of activated HRASV12G and BRAFV600E in promoting melanomagenesis in mice and oncogenic NRAS cooperates with p53 loss to generate melanomas in zebrafish. Cancers that retain expression of wild-type p53 often find alternative ways to subvert p53 function, through either deregulation of upstream modulators and/or inactivation of downstream

effectors. MDM2, which encodes an E3 ubiquitin ligase that control p53 levels and function19, is amplified in human melanomas but only in 3%–5% of documented cases. Recently, MDM4 upregulation has also been identified as a key determinant of impaired p53 function in human melanoma.

Other pathways that could become the targets of therapeutic interventions are the canonical Wnt signaling pathway and/or the transcriptional network regulated by the melanocyte-lineage specific transcription factor MITF. MITF induces gene expression patterns that prompt melanocytes to differentiate and initiate pigment production by activating genes important for melanin biosynthesis (such as *Mc1r*, *Tyr*, *Dct* and *Trp-1*) and melanosome formation (such as *Pmel*). Importantly, deregulation of MITF levels and/or of its transcriptional activity contributes to melanomagenesis. As such a rheostat model for MITF function was suggested in which higher expression of MITF is associated with proliferation and lower MITF levels with migration/invasion and senescence. Amplification/overexpression of *MITF*, found in 10-20% of metastatic melanomas, correlates with decreased 5-year survival rates. One of the key pro-oncogenic functions of MITF relates to its ability to promote melanoma survival by promoting expression of the anti-apoptotic gene BCL-2. Wnt/β-catenin signaling directly regulates the expression of MITF and constitutive activation of Wnt/β-catenin signaling increases the proliferation of melanoma cells, accompanied by MITF-dependent increases in clonogenic growth, implicating this pathway as a promoter of melanoma progression.

Because of its ability to acquire drug resistance, its chemoresistance and because melanoma is a highly dynamic and genetically heterogeneous tumor, novel treatment strategies and combination therapies are urgently needed. Several protein-coding therapeutic targets have indeed been identified for melanoma including components of the MAPkinase pathway such as BRAFV600E, MEK and a modifier of the p53 pathway, MDM4. However, the targeting of these molecules remains only applicable in a restricted number of cases (e.g. against tumors that carry the BRAFV600E mutation or overexpress MDM4 and harbour wild-type TP53).

It would be advantageous to find novel targets that are not restricted to a small subset of cases or dependent on the presence of a particular mutation. Moreover, it would be advantageous if these targets are specific to melanoma cells (and not present in melanocytes), so that their inhibition results in selective killing of melanoma cells.

Summary

As one of the most virulent human cancers, melanoma is capable of distant and lethal metastases when the primary tumor volume is as little as 1 mm3. Presently, there is a dearth of molecular markers that facilitate detecting the differences between benign and malignant melanocytic lesions and assist in predicting their biological behaviors.

Moreover, there is currently no effective long-term treatment for metastatic melanoma. Melanoma is driven by mutations (i.e. BRAFV600E, NRASQ61K) that activate mitogen-activated protein kinase (MAPK) signaling. Inactivation of MEK or ERK are very effective in killing melanoma cells, unfortunately many normal cells also rely on MAPK signaling for their growth and survival, making MEK and ERK-inhibitors very toxic in patients.

Targeting activated BRAFV600E leads to unprecedented, dramatic and rapid tumor regression that relapses after some months, with resistance arising from activating mutations in other factors that bypass the requirement for activated BRAF in MAPK signaling. An alternative strategy, that would bypass the genetic resistance arising from targeting specific components of the MAPK pathway and toxicity associated with MAPK inactivation in normal cells, would be to identify therapeutics that act on melanoma cells only (and not normal cells) and irrespective of how MAPK signaling is activated.

Here we describe a long non-coding RNA, transcribed from the LINC01212 aka RP11-460N16.1 gene, which is expressed in the vast majority of melanoma (and not in normal/non-transformed melanocytes or other tumor types) and that is essential for melanoma cell survival irrespective of how the MAPkinase is activated (BRAF or NRAS mutations or activation of MEK) and whether TP53 is wild-type or mutated.

Knock-down (KD) of the lncRNA induces apoptosis in melanoma cells independent of TP53 status (in contrast to e.g. therapeutic inhibition of MDM4, which is only successful with wild type TP53) and independent of how the MAPkinase pathway is activated.

Without being bound to a particular mechanism, it could be shown that LINC01212 KD leads to downregulation of key components of the canonical Wnt pathway, MITF (and some of its targets such as DCT, TYRP1) and MEK1 and MEK2, concomitant with activation of the p53/p63 signaling pathway and induction of expression of key pro-apoptotic/tumor suppressor p53/p63 targets such as BAX, APAF-1, PUMA, GADD45A or PERP.

This offers significant potential in treating melanoma, also those melanomas not characterized by e.g. the BRAF V600E mutation.

It is an object of the invention to provide inhibitors of functional expression of the LINC01212 gene. Such inhibitors can act at the DNA level, or at the RNA (i.e. gene product) level. As LINC01212 is a non-coding gene, there is no protein product for this gene.

According to a further aspect, the inhibitors of functional expression of LINC01212 are provided for use as a medicament. According to yet further aspects, the inhibitors of functional expression of LINC01212 are provided for use in treatment of cancer, in particular skin cancer (e.g. BCC, SCC). In still further embodiments, the inhibitors are provided for use in treatment of melanoma.

This is equivalent as saying that methods of treating melanoma in a subject in need thereof are provided, comprising administering an inhibitor of functional expression of LINC01212 to said subject.

The nature of the inhibitor is not vital to the invention, as long as it inhibits the functional expression of the LINC01212 gene. According to specific embodiments, the inhibitor is selected from a gapmer, a shRNA, a siRNA, a CRISPR, a TALEN, or a Zinc-finger nuclease.

According to alternative, but not exclusive, specific embodiments, the inhibitor selectively induces apoptosis in melanoma cells. This particularly implies that it induces apoptosis in melanoma cells, but not in normal (non-transformed) melanocytes. According to further specific embodiments, the inhibitor induces apoptosis independent of p53, BRAF, NRAS or MEK status, e.g., independent whether these proteins have particular mutations or not, or independent of their expression levels.

Even though inhibition of LINC01212 is sufficient to achieve a therapeutic effect, i.e. to achieve apoptosis in cancer cells, it is shown herein that a stronger, synergistic effect is achieved when both an inhibitor of LINC01212 and another chemotherapeutic are administered. This is particularly true for B-raf kinase inhibition.

According to a further aspect, methods are provided that may identify whether a tumor is suitable for treatment with an inhibitor of functional expression of LINC01212. These methods typically have the following steps:

- Determining whether expression of LINC01212 is increased in the tumor or a sample of tumor cells;
- Establishing whether the tumor is suitable for treatment, wherein increased expression is indicative of suitability for treatment.

The increase in expression is typically compared to a suitable control, e.g. a population of control cells, such as, but not limited to, skin cells or non-transformed melanocytes. Of note, if the control does not

express LINC01212, then the mere presence of LINC01212 RNA in the sample is equivalent to increased expression.
The methods thus may entail a first step of providing a sample of tumor cells. The determining step may occur purely in vitro, i.e. without a step interacting on the human or animal body.

According to particular embodiments, the tumor is melanoma.

According to specific embodiments, when it is established that the tumor is suitable for treatment, the methods may further comprise a step of administering an inhibitor of functional expression of LINC01212 to the subject in which the tumor is present. This is in order to treat the tumor.

Also provided herein are methods of diagnosing the presence of melanoma in a subject, comprising the steps of:

- Determining the levels of LINC01212 in a sample of said subject;
- Correlating the levels of LINC01212 in said sample with the presence of melanoma.

In such methods, the presence (or increased expression) of LINC01212 is indicative of the presence of melanoma in the subject from whom the sample is taken. Typically, these methods are performed in vitro, although in vivo methods are not necessarily excluded. Determining the levels of LINC01212 will typically be done by determining the levels of LINC01212 RNA in said sample.
The sample can be a tissue sample (e.g. a skin biopt), but as is shown herein, in melanoma patients, LINC01212 also circulates in the blood. Thus, it can also be detected in blood or serum, and the sample can be a blood sample or a serum sample.

The levels of LINC01212 RNA vary with different stages of the disease (Fig. 6). Accordingly, in methods that determine the presence of melanoma, a further step may be included that correlates the levels of LINC01212 to disease severity, disease stage (e.g. stage of melanoma), or disease progression.

Brief description of the Figures

Figure 1 shows an arrayCGH analysis of the long transcript of the LINC01212 gene, downstream of the MITF locus.
Figure 2: expression analysis of the LINC01212 gene in different cell lines with emphasis on melanoma cell lines and normal human epidermal melanocytes (NHEM).

Figure 3: Heat map of the long isoform of LINC01212 expression based on the analysis of RNA-seq data from >200 human melanoma samples from TCGA (SKCM01 non metastatic versus SKCM06 metastatic samples). The top panel shows that the actual transcript is longer than the UCSC annotated lncRNA-525 transcript. The bottom panel shows that the transcript can be detected in > than 85% of both metastatic and non-metastatic human melanoma samples.

Figure 4: heat map of LINC01212 expression in normal melanocytes, human melanoma cell lines and normal adult tissues (top panel). Relative LINC01212 expression (normalized with three different reference genes) in normal tissues, primary melanocytes and human melanoma cell lines (bottom panel).

Figure 5 shows LINC01212 expression in 38 normal human adult tissues, primary melanocytes and melanoma cell lines. Expression data demonstrate the melanoma-specific expression pattern of LINC01212.

Figure 6: Relative LINC01212 expression (normalized with three different reference genes) in normal human melanocytes (NHME) and in RGP (radial growth phase), VGP (vertical growth phase) (isolated by laser capture microdissection) and metastatic human melanoma samples.

Figure 7: A. Relative expression in melanoma cell lines and primary melanocytes shows increased expression of lncRNA525 in melanoma cells. B. High correlation between MITF and LINC01212 (indicated as lnc-MITF) copy number in melanoma cell lines. LINC01212 is co-amplified with MITF explaining, in part, increased LINC01212 expression in melanoma.C. Cellular location of LINC01212 transcript in SKMEL28 melanoma cells, visualized by means of RNA-FISH. LINC01212 trancripts are predominantly expressed in the nucleus although several transcripts are detected in the cytoplasm as well, suggesting that this lncRNA might have nuclear and cytoplasmic functions.

Figure 8: Copy number analysis of the MITF and LINC01212 containing genomic region in more than 200 human melanoma patients (TCGA data) indicates that the LINC01212 locus is present in all of the MITF-containing amplicons (no lines: present in two copies; red lines indicate the presence of more than 2 copies; blue lines indicate deletions, less than two copies).

Figure 9 shows LINC01212 promoter activity as assessed by measuring the H3K27 acetylation landscape. A readily detectable peak is present upstream of the LINC01212 gene indicating that the LINC01212 promoter is active/ON in all but one (MM001) melanoma lines analyzed.

Figure 10. Expression analysis of LINC01212 in the SK-MEL28 melanoma cell line transfected with different gapmers (scrambled or directed against the LINC01212 long transcript).

Figure 11. SK-MEL28 melanoma cell lines treated with the lncRNA inhibitors of Figure 10, showing cell death.

Figure 12. Gapmer inhibition in SK-MEL 28, BRAF V600E, P53 mutant. By measuring caspase 3/7 activity with a luciferase reporter, it could be confirmed that knockdown of the LINC01212-encoded lncRNA in SK-MEL28 melanoma cells resulted in a significant induction of apoptosis.

Figure 13. Gapmer inhibition in MM034 (BRAFV600E, P53 WT) (lower panel) and MM087 (BRAF WT, NRAS WT, P53 Mutant) (top panel) cells.

Figure 14. The SK-mel 28 human melanoma cell line was transfected with si-scramble (sc) or siRNA-targeting LINC01212 (and particularly its long transcript) and with gapmer-scramble (sc) or gapmer 3 and 11 (GAP3 and GAP11). Apoptosis was measured using a Annexin-5/PI assay by FACS 48h after transfection. Representative pictures of the cells are shown.

Figure 15. A stable SK-MEL28 human melanoma cell line engineered to express exogenous LINC01212 was transfected with gapmer-scramble (sc) or 11 (GAP11). Apoptosis was measured using a Annexin-5/PI assay by FACS 48h after transfection. Representative pictures of the cells are shown.

Figure 16: A BRAFV600E human melanoma cell line was transfected with gapmer-scramble (sc) or gapmer 3 (GAP3) and either treated with vehicle (DMSO) or a BRAFV600E-inhibitor. Apoptosis was measured by quantification of Annexin-5-positive cells by FACS 24h after exposure to the BRAFV600E-inhibitor (and 48h after transfection). Representative pictures of the cells are shown.

Figure 17. Full-genome transcriptome gene expression analysis of SKMEL28 cells treated with the different gapmers.

Figure 18. Pathways, significantly downregulated upon LINC01212 knockdown in MM034 short term melanoma cultures. Pathways are identified by means of whole genome mRNA expression profiling upon LINC01212 knockdown and gene set enrichment analysis (GSEA). Pathways are clustered based on pathway overlap. In MM034 cells, LINC01212 knockdown results in a decreased cell cycle activity as evidenced by multiple cell cycle gene sets.

Figure 19. Pathways, significantly upregulated (top) and downregulated (bottom) upon lncRNA525 knockdown in SKMEL28 melanoma cells. Pathways are identified by means of whole genome mRNA expression profiling upon LINC01212 knockdown and gene set enrichment analysis (GSEA). Pathways are clustered based on pathway overlap. In SKMEL28 cells, LINC01212 knockdown results in induction of apoptotic and TP53 pathway genes and repression of MAPK and WNT signaling.

Figure 20. Detection of LINC01212 transcript in serum of melanoma patients. Units are cDNA copies per 2.5 µl serum.

Detailed description

Definitions

The present invention will be described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims. Any reference signs in the claims shall not be construed as limiting the scope. The drawings described are only schematic and are non-limiting. In the drawings, the size of some of the elements may be exaggerated and not drawn on scale for illustrative purposes. Where the term "comprising" is used in the present description and claims, it does not exclude other elements or steps. Where an indefinite or definite article is used when referring to a singular noun e.g. "a" or "an", "the", this includes a plural of that noun unless something else is specifically stated.

Furthermore, the terms first, second, third and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

The following terms or definitions are provided solely to aid in the understanding of the invention. Unless specifically defined herein, all terms used herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Plainsview, New York (1989); and Ausubel et al., Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons, New York (1999), for definitions and terms of the art. The definitions provided herein should not be construed to have a scope less than understood by a person of ordinary skill in the art.

The term “LINC01212”, “long intergenic non-protein coding RNA 1212”, or “RP11-460N16.1” as used herein refers to the gene with accession number ENSG00000240405 in Ensembl, as well as the mRNA that is transcribed from the gene. It can also be identified with Gene ID: 101927152 or the human gene nomenclature identifier HGNC: 49644. As it is a non-protein coding gene, there is no protein product. In humans, the gene is located on the short arm of chromosome 3, from position 70,048,728 to 70,064,469. The gene has 2 annotated transcripts (or splice variants): LINC01212-001 (transcript ID ENST00000483525 in Ensembl) with a length of 2044 bp, and LINC01212-002 (transcript ID ENST00000488861 in Ensembl) with a length of 513 bp. Both transcripts are lincRNA (large intergenic non-coding RNAs), their respective consensus sequences are
CTGAAGTCGCTAGACATTTGAGGAACACATCCGGGGAAGAAGACACAGGTGGCTGGTCATGGAGAGCCCGCTG
GGGGAAGAGCACACAGACAGGCACCGGCAGGCCATTGACCAGCGGGACAAGGTGGGCTCAATGTCATCACAA
GGGTGCTTAAGAGGGAAAGAGGAAGCCATGAGGGTCAGAGTCAAAGGAAGACTTGAAGATACTACACTGCGG
ACTTTAAAGATGAAGGAAGGGGCGAAAACCAAGAATGTGGGAAGCCTCTAGAAGCTAGAGAAGGCAAGGAAA

CAAATTTTCCACTAGAGCCTCCAGAAGGAACACAGCCCTGCTGACCCACTGTAATGTCTGACCTCTAGATGTGTA
AGGGTAGTAAGACTGAAAGTCTCAGAAAGGCACTGTTAAATTCTTATTCCTCAACTATGCAACTCAAAACTGGGG
TCCTCAGCAGTGAGCGAGGGGTGAGAGAAGACACTGTATAAACATGGCACATCCTCCTGGAAAGTCAACTTTAC
TCAAAGCTTTAGAAACCCAGCTCAAACTAGCTGAAGCAGAAAAGACATTACTGCGCCTATAAGTTGAAACTTTGG
AGGGATAAATTTCAGAGGTGTGGCTAGATCCAACGGCTCTCTGGAAAACTCTGTGAAAAAAATGCTTCCTCAAGT
CCAGAAACCAGAAGCTCAGGAAATTGTTGCTTGGTTCCTCTTGTTAGAGGCAGAGATTATTCAACGACCTACAGG
GTAGCGTTTGAACATTGTTACCAGGAATCTTTACTTTGCCATCTTCCAAGTCTGTTCTCCTCAGTATTGGTTTCATT
TACAAGCAGGCCATCTCTGCCCTCATGGCAATGATGGTCTTTACTCCAGGCTTAAGACCCTTACTATCTACTATTTC
CAAAGCGGAGAGAGAACTTCCCACACCTAGTGACCTGTGACACAGGATTTTATCCTTCGTACAGAGGGAATTCA
GTTGGCTAACATAATCTGCCTTCCAATGGAGTAAGAATGTCTGGACTCTTTCCTTCACCTACCCCCAAGACATGGA
GGCGTCTAAAAGATAAATAAAACTTGGCAACTGACCGAAGGAGGAAGAGGGGATTTCAGGCAAAATCAACGCT
GTTCACTACGAGGAGACTTCAGAAAGGTTGCCTGCTTCTGGGGAGCATAGTCCCTGATTCCTCAAGACATACGTT
TATTCTTTTCTTCAATGTCTTTGCCTGCAGTCAAAACAAAACCATTACCTTTAGCCAAGTTCACACATTTCAGCCAA
ATCCATATGCATCGGATCAGTTCTGTAGGTTATGGGTGAGCATGAACATATAAAAGAGGCACCTGCCTGTGCCTA
TCTACTCCATGGAATTTCAAAAGGGGCCACTTATGGAGAATGTCTTTAGGGACAGAACCAACCACCCTGTCTTTC
CTCCAACTCTCAAAGTAACTTCTGGCTTTAATCCTCAAGTGTCTATGCTGGAGTTTAAGAAAAATGTTTTTCATAG
AATTCATGTGTATGATATTGCATGAGTTGTCCATCTTTGTATATATCTCAAGACTTGTGGTGTTAGTTAAAGATTCA
GAGCTCTGTGTCCTGAACACAGAGTAATACCAGCATTACTAAGGATGATCGTGGGATTTTAAAATTCCTCCCTTA
GATAGATCTTACGAACTTATGTTACCAATCAACATAAGTTAAGACAAAAAGAGCAAATTTAGATGTAAAACCATC
TTGGGGCCAGGTGCTGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGCGGGCGGATCACTAGGTC
AGGAGATCGAGACCATCTTGGCCAACACGGTGAAACCCTGTCTCTACTAAAAATACAAAAAAATTAGCCGGGCA
TGGTGGCAGGCGCCTGTAGTCCCAGCTACTAGGGAGGCTGAGGCAGGAGAATGGTGTGAACCCTGGAAGCGG
AGCTTGCAGTGAGCCAAGATCATGCCACTGCACTGCAGCTTGGGCGACAGAGCGAGACTCCGTCTCAAAAAAAA
CCAAACCAACAAACAAACAAACAAAAAAAA (SEQ ID NO: 1)

and

GAGGAAGGCGGGTCCCTGGCTCGGCTCTACCCCCATGGATCTAGGTGGGCTCAATGTCATCACAAGGGTGCTTA
AGAGGGAAAGAGGAAGCCATGAGGGTCAGAGTCAAAGGAAGACTTGAAGATACTACACTGCGGACTTTAAAGA
TGAAGGAAGGGGCGAAAACCAAGAATGTGGGAAGCCTCTAGAAGCTAGAGAAGGCAAGGAAACAAATTTTCCA
CTAGAGCCTCCAGAAGGAACACAGCCCTGCTGACCCACTGTAATGTCTGACCTCTAGATGTGTAAGGGTAGTAA
GACTGAAAGTCTCAGAAAGGCACTGTTAAATTCTTATTCCTCAACTATGCAACTCAAAACTGGGGTCCTCAGCAG
TGAGCGAGGGGTGAGAGAAGACACTGTATAAACATGGCACATCCTCCTGGAAAGTCAACTTTACTCAAAGCTTT
AGAAACCCAGCTCAAACTAGCTGAAGCAGAAAAGACATTACTGCGCCTATAAGTTGAAACTTTGGAGGGA (SEQ ID NO: 2). Note however that, for both sequences, variations in the non-coding exons have been reported in dbSNP, and these variations are envisaged as belonging to the respective transcript IDs.

I.e., unless specifically mentioned otherwise, the term RP11460N16.1 (or LINC01212) encompasses the different isoforms.

Moreover, analysis of publicly available RNA-seq data from TCGA as well as in-house RNA-seq data from short-term melanoma cultures indicate the presence of additional exons located 3' from the annotated LINC01212 exons. *In silico* based reconstitution of the transcript indicates that the full-length isoform is 4063 bp in length.

The sequence of this isoform is:

CTGAAGTCGCTAGACATTTGAGGAACACATCCGGGGAAGAAGACACAGGTGGCTGGTCATGGAGAGCCCGCTG
GGGGAAGAGCACACAGACAGGCACCGGCAGGCCATTGACCAGCGGGACAAGGTGGGCTCAATGTCATCACAA
GGGTGCTTAAGAGGGAAAGAGGAAGCCATGAGGGTCAGAGTCAAAGGAAGACTTGAAGATACTACACTGCGG
ACTTTAAAGATGAAGGAAGGGGCGAAAACCAAGAATGTGGGAAGCCTCTAGAAGCTAGAGAAGGCAAGGAAA
CAAATTTTCCACTAGAGCCTCCAGAAGGAACACAGCCCTGCTGACCCACTGTAATGTCTGACCTCTAGATGTGTA
AGGGTAGTAAGACTGAAAGTCTCAGAAAGGCACTGTTAAATTCTTATTCCTCAACTATGCAACTCAAAACTGGGG
TCCTCAGCAGTGAGCGAGGGGTGAGAGAAGACACTGTATAAACATGGCACATCCTCCTGGAAAGTCAACTTTAC
TCAAAGCTTTAGAAACCCAGCTCAAACTAGCTGAAGCAGAAAAGACATTACTGCGCCTATAAGTTGAAACTTTGG
AGGGATAAATTTCAGAGGTGTGGCTAGATCCAACGGCTCTCTGGAAAACTCTGTGAAAAAAATGCTTCCTCAAGT
CCAGAAACCAGAAGCTCAGGAAATTGTTGCTTGGTTCCTCTTGTTAGAGGCAGAGATTATTCAACGACCTACAGG
GTAGCGTTTGAACATTGTTACCAGGAATCTTTACTTTGCCATCTTCCAAGTCTGTTCTCCTCAGTATTGGTTTCATT
TACAAGCAGGCCATCTCTGCCCTCATGGCAATGATGGTCTTTACTCCAGGCTTAAGACCCTTACTATCTACTATTTC
CAAAGCGGAGAGAGAACTTCCCACACCTAGTGACCTGTGACACAGGATTTTATCCTTCGTACAGAGGGAATTCA
GTTGGCTAACATAATCTGCCTTCCAATGGAGTAAGAATGTCTGGACTCTTTCCTTCACCTACCCCCAAGACATGGA
GGCGTCTAAAAGATAAATAAAACTTGGCAACTGACCGAAGGAGGAAGAGGGGATTTCAGGCAAAATCAACGCT
GTTCACTACGAGGAGACTTCAGAAAGGTTGCCTGCTTCTGGGGAGCATAGTCCCTGATTCCTCAAGACATACGTT
TATTCTTTTCTTCAATGTCTTTGCCTGCAGTCAAAACAAAACCATTACCTTTAGCCAAGTTCACACATTTCAGCCAA
ATCCATATGCATCGGATCAGTTCTGTAGGTTATGGGTGAGCATGAACATATAAAAGAGGCACCTGCCTGTGCCTA
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TATCAAAAGTGGGAAATAGACTTTGACAAAATACTATTCATTAGACCACAGATCATATGGGGATTTCATTAGTTTT
TAGATGCACTCTATTGTTTTGTATAGTTCTTTTCCATTTTATCACCTGTATAGATTTGTGTAACCACCAAGAAGTAA
TTTGTTTTAAGCT (SEQ ID NO: 3)

According to particular embodiments, the LINC01212 gene product refers to the long transcript, i.e. ENST00000483525 or the longer isoform (as exemplified by SEQ ID NOs 2 and 3).

With "functional expression" of LINC01212, it is meant the transcription and/or translation of functional gene product. For non-protein coding genes like LINC01212, "functional expression" can be deregulated on at least two levels. First, at the DNA level, e.g. by absence or disruption of the gene, or lack of transcription taking place (in both instances preventing synthesis of the relevant gene product). The lack of transcription can e.g. be caused by epigenetic changes (e.g. DNA methylation) or by loss of function mutations. A "loss-of-function" or "LOF" mutation as used herein is a mutation that prevents, reduces or abolishes the function of a gene product as opposed to a gain-of-function mutation that confers enhanced or new activity on a protein. LOF can be caused by a wide range of mutation types, including, but not limited to, a deletion of the entire gene or part of the gene, splice site mutations, frame-shift mutations caused by small insertions and deletions, nonsense mutations, missense mutations replacing an essential amino acid and mutations preventing correct cellular localization of the product. Also included within this definition are mutations in promoters or regulatory regions of the LINC01212 gene if these interfere with gene function. A null mutation is an LOF mutation that completely abolishes the function of the gene product. A null mutation in one allele will typically reduce expression levels by 50%, but may have severe effects on the function of the gene product. Note that functional expression can also be deregulated because of a gain of function mutation: by conferring a new activity on the protein, the normal function of the protein is deregulated, and less functionally active protein is expressed. Vice versa, functional expression can be increased e.g. through gene duplication or by lack of DNA methylation.

Second, at the RNA level, e.g. by lack of efficient translation taking place – e.g. because of destabilization of the mRNA (e.g. by UTR variants) so that it is degraded before translation occurs from the transcript. Or by lack of efficient transcription, e.g. because a mutation introduces a new splicing variant.

The term “status” as used in the application with regard to a particular protein, specifically tumor-associated proteins (e.g. p53 status, BRAF status, NRAS status, MEK status, …) refers to the mutational status and/or the expression of these particular proteins. Typically, the term is used in the sense ‘irrespective of’ or ‘independent of’ status, meaning that an effect is observed irrespective of expression levels of, or presence of mutations in, the particular protein.

“Long non-coding RNAs” (long ncRNAs, lncRNA) as used herein are non-protein coding transcripts longer than 200 nucleotides. A particular class of lncRNA are long intergenic ncRNAs (lincRNA), referring to long non-coding RNAs that are transcribed from non-coding DNA sequences between protein-coding genes.

The present application is the first to show specific expression of lncRNAs in melanoma, and that inhibition of such lncRNA can be used to selectively induce apoptosis in these cancer cells.

Accordingly, it is an object of the invention to provide inhibitors of functional expression of the LINC01212 gene. Such inhibitors can act at the DNA level, or at the RNA (i.e. gene product) level. As LINC01212 is a non-coding gene, there is no protein product for this gene.

If inhibition is to be achieved at the DNA level, this may be done using gene therapy to knock-out or disrupt the target gene. As used herein, a “knock-out” can be a gene knockdown or the gene can be knocked out by a mutation such as, a point mutation, an insertion, a deletion, a frameshift, or a missense mutation by techniques known in the art, including, but not limited to, retroviral gene transfer. Another way in which genes can be knocked out is by the use of zinc finger nucleases. Zinc-finger nucleases (ZFNs) are artificial restriction enzymes generated by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain. Zinc finger domains can be engineered to target desired DNA sequences, which enable zinc-finger nucleases to target unique sequence within a complex genome. By taking advantage of endogenous DNA repair machinery, these reagents can be used to precisely alter the genomes of higher organisms. Other technologies for genome customization that can be used to knock out genes are meganucleases and TAL effector nucleases (TALENs, Cellectis bioresearch). A TALEN® is composed of a TALE DNA binding domain for sequence-specific recognition fused to the catalytic domain of an endonuclease that introduces double strand breaks (DSB). The DNA binding domain of a TALEN® is capable of targeting with high precision a large recognition site (for instance 17bp). Meganucleases are sequence-specific endonucleases, naturally occurring “DNA scissors”, originating from a variety of single-celled organisms such as bacteria, yeast, algae and some plant organelles. Meganucleases have long recognition sites of between 12 and 30 base pairs. The recognition site of natural meganucleases can be modified in order to target native genomic DNA sequences (such as endogenous genes).

Another recent genome editing technology is the CRISPR/Cas system, which can be used to achieve RNA-guided genome engineering. CRISPR interference is a genetic technique which allows for sequence-specific control of gene expression in prokaryotic and eukaryotic cells. It is based on the bacterial immune system-derived CRISPR (clustered regularly interspaced palindromic repeats) pathway.

Gene inactivation, i.e. inhibition of functional expression of the gene, may for instance also be achieved through the creation of transgenic organisms expressing antisense RNA, or by administering antisense RNA to the subject. An antisense construct can be delivered, for example, as an expression plasmid, which, when transcribed in the cell, produces RNA that is complementary to at least a unique portion of the cellular LINC01212 lncRNA.

A more rapid method for the inhibition of gene expression is based on the use of shorter antisense oligomers consisting of DNA, or other synthetic structural types such as phosphorothiates, 2’-0-alkylribonucleotide chimeras, locked nucleic acid (LNA), peptide nucleic acid (PNA), or morpholinos. With the exception of RNA oligomers, PNAs and morpholinos, all other antisense oligomers act in eukaryotic cells through the mechanism of RNase H-mediated target cleavage. PNAs and morpholinos bind complementary DNA and RNA targets with high affinity and specificity, and thus act through a simple steric blockade of the RNA translational machinery, and appear to be completely resistant to nuclease attack. An "antisense oligomer” refers to an antisense molecule or anti-gene agent that comprises an oligomer of at least about 10 nucleotides in length. In embodiments an antisense oligomer comprises at least 15, 18 20, 25, 30, 35, 40, or 50 nucleotides. Antisense approaches involve the design of oligonucleotides (either DNA or RNA, or derivatives thereof) that are complementary to an RNA encoded by polynucleotide sequences of LINC01212. Antisense RNA may be introduced into a cell to inhibit translation of a complementary mRNA by base pairing to it and physically obstructing the translation machinery. This effect is therefore stoichiometric. Absolute complementarity, although preferred, is not required. A sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double stranded antisense polynucleotide sequences, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense polynucleotide sequence. Generally, the longer the hybridizing polynucleotide sequence, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex. Antisense oligomers should be at least 10 nucleotides in length, and are preferably oligomers ranging from 15 to about 50 nucleotides in length. In certain embodiments, the oligomer is at least 15 nucleotides, at least 18 nucleotides, at least 20 nucleotides, at least 25 nucleotides, at least 30 nucleotides, at least 35 nucleotides, at least 40 nucleotides, or at least 50 nucleotides in length. A related method uses ribozymes instead of antisense RNA. Ribozymes are catalytic RNA molecules with enzyme-like cleavage properties that can be designed to target specific RNA sequences. Successful target gene inactivation, including temporally and tissue-specific gene inactivation, using ribozymes has been reported in mouse, zebrafish and fruitflies. RNA interference (RNAi) is a form of post-transcriptional gene silencing. The phenomenon of RNA interference was first observed and described in Caenorhabditis elegans where exogenous double-stranded RNA (dsRNA) was shown to specifically and potently disrupt the activity of genes containing homologous sequences through a mechanism that induces rapid degradation of the target RNA. Several reports describe the same catalytic phenomenon in other organisms, including experiments demonstrating spatial and/or temporal control of gene inactivation, including plant (Arabidopsis thaliana), protozoan (Trypanosoma bruceii), invertebrate (Drosophila melanogaster), and vertebrate species (Danio rerio and Xenopus laevis). The mediators of sequence-specific messenger RNA degradation are small interfering RNAs (siRNAs) generated by ribonuclease III cleavage from longer dsRNAs. Generally, the length of siRNAs is between 20-25 nucleotides (Elbashir et al. (2001) Nature 411, 494 498). The siRNA typically comprise a sense RNA strand and a complementary antisense RNA strand annealed together by standard Watson Crick base pairing interactions (hereinafter “base paired”). The sense strand comprises a nucleic acid sequence that is identical to a target sequence contained within the target mRNA. The sense and antisense strands of the present siRNA can comprise two complementary, single stranded RNA molecules or can comprise a single molecule in which two complementary portions are base paired and are covalently linked by a single stranded “hairpin” area (often referred to as shRNA). The term “isolated” means altered or removed from the natural state through human intervention. For example, an siRNA naturally present in a living animal is not "isolated," but a synthetic siRNA, or an siRNA partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated siRNA can exist in substantially purified form, or can exist in a non-native environment such as, for example, a cell into which the siRNA has been delivered.

The siRNAs of the invention can comprise partially purified RNA, substantially pure RNA, synthetic RNA, or recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siRNA or to one or more internal nucleotides of the siRNA, including modifications that make the siRNA resistant to nuclease digestion.

One or both strands of the siRNA of the invention can also comprise a 3′ overhang. A "3′ overhang" refers to at least one unpaired nucleotide extending from the 3′ end of an RNA strand. Thus, in one embodiment, the siRNA of the invention comprises at least one 3′ overhang of from one to about six nucleotides (which includes ribonucleotides or deoxynucleotides) in length, preferably from one to about five nucleotides in length, more preferably from one to about four nucleotides in length, and particularly preferably from about one to about four nucleotides in length.

In the embodiment in which both strands of the siRNA molecule comprise a 3′ overhang, the length of the overhangs can be the same or different for each strand. In a most preferred embodiment, the 3′ overhang is present on both strands of the siRNA, and is two nucleotides in length. In order to enhance the stability of the present siRNAs, the 3′ overhangs can also be stabilized against degradation. In one embodiment, the overhangs are stabilized by including purine nucleotides, such as adenosine or guanosine nucleotides.

Alternatively, substitution of pyrimidine nucleotides by modified analogues, e.g., substitution of uridine nucleotides in the 3′ overhangs with 2′ deoxythymidine, is tolerated and does not affect the efficiency of RNAi degradation. In particular, the absence of a 2′ hydroxyl in the 2′ deoxythymidine significantly enhances the nuclease resistance of the 3′ overhang in tissue culture medium.

The siRNAs of the invention can be targeted to any stretch of approximately 19 to 25 contiguous nucleotides in any of the target LINC01212 RNA sequences (the "target sequence"), of which examples are given in the application. Techniques for selecting target sequences for siRNA are well known in the art. Thus, the sense strand of the present siRNA comprises a nucleotide sequence identical to any contiguous stretch of about 19 to about 25 nucleotides in the target mRNA.

The siRNAs of the invention can be obtained using a number of techniques known to those of skill in the art. For example, the siRNAs can be chemically synthesized or recombinantly produced using methods known in the art. Preferably, the siRNA of the invention are chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. The siRNA can be synthesized as two separate, complementary RNA molecules, or as a single RNA molecule with two complementary regions. Commercial suppliers of synthetic RNA molecules or synthesis reagents include Proligo (Hamburg, Germany), Dharmacon Research (Lafayette, Colo., USA), Pierce Chemical (part of Perbio Science, Rockford, Ill., USA), Glen Research (Sterling, Va., USA), ChemGenes (Ashland, Mass., USA) and Cruachem (Glasgow, UK).

Alternatively, siRNA can also be expressed from recombinant circular or linear DNA plasmids using any suitable promoter. Suitable promoters for expressing siRNA of the invention from a plasmid include, for example, the U6 or H1 RNA pol III promoter sequences and the cytomegalovirus promoter. Selection of other suitable promoters is within the skill in the art. The recombinant plasmids of the invention can also comprise inducible or regulatable promoters for expression of the siRNA in a particular tissue or in a particular intracellular environment. The siRNA expressed from recombinant plasmids can either be isolated from cultured cell expression systems by standard techniques, or can be expressed intracellularly, e.g. in breast tissue or in neurons.

The siRNAs of the invention can also be expressed intracellularly from recombinant viral vectors. The recombinant viral vectors comprise sequences encoding the siRNAs of the invention and any suitable promoter for expressing the siRNA sequences. Suitable promoters include, for example, the U6 or H1 RNA pol III promoter sequences and the cytomegalovirus promoter. Selection of other suitable promoters is within the skill in the art. The recombinant viral vectors of the invention can also comprise inducible or regulatable promoters for expression of the siRNA in the tissue where the tumour is localized.

As used herein, an “effective amount” of the siRNA is an amount sufficient to cause RNAi mediated degradation of the target mRNA, or an amount sufficient to inhibit the progression of metastasis in a subject. RNAi mediated degradation of the target mRNA can be detected by measuring levels of the target mRNA or protein in the cells of a subject, using standard techniques for isolating and quantifying mRNA or protein as described above.

One skilled in the art can readily determine an effective amount of the siRNA of the invention to be administered to a given subject, by taking into account factors such as the size and weight of the subject; the extent of the disease penetration; the age, health and sex of the subject; the route of administration; and whether the administration is regional or systemic. Generally, an effective amount of the siRNA of the invention comprises an intracellular concentration of from about 1 nanomolar (nM) to about 100 nM, preferably from about 2 nM to about 50 nM, more preferably from about 2.5 nM to about 10 nM. It is contemplated that greater or lesser amounts of siRNA can be administered.

Recently it has been shown that morpholino antisense oligonucleotides in zebrafish and frogs overcome the limitations of RNase H-competent antisense oligonucleotides, which include numerous non-specific effects due to the non-target-specific cleavage of other mRNA molecules caused by the low stringency requirements of RNase H. Morpholino oligomers therefore represent an important new class of antisense molecule. Oligomers of the invention may be synthesized by standard methods known in the art. As examples, phosphorothioate oligomers may be synthesized by the method of Stein et al. (1988) Nucleic Acids Res. 16, 3209 3021), methylphosphonate oligomers can be prepared by use of controlled pore glass polymer supports (Sarin et al. (1988) Proc. Natl. Acad. Sci. USA. 85, 7448-7451). Morpholino oligomers may be synthesized by the method of Summerton and Weller U.S. Patent Nos. 5,217,866 and 5,185,444.

Another particularly form of antisense RNA strategy are gapmers. A gapmer is a chimeric antisense oligonucleotide that contains a central block of deoxynucleotide monomers sufficiently long to induce RNase H cleavage. The central block of a gapmer is flanked by blocks of 2′-O modified ribonucleotides or other artificially modified ribonucleotide monomers such as bridged nucleic acids (BNAs) that protect the internal block from nuclease degradation. Gapmers have been used to obtain RNase-H mediated cleavage of target RNAs, while reducing the number of phosphorothioate linkages. Phosphorothioates possess increased resistance to nucleases compared to unmodified DNA. However, they have several disadvantages. These include low binding capacity to complementary nucleic acids and non-specific binding to proteins that cause toxic side-effects limiting their applications. The occurrence of toxic side-effects together with non-specific binding causing off-target effects has stimulated the design of new artificial nucleic acids for the development of modified oligonucleotides that provide efficient and specific antisense activity in vivo without exhibiting toxic side-effects. By recruiting RNase H, gapmers selectively cleave the targeted oligonucleotide strand. The cleavage of this strand initiates an antisense effect. This approach has proven to be a powerful method in the inhibition of gene functions and is emerging as a popular approach for antisense therapeutics. Gapmers are offered commercially, e.g. LNA longRNA GapmeRs by Exiqon, or MOE gapmers by Isis pharmaceuticals. MOE gapmers or “2′MOE gapmers” are an antisense phosphorothioate oligonucleotide of 15-30 nucleotides wherein all of the backbone linkages are modified by adding a sulfur at the non-bridging oxygen (phosphorothioate) and a stretch of at least 10 consecutive nucleotides remain unmodified (deoxy sugars) and the remaining nucleotides contain an O′-methyl O′-ethyl substitution at the 2′ position (MOE).

According to a further aspect, the inhibitors of functional expression of LINC01212 are provided for use as a medicament. According to yet further aspects, the inhibitors of functional expression of LINC01212 are provided for use in treatment of cancer, in particular skin cancer. In still further embodiments, the inhibitors are provided for use in treatment of melanoma.

This is equivalent as saying that methods of treating melanoma in a subject in need thereof are provided, comprising administering an inhibitor of functional expression of LINC01212 to said subject.

The nature of the inhibitor is not vital to the invention, as long as it inhibits the functional expression of the LINC01212 gene. According to specific embodiments, the inhibitor is selected from an inhibitory RNA technology (such as a gapmer, a shRNA, a siRNA), a CRISPR, a TALEN, or a Zinc-finger nuclease.

According to alternative, but not exclusive, specific embodiments, the inhibitor selectively induces apoptosis in melanoma cells. This particularly implies that it induces apoptosis in melanoma cells, but not in normal (non-transformed) melanocytes. According to further specific embodiments, the inhibitor induces apoptosis independent of p53, BRAF, NRAS or MEK status, e.g., independent whether these proteins have particular mutations or not, or independent of their expression levels.

Even though inhibition of LINC01212 is sufficient to achieve a therapeutic effect, i.e. to achieve apoptosis in cancer cells, it is shown herein that a stronger, synergistic effect is achieved when both an inhibitor of LINC01212 and another chemotherapeutic are administered. This is particularly true for B-raf kinase inhibition (Example 3, Fig. 16). However, without being bound to a particular mechanism, the fact that LINC01212 inhibition induces apoptosis of melanoma cells independent of B-raf, N-ras or p53 status, as well as results in inhibition of all known survival pathways (see Example 4), indicates that this synergistic effect will be observed for other chemotherapeutics as well (particularly those therapeutics that interact with those targets or are sensitive to resistance using the survival pathways). Thus, other chemotherapeutics such as MEK inhibitors, cisplatin and melphalan are also explicitly envisaged. Additionally, the chemotherapeutic that can be used to obtain a synergistic effect can be one or more chemotherapeutic agents selected from a microtubule active agent, an alkylating agent, an anti-neoplastic anti-metabolite, a platin compound, a Raf or MEK kinase inhibitor, a topoisomerase I inhibitor, a topoisomerase II inhibitor, a VEGF inhibitor, a PI3/AKT kinase inhibitor, a tyrosine kinase inhibitor, an EGFR kinase inhibitor, an mTOR kinase inhibitor, an insulin-like growth factor I inhibitor, a HDAC inhibitor, a proteasome inhibitor, and ionizing radiation. The synergistic effect can be obtained through simultaneous, concurrent, separate or sequential use for preventing or treating melanoma.

According to a further aspect, methods are provided that may identify whether a tumor is suitable for treatment with an inhibitor of functional expression of LINC01212. These methods typically have the following steps:

- Determining whether expression of LINC01212 is increased in the tumor or a sample of tumor cells;
- Establishing whether the tumor is suitable for treatment, wherein increased expression is indicative of suitability for treatment.

The methods thus may entail a first step of providing a sample of tumor cells. The determining step may occur purely in vitro, i.e. without a step interacting on the human or animal body.

According to particular embodiments, the tumor is a skin cancer, e.g. BCC, SCC or melanoma. According to further particular embodiments, the tumor is melanoma.

Increased levels of LINC01212 gene product (i.e., typically lncRNA) are typically increased versus a control. The skilled person is capable of picking the most relevant control. This will typically also depend on the nature of the disease studied, the sample(s) that is/are available, and so on. Suitable controls include, but are not limited to, similar samples from subjects not having a tumor, the average levels in a control group (or control cells, e.g. melanocytes), or a set of clinical data on average LINC01212 gene product levels in the tissue from which the sample is taken. As is evident from the foregoing, the control may be from the same subject, or from one or more different subjects or derived from clinical data. Optionally, the control is matched for e.g. sex, age etc.

With 'increased' levels of LINC01212 gene product as mentioned herein, it is meant levels that are higher than are normally present. Typically, this can be assessed by comparing to control. According to particular embodiments, increased levels of LINC01212 are levels that are 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, 100%, 150%, 200% or even more high than those of the control. According to further particular embodiments, it means that LINC01212 gene product is expressed or present, whereas it normally (or in control) is absent. In other words, in these embodiments determining the increased expression of LINC01212 gene product is equivalent to detecting the presence of LINC01212 gene product. Typically, in such cases, a control will be included to make sure the detection reaction worked properly. The skilled person will appreciate that the exact levels by which LINC01212 gene product needs to be higher in order to allow a reliable and reproducible diagnosis may depend on the type of sample tested and of which product (lncRNA) the levels are assessed. However, assessing the correlation itself is fairly straightforward.

Instead of looking at increased levels compared to a healthy control, the skilled person will appreciate that the reverse, comparing to a control with disease, can also be done. Thus, if the LINC01212 gene product levels measured in the sample are similar to those of a sample with a tumor (melanoma), (or are e.g. comparable to LINC01212 gene product levels found in a clinical data set of cancer patients), this may be considered equivalent to increased LINC01212 gene product levels compared to a healthy control, and be correlated to an increased suitability of treatment. Of course, LINC01212 gene product levels may be compared to both a negative and a positive control in order to increase accuracy of the diagnosis.

According to specific embodiments, when it is established that the tumor is suitable for treatment, the methods may further comprise a step of administering an inhibitor of functional expression of LINC01212 to the subject in which the tumor is present. This in order to treat the tumor.

Also provided herein are methods of diagnosing the presence of melanoma in a subject, comprising the steps of:

- Determining the levels of LINC01212 (or LINC01212 gene product) in a sample of said subject;
- Correlating the levels of LINC01212 in said sample with the presence of melanoma.

In such methods, the presence (or increased expression) of LINC01212 is indicative of the presence of melanoma in the subject from whom the sample is taken. Typically, these methods are performed in vitro, although in vivo methods are not necessarily excluded. Determining the levels of LINC01212 will typically be done by determining the levels of LINC01212 RNA in said sample. The same considerations regarding samples and controls apply as described above.
The sample can be a tissue sample (e.g. a skin biopt), but as is shown herein, in melanoma patients, LINC01212 also circulates in the blood. Thus, it can also be detected in blood or serum, and the sample can be a blood sample or a serum sample.

The levels of LINC01212 RNA vary with different stages of the disease (Fig. 6). Accordingly, in methods that determine the presence of melanoma, a further step may be included that correlates the levels of LINC01212 to disease severity, disease stage (e.g. stage of melanoma), or disease progression.

It is to be understood that although particular embodiments, specific configurations as well as materials and/or molecules, have been discussed herein for cells and methods according to the present invention, various changes or modifications in form and detail may be made without departing from the scope and spirit of this invention. The following examples are provided to better illustrate particular embodiments, and they should not be considered limiting the application. The application is limited only by the claims.

Examples

Example 1. Identification of a melanoma-specific lncRNA

Long non-coding RNAs (lncRNAs) are the most abundant class of ncRNA molecules and are emerging as an important regulatory layer of the transcriptome. Currently, expression and function of lncRNAs during human disease and development is largely unexplored. In part, this is due to the fact that few platforms are available that allow sensitive and specific high-throughput lncRNA expression profiling.

1718 MIQE-validated lncRNA RT-qPCR assays, were spotted in triplicate on a SmartChip (5184 wells). lncRNA expression is measured by RT-qPCR in 100 nl reactions. Triplicate expression values are combined based on the median Cq which is insensitive to outliers. lncRNA expression data is normalized using the global mean (Biogazelle's qbase+ software).

Expression of 1718 lncRNAs was measured by means of RT-qPCR in 60 cancer cell lines (NCI60 cancer cell line panel, representing 9 different tumour entities). This revealed cancer-specific lncRNA expression profiles. Several lncRNAs were identified with a highly specific expression pattern in the 9 melanoma cell lines of this panel. Of note, one of these melanoma specific lncRNAs, ENST00000483525 (aka lncRNA-525), product of the LINC01212 gene, is located immediately downstream of microphthalmia-associated transcription factor (MITF), a lineage specific oncogene in melanoma (Figure 1).

When assessing expression of the lncRNA in different cells, it is striking that high expression levels are only observed in short term melanoma cultures, melanoma cell lines of the NCI60 panel, and metastatic melanomas. In contrast, no significant expression is observed in Normal Human Epidermal Melanocytes (NHEM) – see Figure 2. Further analysis showed that the lncRNA is specifically expressed in more than 85% of human melanomas (Figure 3) but not in normal human melanocytes or other normal adult tissues (Figure 4 and 5), nor in other cancer cell lines (not shown).

While the LINC01212 gene is not expressed in normal human melanocytes (NHEM), its expression is up-regulated very early during melanomagenesis (but is not expressed in benign nevi); its expression is specifically increased during the transition from Radial Growth Phase (RGP) to Vertical Growth Phase – which coincides with the switch from benign (immortalized) to fully transformed melanoma (Figure 6). It is found overexpressed in some melanomas regardless of stage - from early melanoma lesions (stage I – primary melanomas) to stage IV melanomas (data not shown). This observation raises the possibility that it could serve as a biomarker for early detection of the benign to malignant switch.

Example 2. Mechanisms of overexpression

To check the cause for this overexpression of the lncRNA, a copy number analysis was performed in nevi, different melanoma cell lines (including NCI60 melanoma cell lines), short-term melanoma cultures and primary melanocytes (NHEM). It could be shown that, in some cases of melanoma, a rare amplification (more precisely, a co-amplification with the MITF locus) may explain the observed overexpression. The LINC01212 gene is located immediately downstream of the *MITF* locus and is co-amplified with *MITF* in a fraction of human melanoma. Gene amplification of the LINC01212 locus can therefore explain, in part, its up-regulation in melanoma (Figures 7 and 8).

Other epigenetic mechanisms are likely to contribute to LINC01212 up-regulation in melanoma. We have obtained evidence that the LINC01212 promoter is methylated in NHME and non-melanoma cell lines and is de-methylated in the majority of short-term melanoma cultures analyzed, pointing to another mechanism of LINC01212 expression in melanoma. Experiments aimed at understanding the mechanisms that contribute to demethylation of the LINC01212 promoter during melanomagenesis are ongoing.

Consistent with the above evidence of increased promoter activity of the LINC01212 gene by demethylation, H3K27Acetylation ChIP-seq experiments (Figure 9) and FAIRE-seq on a series of short-term culture melanoma lines corroborated the increased promoter activity.

Example 3. Inhibition of the LINC01212 gene product induces apoptosis specifically in melanoma cells.

To assess the functional relevance of the observed overexpression of the LINC01212-encoded lncRNA, its expression was inhibited using gapmers. LNA gapmers are potent antisense oligonucleotides used for highly efficient inhibition of mRNA and lncRNA function. Two different gapmers were designed to target the lncRNA. The SK-MEL28 melanoma cell line was chosen as a test cell line, since it is part of the

NCI60 panel, and shows high expression of the LINC01212-encoded lncRNA (as a result of a copy number gain).

The gapmers effectively succeeded in reducing lncRNA expression in these cells, whereas a control scrambled gapmer had no effect (Figure 10).

Remarkably, a lot of the cells treated with the lncRNA inhibitors died (Figure 11). By measuring caspase 3/7 activity with a luciferase reporter, it could be confirmed that knockdown of the LINC01212-encoded lncRNA in SK-MEL28 melanoma cells resulted in a significant induction of apoptosis (Figure 12).

These findings were further validated in two additional melanoma short term culture systems with differential TP53 status (i.e. wild type and mutant). Apoptosis induction was observed with both gapmers in both short term cultures suggesting that the observed phenotype is independent of TP53 status, but also independent of BRAF status (Figure 13). To our knowledge, this is the first inhibitor that can achieve this.

Thus, strikingly, knocking-down this lncRNA, using siRNA or LNA-antisense oligonucleotides, invariably leads to dramatic apoptotic cell death (Figure 11 and 14) irrespective of the BRAF, NRAS (or TP53) status.

Similar results were obtained using melanoma lines with different genetic backgrounds such as for instance MM057 (NRAS Q61L) or MM087 (TP53 mutation). Cells that do not express LINC01212 such as NHME, HCT116, and MM001 were not affected by these treatments.

Importantly, overexpression of the annotated long LINC01212 transcript in NMHE and the melanoma SK-MEL28 cells resulted in an increase in cell proliferation and decrease in the basal level of apoptotic cell death (data not shown). Enforced expression of this transcript also rescues at least partly the increase in apoptosis observed upon LINC01212 KD with Gapmer 11 (Figure 15). Given the FISH analysis indicated that exogenous LINC01212 was mainly localized to the cytoplasm (Figure 7C) these preliminary data indicate that LINC01212 functions (to protect melanoma cells from apoptosis) at least partly by acting in *trans* in the cytoplasm.

Importantly, knockdown of LINC01212 sensitizes BRAFV600E-melanoma cells to a BRAF-inhibitor currently used in the clinic, PLX3042 (Figure 16). Without being bound to a particular mechanism, the fact that LINC01212 inhibition induces apoptosis of melanoma cells independent of B-raf, N-ras or p53 status, as well as results in inhibition of all known survival pathways (see Example 4), indicates that this synergistic effect will be observed for other chemotherapeutics as well (particularly those therapeutics that interact with those targets or are sensitive to resistance using the survival pathways). Preliminary data confirm this, experiments on synergistic effects with combinations with MEK inhibitors, cisplatin and melphalan are currently ongoing.

Example 4. Pathway and interaction analysis.

A full-genome transcriptome gene expression analysis was performed in these short term melanoma culture cells in order to identify pathways that could account for the observed biological effect (induction of apoptosis). Remarkably, all known melanoma survival pathways are inactivated upon knockdown of the LINC01212-encoded lncRNA: WNT:Bcat, MAPK (through downregulation of both MEK1 and MEK2) and MITF (Figure 17-19). As melanoma is known to be particularly refractory to cancer therapy, this concomitant inactivation of all survival pathways offers high therapeutic potential. In addition, it appears that a p53/P63 pro-apoptotic signature is induced (even in cells harbouring TP53 inactivation mutations).

Ongoing experiments aimed at identifying the total/nuclear/cytoplasmic 525 interactomes should shed light on the molecular mechanisms underlying 525 melanoma protective function. Identification of polypeptides interacting with the endogenously expressed LINC01212 transcript in SK-MEL28 cells by MS in ongoing.

Example 5. Expression of LINC01212 can be detected in serum

Given the upregulation very early during melanomagenesis, coinciding with the switch from benign (immortalized) to fully transformed melanoma (Figure 6), the potential of LINC01212 as a melanoma biomarker was further explored. Particularly, it was evaluated whether LINC01212 could be detected in serum from melanoma patients, as blood or serum is easy to collect as a sample. As shown in Figure 20, LINC01212 can be found in serum of melanoma patients. In contrast, the LINC01212 transcript cannot be detected in the serum of healthy volunteers.

Conclusions

Given its melanoma-specific expression profile these data indicate that therapeutics that target this lncRNA could serve as melanoma-specific MAPK inhibitors, irrespective of how MAPK signaling is activated.

- LINC01212 is a melanoma-specific lncRNA that could serve as an early diagnostic marker of malignant transformation.
- LINC01212 could actively contribute to melanomagenesis in a previously unrecognized manner, particularly in samples with MITF co-amplification.
- LINC01212 is a key therapeutic target in melanoma.
- LINC01212 inhibition is a good candidate for combination therapy with other chemotherapeutics, such as BRAF-inhibitors.

Further development includes additional validation of the phenotype in vitro, including cell lines with differential KRAS and BRAF status and in vivo using xenografts and melanoma mouse models. Also, phenotypic and molecular characterization of primary melanocytes with LINC01212 overexpression and further molecular characterization of melanoma cell lines with LINC01212 overexpression or knockdown will be performed. Further validation of LNC01212 transcript as a serum marker for melanoma is currently ongoing, by testing in a larger patient population. Experiments aimed at elucidating the underlying mechanism by which downregulation of this lncRNA induces apoptosis will also be performed.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

Claims

1. An inhibitor of functional expression of LINC01212, wherein the inhibitor is selected from a group consisting of a gapmer, an shRNA and an siRNA.

2. The inhibitor according to claim 1, wherein the inhibitor selectively induces apoptosis in melanoma cells.

3. The inhibitor according to claim 1 or claim 2, wherein the inhibitor induces apoptosis independent of TP53, BRAF, NRAS or MEK status.

4. A method of treating cancer in a subject in need thereof, the method comprising administering to said subject the inhibitor according to any one of claims 1 to 3.

5. The method according to claim 4, wherein the cancer is melanoma.

6. The method of any one of claims 4 to 5, further comprising administering an additional chemotherapeutic agent to the subject.

7. The method according to claim 6, wherein the additional chemotherapeutic agent is a Raf kinase inhibitor.

8. The method according to claim 7, wherein the Raf kinase inhibitor is a B-raf kinase inhibitor.

9. A method of identifying a tumor suitable for treatment with an inhibitor of functional expression of LINC01212, the method comprising:
   - Determining whether expression of LINC01212 is increased in the tumor or a sample of tumor cells; and
   - Establishing whether the tumor is suitable for treatment, wherein increased expression is indicative of suitability for treatment.

10. The method according to claim 9, wherein the tumor is melanoma.

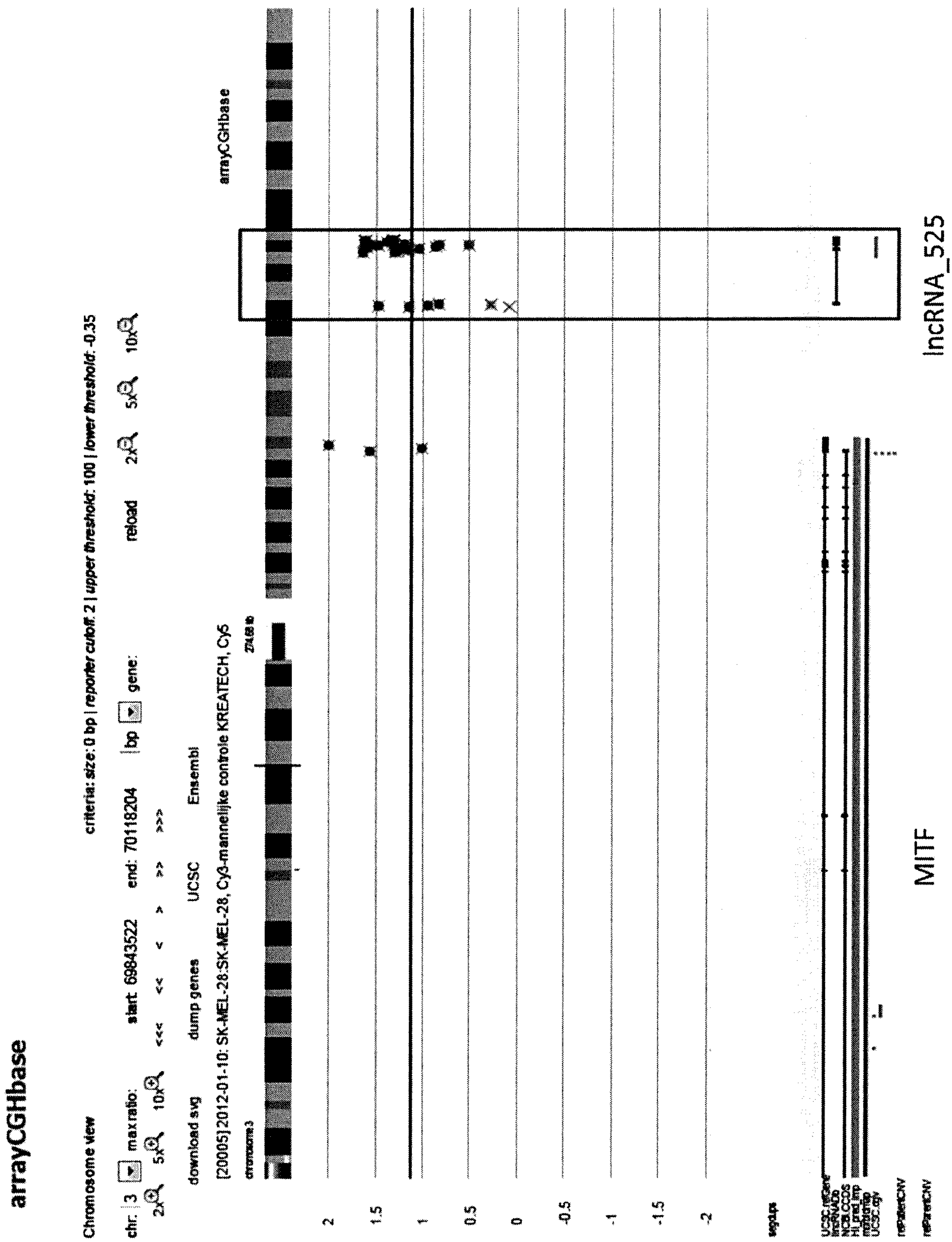
11. A method of determining the presence of melanoma in a subject, the method comprising:
    - Determining the levels of LINC01212 in a sample of said subject; and
    - Correlating the levels of LINC01212 in said sample with the presence of melanoma.

12. The method according to claim 11, wherein the sample is a blood or serum sample.

13. The method according to claim 10 or claim 11, further comprising correlating the levels of LINC01212 in said sample with the stage of melanoma.

14. The use of an inhibitor of any one of claims 1 to 3 for the manufacture of a medicament for the treatment of cancer in a subject in need thereof.

Figure 1


arrayCGHbase
Chromosome view
criteria: size: 0 bp | reporter cutoff: 2 | upper threshold: 100 | lower threshold: -0.35
chr. 3 max ratio:
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reload
download svg dump genes UCSC Ensembl
[20005] 2012-01-10: SK-MEL-28:SK-MEL-28, Cy3-mannelijke controle KREATECH, Cy5
arrayCGHbase
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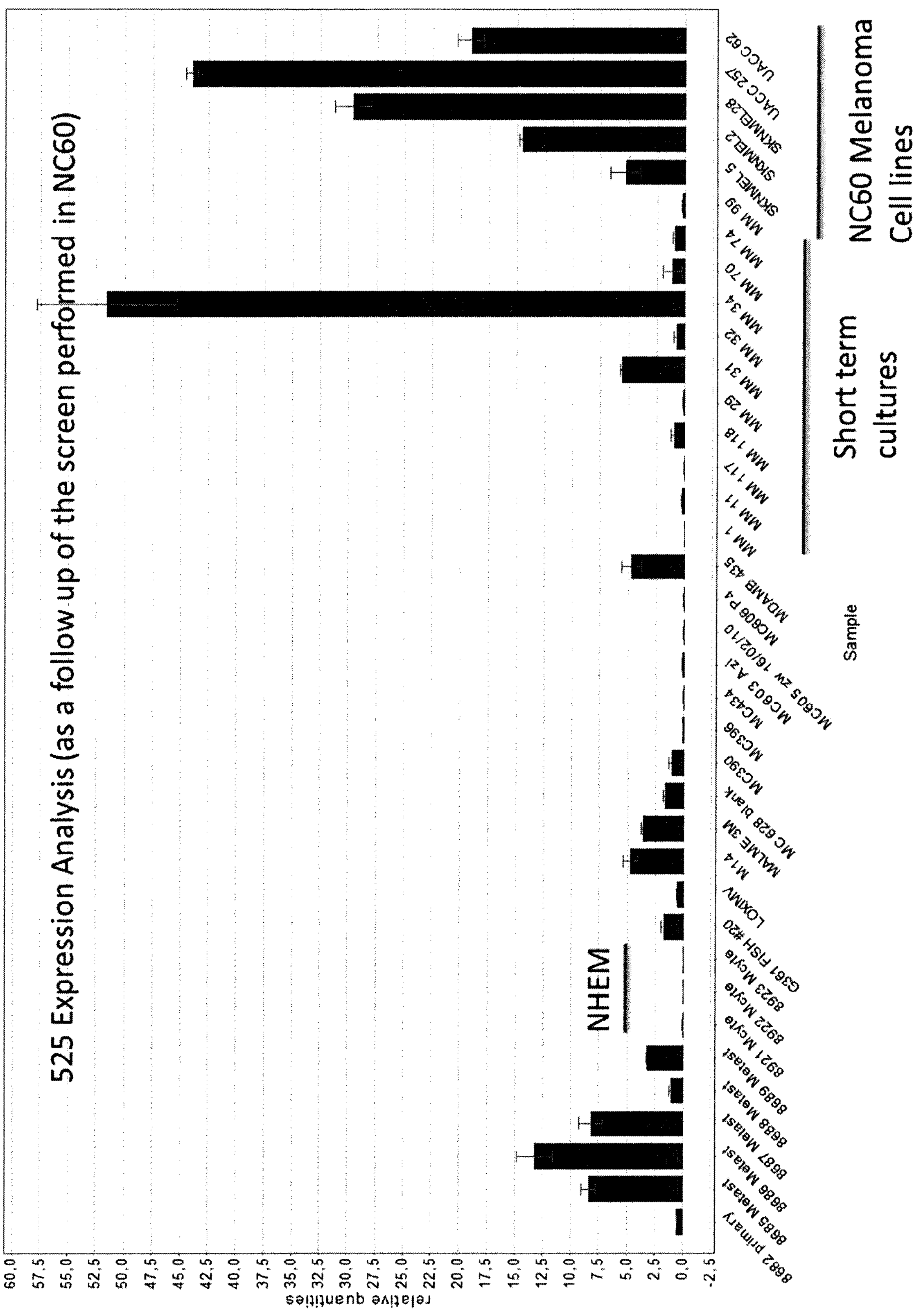
1
0.5
0
-0.5
-1
-1.5
-2
lncRNA_525
MITF

Figure 2


525 Expression Analysis (as a follow up of the screen performed in NC60)
relative quantities
Sample
NHEM
Short term cultures
NC60 Melanoma Cell lines
8882 primary
8885 Metast
8886 Metast
8887 Metast
8888 Metast
8889 Metast
8921 Mcyte
8922 Mcyte
8923 Mcyte
G361 FISH #20
LOXIMV
M14
MALME 3M
MC 628 blank
MC390
MC398
MC434
MC603 A zl
MC605 zw 16/02/10
MC606 P4
MDAMB 435
MM 1
MM 11
MM 117
MM 118
MM 29
MM 31
MM 32
MM 34
MM 70
MM 74
MM 99
SKMEL 5
SKMEL2
SKMEL28
UACC 257
UACC 62
60,0
57,5
55,0
52,5
50,0
47,5
45,0
42,5
40,0
37,5
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30,0
27,5
25,0
22,5
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0,0
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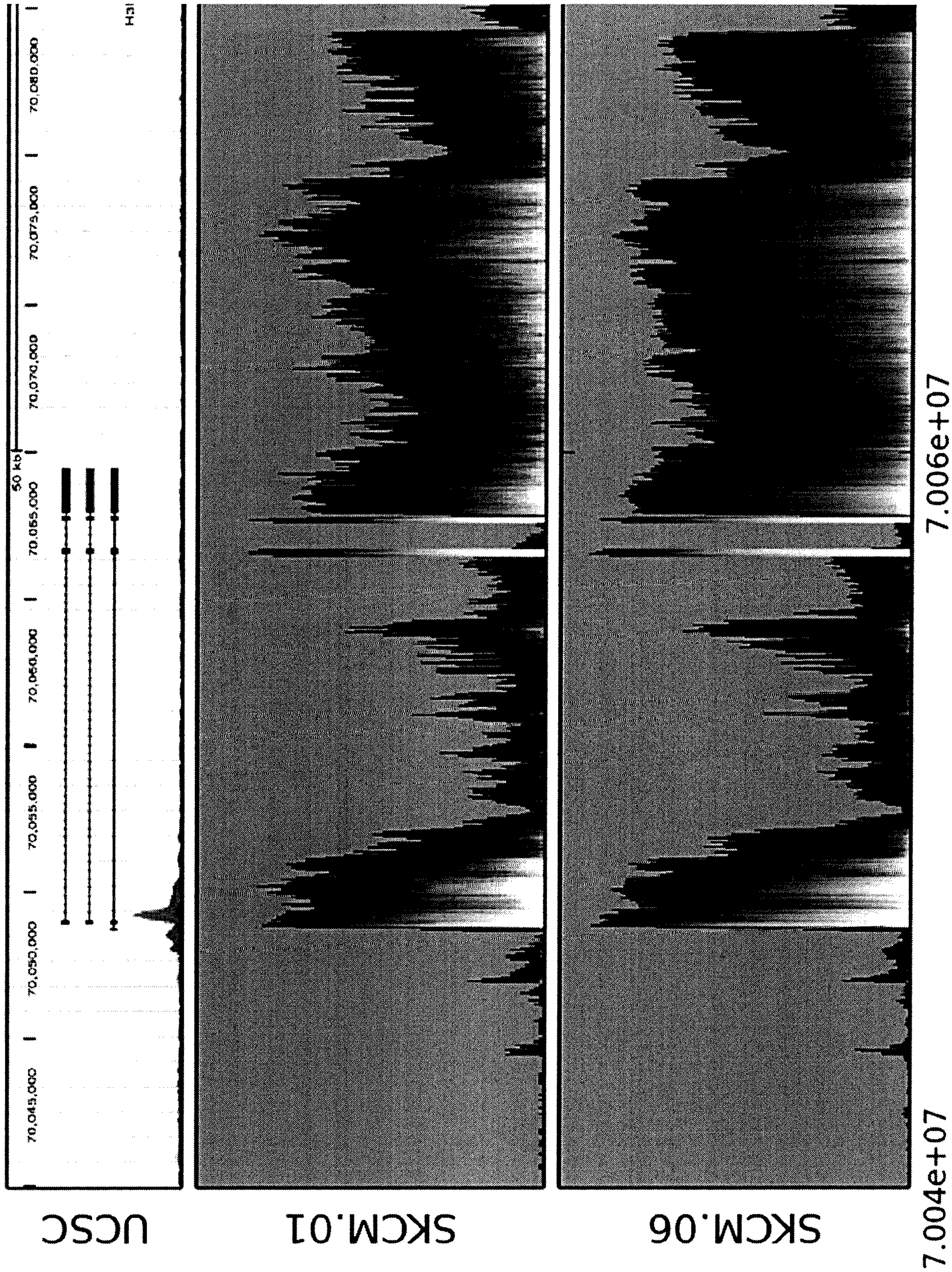
Figure 3



UCSC
SKCM.01
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7.006e+07
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70,080,000

Figure 3 continued

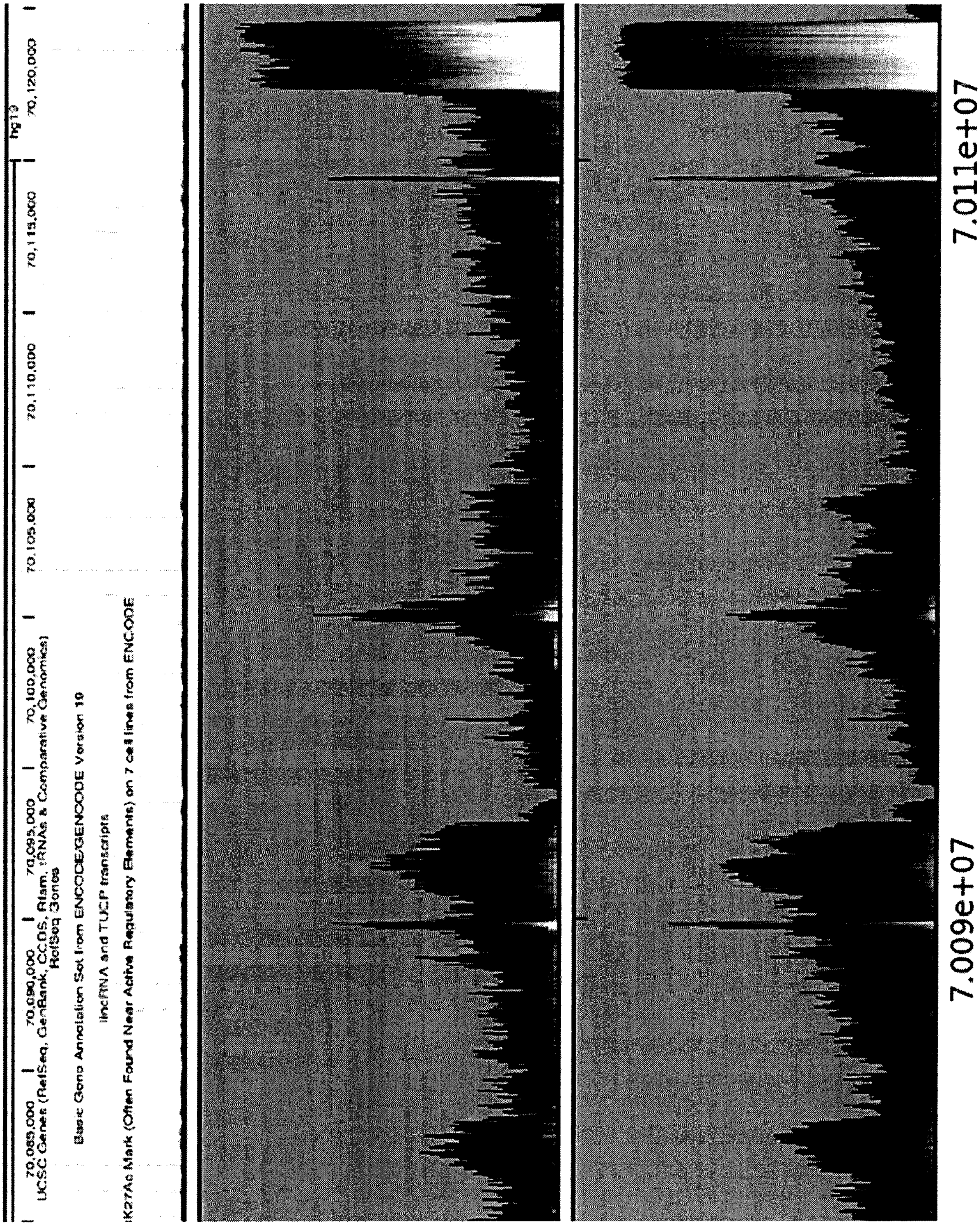


Figure 3 continued

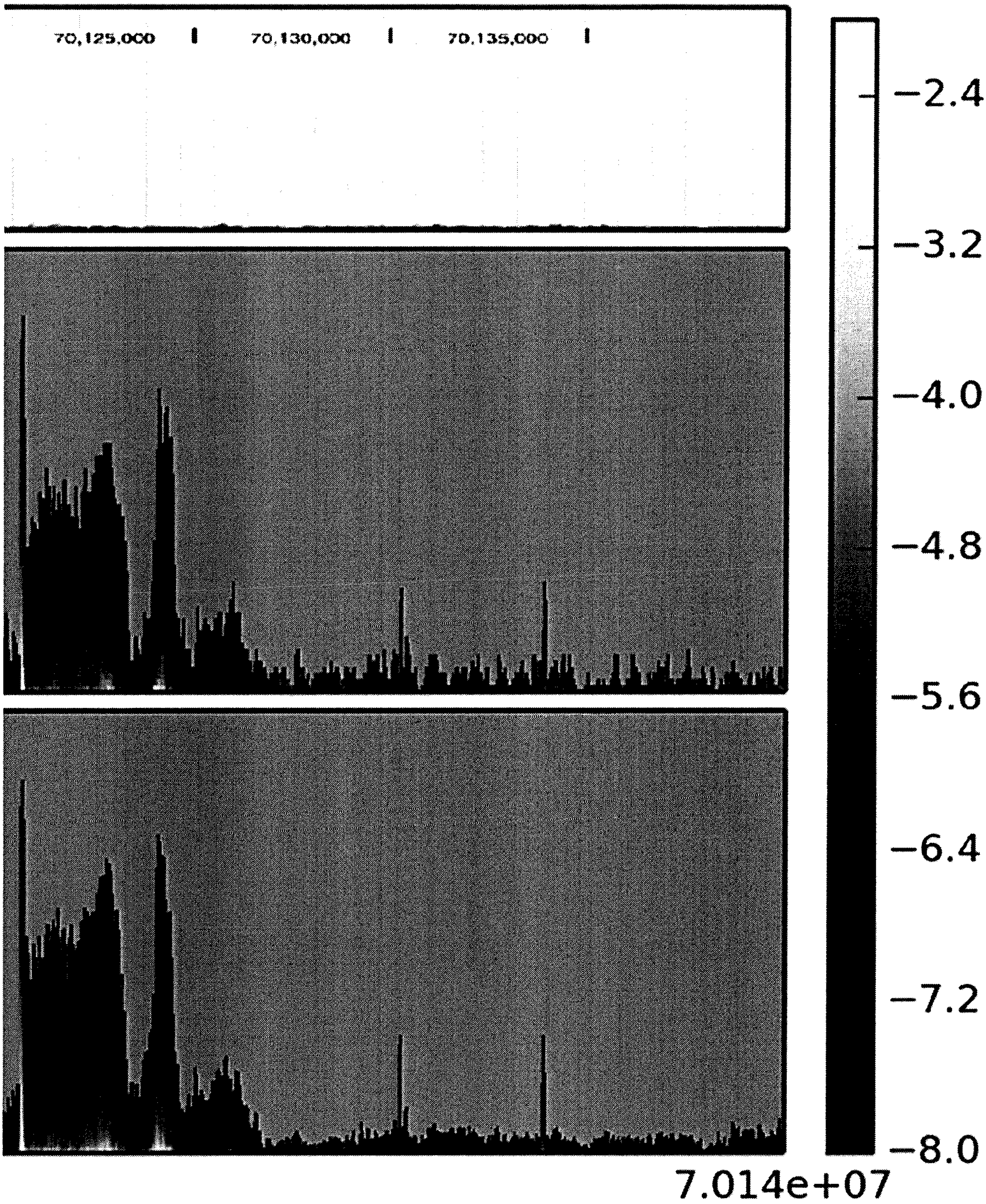


Figure 3 continued

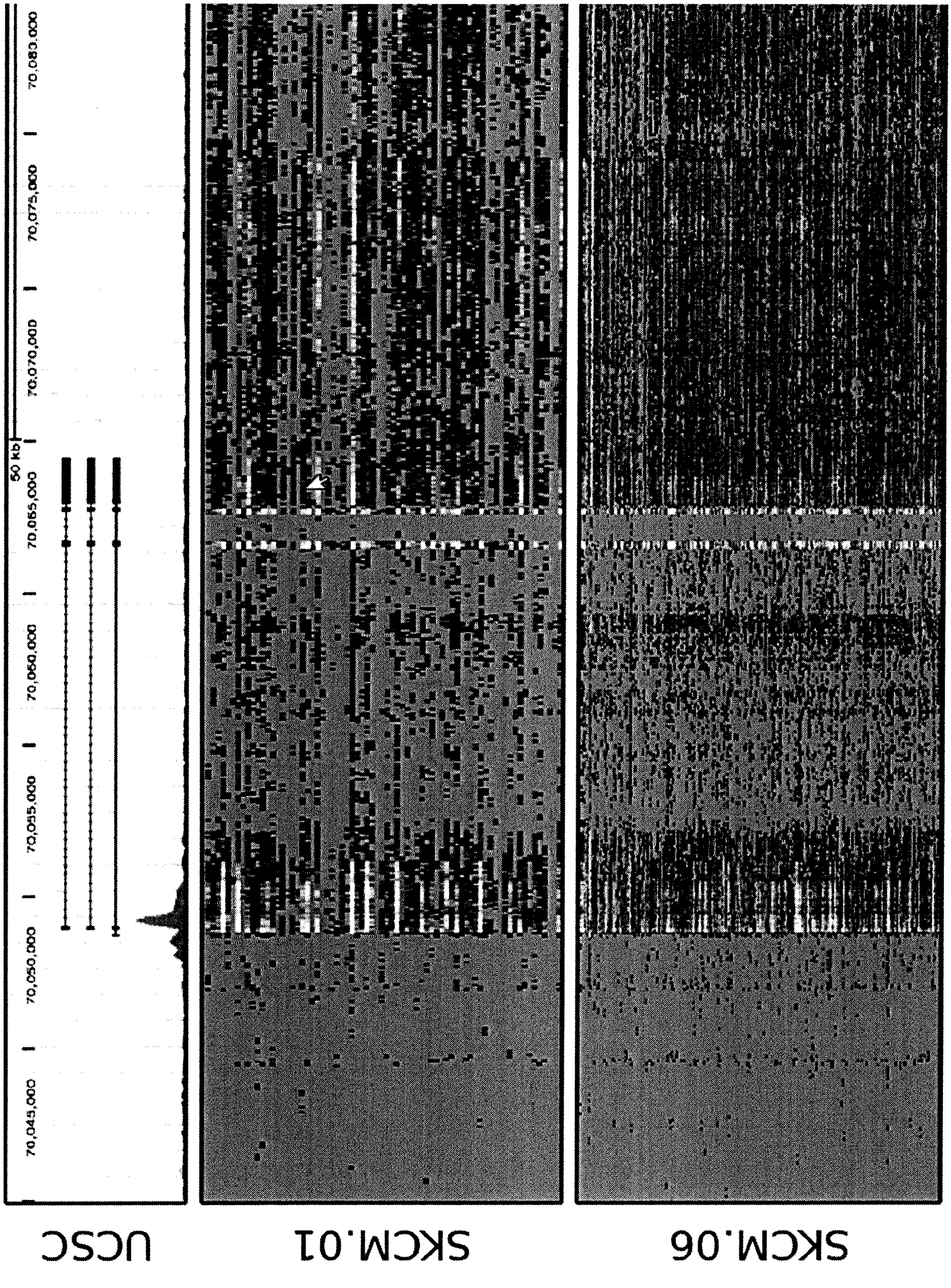


Figure 3 continued

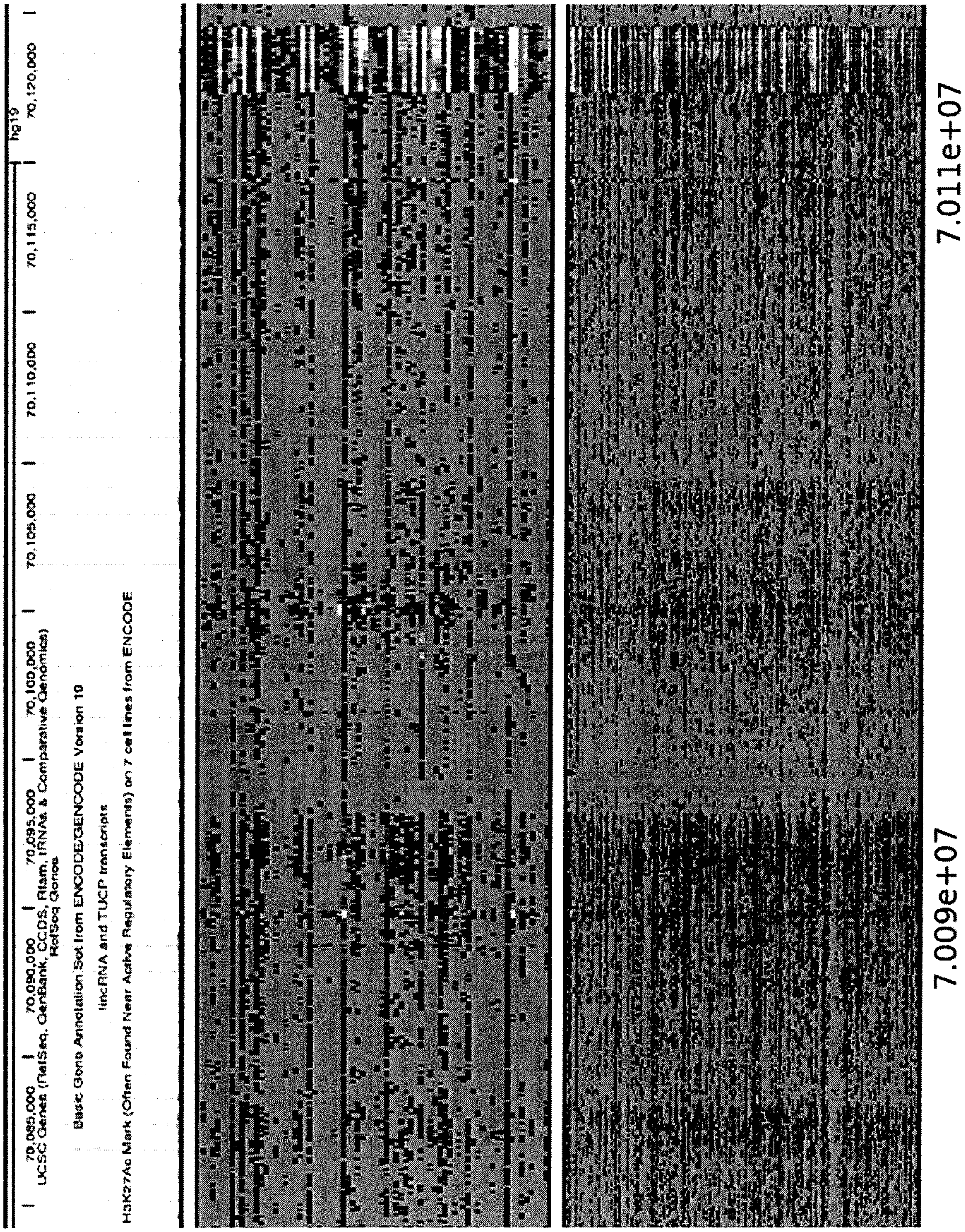


Figure 3 continued

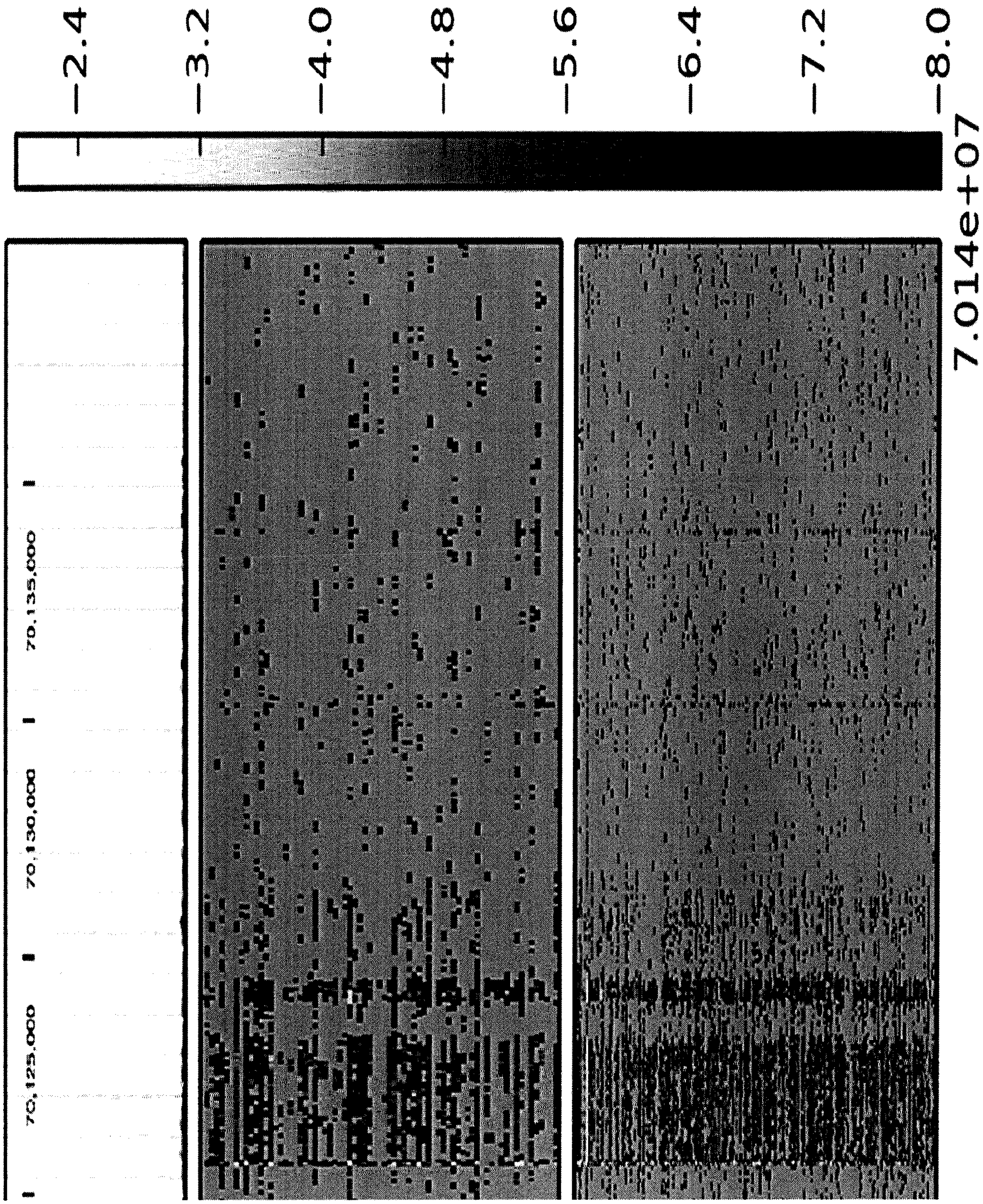
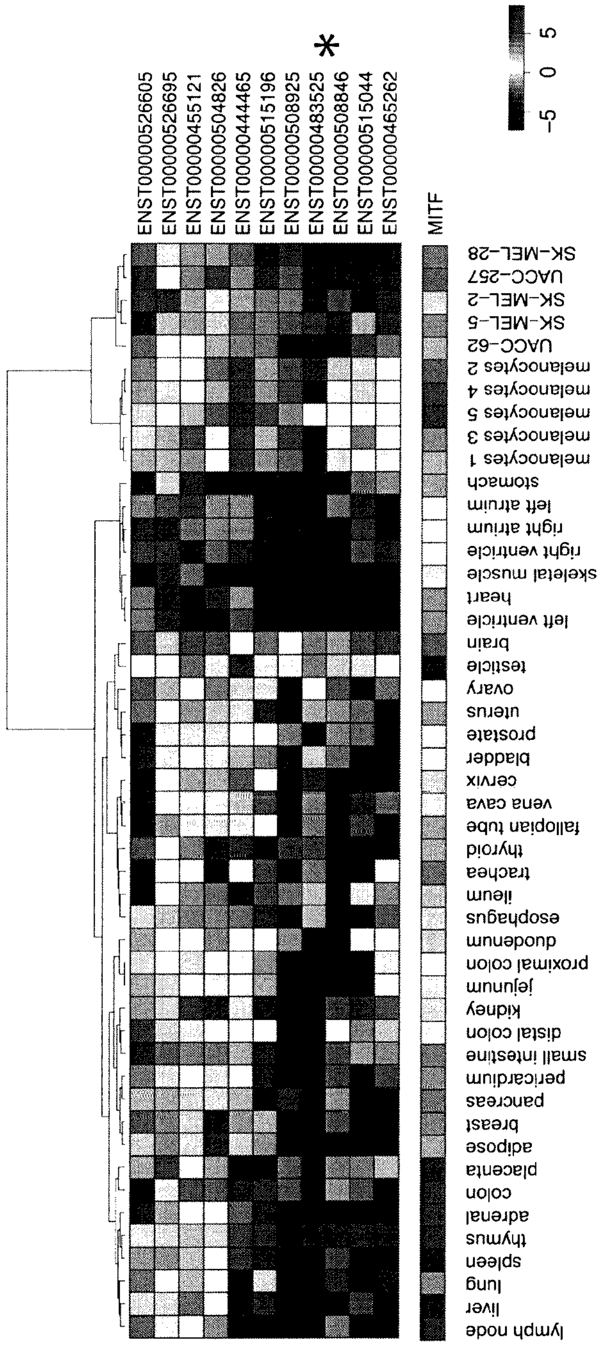


Figure 4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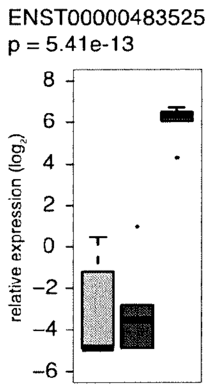
MITF
-5
0
5
SK-MEL-28
UACC-257
SK-MEL-2
SK-MEL-5
UACC-62
melanocytes 2
melanocytes 4
melanocytes 5
melanocytes 3
melanocytes 1
stomach
left atrium
right atrium
right ventricle
skeletal muscle
heart
left ventricle
brain
testicle
ovary
uterus
prostate
bladder
cervix
vena cava
fallopian tube
thyroid
trachea
ileum
esophagus
duodenum
proximal colon
jejunum
kidney
distal colon
small intestine
pericardium
pancreas
breast
adipose
placenta
colon
adrenal
thymus
spleen
lung
liver
lymph node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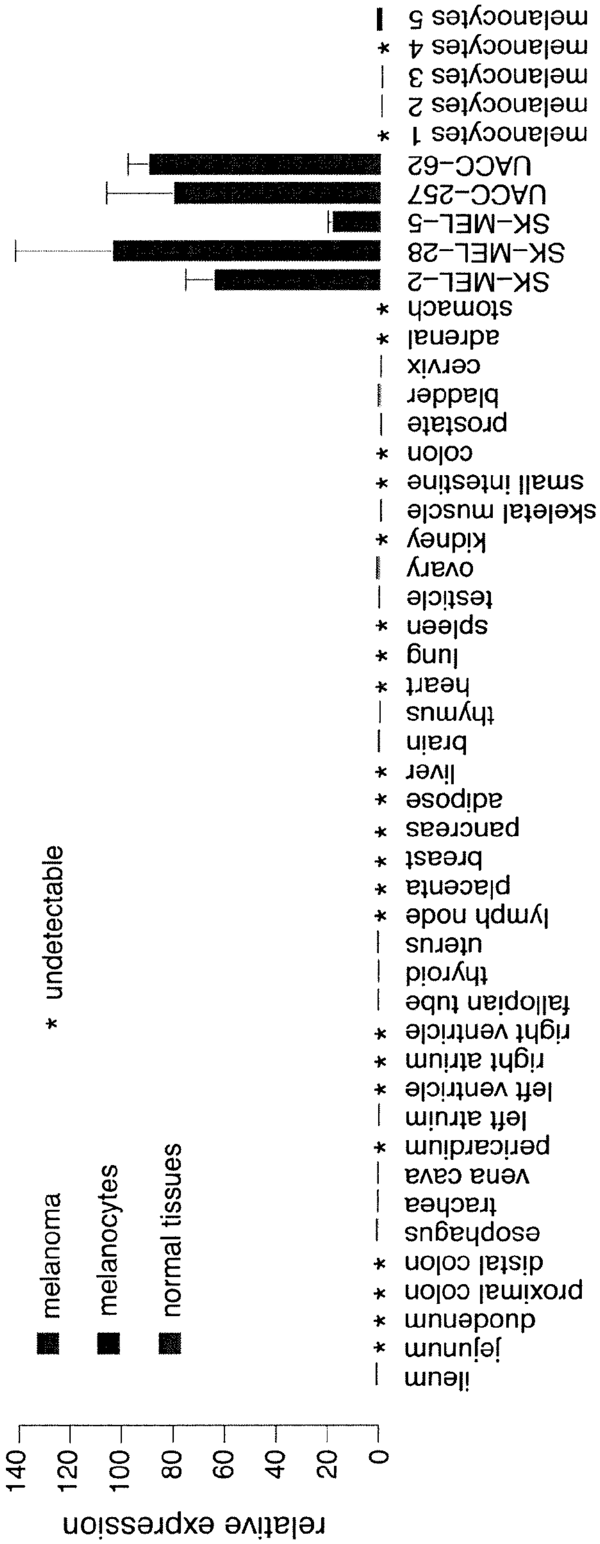
6
4
2
0
−2
−4
−6

Figure 5


melanoma
melanocytes
normal tissues
* undetectable
relative expression
0
20
40
60
80
100
120
140
ileum
jejunum
duodenum
proximal colon
distal colon
esophagus
trachea
vena cava
pericardium
left atrium
left ventricle
right atrium
right ventricle
fallopian tube
thyroid
uterus
lymph node
placenta
breast
pancreas
adipose
liver
brain
thymus
heart
lung
spleen
testicle
ovary
kidney
skeletal muscle
small intestine
colon
prostate
bladder
cervix
adrenal
stomach
SK-MEL-2
SK-MEL-28
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UACC-257
UACC-62
melanocytes 1
melanocytes 2
melanocytes 3
melanocytes 4
melanocytes 5

Figure 6

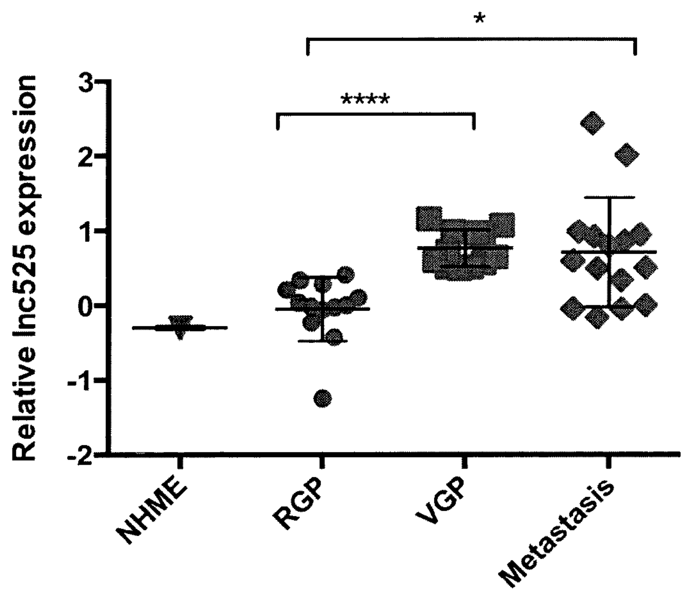


Figure 7

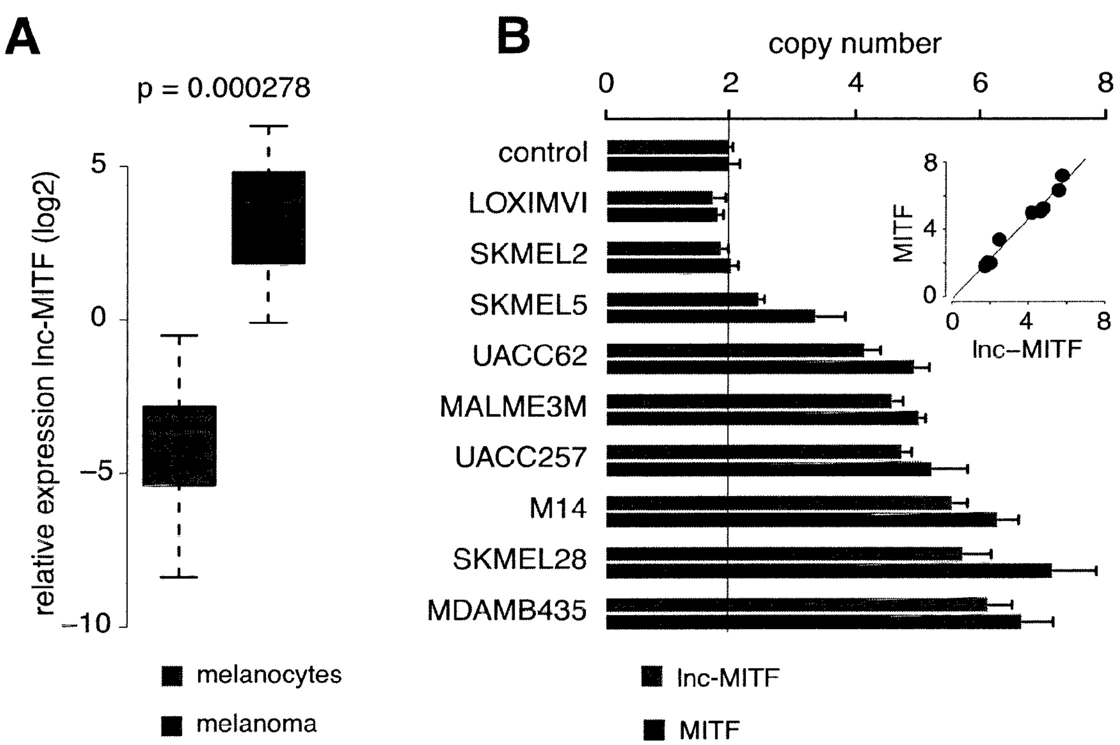


Figure 7 continued

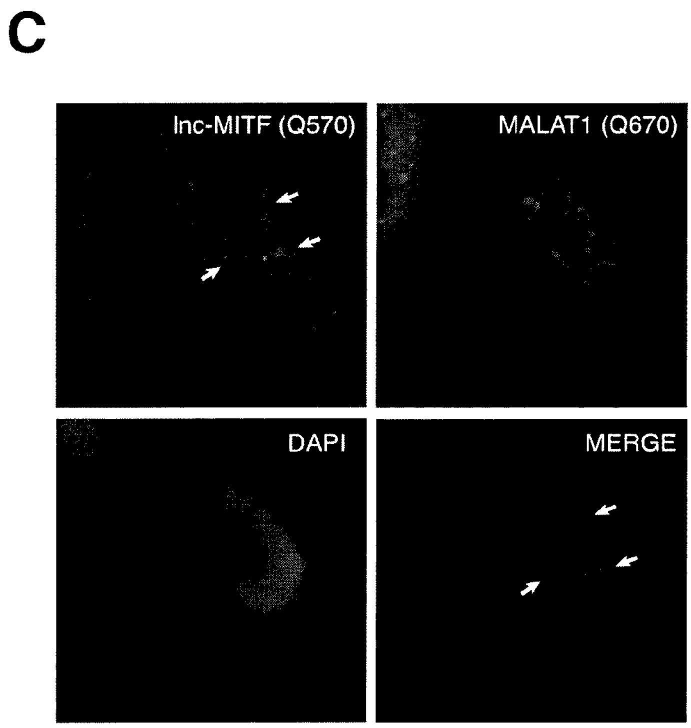


Figure 8

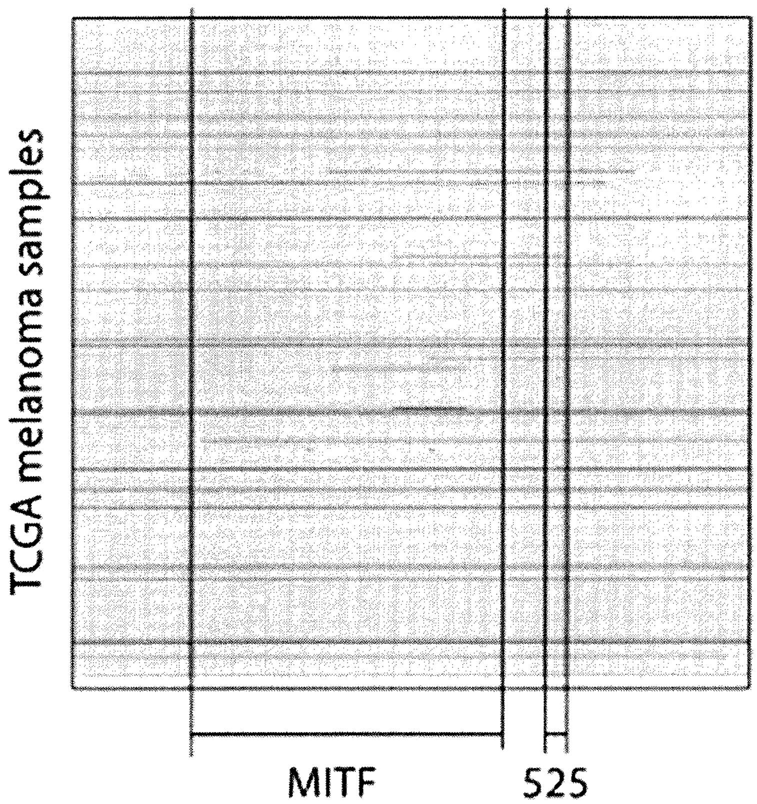
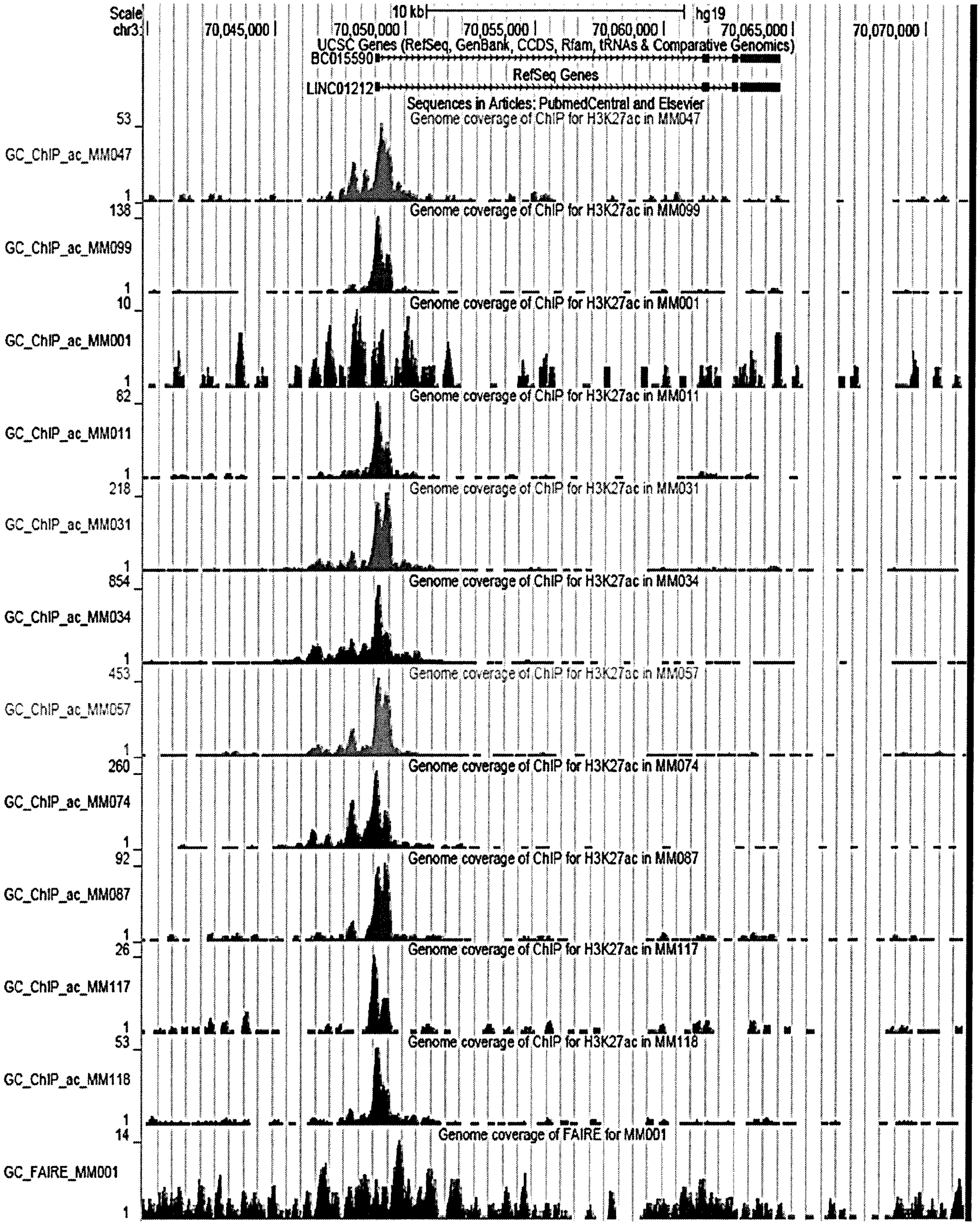


Figure 9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hg19
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70,060,000
70,065,000
70,070,000
UCSC Genes (RefSeq, GenBank, CCDS, Rfam, tRNAs & Comparative Genomics)
BC015590
RefSeq Genes
LINC01212
Sequences in Articles: PubmedCentral and Elsevier
Genome coverage of ChIP for H3K27ac in MM047
GC_ChIP_ac_MM047
Genome coverage of ChIP for H3K27ac in MM099
GC_ChIP_ac_MM099
Genome coverage of ChIP for H3K27ac in MM001
GC_ChIP_ac_MM001
Genome coverage of ChIP for H3K27ac in MM011
GC_ChIP_ac_MM011
Genome coverage of ChIP for H3K27ac in MM031
GC_ChIP_ac_MM031
Genome coverage of ChIP for H3K27ac in MM034
GC_ChIP_ac_MM034
Genome coverage of ChIP for H3K27ac in MM057
GC_ChIP_ac_MM057
Genome coverage of ChIP for H3K27ac in MM074
GC_ChIP_ac_MM074
Genome coverage of ChIP for H3K27ac in MM087
GC_ChIP_ac_MM087
Genome coverage of ChIP for H3K27ac in MM117
GC_ChIP_ac_MM117
Genome coverage of ChIP for H3K27ac in MM118
GC_ChIP_ac_MM118
Genome coverage of FAIRE for MM001
GC_FAIRE_MM001
53
138
10
82
218
854
453
260
92
26
53
14
1

Figure 9 continued

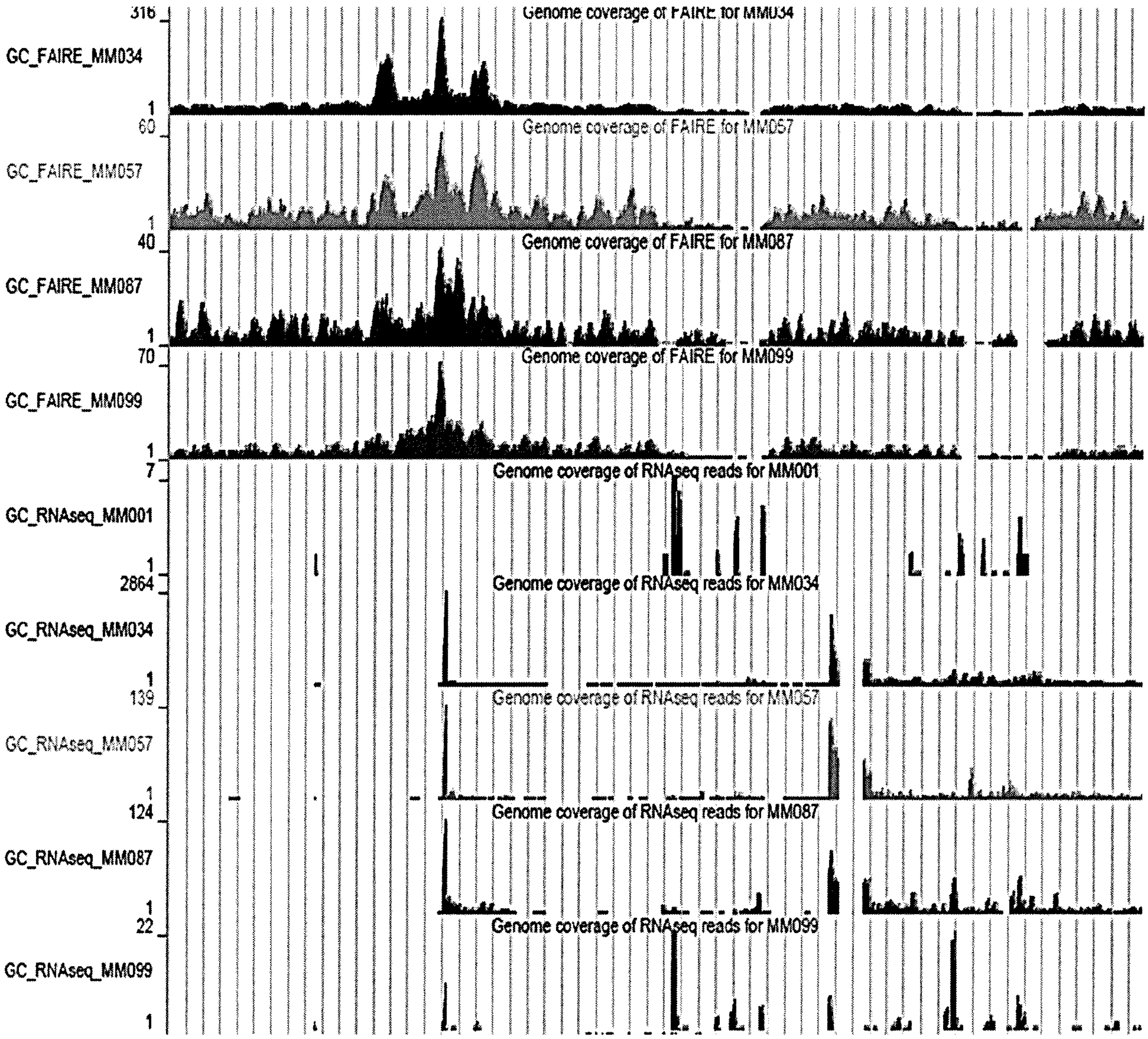


Figure 9 continued

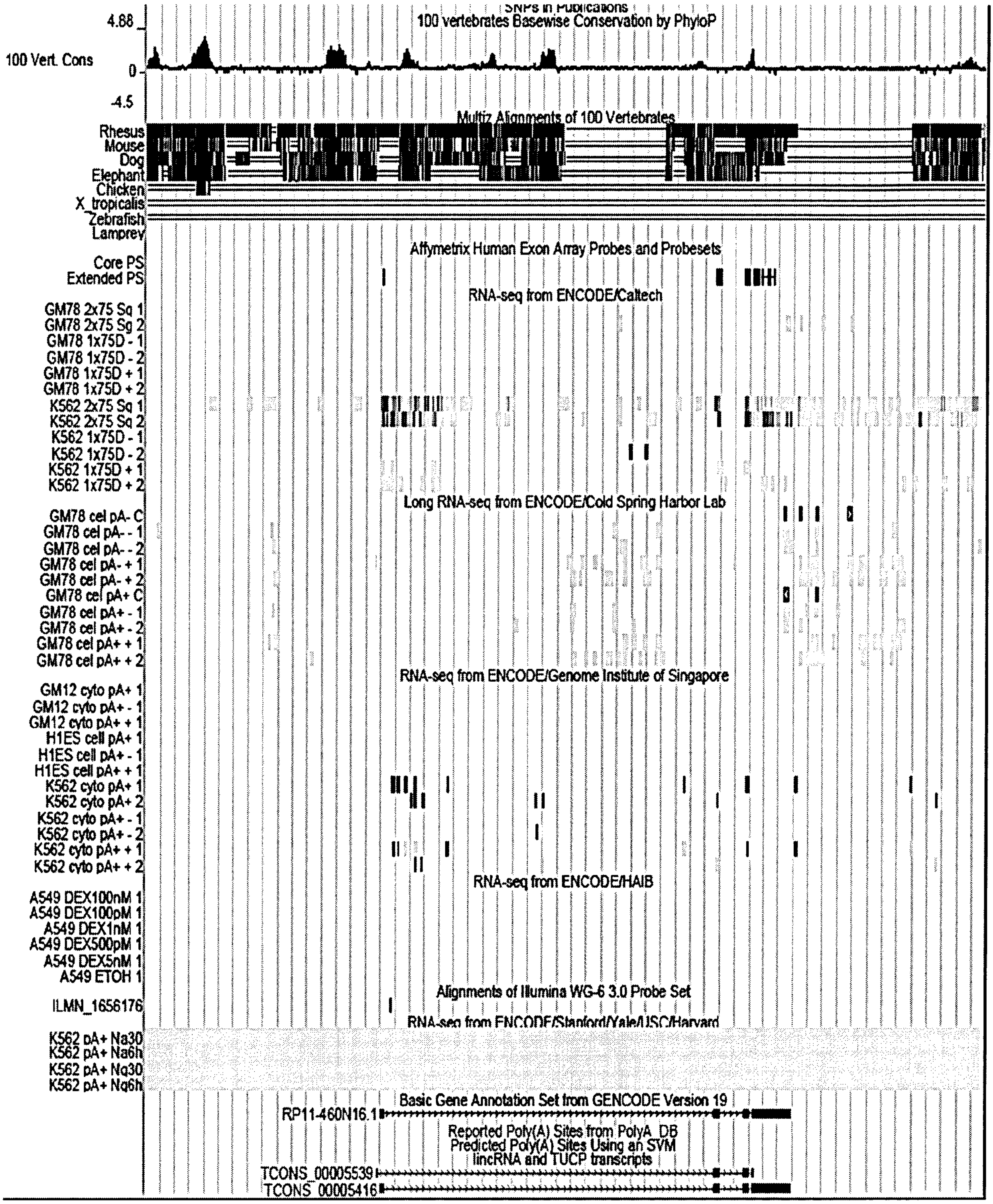


Figure 10

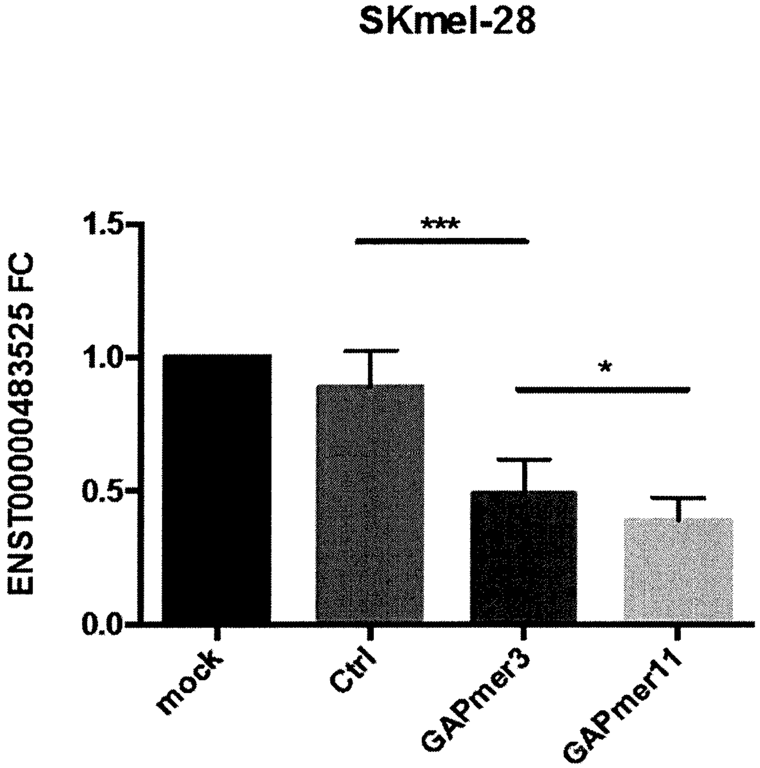


Figure 11

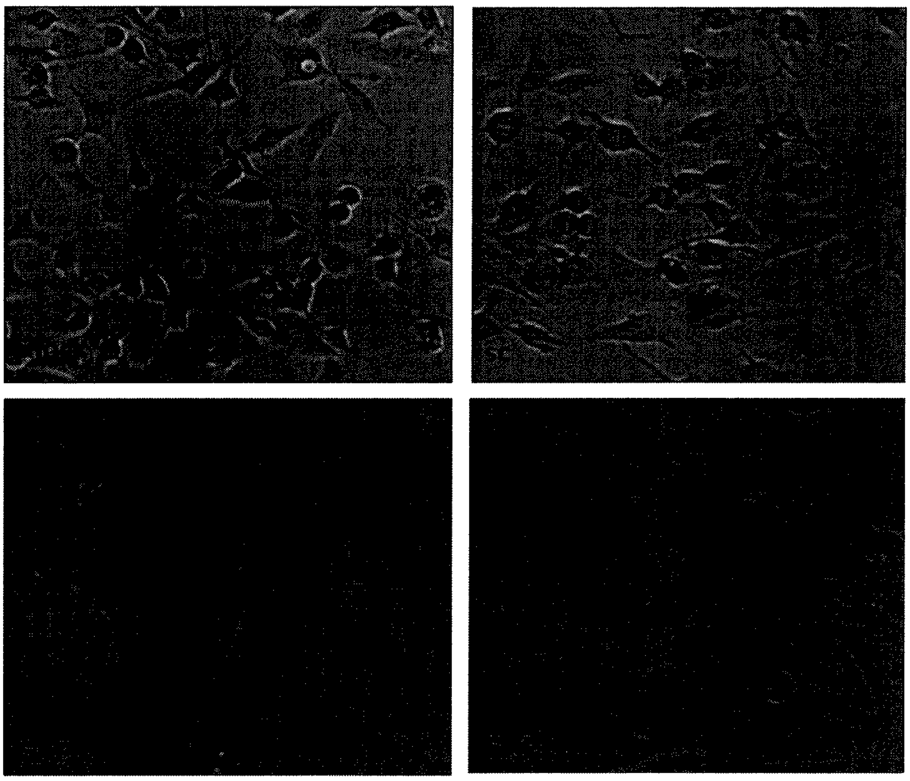
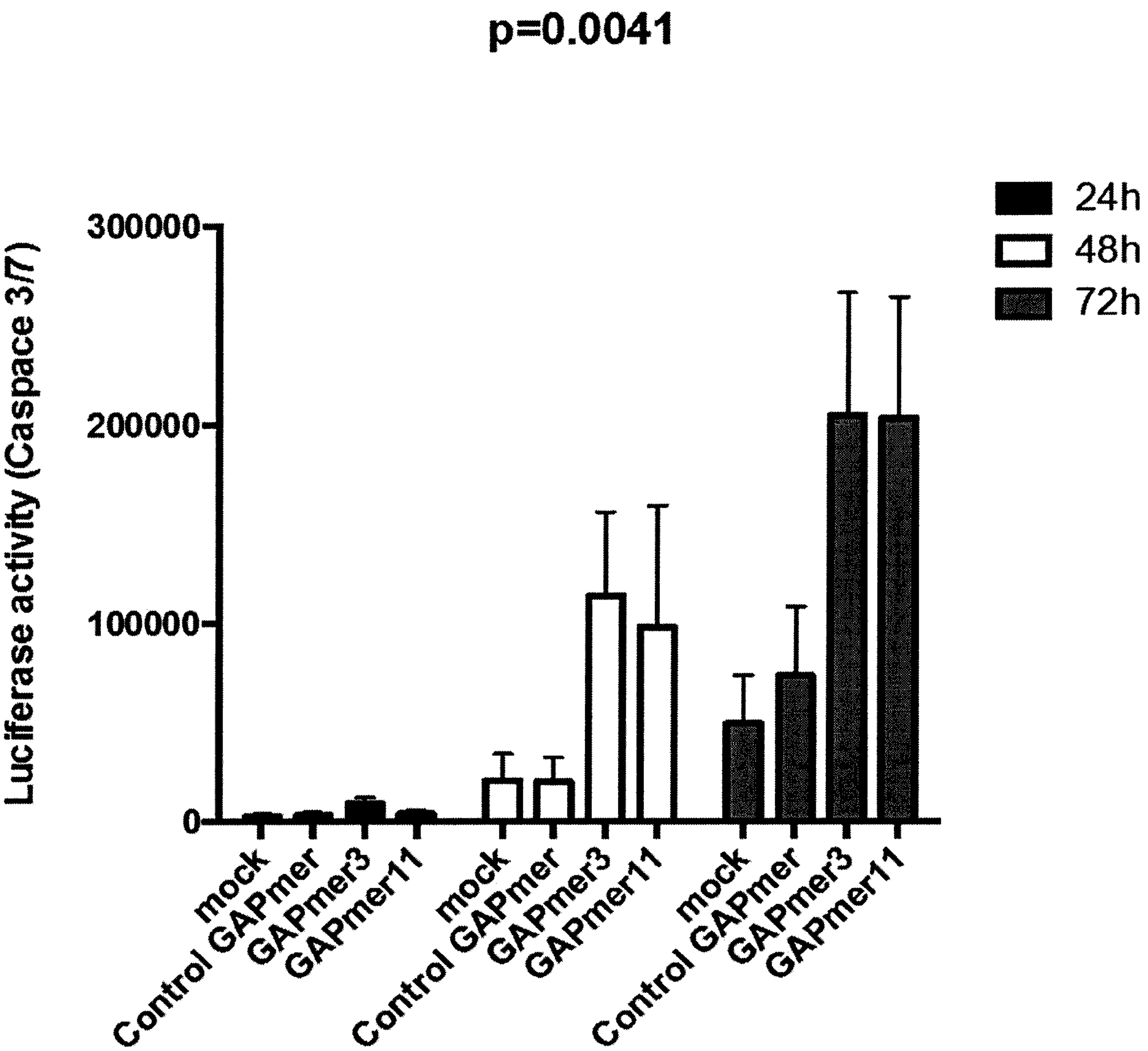


Figure 12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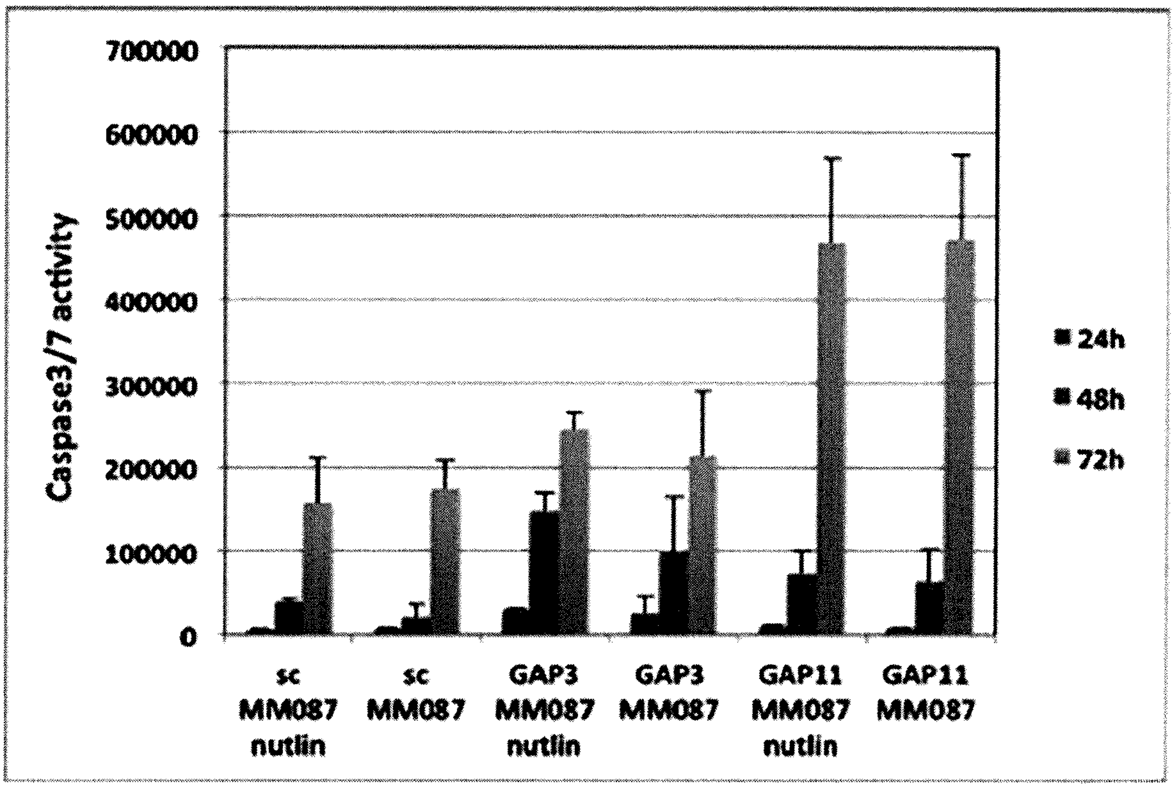
24h
48h
72h
Luciferase activity (Caspace 3/7)
300000
200000
100000
0
mock
Control GAPmer
GAPmer3
GAPmer11
mock
Control GAPmer
GAPmer3
GAPmer11
mock
Control GAPmer
GAPmer3
GAPmer11

Figure 13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600000
500000
400000
300000
200000
100000
0
Caspase3/7 activity
24h
48h
72h
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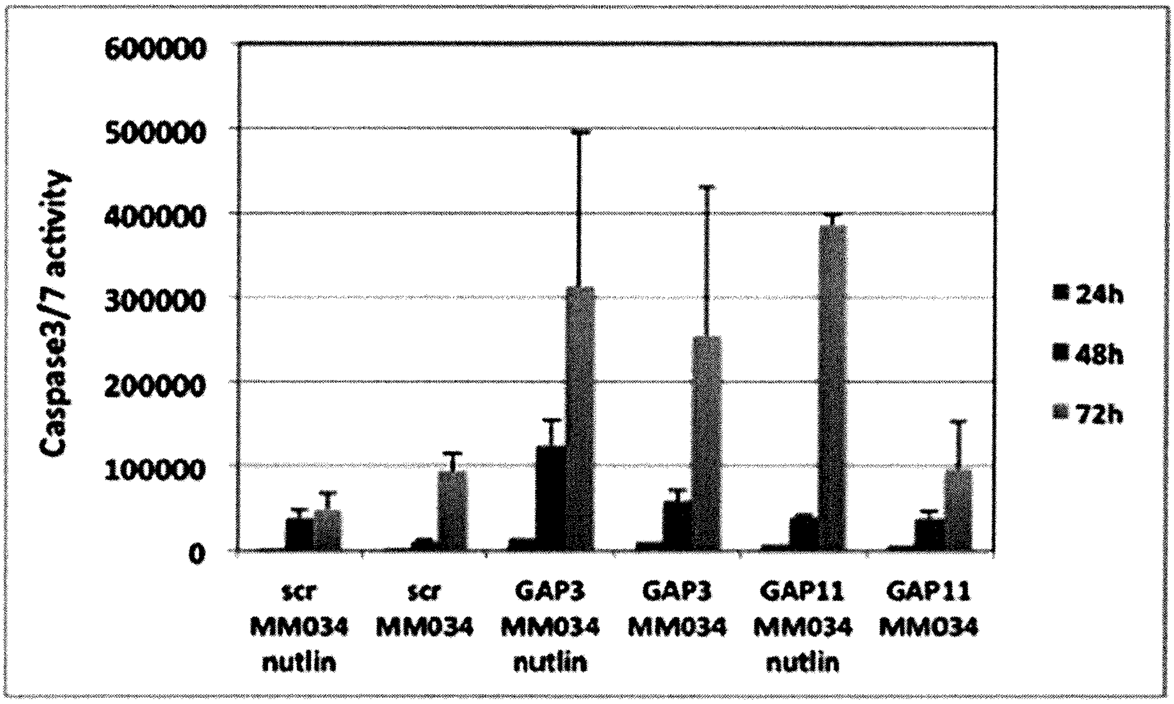
sc MM087
GAP3 MM087 nutlin
GAP3 MM087
GAP11 MM087 nutlin
GAP11 MM087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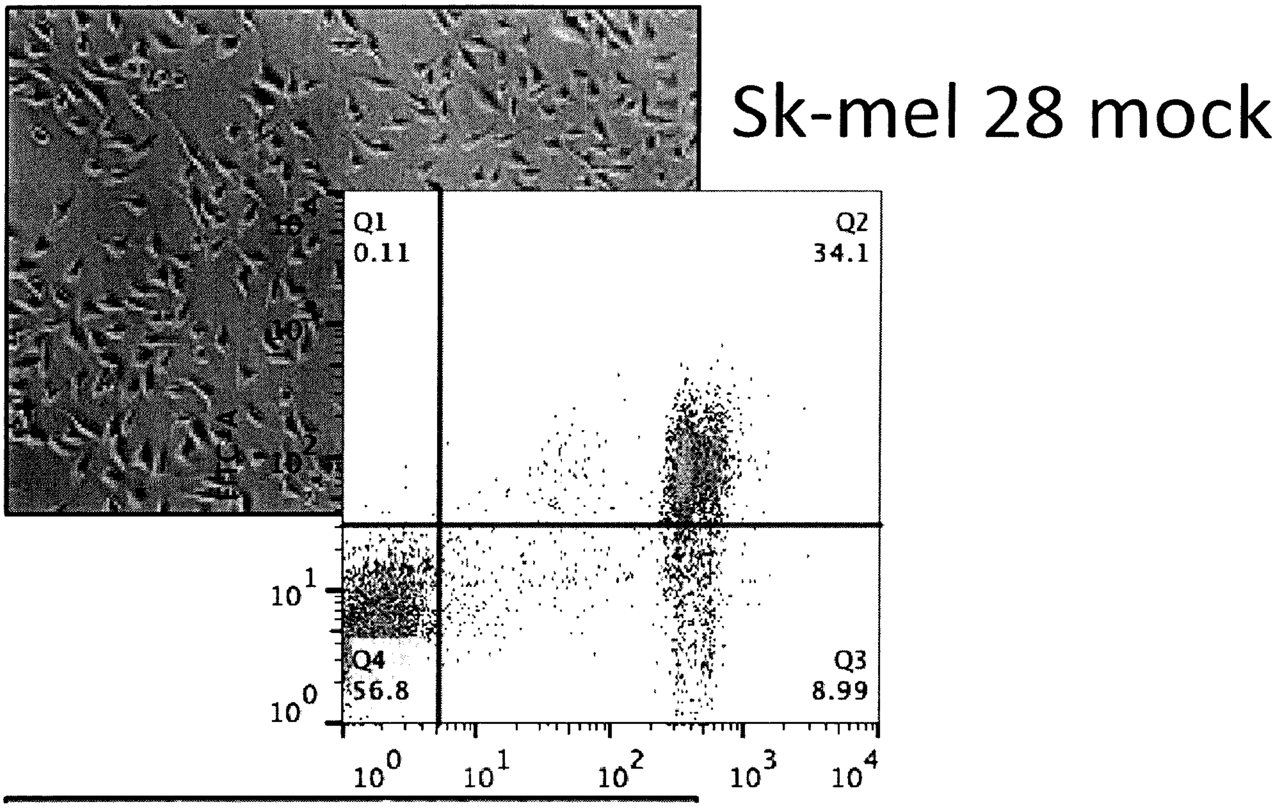
500000
400000
300000
200000
100000
0
Caspase3/7 activity
24h
48h
72h
scr MM034 nutlin
scr MM034
GAP3 MM034 nutlin
GAP3 MM034
GAP11 MM034 nutlin
GAP11 MM034

Figure 14


Sk-mel 28 mock
Q1
0.11
Q2
34.1
Q4
56.8
Q3
8.99

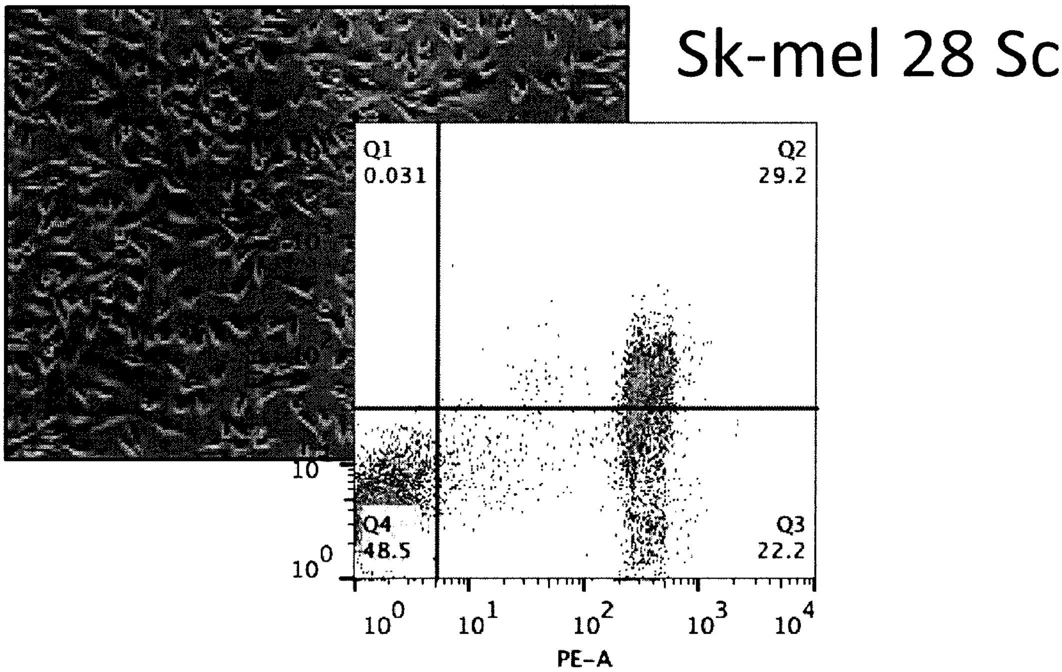
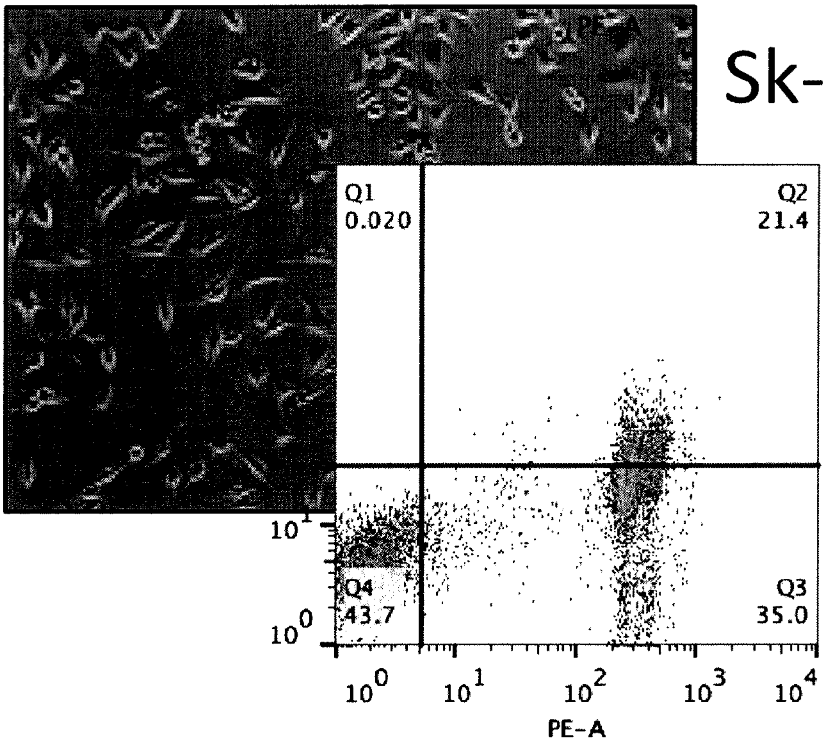
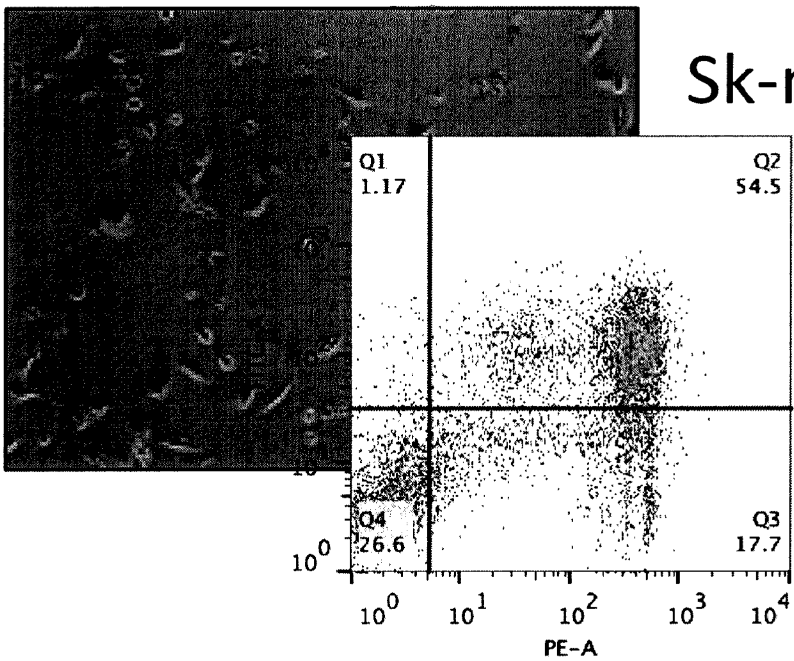

Sk-mel 28 Sc
Q1
0.031
Q2
29.2
Q4
48.5
Q3
22.2
PE-A

Figure 14 continued

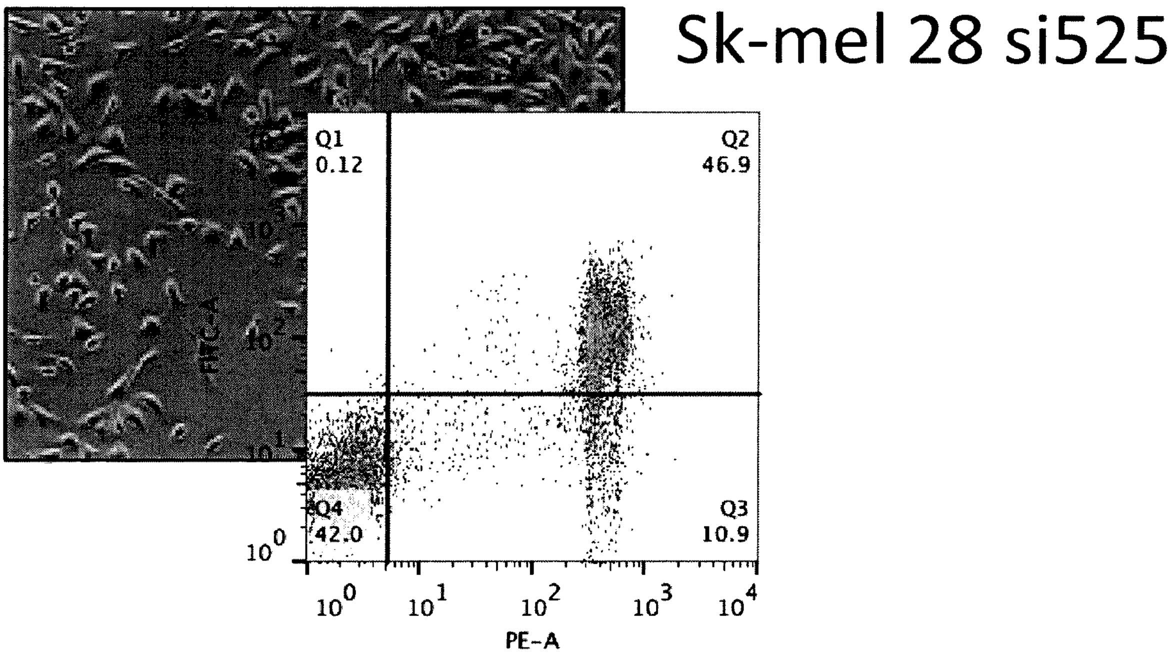


Sk-mel 28 sc



Sk-mel 28 GAP3

Figure 14 continued



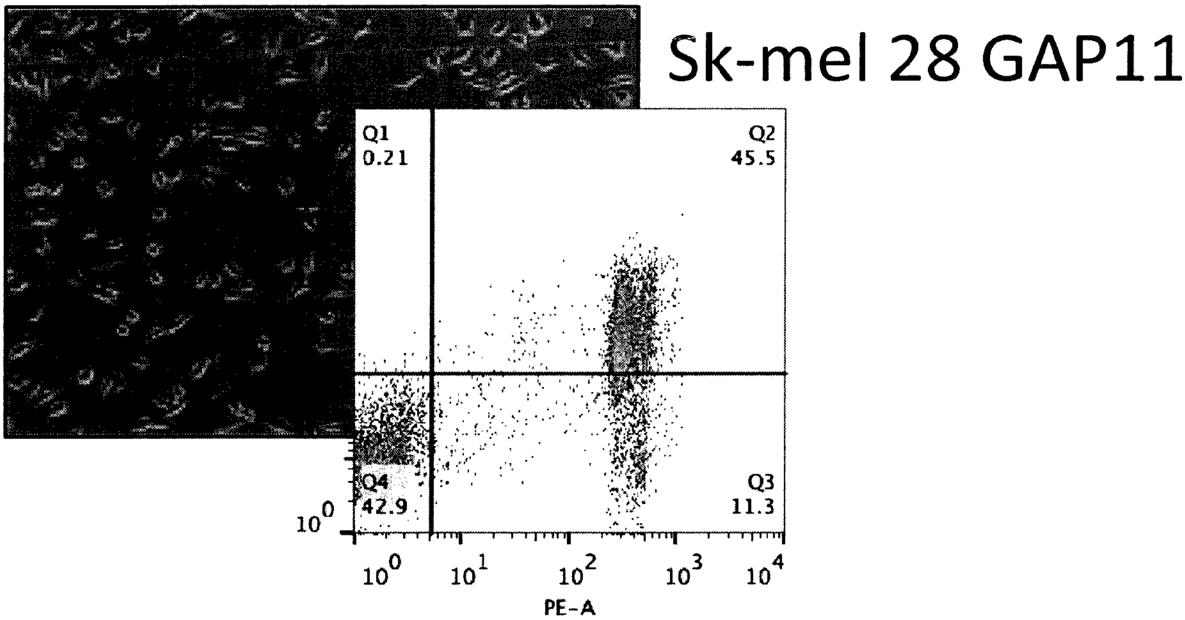
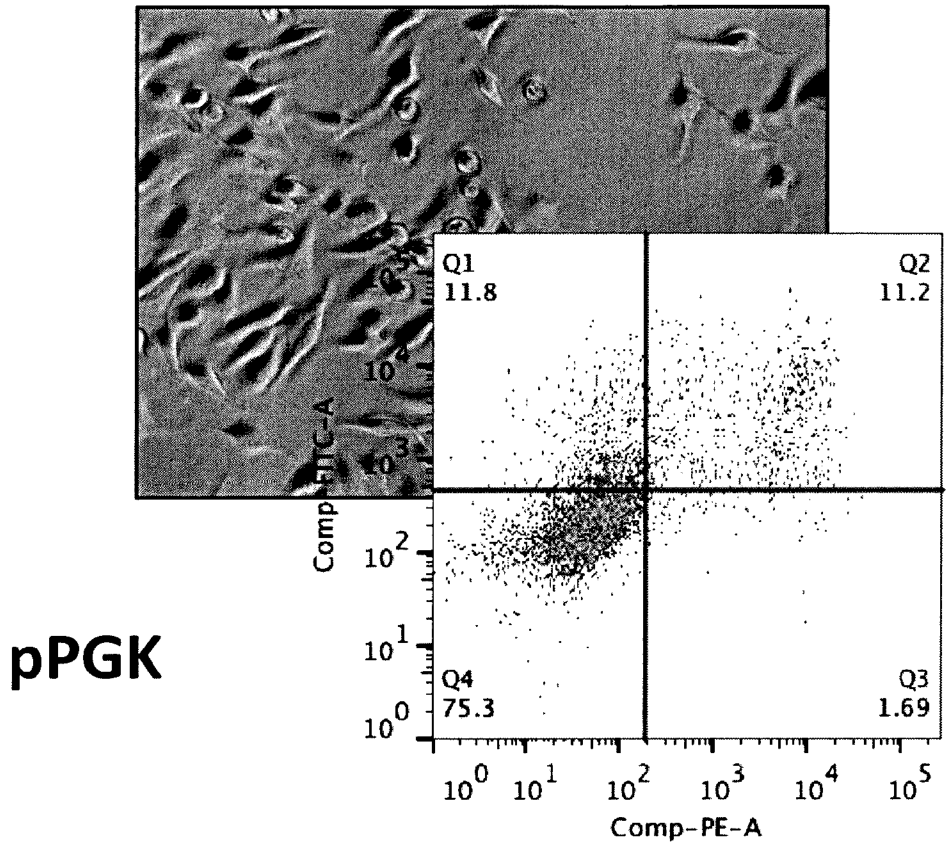


Figure 15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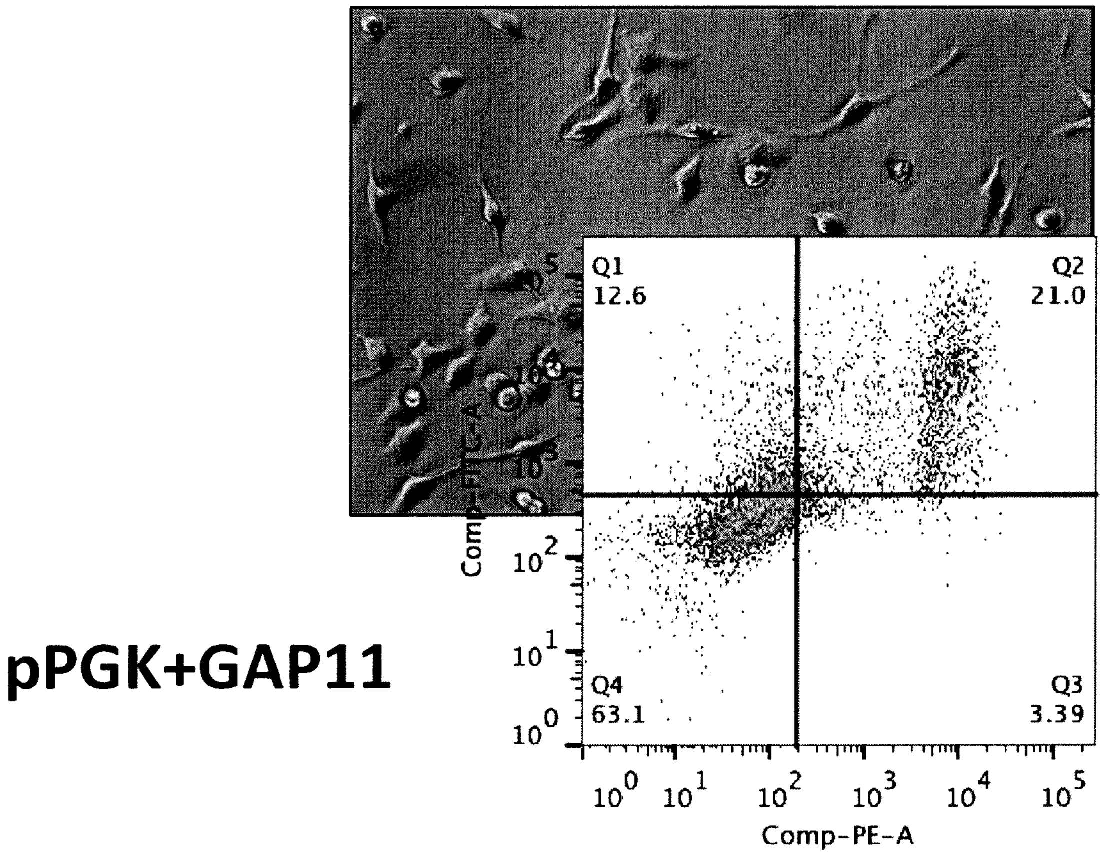
Q2
11.2
Q4
75.3
Q3
1.69
Comp-FITC-A
Comp-PE-A
pPGK


Q1
12.6
Q2
21.0
Q4
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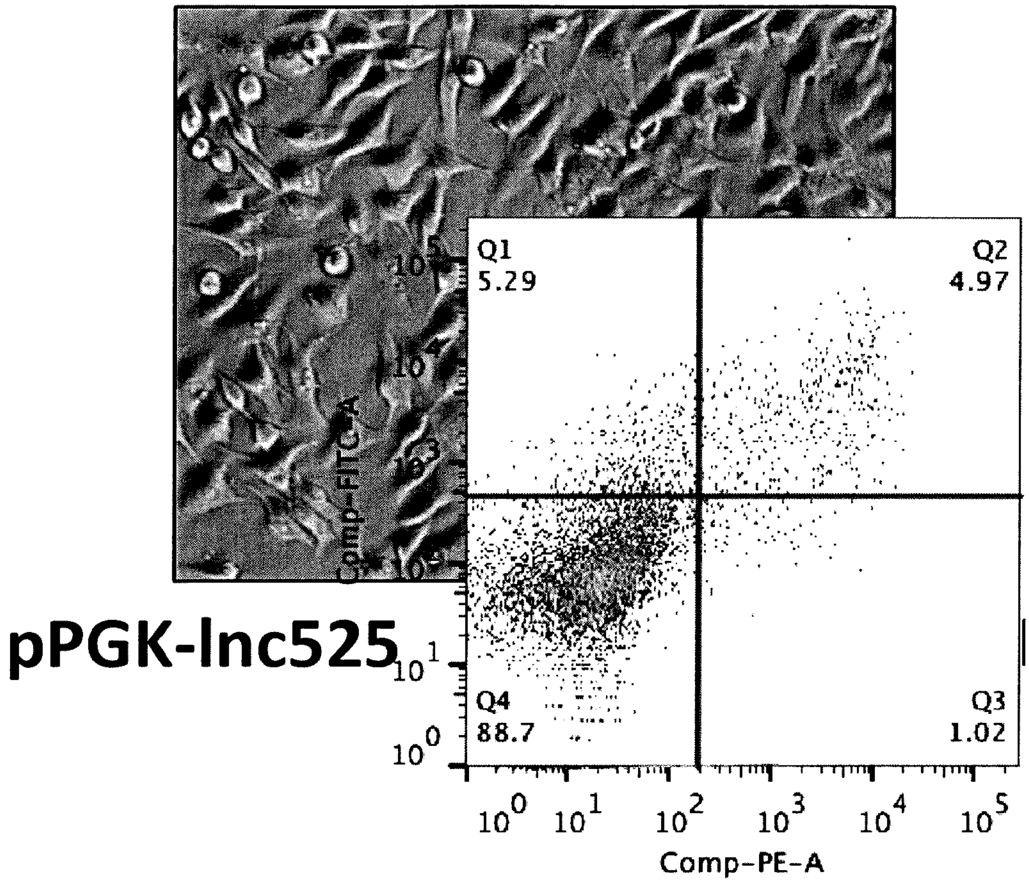
Q3
3.39
Comp-FITC-A
Comp-PE-A
pPGK+GAP11

Figure 15 continued



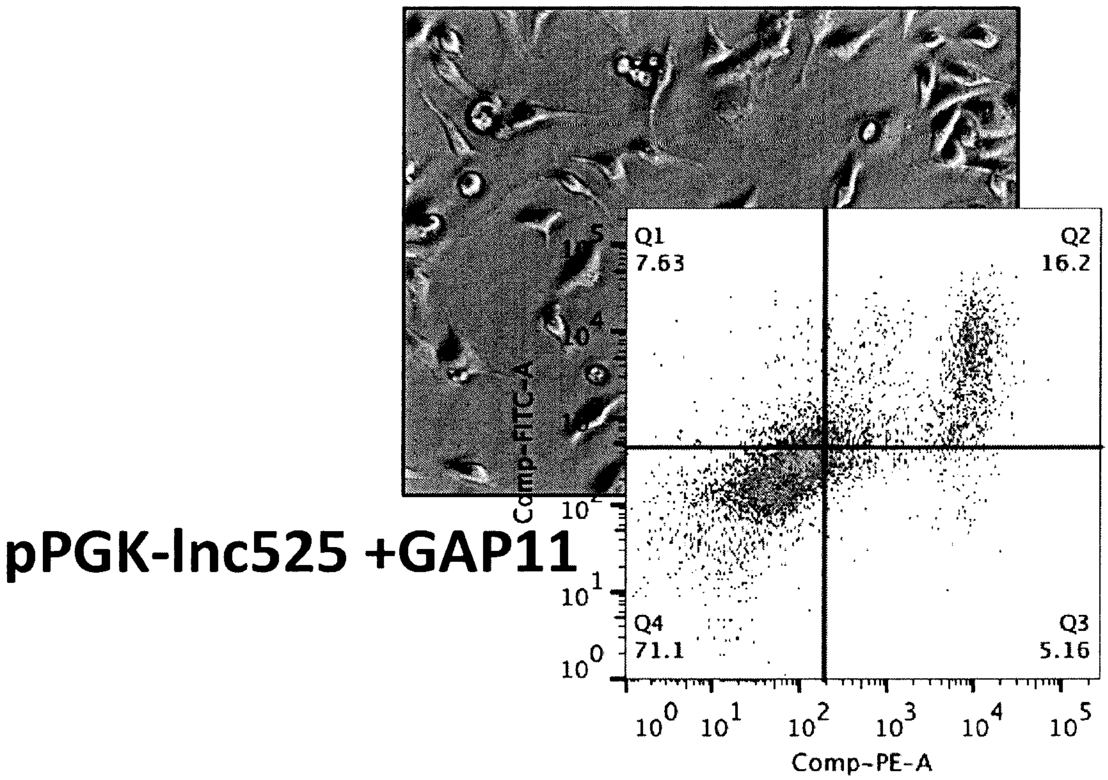
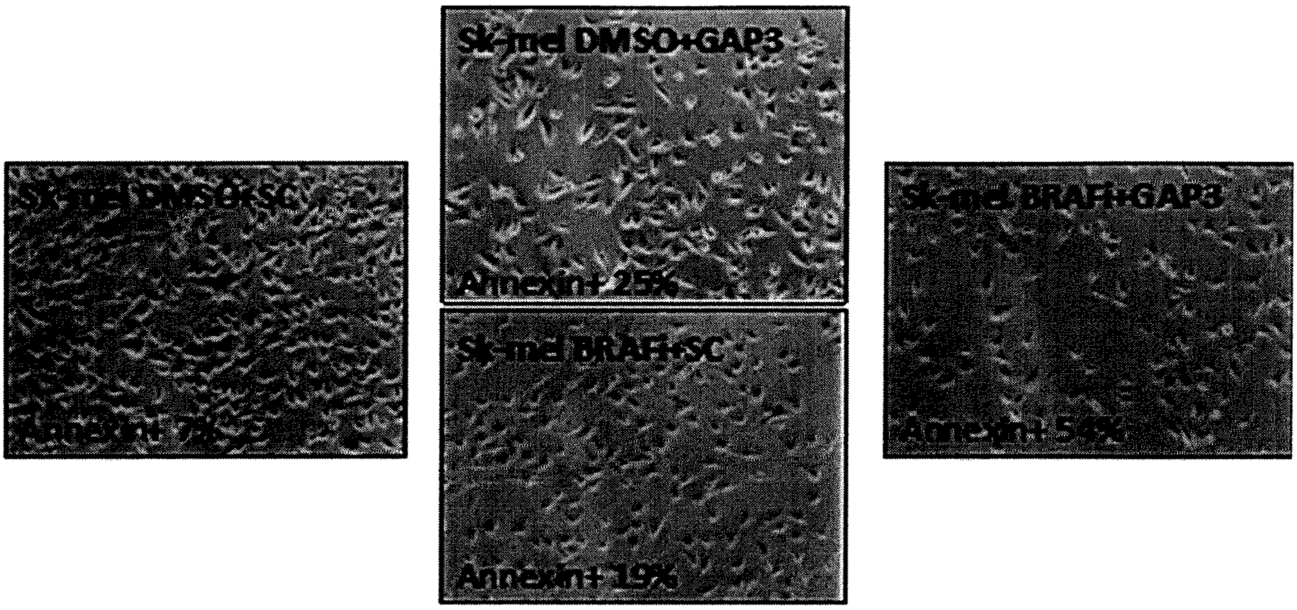


Figure 16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Annexin+ 25%
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Figure 17

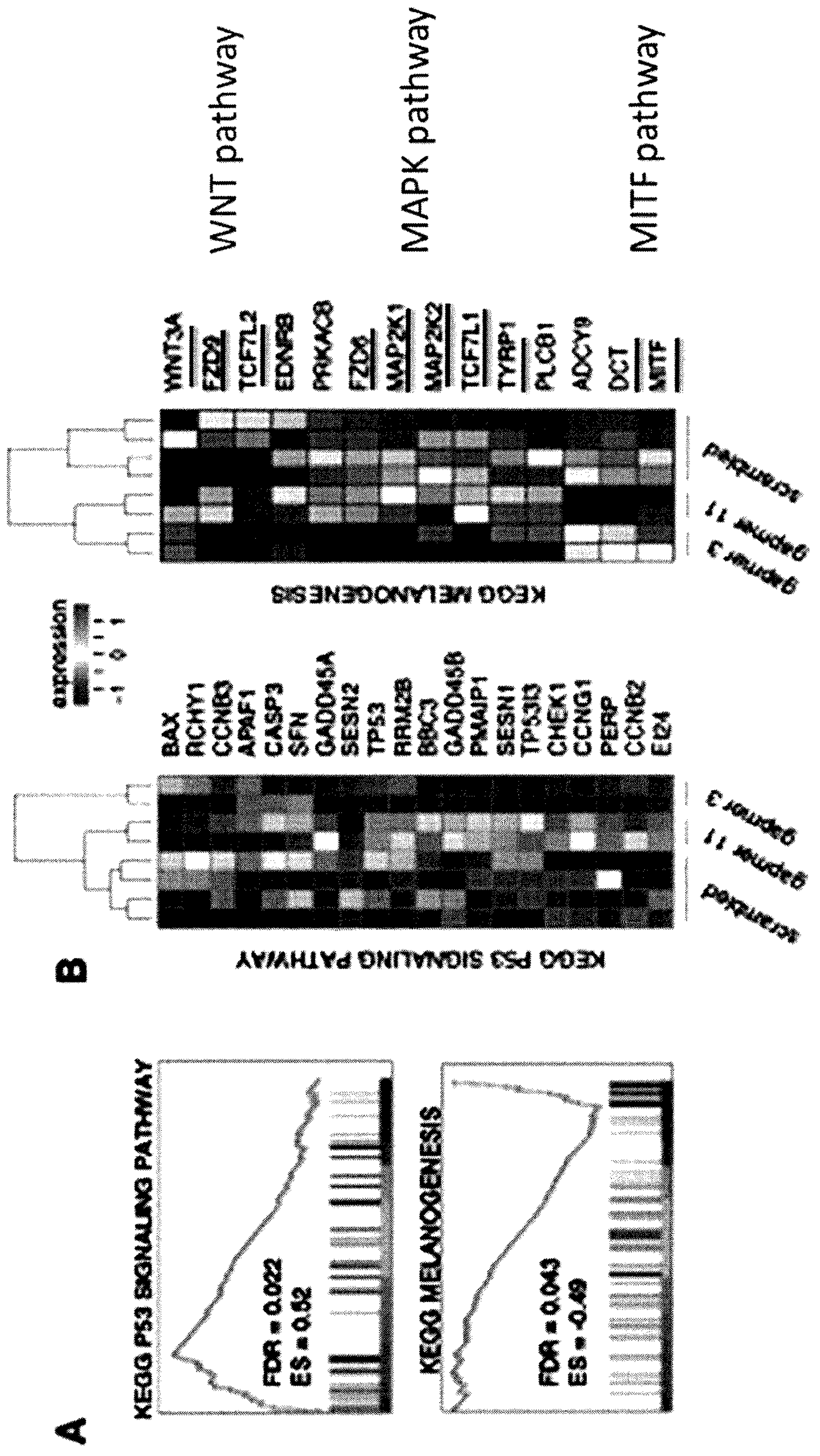
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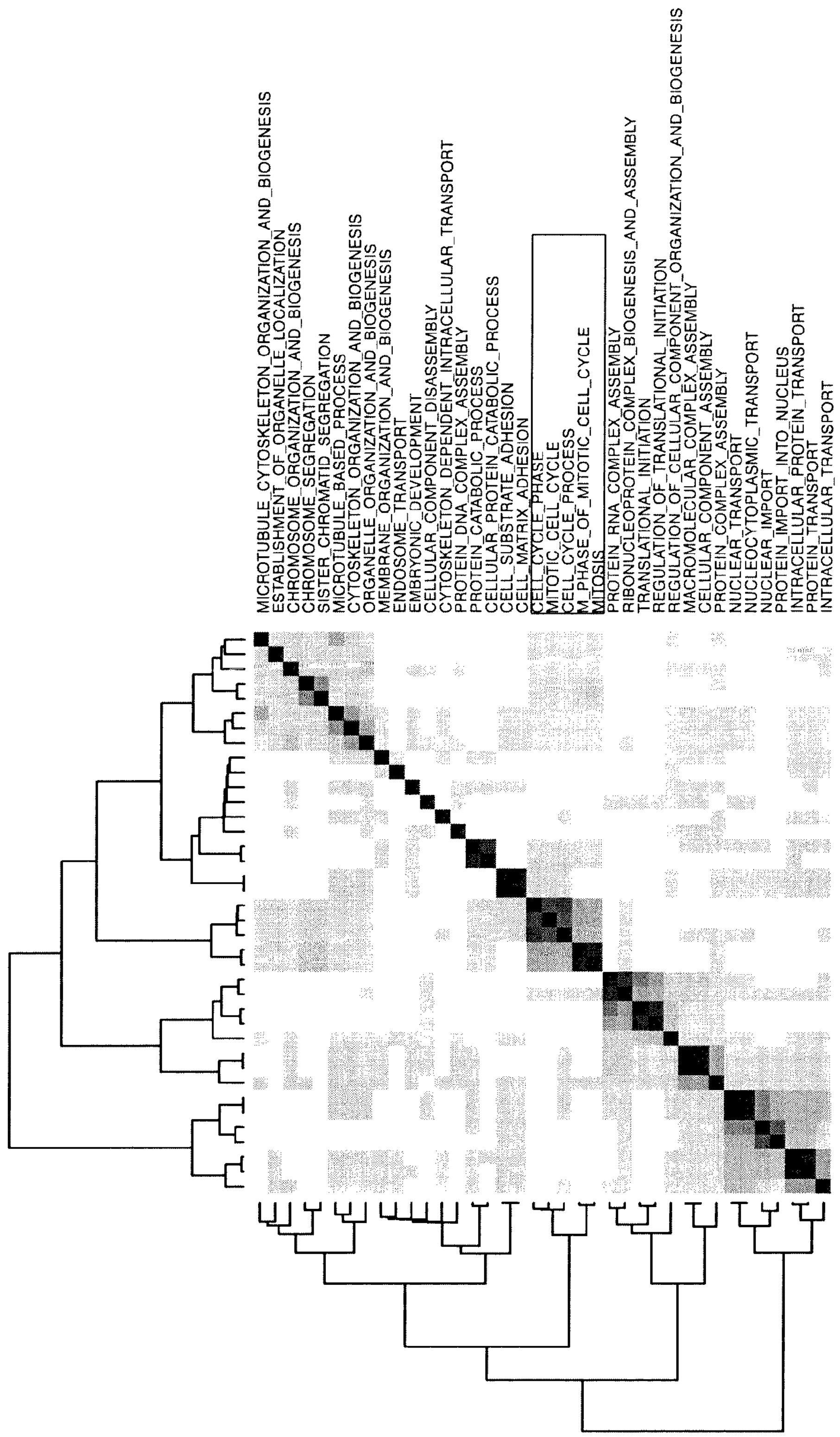
Figure 18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ENDOSOME_TRANSPORT
EMBRYONIC_DEVELOPMENT
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CYTOSKELETON_DEPENDENT_INTRACELLULAR_TRANSPORT
PROTEIN_DNA_COMPLEX_ASSEMBLY
PROTEIN_CATABOLIC_PROCESS
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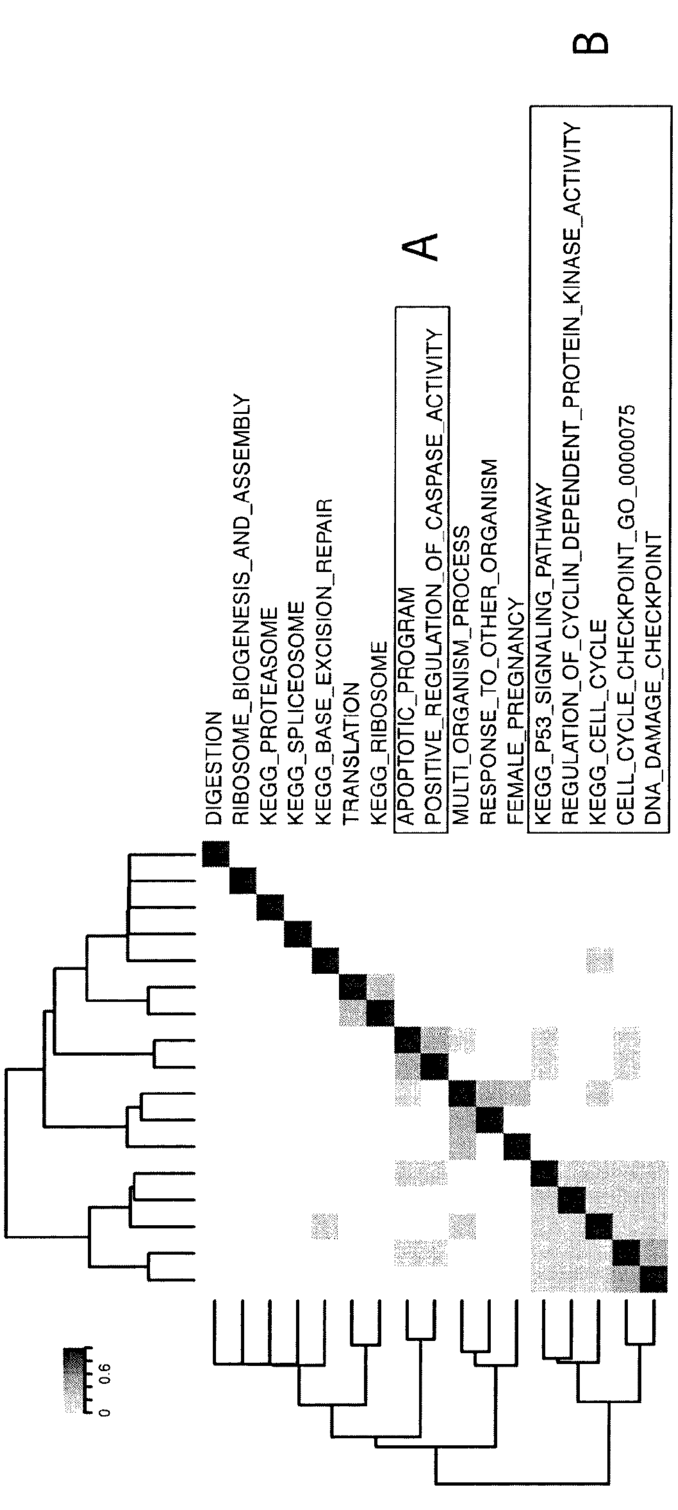
Figure 19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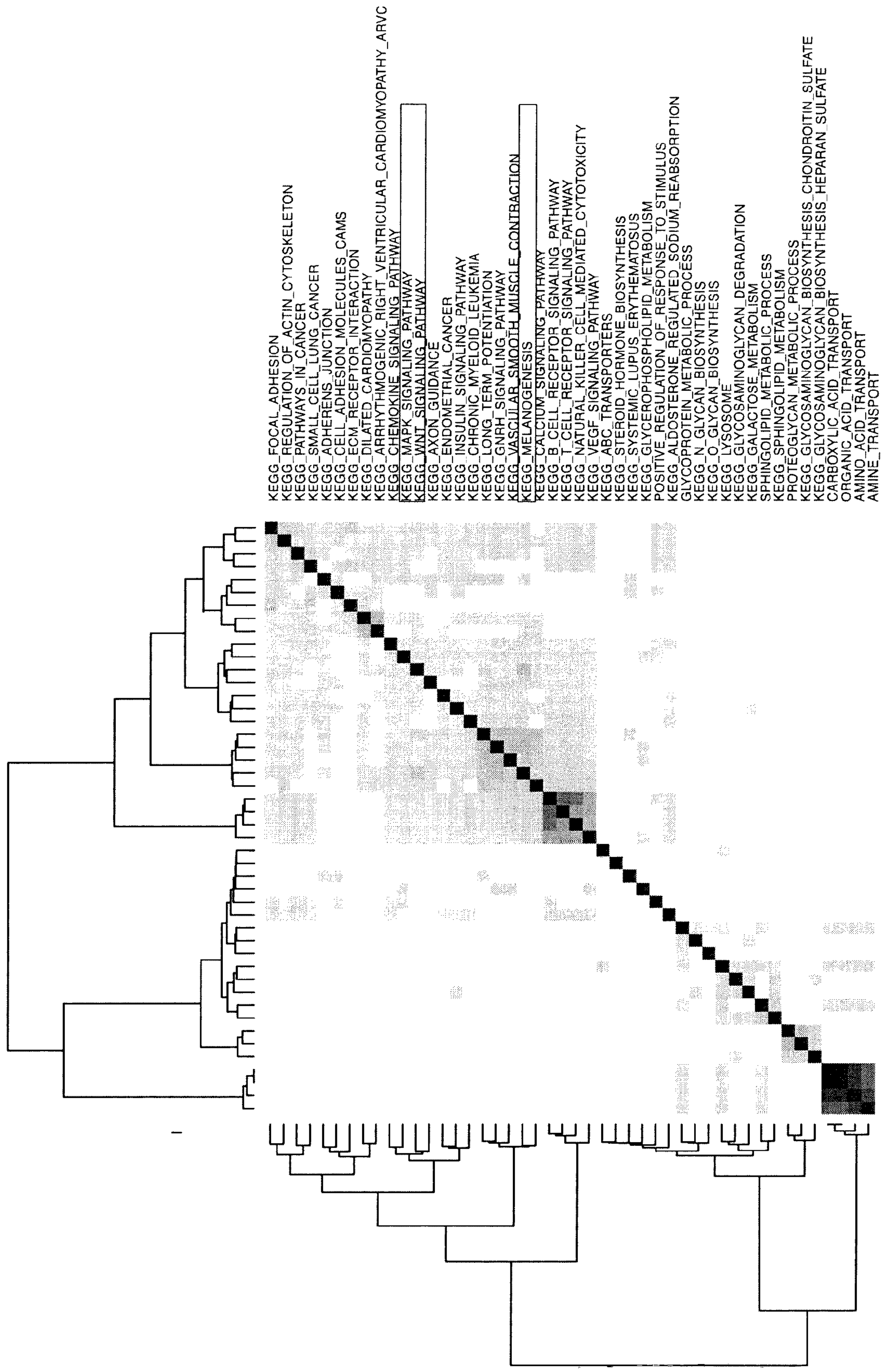
A
B

Figure 19 continued



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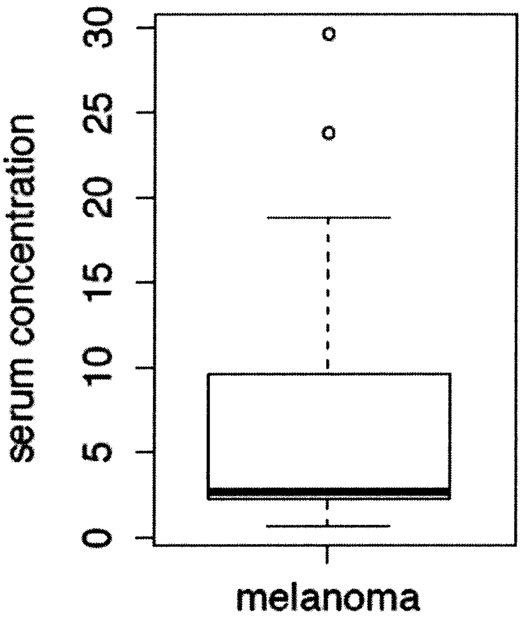

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