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## HOX transcription factors are potential targets and markers in malignant mesothelioma

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1 **HOX transcription factors are potential targets and markers in malignant**  
2 **mesothelioma**

3

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24

25 **Keywords:** Mesothelioma; *HOX* genes; HXR9; HOXB4; overall survival

26 **Abstract**

27

28 **Background** The *HOX* genes are a family of homeodomain-containing transcription  
29 factors that determine cellular identity during development and which are dys-  
30 regulated in some cancers. In this study we examined the expression and oncogenic  
31 function of *HOX* genes in mesothelioma, a cancer arising from the pleura or  
32 peritoneum which is associated with exposure to asbestos.

33 **Methods** We tested the sensitivity of the mesothelioma-derived lines MSTO-211H,  
34 NCI-H28, NCI-H2052, and NCI-H226 to HXR9, a peptide antagonist of HOX protein  
35 binding to its PBX co-factor. Apoptosis was measured using a FACS-based assay  
36 with Annexin, and *HOX* gene expression profiles were established using RT-QPCR  
37 on RNA extracted from cell lines and primary mesotheliomas. The *in vivo* efficacy of  
38 HXR9 was tested in a mouse MSTO-211H flank tumor xenograft model.

39 **Results** We show that *HOX* genes are significantly dysregulated in malignant  
40 mesothelioma. Targeting *HOX* genes with HXR9 caused apoptotic cell death in all of  
41 the mesothelioma-derived cell lines, and prevented the growth of mesothelioma  
42 tumors in a mouse xenograft model. Furthermore, the sensitivity of these lines to  
43 HXR9 correlated with the relative expression of *HOX* genes that have either an  
44 oncogenic or tumor suppressive function in cancer. The analysis of *HOX* expression  
45 in primary mesothelioma tumors indicated that these cells could also be sensitive to  
46 the disruption of HOX activity by HXR9, and that the expression of *HOXB4* is  
47 strongly associated with overall survival.

48 **Conclusion** *HOX* genes are a potential therapeutic target in mesothelioma, and  
49 *HOXB4* expression correlates with overall survival.

50

51

## 52 **Background**

53

54 The *HOX* genes are a family of transcription factors characterized by highly  
55 conserved DNA- and co-factor binding domains. This conservation has been driven  
56 by their roles in some of the most fundamental patterning events that underlie early  
57 development[1]. Most notable of these is the patterning of the anterior to posterior  
58 axis, for which a precise spatial and temporal order in the expression of *HOX* genes is  
59 required. This is achieved in part through a chromosomal arrangement whereby *HOX*  
60 genes are present in closely linked clusters allowing the sharing of common enhancer  
61 regions. In mammals there are four such clusters (A-D), containing a total of 39 *HOX*  
62 genes[1]. The relative position of each *HOX* gene 3' to 5' within the cluster is  
63 reflected in a number of key attributes, including the spatial and temporal order of  
64 expression, whereby the 3' most genes are expressed earlier than their 5' neighbors.  
65 The nomenclature of the *HOX* genes reflects this precise chromosomal ordering, with  
66 members of each cluster being numbered with respect to the 3' end, thus for example,  
67 the 3' most member of cluster B is *HOXB1*[2].

68

69 The 3' to 5' order of *HOX* genes is reflected not only in their expression patterns but  
70 also in their DNA binding specificities and co-factor interactions. For example, the  
71 products of the 3' *HOX* genes (1 to 9) bind to another transcription factor, PBX,  
72 which modifies their binding specificity to DNA[3], influences their  
73 nucleocytoplasmic distribution[3], and also determines whether a HOX protein will  
74 activate or repress transcription of downstream target genes[4]. This interaction with  
75 PBX is mediated through a highly conserved hexapeptide region on HOX proteins 1-9

76 that binds to a cleft in PBX[3, 5]. Once PBX has bound it can recruit other specific  
77 co-factors, including MEIS, which can then further modify HOX activity[6].

78  
79 Although *HOX* genes were initially characterized as key developmental genes, they  
80 also function in adult stem cells to promote proliferation[7], and subsequently in their  
81 progeny to confer lineage-specific identities[8]. Furthermore, *HOX* genes are strongly  
82 dys-regulated in cancer, and generally exhibit greatly increased expression. This  
83 differential change in expression in cancer may reflect the apparent ability of some  
84 *HOX* genes to function as tumor suppressors and some as oncogenes. Thus for  
85 example, *HOXA5* acts as a tumor suppressor in breast cancer by stabilizing P53[9],  
86 whilst forced expression of *HOXB6* can immortalize fibroblast cells[10]. Further  
87 examples of this phenomenon are listed in Table 1.

88  
89 The dys-regulation of *HOX* genes has been demonstrated in a range of cancers, and in  
90 some it has been shown to be a potential therapeutic target through the use of a  
91 peptide, HXR9. HXR9 prevents PBX binding to HOX and triggers apoptosis in  
92 malignant cells, whilst sparing normal adult cells[11-17]. Although these studies  
93 include non-small cell lung cancer (NSCLC)[16], they do not encompass  
94 mesothelioma, a malignancy of the mesothelium cells which is most frequently found  
95 in the lung and is associated with long term exposure to asbestos[18]. Mesothelioma  
96 has limited treatment options and generally a very poor prognosis[18], and therefore  
97 finding novel therapeutic approaches in this disease is an important goal. In this study  
98 we show that *HOX* dys-regulation is present in cell lines derived from mesothelioma,  
99 and in primary tumors, usually with a significant increase in the expression of those  
100 *HOX* genes that behave as oncogenes. Furthermore, antagonism of the HOX / PBX

101 interaction in these cell lines triggers apoptosis, with malignant cells generally being  
102 considerably more sensitive to HXR9 than cells derived from non-malignant  
103 mesothelium cells.

104

## 105 **Materials and Methods**

106

### 107 *Cell lines and culture*

108 The cell lines used in this study are listed in Table 2. They were obtained from the  
109 ATCC through LGC Standards Ltd (UK), and were cultured according to the  
110 instructions on the LGC Standards website.

111

### 112 *Synthesis of HXR9 and CXR9 peptides*

113 HXR9 is an 18 amino acid peptide consisting of the previously identified hexapeptide  
114 sequence that can bind to PBX and nine C-terminal arginine residues (R9) that  
115 facilitate cell entry. The N-terminal and C-terminal amino bonds are in the D-isomer  
116 conformation, which has previously been shown to extend the half-life of the peptide  
117 to 12 hours in human serum[14]. CXR9 is a control peptide that lacks a functional  
118 hexapeptide sequence but which includes the R9 sequence. The sequences of these  
119 peptides have been published previously[13]. All peptides were synthesized using  
120 conventional column based chemistry and purified to at least 80% (Biosynthesis Inc.,  
121 USA).

122

### 123 *Imaging of cell cultures*

124 Cells were plated in 6-well plates using 2 ml of medium and allowed to recover for at  
125 least 24 hours. When approximately 60% confluent, cells were treated with the active  
126 peptide HXR9 (60  $\mu$ M) or the control peptide CXR9 (60  $\mu$ M) for 3 hours.

127

#### 128 *Immunohistochemistry for HOXA4, HOXA9, and HOXB4*

129 Expression of HOXA4, HOXA9, and HOXB4 in mesothelioma and normal  
130 mesothelium tissue was investigated using 3  $\mu$ m-thick, formalin fixed, paraffin  
131 embedded tissue array sections (MS081, US Biomax, Rockville, MD, USA).  
132 Immunohistochemical analysis was performed using a monoclonal rabbit anti-  
133 HOXB4 antibody (ab676093, 1:100 dilution, Abcam, Cambridge, UK), a polyclonal  
134 rabbit anti-HOXA4 antibody (ab131049, 1:500 dilution, Abcam, Cambridge, UK),  
135 and a polyclonal rabbit anti-HOXA9 antibody (ab191178, 1:75 dilution, Abcam,  
136 Cambridge, UK). The ABC detection method with peroxidase block  
137 (DakoCytomation) was used for all of these primary antibodies. Antigen retrieval was  
138 performed using pH 9.0 Tris/EDTA buffer (DakoCytomation) and heating in a  
139 microwave for 23 minutes.

140

#### 141 *Analysis of cell death and apoptosis*

142 Cells were treated with HXR9 or CXR9 as described above. Cell viability was  
143 assessed using the MTS assay (Promega) according to the manufacturer's  
144 instructions. Cells were harvested by incubating in trypsin-EDTA (Sigma) at 37°C  
145 until detached and dissociated. Apoptotic cells were identified using flow cytometry  
146 (Beckman Coulter Epics XL Flow) and the Annexin V-PE apoptosis detection kit (BD  
147 Pharmingen) as described by the manufacturer's protocol. Caspase-3 activity was

148 measured using the EnzCheck Caspase-3 Assay Kit (Molecular Probes), using the  
149 protocol defined by the manufacturer.

150

#### 151 *RNA purification and reverse transcription*

152 Total RNA was isolated from cells using the RNeasy Plus Mini Kit (Qiagen) by  
153 following the manufacturer's protocol. The RNA was denatured by heating to 65°C  
154 for 5 minutes. cDNA was synthesized from RNA using the Cloned AMV First Strand  
155 Synthesis Kit (Invitrogen) according to the manufacturer's instructions.

156

#### 157 *Quantitative PCR*

158 Quantitative PCR was performed using the Stratagene MX3005P real-time PCR  
159 machine and the Brilliant SYBR Green QPCR Master Mix (Stratagene). The  
160 following primers were designed to facilitate the unique amplification of *β-actin*, *c-*

161 *Fos*, and each *HOX* gene:

162 HsBeta-ActinF: 5' ATGTACCCTGGCATTGCCGAC 3'  
163 HsBeta-ActinR: 5' GACTCGTCATACTCCTGCTTG 3'  
164 HscFos1F: 5' CCAACCTGCTGAAGGAGAAG 3'  
165 HscFos1R: 5' GCTGCTGATGCTCTTGACAG 3'  
166 HsHOXA1F: 5' CTGGCCCTGGCTACGTATAA 3'  
167 HsHOXA1R: 5' TCCAACCTTCCCTGTTTTGG 3'  
168 HsHOXA4F: 5' CCCTGGATGAAGAAGATCCA 3'  
169 HsHOXA4R: 5' AATTGGAGGATCGCATCTTG 3'  
170 HsHOXA5F: 5' CCGGAGAATGAAGTGGAAAA 3'  
171 HsHOXA5R: 5' ACGAGAACAGGGCTTCTTCA 3'  
172 HsHOXA9F: 5' AATAACCCAGCAGCCAACCTG 3'  
173 HsHOXA9R: 5' ATTTTCATCCTGCGGTTCTG 3'  
174 HsHOXB3F: 5' TATGGCCTCAACCACCTTTC 3'  
175 HsHOXB3R: 5' AAGCCTGGGTACCACCTTCT 3'  
176 HsHOXB4F: 5' TCTTGGAGCTGGAGAAGGAA 3'  
177 HsHOXB4R: 5' GTTGGGCAACTTGTGGTCTT 3'  
178 HsHOXB5F: 5' AAGGCCTGGTCTGGGAGTAT 3'  
179 HsHOXB5R: 5' GCATCCACTCGCTCACTACA 3'  
180 HsHOXB6F: 5' ATTTCCCTTCTGGCCCTCACT 3'  
181 HsHOXB6R: 5' GGAAGGTGGAGTTCACGAAA 3'  
182 HsHOXB9F: 5' TAATCAAAGACCCGGCTACG 3'  
183 HsHOXB9R: 5' CTACGGTCCCTGGTGAGGTA 3'

184 HsHOXC4F: 5' CGCTCGAGGACAGCCTATAC 3'  
185 HsHOXC4R: 5' GCTCTGGGAGTGGTCTTCAG 3'  
186 HsHOXC8F: 5' CTCAGGCTACCAGCAGAACC 3'  
187 HsHOXC8R: 5' TTGGCGGAGGATTTACAGTC 3'

188

189 *Mice and in vivo trial*

190 All animal experiments were conducted in accordance with the United Kingdom Co-  
191 ordinating Committee on Cancer Research (UKCCCR) guidelines for the Welfare of  
192 Animals in Experimental Neoplasia and were approved by the University of Surrey  
193 Research Ethics Committee. The mice were kept in positive pressure isolators in 12  
194 hour light / dark cycles and food and water were available *ad libitum*.

195 Athymic nude mice were inoculated subcutaneously with a suspension of 2.5  
196  $\times 10^6$  MSTO-211H cells in culture media (100  $\mu$ l). Once tumors reached volumes of  
197 approximately 100 mm<sup>3</sup>, mice were injected IP with PBS or 25 mg/Kg HXR9 in PBS  
198 (injection volume 100  $\mu$ l), every 4 days. The mice were sacrificed after 36 days and  
199 the tumors were excised for RNA extraction, as previously described[12]. Each  
200 treatment group contained 10 mice. The mice were monitored carefully for signs of  
201 distress, including behavioral changes and weight loss.

202

203 *Patient characteristics*

204 Primary mesothelioma samples were obtained from 16 male and 5 female patients.  
205 The median patient age at diagnosis was 63.9 years (range, 38.2–79.53 years) and  
206 median survival was 9.04 months (range, 0.23–81.85 months). Recruitment was via a  
207 specialized multidisciplinary thoracic oncology clinic, involving thoracic surgeons,  
208 radiation oncologists, and medical oncologists. Histopathology and imaging review  
209 was undertaken for all patients. Patients underwent tumor resection at the Department  
210 of Thoracic Surgery, Guy's & St Thomas' NHS Foundation Trust. Tumor samples  
211 were confirmed as mesothelioma by pathological examination and categorized as a

212 sarcomatoid, biphasic, or epithelial type using an antibody panel that included  
213 BerEP4, CEA, TTF1, Calretinin, WT1, CK5, MNF116, and EMA. Tissues and data  
214 were released for study from the KHP Cancer Biobank in accordance with NHS REC  
215 approval number 07/H0804/91.

216

### 217 *Statistical analysis*

218 All values are given as the mean of three independent experiments and error bars  
219 show the standard error of the mean. Categorical variables were compared using  
220 Student's t-test or a one-way ANOVA. Survival curves were generated using the  
221 Kaplan-Meier method and compared using the log-rank test. A p value <0.05 was  
222 considered to be significant.

223

## 224 **Results**

225

### 226 *HOX gene expression in mesothelioma-derived cell lines and primary tumors*

227 In order to assess the expression of *HOX* genes in mesothelioma we used QPCR to  
228 measure RNA levels in four cell lines derived from this malignancy: NCI-H28, NCI-  
229 H2052, NCI-H226, and MSTO-211H, together with Met-5A which is derived from  
230 non-malignant mesothelium cells (Table 2). *HOX* gene expression was also studied in  
231 primary mesothelioma tumors. The expression of *HOX* genes within each cell line and  
232 between cell lines varied considerably, with MSTO-211H and Met-5A generally  
233 having far higher expression than the other cell lines. The only *HOX* genes expressed  
234 uniquely by a single cell line were *HOXC12* and *HOXD12*, in Met-5A. Analysis of  
235 *HOX* genes that are known to have oncogenic or tumor suppressive functions (Table  
236 1) likewise reveals considerable variation, although Met-5A showed higher

237 expression of the potential tumor suppressor genes *HOXA4* and *HOXA5* compared to  
238 the malignant cell lines (Fig 1a). We also assessed the expression of these *HOX* genes  
239 in 21 primary tumors using RT-QPCR, as well the protein expression of the three  
240 most strongly expressed, *HOXA4*, *HOXA9*, and *HOXB4* at the protein level using  
241 immunohistochemistry (Fig 1b).

242

#### 243 *High HOXB4 tumor expression is associated with poor overall survival*

244 We looked for associations between the RNA expression levels of the different *HOX*  
245 genes and patient survival. The tumors of patients surviving less than 6 months had a  
246 significantly higher expression of *HOXB4* ( $p = 0.0166$ ; Fig 1c), and likewise a  
247 Kaplan-Meier analysis of overall survival (OS) showed that high *HOXB4* tumor  
248 expression was associated with a significantly shorter OS ( $p = 0.041$ ; Fig 1d).

249

#### 250 *HXR9 is cytotoxic to mesothelioma cells*

251 Given the high level of *HOX* expression in the mesothelioma cell lines, we treated  
252 cells with the *HOX* / *PBX* inhibitor HXR9 that has previously been shown to block  
253 *HOX* / *PBX* interactions and trigger apoptosis in a number of other cancers[11-17].  
254 Use of a fluorescently labeled version of HXR9 demonstrated that it can be taken up  
255 by the cell lines studied here (Fig 2a), and the MTS assay for cell viability revealed  
256 that HXR9 is cytotoxic in all five cell lines (Fig 2b,c; Table 2). The non-malignant  
257 line Met-5A is amongst the least sensitive with an  $IC_{50}$  of  $98\mu M$ , whilst the NCI-H28  
258 cell line is the most sensitive with an  $IC_{50}$  of  $18\mu M$  (Fig 2c, Table 2).

259

#### 260 *HXR9 triggers apoptosis*

261 Previous studies have suggested that the mechanism of cell death when HOX function  
262 is blocked by HXR9 is primarily through apoptosis[11-17]. To establish whether this  
263 is also the case of the mesothelioma derived cell lines, a standard FACS based assay  
264 for apoptosis-associated cell membrane changes was used. This involves the use of  
265 Annexin V that binds to membrane components usually located on the cytoplasmic  
266 side but which relocates to the external surface during apoptosis[19], and a  
267 fluorescent dye (7AAD) which binds to DNA but can only enter cells when  
268 membrane integrity has been lost. This assay revealed that all the mesothelioma cell  
269 lines underwent apoptosis when treated with HXR9 at the relevant IC50 (Fig 3), with  
270 the non-malignant cell line Met-5A showing the lowest level of apoptosis and NCI-  
271 H2052 the highest (Fig 3c).

272

273 The induction of apoptosis by HXR9 is thought to depend, at least in part, upon a  
274 rapid increase in *cFos* expression[14], and QPCR analysis of the HXR9 treated cells  
275 correspondingly showed a significant increase in *cFos* in all of the cell lines, with the  
276 smallest increase in Met-5A and the largest increase in the most sensitive cell line,  
277 NCI-H28 (Fig 4a). Correspondingly NCI-H28 also showed the greatest increase in  
278 Caspase 3 activity (a protease involved in the apoptotic pathway; Fig 4b), whilst Met-  
279 5A failed to show any significant increase in caspase activity (Fig 4c).

280

281 *Sensitivity to HXR9 correlates with the expression of specific HOX genes*

282 The expression of *HOX* genes with previously identified oncogenic or tumor  
283 suppressor properties (Table 1; Fig 1), raises the possibility that the expression profile  
284 of these genes could determine the sensitivity of cells to HXR9. To assess this we  
285 divided *HOX* genes into two groups – those with potential oncogenic functions, and

286 those with possible tumor suppressor functions. An expression ratio was obtained by  
287 dividing the total expression of genes in the former group with that in the latter ('O/S  
288 ratio'). This revealed that the most sensitive cell line, NCI-H28, has the highest O/S  
289 ratio, whilst Met-5a and the least sensitive malignant line, NCI-H226, have the lowest  
290 O/S ratios (Fig 5a). Plotting these ratios against the IC50 for each cell line suggest a  
291 positive correlation between the O/S ratio and sensitivity (Fig 5b). Furthermore, the  
292 calculated O/S ratios for the primary mesothelium tumors indicate that these cells  
293 could also be sensitive HXR9 (Fig 5b).

294

#### 295 *HXR9 blocks the growth of mesothelioma tumors in vivo*

296 In order to determine whether HXR9 could also block tumor growth *in vivo*, we  
297 established a xenograft mouse flank model using the MSTO-211H cell line. Mice  
298 were injected IP with either PBS or 25 mg/Kg HXR9 in PBS every 4 days after  
299 tumors had grown to a mean volume of 100 mm<sup>3</sup>. HXR9 significantly retarded tumor  
300 growth compared to PBS alone (Fig 6a). In tumors from mice injected with PBS only,  
301 we found a significant, linear relationship between the expression of *HOXB4* and final  
302 tumor size ( $r^2 = 0.8278$ ;  $p = 0.0321$ ; Fig 6b).

303

#### 304 **Discussion**

305

306 The dys-regulation of *HOX* genes in cancer is now well established, and in many  
307 cases a putative function for individual *HOX* genes has been established[20]. Despite  
308 a high degree of sequence and regulatory conservation between *HOX* genes, there is  
309 apparently a wide range of cancer specific functions which include both oncogenic  
310 and tumor suppressing activities. Thus for example the fifth gene of the *HOXA*

311 complex, *HOXA5*, acts primarily as a tumor suppressor in breast cancer through  
312 stabilizing p53[9], whilst its closely related counterpart in the *HOXB* cluster, *HOXB5*,  
313 can be defined as an oncogene as it can immortalize fibroblast cells upon  
314 transfection[21].

315

316 None of these studies have as yet addressed whether *HOX* genes are dys-regulated in  
317 mesothelioma, but here we show that cell lines derived from mesothelioma as well as  
318 primary mesothelioma cells have distinctly different *HOX* expression patterns from  
319 the Met-5a cell line that is derived from normal mesothelium. One of the most  
320 striking differences is the expression of *HOXC12* and *HOXD12* by Met-5a but not by  
321 any of the mesothelioma cell lines. *HOXC12* is repressed in follicular lymphoma  
322 through hypermethylation of its promoter, and has also been implicated in the  
323 differentiation of follicle cells[22], both of which suggest a possible function in tumor  
324 suppression. Likewise, the function of *HOXD12* has not been defined, but it has been  
325 shown to be silenced in melanoma cells through the methylation of its promoter[23].

326

327 Another oncogenic *HOX* gene that we found to be up-regulated in primary  
328 mesothelioma tumors was *HOXB4*. High *HOXB4* expression levels were associated  
329 with shorter OS, suggesting that *HOXB4* expression is a potential prognostic factor in  
330 this malignancy. We also found that there was a positive, linear relationship between  
331 *HOXB4* expression and tumor growth a mouse model of human mesothelioma. Given  
332 the functional redundancy amongst *HOX* proteins, this finding that *HOXB4* was the  
333 only *HOX* gene among the 39-strong family to have any prognostic significance  
334 seems unexpected. However, there are a number of other cancers for which a single  
335 *HOX* gene alone acts as a prognostic marker, and the identity of the *HOX* gene in each

336 case varies from one malignancy to another. Examples include *HOXC6* in gastric  
337 cancer, *HOXB8* in ovarian cancer, and *HOXD3* in breast cancer[24]. This might  
338 reflect the embryonic origins of different cancer types, as *HOX* gene expression in  
339 adult cells tends to reflect their developmental origin[25]. From a practical view point,  
340 there are currently no reliable markers of OS in mesothelioma[26], and the use of  
341 *HOXB4* as a prognostic marker in this context therefore justifies further evaluation.

342

343 In this study we have found that the ratio of expression between *HOX* genes with a  
344 putative oncogenic function and those that have tumor suppressor activity ('O/S  
345 ratio') predicts which mesothelioma cell lines are most sensitive to HXR9, a peptide  
346 that prevents *HOX* proteins binding to *PBX* and has been shown to cause apoptosis in  
347 other malignancies[11-17]. The O/S ratio may indicate the degree to which malignant  
348 cells are dependent on the activity of oncogenic *HOX* genes for their proliferation and  
349 survival, a concept similar to the idea of 'oncogene addiction'[27], which would  
350 explain their sensitivity to HXR9. The extent to which this is true is yet to be  
351 determined, but at a more practical level the O/S ratio might act as a biomarker for the  
352 sensitivity of mesothelioma cells to HXR9, and could ultimately be used to select  
353 patients that might benefit from this therapeutic approach.

354

355

### 356 **Competing interests**

357 The authors declare that they have no competing interests

358

### 359 **Authors' contributions**

360 RM designed and oversaw the study and wrote the manuscript draft. GS conducted  
361 the *in vivo* study. CG oversaw the collection of tumour samples (London) and helped  
362 analyse the data. ZT oversaw the collection of tumour samples (Cardiff) and helped  
363 analyse the data. JS oversaw the collection of tumour samples (London) and helped  
364 analyse the data. KH helped design the study and write the manuscript. HP helped  
365 design the study, write the manuscript, and analyse the data.

366

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370

371

372

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374

375

376

377

378

379 **Table 1** *HOX* genes with potential oncogenic or tumor suppressor functions

380

<b>Gene</b>	<b>O / S</b>	<b>Evidence</b>	<b>Reference</b>
<i>HOXA1</i>	O	Transforms non-malignant mammary epithelial cells	[28]
<i>HOXA9</i>	O	Key oncogene in leukemia	[29]
<i>HOXB3</i>	O	Pro-survival and proliferation gene in leukemia	[29]
<i>HOXB4</i>	O	Pro-survival and proliferation gene in leukemia	[29]
<i>HOXB5</i>	O	Transfection can immortalize fibroblast cells	[21]
<i>HOXB6</i>	O	Transfection can immortalize myelomonocytic cells	[10]
<i>HOXB9</i>	O	Promotes tumorigenesis in breast cancer	[30]
<i>HOXC4</i>	O	High expression in malignant prostate cells	[31]

<i>HOXA4</i>	S	Blocks spread of ovarian cancer cells	[32]
<i>HOXA5</i>	S	Identified as a tumor suppressor gene in breast ca	[9]
<i>HOXC8</i>	S	Expression inversely related to progression	[33]
<i>HOXC12</i>	S	Promotes cell differentiation in follicular lymphoma	[22]
<i>HOXD12</i>	S	Silenced in melanoma cells	[23]

381

382 O, *HOX* gene with oncogenic activity; S, *HOX* gene with tumor suppressor activity

383

384

385 **Table 2** Mesothelioma-derived cell lines used in this study

386

<b>Cell line</b>	<b>Source</b>	<b>IC50 HXR9 (μM)</b>	<b>Ref</b>
Met-5a	Normal mesothelium cells from pleural fluid	98	[34]
NCI-H28	Pleural effusion	18	ATCC
MSTO-211H	Biphasic mesothelioma (fibroblast morphology)	28	[35]
NCI-H2052	Pleural effusion (epithelial morphology)	45	ATCC
NCI-H226	Squamous carcinoma; mesothelioma (epithelial morphology). This cell line was derived from non-small cell lung cancer, although it was subsequently found to have a number of mesothelioma-related properties, including the expression of mesothelin.	107	ATCC, [36]

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397 **Figure legends**

398

399 **Figure 1.** Expression of *HOX* genes in cell lines derived from mesothelioma (a) and  
400 (b) primary mesothelioma tumors. These genes were previously shown to function as  
401 either oncogenes or tumor suppressors (see Table 1 for more detail). The relative  
402 levels of RNA for each gene are shown as a ratio with *Beta-actin* ( $\times 10000$  for NCI-  
403 H28, NCI-H2052 and NCI-H226,  $\times 100$  for primary mesothelioma tumors, Met-5A,  
404 and MSTO-211). For the cell lines (a) each value is the mean of three experiments,  
405 and error bars show the SEM. For the primary tumors (b) the expression of each *HOX*  
406 gene is shown for each individual tumor. The values shown are the mean of three  
407 technical repeats. No error bars are included in order to simplify the figure, although  
408 all repeats were within 10% of the mean value. For three of the *HOX* genes,  
409 (*HOXA4*, *HOXA9*, and *HOXB4*), the protein expression was also determined using  
410 immunohistochemistry and an example of each staining from a single tumor is shown.  
411 Scale bar: 20  $\mu\text{m}$ . Neg, negative – no primary antibody. (c) *HOXB4* tumor expression,  
412 as determined using quantitative real-time PCR, is significantly higher amongst  
413 patients surviving for less than 6 months after diagnosis (values on the y-axis are the  
414 ratio of *HOXB4* to *Beta-actin* expression  $\times 10000$ ). (d) *HOXB4* expression is  
415 associated with a shorter overall survival. Kaplan-Meier survival curves for patients  
416 with high- and low-*HOXB4* expressing tumors ( $p = 0.041$ ). The cut-off point between  
417 high- and low-expression was determined as the midpoint between the mean values of  
418 *HOXB4* expression shown in (c), which was 53.

419

420 **Figure 2.** HXR9 is cytotoxic in mesothelioma-derived cell lines. (a) Fluorescent  
421 micrograph of NCI-H28 cells treated with 18 $\mu\text{M}$  FITC-HXR9 (green) showing uptake  
422 into the nucleus and cytoplasm. Cell nuclei are stained blue. Scale bar: 5  $\mu\text{m}$ . (b)  
423 Sample dose response curves for HXR9 and CXR9 treatment of NCI-H28 and Met-

424 5A cell lines. (c) IC50 values for HXR9 in mesothelioma-derived cell lines. All  
425 incubations with HXR9 were for two hours. Each value is the mean of 5 experiments,  
426 error bars show the SEM. The NCI-H28, MSTO-211H, and NCI-H2052 cells were all  
427 significantly more sensitive to killing by HXR9 than Met-5a (\*\*,  $p < 0.01$ ; \*\*\*,  $p <$   
428 0.001).

429

430 **Figure 3.** HXR9 triggers apoptosis in treated cells. The mechanism of cell death was  
431 analyzed using a FACS-based Annexin / 7AAD method to assess early and late  
432 apoptosis. (a) Sample dot plots for NCI-H28 cells treated with 18  $\mu$ M HXR9 for two  
433 hours. Viable cells sort to the lower left hand quadrant (low Annexin / 7AAD  
434 staining), whilst cells in early and late apoptosis sort to the lower and upper right hand  
435 quadrants, respectively. Necrotic cells are in the upper left hand quadrant. (b)  
436 Apoptosis in NCI-H28 cells either untreated or incubated with 18  $\mu$ M HXR9 or  
437 CXR9 for two hours. The values are the means of three experiments, error bars show  
438 the SEM. Treatment with HXR9 causes a significant increase in apoptosis (\*,  $p <$   
439 0.05). (c) Summary of apoptosis data for all five cell lines. V – viable cells, EA – cells  
440 in early apoptosis, LA – cells in late apoptosis, N-necrotic cells. The values are the  
441 means of three experiments, error bars show the SEM. \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$   
442 relative to the corresponding values for Met-5a.

443

444 **Figure 4.** Mechanisms of cell death. (a) Induction of *cFos* in mesothelioma-derived  
445 cell lines. The amount of *cFos* RNA was determined by QPCR in cells either  
446 untreated or treated with HXR9 or CXR9 for two hours at the IC50 for each.  
447 Expression is shown relative to *Beta-actin* ( $\times 10000$ ). The values are the means of  
448 three experiments, error bars show the SEM. \*\*\* indicates a  $p < 0.001$  compared to

449 *cFos* expression in untreated cells. (b) Caspase 3 activation in NCI-H28 cells and  
450 Met-5A cells (c). The values are the means of three experiments, error bars show the  
451 SEM. \* indicates a  $p < 0.05$  compared to caspase 3 activity in untreated cells.

452

453 **Figure 5.** (a) Ratios of oncogenic to tumor suppressor *HOX* gene expression (O/S  
454 ratio) in mesothelioma derived cell lines. The values are the means of three  
455 experiments, error bars show the SEM. \*\*\*\* denotes  $p < 0.001$  compared to the O/S  
456 ratio in the non-malignant mesothelium cell line Met-5A; (+) denotes that no  
457 expression of tumor suppressor *HOX* genes was detected in NCI-H28 so the ratio  
458 could not be calculated. (b) Correlation between sensitivity to HXR9 and O/S ratio.  
459 The IC<sub>50</sub> for killing by HXR9 is plotted against the O/S ratio for each cell line (black  
460 dots), revealing a possible negative correlation between the two. This relationship can  
461 be modeled using a third order polynomial equation ( $r^2 = 1$ ), which is shown as a solid  
462 black line. The O/S ratio of each primary tumor was used to calculate its predicted  
463 sensitivity to HXR9 (red dots).

464

465 **Figure 6.** HXR9 blocks the growth of mesothelioma tumors *in vivo*. (a) The growth of  
466 MSTO-211H tumors in xenograft mice injected IP every 4 days with PBS or 25  
467 mg/Kg HXR9, for a total of 5 times. P values were calculated using a Student's t-test  
468 for each time point, "\*" indicates statistical significance ( $p = 0.008$ ,  $p = 0.037$ , and  $p =$   
469  $0.041$  for days 30, 34, and 37, respectively. (b) *HOXB4* expression in the excised  
470 tumors from PBS-treated mice, as determined by QRT-PCR. There was a linear  
471 relationship between tumor size and *HOXB4* expression ( $r^2 = 0.8278$ ;  $p = 0.0321$ ).

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Figure 1a

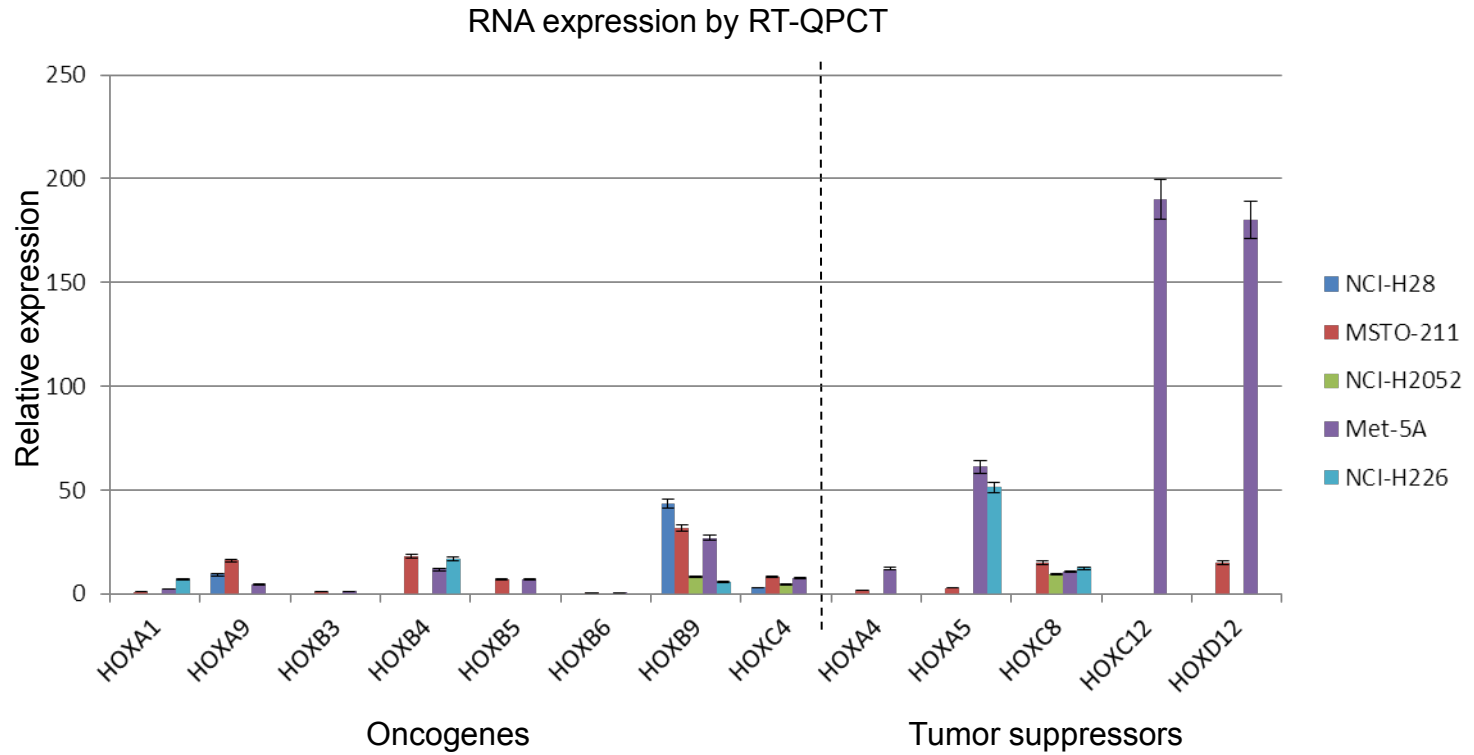




Figure 1c

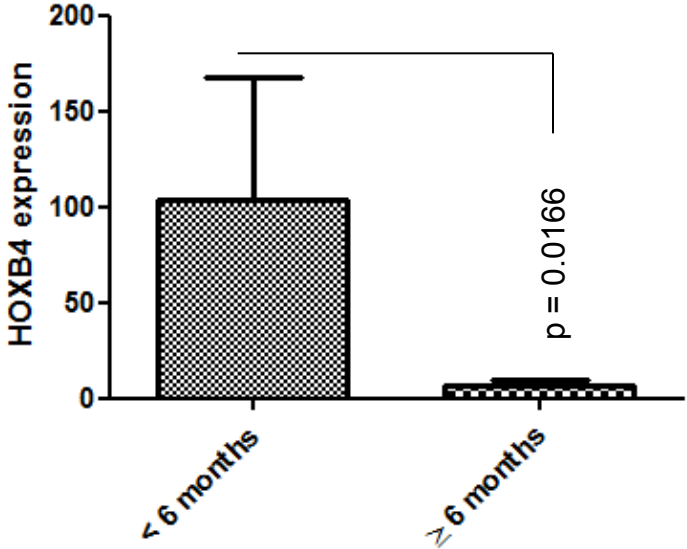


Figure 1d

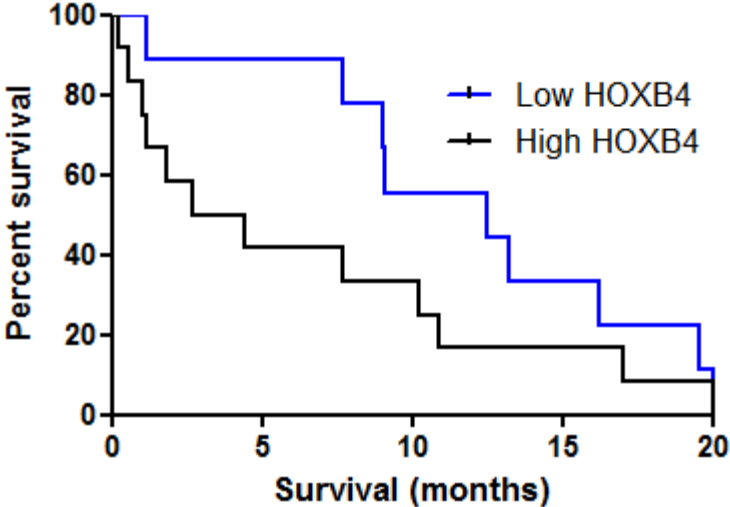


Figure 2

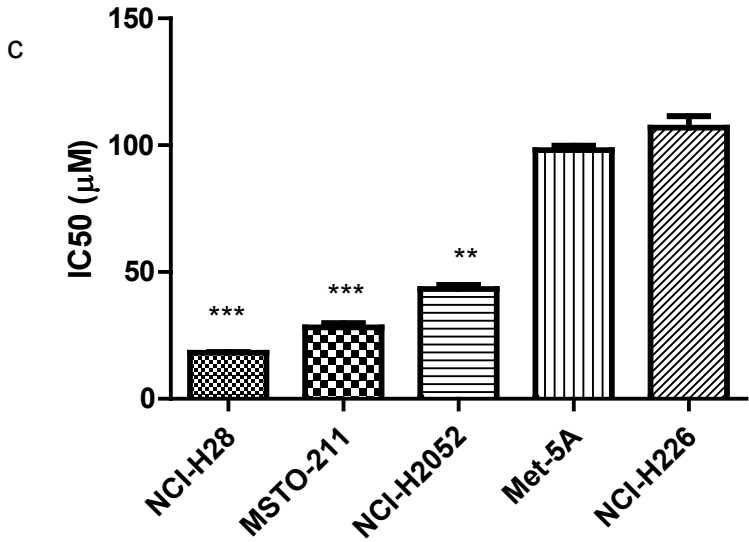
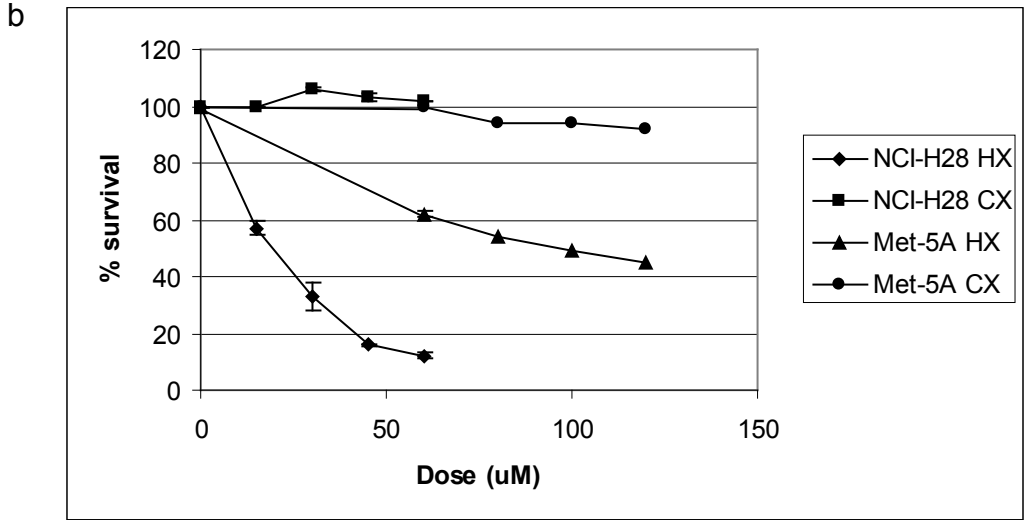
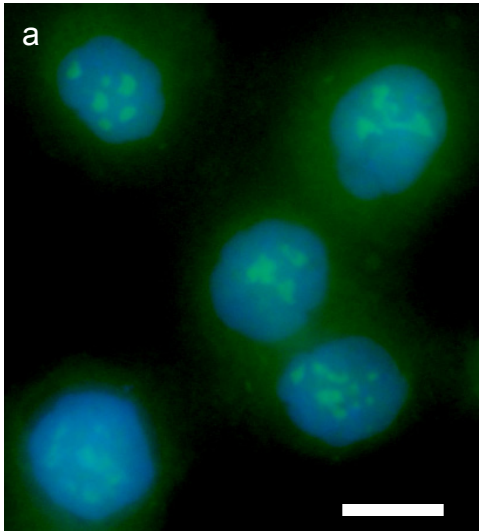


Figure 3

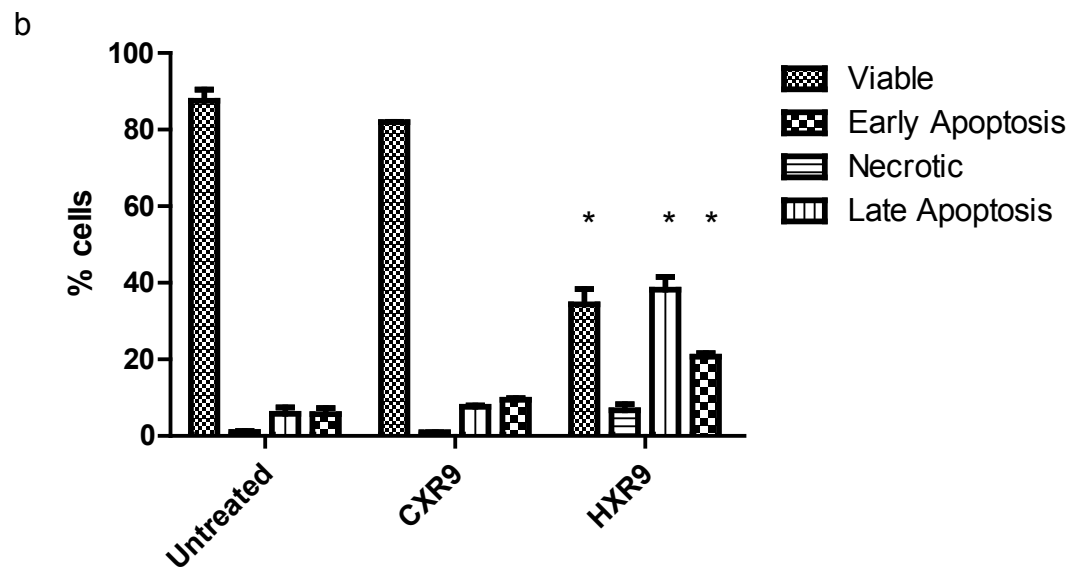
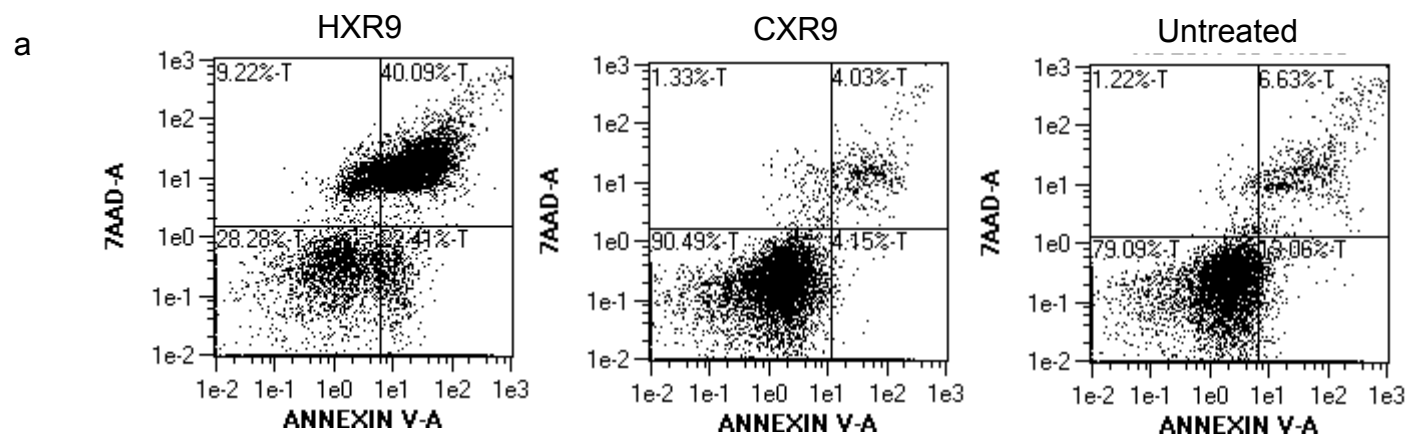


Figure 3

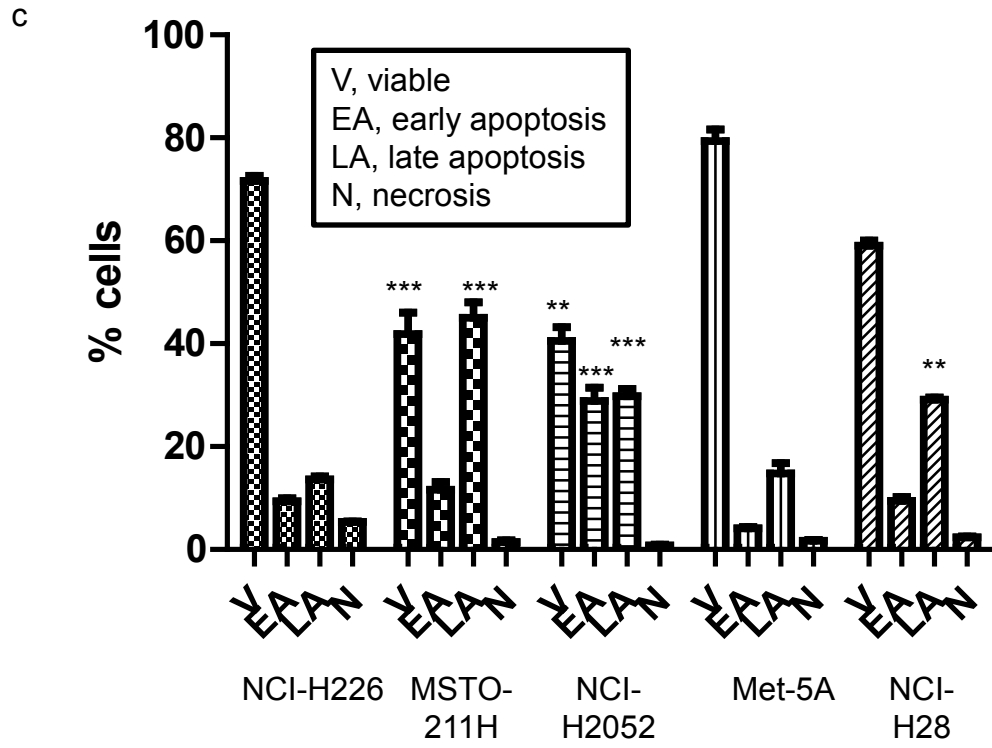


Figure 4

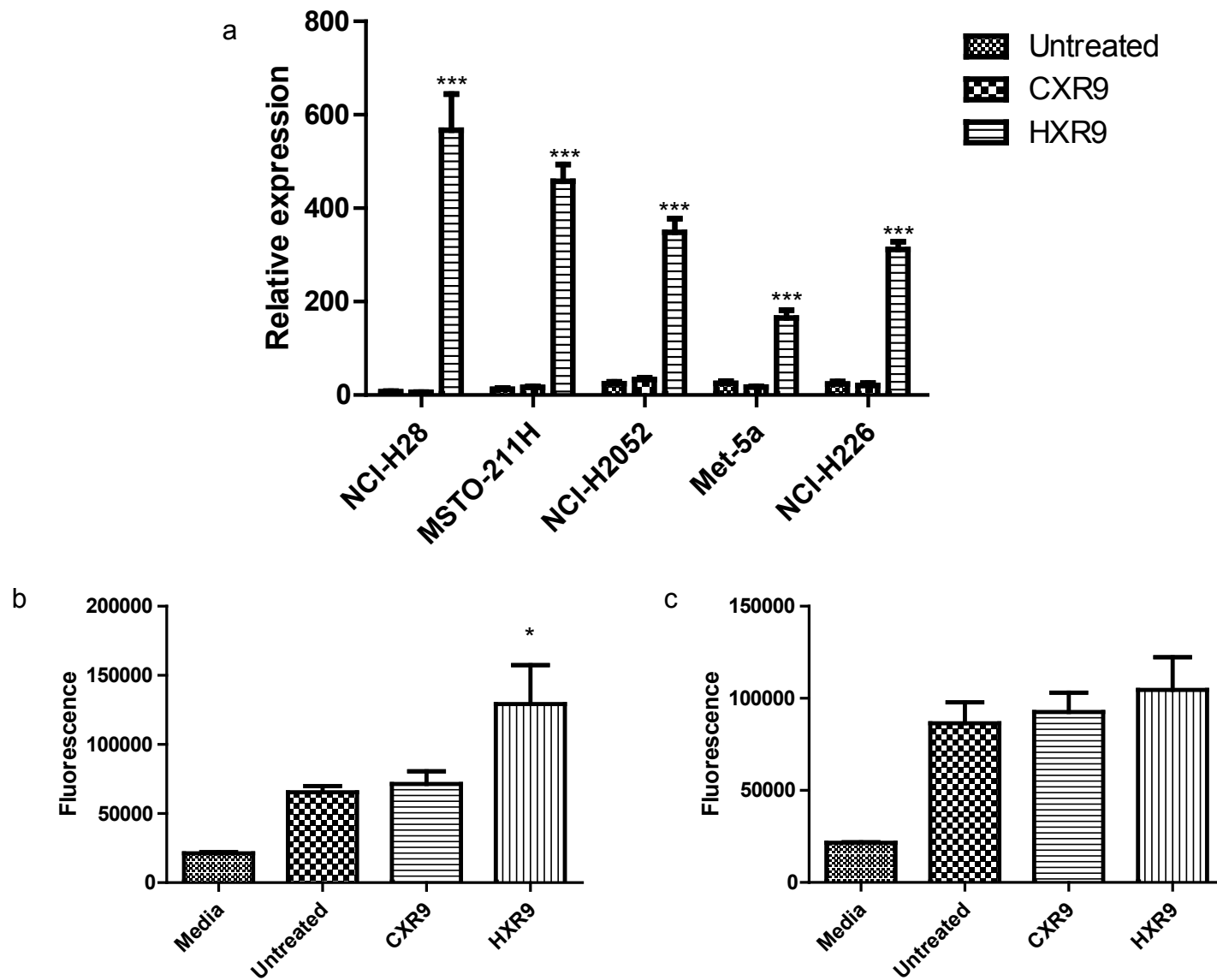


Figure 5

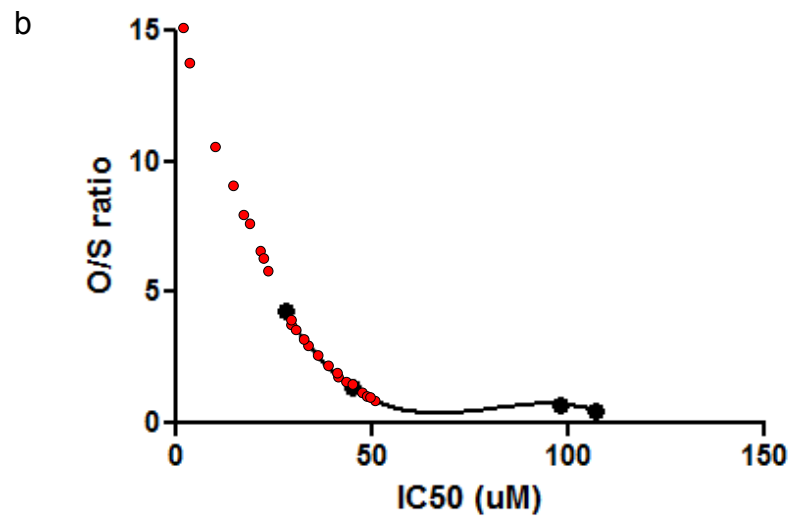
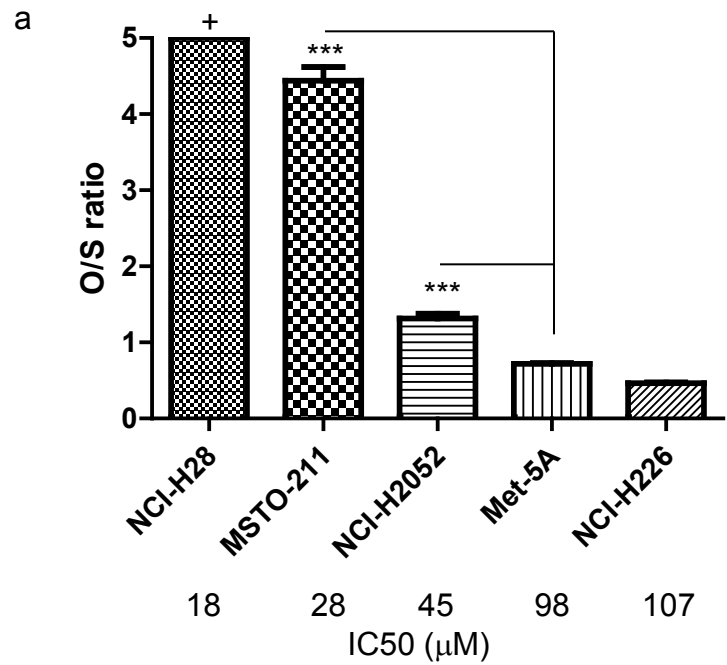
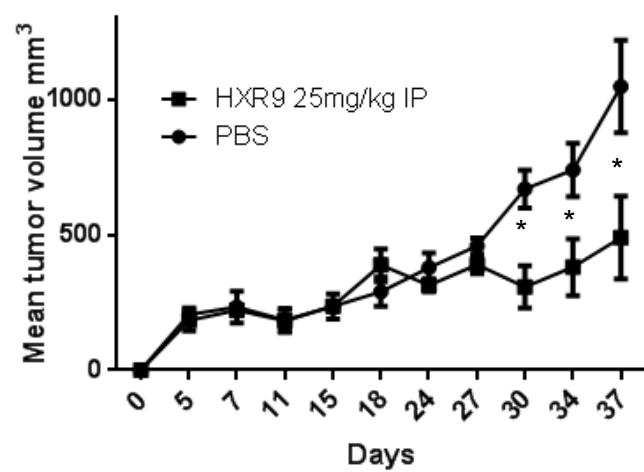


Figure 6

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b

