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Cell-type-specific regulation of distinct sets of gene targets by Pax3 and Pax3/FKHR

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The oncogenic fusion protein, Pax3/FKHR, is a more potent transcription factor relative to its normal counterpart, Pax3. Since Pax3 induced a mesenchymal to epithelial transition (MET) in human SaOS-2 osteosarcomas, we hypothesized that Pax3/FKHR would also induce a morphological change in SaOS-2 cells. We demonstrate here that Pax3/FKHR more potently induces a MET in SaOS-2 cells than Pax3. This greater potency was further evident where Pax3/FKHR, but not Pax3, induced a morphological alteration in U2-OS osteosarcoma cells. By microarray analysis, we determined that Pax3/FKHR altered the expression of gene targets in a manner quantitatively and qualitatively distinct from Pax3. Three classes of genes were identified: (i) genes induced or repressed by Pax3 and Pax3/FKHR, (ii) genes induced or repressed by Pax3/FKHR but not Pax3 and (iii) genes induced by Pax3/FKHR but repressed by Pax3. Chromatin immunoprecipitations confirmed the direct binding of Pax3/FKHR to the promoter region of several factors including *cannabinoid receptor-1*, *EPHA2* and *EPHA4*. Verification of the microarray data also revealed coordinate alteration in the expression of factors involved in BMP4 signalling. Regulation of gene expression by Pax3 and Pax3/FKHR is, however, cell-type specific. *BMP4* expression, for example, was repressed by both Pax3 and Pax3/FKHR in SaOS-2 cells, while in the rhabdomyosarcoma, RD, Pax3/FKHR, but not Pax3, induced *BMP4* expression. Thus, our data reveal that Pax3/FKHR regulates a distinct but overlapping set of genes relative to Pax3 and that the global set of Pax3 and Pax3/FKHR gene targets is cell-type specific.
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Introduction

Pax3 transcriptional activity is required for normal embryonic development of both humans and mice. Mutations in *PAX3* in humans gives rise to Waardenburg syndrome (Hoth *et al*., 1993; Baldwin *et al*., 1994), which is characterized by pigmentation, craniofacial, limb and hearing defects. Genetic analysis of *splotch* (*sp*) mice (Tassabehji *et al*., 1994), which harbour a null mutation in *Pax3*, further revealed a role for this transcription factor in the development of the neural tube, peripheral nervous system (Tremblay *et al*., 1995) and limb musculature (Bober *et al*., 1994; Epstein *et al*., 1996; Conway *et al*., 1997b), as well as in a number of tissues, which require specific migrating neural crest cell populations including the heart and the dermis (Conway *et al*., 1997a).

Pax3 encodes an N-terminal paired domain (PD) DNA-binding motif as well as a C-terminal homeodomain (HD; Chalepakis *et al*., 1994a). Detailed mutational analysis of murine Pax3 and Pax3 mutants from Waardenburg patients revealed the reciprocal influence of these two domains on their respective DNA-binding activities (Fortin *et al*., 1997). For example, specific point mutations in the PD which abrogated DNA-binding activity of this motif also altered binding to DNA of the adjacent HD, in some cases abrogating binding completely. Likewise, the R53G mutation in the HD prevents DNA binding by both the HD and the adjacent PD. Transcriptional activity of Pax3 is also modulated by other motifs, including the region between the two DNA-binding domains, the octapeptide domain (Chalepakis *et al*., 1994a), which modulates homodimerization, and the C-terminal transactivation domain (Cao and Wang, 2000). Pax3 activity is further regulated through binding of other nuclear factors. For example, both Daxx (Hollenbach *et al*., 1999) and pRB (Wiggan *et al*., 1998) associate with Pax3, repressing Pax3-dependent transcription in a dose-dependent manner.

Altered Pax3 activity is associated with malignant transformation in muscle (Anderson *et al*., 1999; Xia *et al*., 2002) and in skin (Vachtenheim and Novotna, 1999; Blake and Ziman, 2003; Poser and Bosserhoff, 2004). For example, one reciprocal translocation (t(2;13)(q35;q14)) characteristic of human alveolar rhabdomyosarcomas (ARMS) results in fusion of the

transactivation domain of the Forkhead-family protein, Forkhead-Box O1a (FOXO1a; formerly known as ForkHead (FKHR); Kaestner *et al*., 2000), to the N-terminus of Pax3 (Shapiro *et al*., 1993). The resultant fusion protein, Pax3/FKHR, exhibits greater transcriptional activity than Pax3 (Fredericks *et al*., 1995) and is refractory to repression of its activity by Daxx (Hollenbach *et al*., 1999). Pax3/FKHR induces cellular transformation, demonstrated following its expression in chick embryo fibroblasts (Scheidler *et al*., 1996), NIH3T3 cells (Lam *et al*., 1999) or the human rhabdomyosarcoma cell line, RD (Anderson *et al*., 2001). Using a reciprocal approach, expression of Pax3 fused to the repression domain of KRAB suppressed the transformed phenotype in rhabdomyosarcomas expressing Pax3/FKHR (Fredericks *et al*., 2001). That the transformed phenotype in rhabdomyosarcomas requires Pax3 or Pax3/FKHR expression was demonstrated using siRNA (Elbashir *et al*., 2001), repression of Pax3 or Pax3/FKHR expression, resulting in programmed cell death (Bernasconi *et al*., 1996). However, confounding the notion that Pax3/FKHR is a potent oncogene are *sp* mice with a 'knocked-in' allele of Pax3/FKHR (Lagutina *et al*., 2002; Relaix *et al*., 2003). These animals exhibit a partial rescue of the *sp* phenotype but do not develop tumours, potentially due to the low levels of expression of Pax3/FKHR.

The structural basis for the oncogenic potential of Pax3/FKHR relative to Pax3 is not well defined. Unlike the important interactions between the PD and HD of Pax3 required for normal activity, transformation of cells in culture requires only the HD, an intact PD apparently dispensable in this assay (Lam *et al*., 1999). Pax3/FKHR is also refractory to the inhibitory effects of repressors such as Daxx (Hollenbach *et al*., 1999) and exhibits greater transcriptional activation activity than wild-type Pax3 (Fredericks *et al*., 1995). The oncogenic potential of Pax3/FKHR may also stem from its ability to regulate the expression of factors unaffected by Pax3. For example, Pax3/FKHR, but not Pax3, specifically induces PDGFαR promoter activity and expression (Epstein *et al*., 1998). Consistent with the transforming activity of Pax3/FKHR requiring only the HD, activation of the PDGFαR promoter required a DNA sequence identical to that recognized by paired-like HDs. Thus, while Pax3/FKHR can bind to and induce promoter activity through PD consensus sequences, the HD appears to be utilized in some contexts to drive gene expression. These data suggest that transformation by Pax3/FKHR may occur through altered potency of its transcriptional activity and through the regulation of unique gene targets not recognized by Pax3.

We determined previously that Pax3 induces a mesenchymal to epithelial transition (MET) in number of cell lines, including the human osteosarcoma, SaOS-2 (Wiggan and Hamel, 2002; Wiggan *et al*., 2002). This transition is accompanied by significant alterations in cytoskeletal structure. Specifically, dense peripheral bands of actin replace stress fibres, while microtubules become oriented in the *z*-plane as these cells form polarized cell–cell contacts. We have now compared potential gene targets of Pax3 with those of its oncogenic counterpart, Pax3/FKHR, as well as determined the effect of Pax3/FKHR on SaOS-2 cell morphology. These data reveal that Pax3/FKHR induced a morphological transition in these cells with significantly increased kinetics relative to Pax3-expressing cells. We showed further that Pax3/FKHR alters the expression of a distinct but overlapping set of factors relative to those regulated by Pax3, and that for some of these factors, their regulation is cell-type specific.

Results

Pax3/FKHR is a more potent activator of MET than Pax3

Expression of Pax3 in the human osteosarcoma cell line, SaOS-2, causes a MET (Wiggan and Hamel, 2002; Wiggan *et al*., 2002). During this transition, cells condense, formed compact aggregates and become polarized. We predicted that the oncogenic variant of Pax3, Pax3/FKHR, would also induce this transition. SaOS-2 cells, which do not express endogenous Pax3, were infected with adenovirus expressing green fluorescent protein (GFP) and either βgalactosidase (βgal), flag-tagged Pax3 ($Pax3^{Flag}$) or HA-tagged Pax3/FKHR ($Pax3/FKHR^{HA}$). Expression of either Pax3 or Pax3/FKHR was detectable by Western blot within 1 h postinfection (Figure 1a). Using primers common to both Pax3 and Pax3/FKHR, RT–PCR analysis revealed that Pax3 message was expressed at levels significantly higher than Pax3/FKHR (Figure 1b). This difference in mRNA levels was similarly reflected in the levels of protein detected at 48 h, although differences in the efficiency of recognition by the α-Pax3 antibody of Pax3 versus Pax3/FKHR was not determined (Figure 1c).

Figure 2a illustrates that Pax3/FKHR causes a similar MET to that of Pax3 in SaOS-2 cells. However, despite the apparent lower levels of Pax3/FKHR relative to Pax3, the Pax3/FKHR-induced transition occurs with increased kinetics, resulting in a phenotype distinct from that induced by Pax3. Specifically, by 24 h, Pax3/FKHR-expressing cells are reduced in size (data not shown) and by 48 h they have aggregated, forming tight clusters in elongated chains. By 72 h, the Pax3/FKHR-expressing cells have compacted further and numerous small processes ('microspikes') are visible. In contrast to this rapid transition induced by Pax3/FKHR, cells expressing Pax3 aggregated with significantly slower kinetics, with tight clusters of cells forming only after 72 h. Phenotypically, the Pax3-expressing cells never appear as compact as those expressing Pax3/FKHR.

Immunohistochemical analysis determined that similar alterations of cytostructural elements were induced by Pax3/FKHR and Pax3 (Figure 3). By 48 h, Pax3-expressing cells had lost their prominent stress fibres (Figure 3a) and formed dense peripheral bands (Figure 3b). This transition is apparent for Pax3/

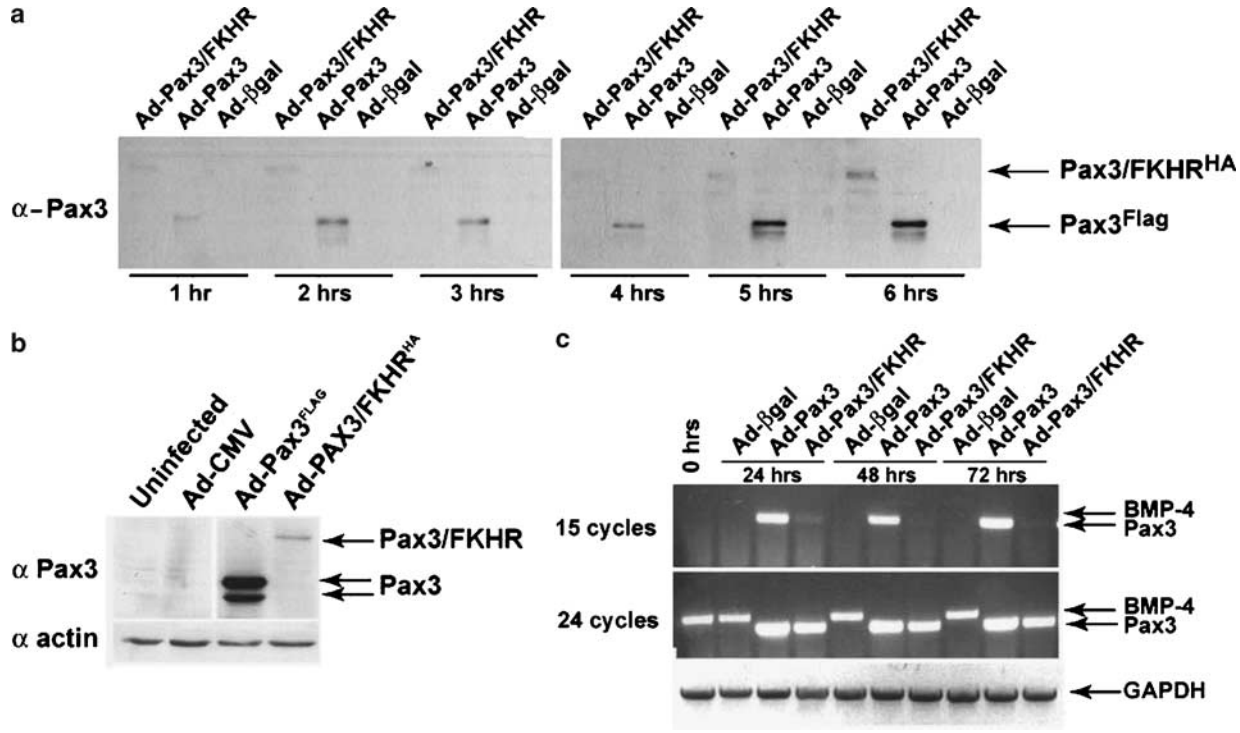

Figure 1 Ectopic expression of Pax3 and Pax3/FKHR. (**a**) Western blot analysis of SaOS-2 cells infected with adenoviruses expressing βgal, Pax3 or Pax3/FKHR. Whole-cell lysates were prepared at various times following infection and Pax3 or Pax3/FKHR detected using an α-Pax3 antibody. Pax3 and Pax3/FKHR protein are detectable within 1 h postinfection. (**b**) Detection of adenovirus-expressed Pax3 and Pax3/FKHR in SaOS-2 cells 48 h postinfection. (**c**) RT–PCR detection of Pax3, Pax3/FKHR and BMP4 message. RNA was isolated 24, 48 or 72 h after infection with adenovirus expressing βgal, Pax3 or Pax3/FKHR. Pax3 and Pax3/FKHR were detected using primers common to both sequences. At 15 cycles, Pax3 is detectable at all time points, but Pax3/FKHR message is just at the level of detection. However, 24 cycles confirm that despite being present at significantly lower levels, Pax3/FKHR message is present at all time points. RT–PCR at 24 cycles also reveals that both Pax3 and Pax3/FKHR repress expression of BMP4 message (see below)

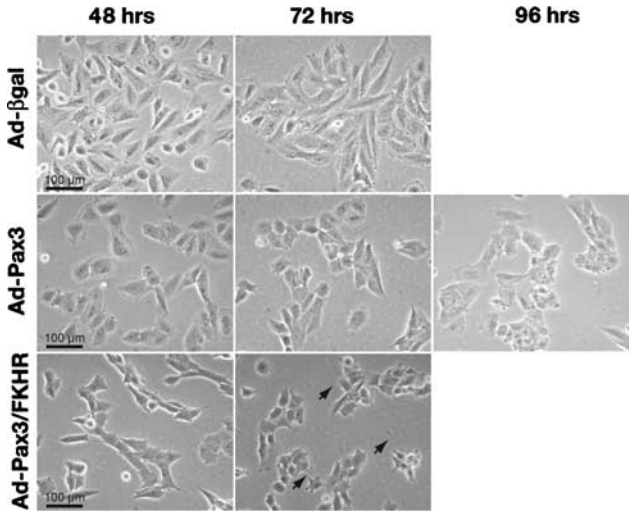

Figure 2 Pax3/FKHR induces a rapid morphological transition in SaOS-2 cells. SaOS-2 cells were infected with adenovirus expressing βgal, Pax3 or Pax3/FKHR. Within 48 h postinfection, cells expressing Pax3/FKHR have reduced in size and aggregated. Small projections ('microspikes'; black arrows) are evident in Pax3/FHKR-expressing cells. Similar morphological transition is evident for Pax3-expressing cells only after 72 h

FKHR-expressing cells (Figure 3c). However, a more significant decrease in cell surface area as well as the formation of chains and aggregates is also evident in the Pax3/FKHR-expressing cells. Detection of β-tubulin (Figure 3d–f) further illustrates these alterations following Pax3/FKHR expression.

We determined next that Pax3/FKHR induced a MET in cells resistant to a Pax3-induced MET. For example, since pRB represses the activity of Pax3 (Wiggan *et al*., 1998), we predicted that Pax3-dependent induction of a MET in the pRB-positive human osteosarcoma, U2-OS, would be retarded. As Figure 4a and b illustrates, a small but significant reduction in the size of U2-OS cells occurs following expression of Pax3. However, the cells fail to develop the characteristics of Pax3-induced SaOS-2 cells. In contrast, expression of Pax3/FKHR induced U2-OS to form tight aggregates of highly compact cells resembling Pax3-expressing SaOS-2 cells.

Thus, Pax3/FKHR induces a morphological change in SaOS-2 cells with accelerated kinetics relative to Pax3 and induces a morphological transition in a pRB-expressing cell line that is resistant to the effects of Pax3 activity.

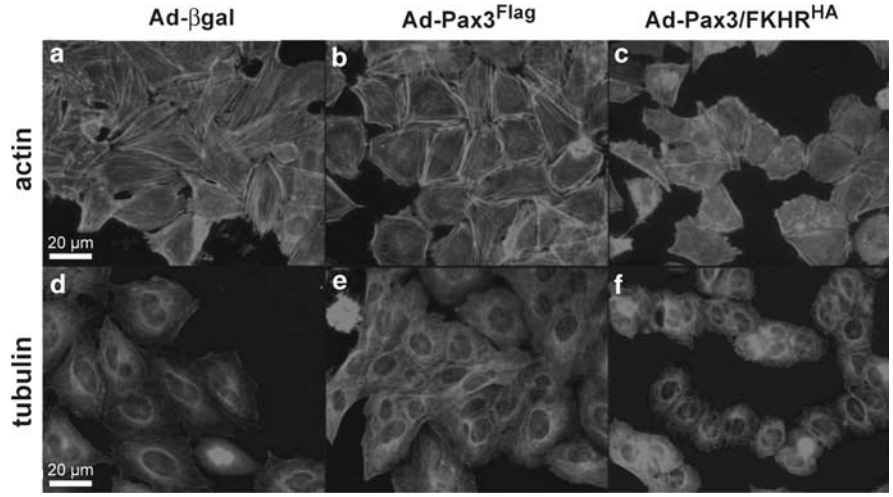

Figure 3 Pax3- and Pax3/FKHR-induced rearrangement of the SaOS-2 cytoskeleton. By 48 h postinfection, stress fibres in Ad-βgal-infected SaOS-2 cells (**a**) are replaced by peripheral bands in Ad-Pax3 (**b**) or Ad-Pax3/FKHR- (**c**) infected cells. Tubulin staining reveals the compaction of the cells expressing Pax3 (**e**) or Pax3/FKHR (**f**) relative to control-infected cells (**d**). At 48 h, the formation of chains of Pax3/FKHR-expressing cells is also clearly evident. Bars = 20 μm

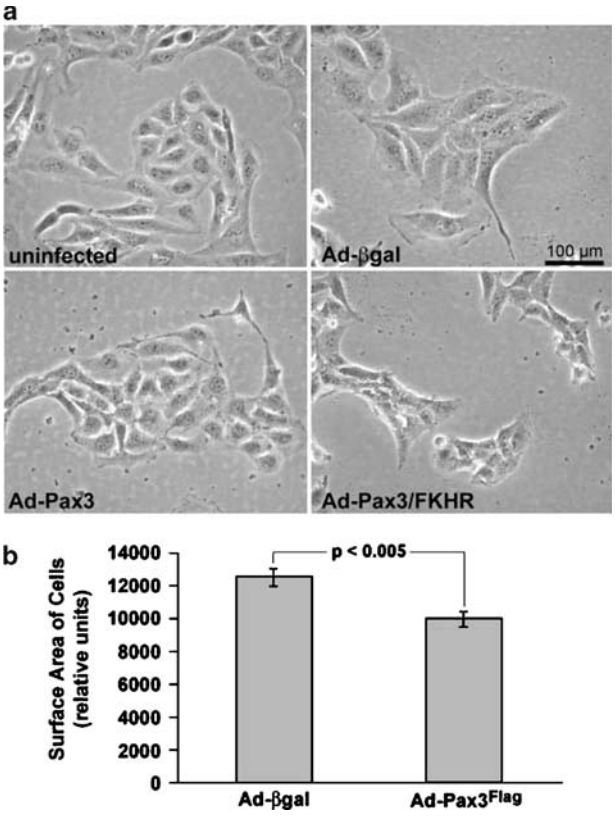

Figure 4 Pax3/FKHR but not Pax3 induces a morphological change in U2-OS cells. (**a**) Human U2-OS osteosacromas were infected with adenovirus expressing βgal, Pax3 or Pax3/FKHR. After 72 h, Pax3/FKHR (bottom right panel) had induced cells to become compact and to aggregate. U2-OS cells expressing Pax3 did not aggregate but were reduced in size. Bars = 100 μm. (**b**) Quantitation of the surface area of Pax3- and βgal-expresssing cells revealed a small but significant reduction in the size of U2-OS cells. Pax3/FKHR-expressing cells were clearly smaller but could not be accurately measured due to cell boundaries being indistinguishable

Distinct but overlapping set of factors are regulated by Pax3 and Pax3/FKHR

Pax3/FKHR is a more potent transcriptional activator than Pax3 (Fredericks *et al*., 1995; Bennicelli *et al*., 1996). The presence of the FKHR domain also alters the specificity of DNA sequence recognition, consistent with its ability to differentially regulate at least one gene (PDGFαR; Epstein *et al*., 1998). Thus, we determined whether Pax3/FKHR regulated a distinct set of factors relative to Pax3 during the induced MET in SaOS-2 cells. RNA isolated from control SaOS-2 cells or cells expressing βgal or Pax3/FKHR 48 h postinfection was subjected to microarray analysis using the Affymetrix U133A Human Genome Microarray (Figure 5, Transition 1). Repetition of this analysis was performed using RNA isolated in an independent transition 12 months later (Figure 5, Transition 2). This second transition included RNA from cells expressing Pax3. Comparison of the arrays revealed that alterations in gene expression by Pax3/FKHR were highly reproducible. All factors with altered expression in the first transition were similarly altered in the second experiment (see Supplementary Data). To verify the results of the microarray, 30 factors whose expression were strongly induced or repressed were subjected to semiquantitative RT–PCR analysis (see Figure 6a and data not shown). Without exception, factors determined by microarray analysis to be induced or repressed by Pax3/FKHR were similarly altered at the level of message.

Analysis of putative targets of Pax3/FKHR and/or Pax3 revealed three classes of factors: (i) factors induced or repressed by both Pax3/FKHR and Pax3, (ii) factors induced by Pax3/FKHR but not Pax3 and (iii) factors induced by Pax3/FKHR but repressed by Pax3. Figure 6a illustrates the case of factors induced (see Supplementary Data; Table 1) or repressed (see Supplementary Data; Table 2) by Pax3/FKHR and Pax3. One

		uninfected	Ad-βgal	Ad-Pax3/FKHR			uninfected	Ad-βgal	Ad-Pax3/FKHR		Ad-Pax3	
		sig.	sig.	sig.	FC		sig.	sig.	sig.	FC	sig.	FC
activation by Pax3/FKHR	1	9.4	9.1	1130.3	6.9	PLN	17.9	15.9	162.5	4.3	16.3	0
	2	64.3	139.5	4028.3	6.1	CNR1	120.9	67.6	3305.5	5.6	347	2.2
	3	31.8	64.2	1271.5	5.4	CITED1	35	50.7	1418.5	6	404.9	3.8
	4	7.2	7.5	409.2	5.4	KITLG	6.8	99.9	1925.5	8.2	110	3.8
	5	46.1	42.5	1334.3	5	ACADL	104.7	15.3	1935.3	4.9	205.3	1.8
	6	13.2	85.2	551.2	4.9	CALCRL	25.4	26.7	765.6	5.6	36.6	0.9
	7	8.4	7.3	481	4.7	STX11	6.5	12	354.8	5.4	15	0.8
	8	15	18.6	1895.2	4.5	EPHA4	17.8	31.6	668	5.2	123.9	2.6
	9	17.2	10.4	262.7	4.2	PLXNC1	14.3	8.4	574.6	5.9	105.1	2.8
	10	13.5	22.8	235.5	4	DMD	72.4	67.2	510.8	4.3	171.6	2.8
	11	74.4	145	1010	3.8	PIGPC1	179.4	163.2	2234.3	4.6	271.9	1.5
	12	252.4	344.6	3038.3	3.8	SCML1	451.8	152.3	3267.5	3.3	770	1.5
	13	432.4	427.3	6606.2	3.6	PRKAR2B	445.8	272.6	5822.8	4.3	1722.5	2.8
	14	258.6	315	3113	3.5	SLIT2	181.7	55.1	1719.8	3.6	171	0.8
	15	13.4	30.8	190.5	3.4	SLIT3	18	42.4	112.4	3.4	10.5	0.1
	16	362.2	238.8	2554.7	3.1	TWSG1	404.1	484.9	2030.3	3	1212.6	2.3
	17	83.2	109.7	366.4	2.9	PCDH7	15.6	27.9	151.1	4.1	18.6	0.8
repression by Pax3/FKHR	18	1801	687.7	67.4	-4.9	VEGF	351.1	398.3	62.2	-1.8	233	0.1
	19	848.6	1238.4	18.8	-4.2	EPHB2	709.9	1235.6	163.6	-1.5	338.5	-0.3
	20	404.8	370.3	19.6	-4	MOT8	841.6	682.1	28.8	-3.9	458.1	-0.6
	21	5944	4227.6	281.4	-3.9	EPHA2	2679.4	3372.3	101.7	-2.8	1187.3	-0.1
	22	1098	828.7	99.7	-3.8	FLNB	679.6	771.3	119.5	-1.3	415	-0.1
	23	1373	1963.3	120.7	-3.8	IL11	1051.6	718.8	150.3	-2	376.9	-0.4
	24	1146	837.4	92.5	-3.5	SEMA3B	414.1	629.5	26.7	-3.6	190.2	-0.6
	25	268.1	295	14.3	-3.4	WNT11	586.9	348.9	21.2	-4.6	66.2	-2.5
	26	4379	5053.4	641.1	-2.8	JAG2	2209.8	2563.8	325	-2	878	-0.5
	27	541.6	720.7	33.2	-2.7	LIF	329.6	309.8	129.1	-0.6	247.6	0.3
	28	1207	929	459.1	-2.4	BMP1	594.4	949	330.6	-0.1	332.5	-0.4
	29	1016	1996.1	311.6	-1.9	BMP4	3619	3326.6	117.3	-4.1	1673.5	-0.3
		Transition 1					Transition 2					

Figure 5 Microarray analysis of altered gene expression induced by Pax3 and Pax3/FKHR. RNA from uninfected SaOS-2 cells or cells infected with virus expressing βgal or Pax3/FKHR was used to probe the Affymetrix Human U133A microarray (Transition 1). An independent experiment was performed 12 month later with the addition of RNA from Pax3-expressing SaOS-2 cells (Transition 2). Depicted are representative factors which were either induced (rows 1–17) or repressed (rows 18–29) by Pax3/FKHR in the first transition (results from complete array available at http://individual.utoronto.ca/paul_hamel). Values are signals relative to internal controls for the Human U133A microarray. FC – fold change

of the factors most highly induced by both Pax3/FKHR and Pax3 was *cannabinoid receptor-1* (*CNR1*; 6.1-fold induction). Semiquantitative RT–PCR revealed that *CNR1* was strongly activated by Pax3/FKHR within 24 h, while Pax3-dependent induction was typically evident only after 48 h. The multiplex RT–PCR in Figure 6a confirms that *bone morphogenetic protein 4* (*BMP4*) was strongly repressed within 24 h of infection with the Ad-Pax3/FKHR virus and by 48 h with Ad-Pax3. In all cases in which both Pax3/FKHR and Pax3 induced a particular factor, the kinetics and level of induction were greater for Pax3/FKHR relative to Pax3.

A large number of factors were induced by Pax3/FKHR but not by Pax3 (see Supplementary Data; Tables 1 and 2). For example, the cardiac calcium flux regulator, *phospholamban* (*PLN*), was strongly induced (6.2-fold induction) by Pax3/FKHR but was unaffected by Pax3 (Figure 6b). Figure 6b also illustrates an example of factors, which were induced following expression of Pax3/FKHR but were repressed in the

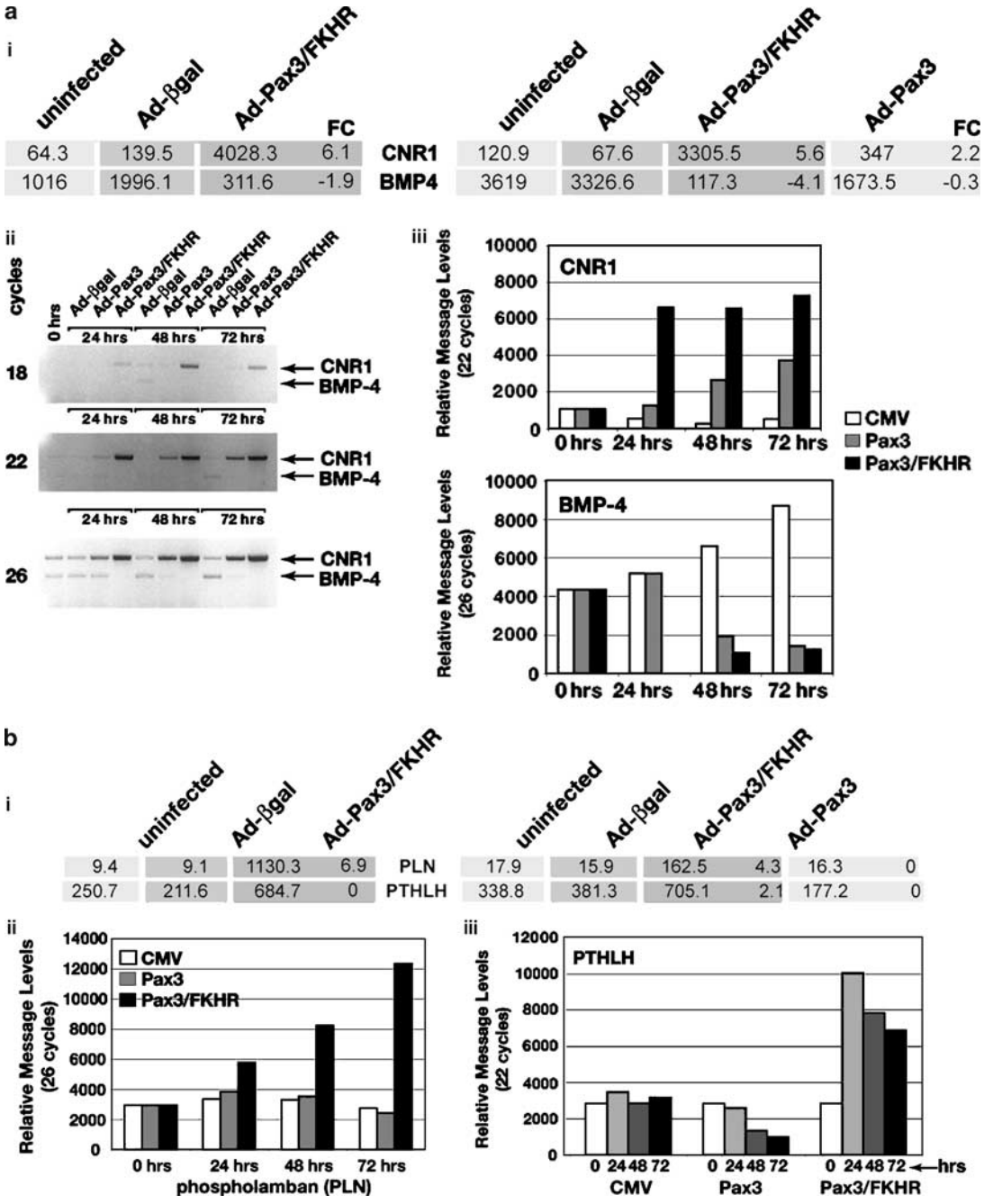

Figure 6 Targets of Pax3 and/or Pax3/FKHR activity. Representative factors identified by microarray analysis were subjected to semiquantitative RT–PCR analysis. Examples of factors (7A) induced (*CNR1*) or repressed (*BMP4*) by both Pax3/FKHR and Pax3, (7B) induced by Pax3/FKHR but unaffected by Pax3 (*PLN*) factors induced by Pax3/FKHR but repressed by Pax3 (*PTHLH*). Part (i) in each figure represents normalized data from microarray analysis. Part (ii) in (7A) illustrates multiplex RT–PCR for *CNR1* and *BMP4*. For each factor, variable number of PCR cycles in the linear range were used on RNA from cells 24, 48 and 72 h postinfection. Gels were digitized and the intensity of bands determined using NIH Image. Relative levels of message were plotted for each time point

presence of Pax3 (see Supplementary Data; Table 3). Parathyroid hormone-like hormone (*PTHLH*) participates in the development of a number of tissues. Importantly, PTHLH expression in breast tumours (Bouizar *et al*., 1993) and rhabdomyosarcomas (Blake and Ziman, 2003) is responsible for metastases of these tumours to bone. While the microarray analysis detected only a modest induction by Pax3/FKHR or repression by Pax3, the densitometry of the RT–PCR reactions consistently produced greater than twofold induction by Pax3/FKHR or repression by Pax3, respectively, of *PTHLH* message.

Together, these data reveal a global alteration in gene expression during the morphological transition induced

by Pax3/FKHR and Pax3 in SaOS-2 cells. However, these alterations in Pax3/FKHR-expressing cells relative to those expressing Pax3 were quantitatively and qualitatively distinct.

Based on the global changes in gene expression observed, we hypothesized that coordinated changes in the expression of factors within defined genetic pathways would occur. As Figure 7 illustrates, coordinated alteration in the expression of factors involved in BMP4 signalling occurred. Specifically, *BMP4* levels were repressed by both Pax3 and Pax3/FKHR. Further, the BMP4 agonist, *BMP1* (Wardle *et al*., 1999a, b), was also repressed, while an antagonist of BMP4, *twisted gastrulation* (*TWGS*; Yu *et al*., 2000; Chang *et al*., 2001; Ross *et al*., 2001; Scott *et al*., 2001), was induced in these cells. Since BMP4 signalling was repressed, we predicted and determined that one potential target of BMP4 signalling, *Dlx3* (Luo *et al*., 2001; Park and Morasso, 2002), was also repressed following Pax3 and Pax3/FKHR expression.

Direct transcriptional targets of Pax3 and Pax3/FKHR

Altered gene expression profiles in SaOS-2 cells following expression of Pax3 or Pax3/FKHR may arise due to indirect regulation of promoter activity. We began to define direct targets of Pax3 and Pax3/FKHR by blocking protein synthesis using cycloheximide (Figure 8). Cells were infected with virus expressing βgal, Pax3 or Pax3/FKHR, and cycloheximide was added 1, 2 or 3 h postinfection to prevent further protein synthesis. Cell harvested after 24 h of cycloheximide treatment maintained low levels of Pax3 or Pax3/FKHR protein relative to untreated cells (Figure 8a). Changes in the levels of expression of factors induced by Pax3 or Pax3/FKHR were determined using RNA isolated after

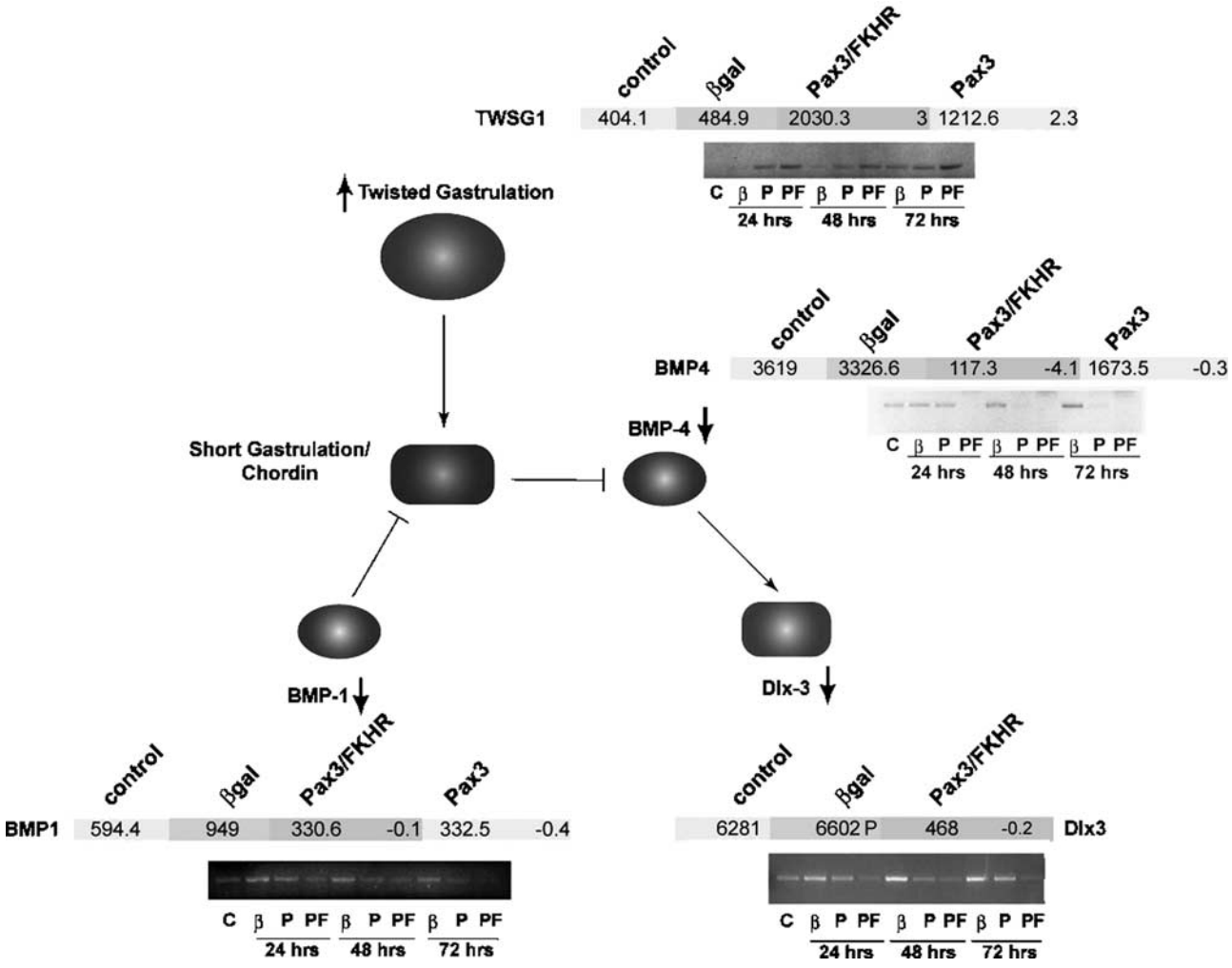

Figure 7 Pax3 and Pax3/FKHR coordinately alter expression of factors in the BMP4 pathway. Expression of factors which influence BMP4 signalling, such as *BMP4*, *TWSG* and *BMP1*, or targets of BMP4 signalling, such as *Dlx3*, are coordinately altered in SaOS-2 cells. Both microarray analysis and RT–PCR revealed that Pax3 and Pax3/FKHR caused an overall decrease in the level of factors which promoted BMP4 signalling, a reduction of one potential downstream target, *Dlx3*, as well as an increase in the BMP4 antagonist, *TWSG*. Microarray data from the second transition are depicted in rows above the agarose gels with RT–PCR products. C = control SaOS-2; β = Ad-βgal-infected cells; P = Ad-Pax3-infected cells; PF = Ad-Pax3/FKHR-infected cells

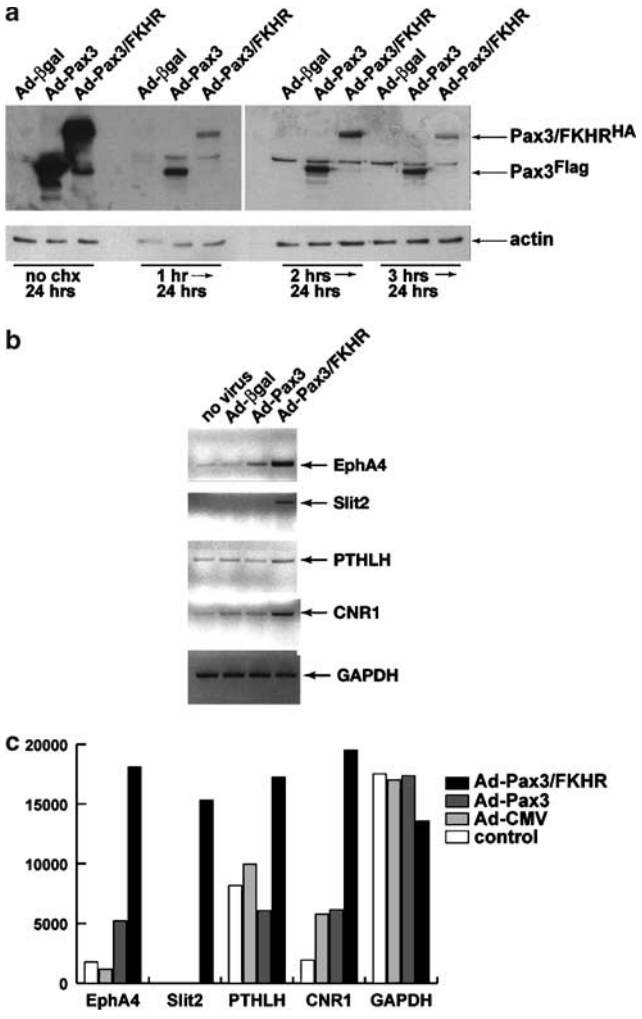

Figure 8 Determination of proximal Pax3 and Pax3/FKHR gene targets. (**a**) SaOS-2 cells were infected with Ad-βgal-, Ad-Pax3- or Ad-Pax3/FKHR-expressing viruses and cycloheximide added 1, 2 or 3 h postinfection. Cells were harvested after 24 h and Pax3 and Pax3/FKHR levels compared to their levels in untreated cells (first three lanes). A block to protein synthesis is evident by the low levels of Pax3 and Pax3/FKHR maintained in the presence of cycloheximide. (**b**) RNA was isolated from cells treated with cycloheximide 2 h after infection by the three viruses. RT–PCR analysis determine if altered expression of specific factors occurred in the absence of protein synthesis 2 h after initial Pax3 or Pax3/FKHR expression. (**c**) Quantification of altered expression patterns of *EphA4*, *Slit2*, *PTHLH* and *CNR1* reveal that message for all four factors are induced by Pax3/FKHR in the absence of protein synthesis 2 h following infection. A small increase in *EphA4* was reproducibly obtained in Pax3-expressing cells treated with cycloheximide. These data suggest that these factors are direct targets of Pax3/FKHR in SaOS-2 cells

24 h from cells treated with cycloheximide 2 h after infection (Figure 8b–c). *EPHA4* was induced by both Pax3 and Pax3/FKHR, while *SLIT2* and *CNR1* appeared to be induced only by Pax3/FKHR under these conditions. *PTHLH* was induced by Pax3/FKHR, while a small, reproducible decrease of *PTHLH* levels in Pax3-expressing cells was also observed. These data suggest, therefore, that these factors may be direct targets of Pax3/FKHR and, for some, Pax3.

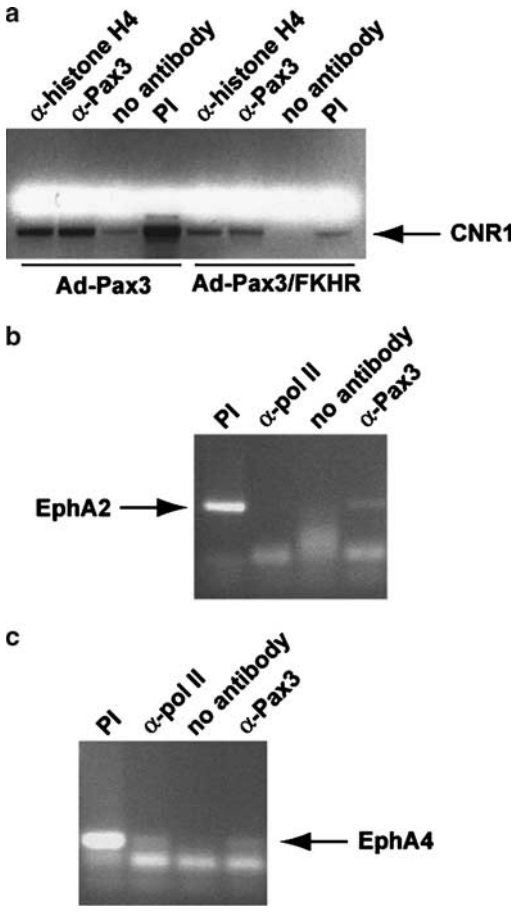

Figure 9 Pax3 and Pax3/FKHR binding to the 5′ region of gene targets. Pax3 (**a**) or Pax3/FKHR (**a**–**c**), crosslinked to DNA was immunoprecipitated from SaOS-2 cells. The associated DNA was amplified with primers specific for the putative promoter region of the (**a**) *CNR1* gene, (**b**) *EphA2* gene or the (**c**) *EphA4* gene. Anti-histone H4 was used as a positive control for *CNR1*. Antibody to RNA polymerase II (α-pol II) was used for *EphA2* and *EphA4* in order to assess the transcriptional activity from these promoters. PI = preimmune input

Confirmation of direct regulation by Pax3 or Pax3/FKHR was obtained by performing chromatin immunoprecipitations (Figure 9). *CNR1* was chosen initially since inspection of the 5′ region flanking the transcriptional start site of its annotated sequence from the human genome revealed a Pax3 consensus site (reverse orientation) at –357. As Figure 9a illustrates, both Pax3 and Pax3/FKHR co-immunoprecipitated a crosslinked genomic DNA fragment adjacent to this Pax3 DNA-binding consensus sequence in the putative *CNR1* promoter region. Similarly, *EPHA4*, which is strongly induced by Pax3/FKHR, and *EPHA2*, which is suppressed, encodes Pax3 DNA-binding consensus sequences at −83 and −76, respectively. For *EPHA4*, this site is identical in sequence and position to the murine *EphA4* promoter, while the PD site for the murine *EphA2* promoter is positionally identical and differs by a single nucleotide. Using the α-Pax3 antibody for a ChIP on Pax3/FKHR-expressing cells, *EPHA4*- and *EPHA2*-specific fragments were isolated from both

putative promoters. Repression of transcription from the *EPHA2* promoter but not the *EPHA4* promoter was also evident since amplification of a band for *EPHA4* but not *EPHA2* band was observed for the α-polymerase II (α-pol II) antibody.

Cell-type-specific gene regulation by Pax3 and Pax3/FKHR

The spectrum of genes we identified by microarray analysis did not overlap significantly with the results from previous studies attempting to identify Pax3 or Pax3/FKHR target genes (Khan *et al*., 1998, 1999; Mayanil *et al*., 2001). Thus, we tested the hypothesis that altered expression of some gene targets would be cell-type specific. Pax3 and Pax3/FKHR were ectopically expressed in the human rhabdomyosarcoma, RD, which expresses low levels of endogenous Pax3 and Pax7. Changes in the expression of specific factors were compared to endogenous levels of expression in the rhabdomyosarcoma, RH30, which expresses low levels of endogenous Pax3/FKHR (Figure 10). As expected, *CNR1* was induced by both Pax3 and Pax3/FKHR in RD cells, similar to its induction in SaOS-2 cells. *CNR1* was also constitutively expressed in RH30 cells. Likewise, induction of *ephrin B2* (*EFNB2*) by Pax3/FKHR, but not Pax3, as well as expression in RH30 cells was observed, similar to *EFNB2* expression in SaOS-2 cells. However, a number of gene targets were distinctly regulated in RD cells relative to SaOS-2 and RH30. For example, in contrast to SaOS-2 cells where both Pax3 and Pax3/FKHR repressed *BMP4* expression, it was strongly induced by Pax3/FKHR, but not Pax3, in RD cells. In the case of *SLIT2*, while strong induction by Pax3/FKHR occurred in SaOS-2 cells, neither Pax3 nor Pax3/FKHR affected *Slit2* levels in RD cells, although expression was observed in RH30. For *EPHA4*, Pax3/FKHR, but not Pax3, induced expression unlike in SaOS-2 cells where *EPHA4* was induced by both factors. These data reveal, therefore, that regulation of at least some Pax3 and Pax3/FKHR gene targets is dependent on the specific cell type in which these transcription factors are expressed.

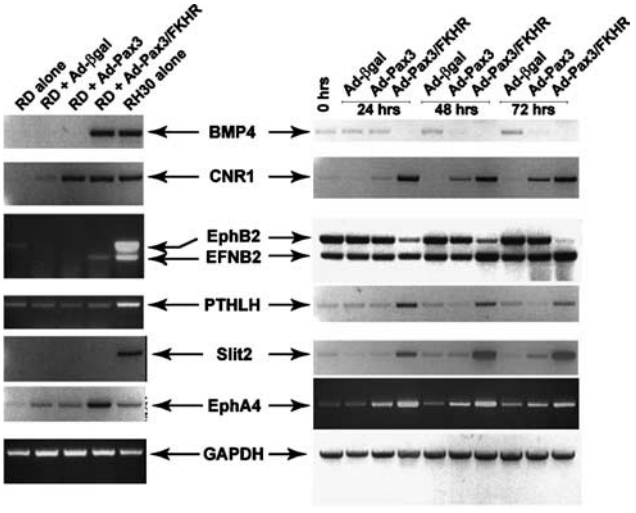

Figure 10 Cell-specific gene regulation by Pax3 and Pax3/FKHR. RD cells, which express low levels of Pax3 and Pax7, were infected with adenovirus expressing βgal, Pax3 or Pax3/FKHR. Expression levels of *BMP4*, *CNR1*, *EphB2*, *EFNB2*, *PTHLH*, *Slit2* and *EphA4* were then determined by RT–PCR and compared to their expression in the Pax3/FKHR-expressing RH30 rhabdomyosarcoma as well as to SaOS-2 cells. While Pax3 and Pax3/FKHR regulated a number of factors in RD cells similar to that observed in SaOS-2, such as *CNR1* and *EFNB2*, regulation of other factors was distinct. For example, *BMP4*, which was induced by Pax3/FKHR in RD cells rather than repressed while *Slit2* was not altered in RD cells in the presence of either Pax3 or Pax3/FKHR

Discussion

We demonstrated previously that Pax3 induces a MET in SaOS-2 cells (Wiggan and Hamel, 2002; Wiggan *et al*., 2002). As illustrated here, expression of the oncogenic variant of Pax3, Pax3/FKHR, was a considerably more potent driver of this transition than Pax3. This increased activity was reflected by the observation that, while U2-OS cells showed only a limited response to the expression of Pax3, Pax3/FKHR induced morphological changes and aggregation similar to SaOS-2 cells.

The stronger activity of Pax3/FKHR was consistent with previous data demonstrating the increased transcriptional activity of the oncogenic (Scheidler *et al*., 1996) fusion protein (Fredericks *et al*., 1995). The microarray analysis presented here revealed that a large number of factors were either induced or repressed by Pax3/FKHR and Pax3. In all cases, where both Pax3/FKHR and Pax3 induced expression, stronger induction occurred with Pax3/FKHR. Consistent with the accelerated kinetics of the morphological transition induced by Pax3/FKHR relative to Pax3, induction or repression of target genes by Pax3/FKHR was also accelerated.

We identified a small number of factors induced by Pax3/FKHR but repressed by Pax3. Significantly, *PTHLH* (also known as *parathyroid-related peptide* (*PTHrP*)) behaved in this manner. While important in the embryonic development of a number of tissues, including bone (Vortkamp *et al*., 1996; Farquharson *et al*., 2001) and the mammary gland (Dunbar and Wysolmerski, 1999; Foley *et al*., 2001), PTHLH expression can also result in metastasis to bone in some breast carcinomas (Kissin *et al*., 1993; Sebag *et al*., 1994; Bundred *et al*., 1996) and rhabdomyosarcomas (Kawasaki *et al*., 1998). PTHLH expression further gives rise to humoral hypercalcaemia (Bundred *et al*., 1996; Guise *et al*., 1996; Kawasaki *et al*., 1998), a result of increased bone resorption due to PTHLH-dependent induction of osteoclast activity (Chirgwin and Guise, 2000). We also identified four potential PD binding sites within the first 550 bp upstream of the *PTHLH* gene annotated in the human genome sequence (unpublished observation). Thus, the Pax3/FKHR-specific induction of *PTHLH* may account, in part, for the some of the specific properties of ARMS. Likewise, we identified Pax3/FKHR-specific targets known to be involved in

metastasis. For example, *SLIT2*, identified originally as a factor involved in axonal guidance (Hu, 1999; Nguyen Ba-Charvet *et al*., 1999), is expressed by a number of transformed cells. In this context, Slit2 recruits vascular endothelial cells, thus promoting angiogenesis as well as establishing a blood supply at distant metastatic tumour sites (Wang *et al*., 2003).

We also focused on Pax3 or Pax3/FKHR target genes known or likely to be genetically downstream of Pax3. For example, Pax3 is required for proper epithelialization of the developing somite (Schubert *et al*., 2001). *EPHA4*, which is normally expressed in the anterior portion of newly formed somites, is misexpressed in Pax3-deficient embryos (Schubert *et al*., 2001). *EPHA4* is induced by both Pax3 and Pax3/FKHR in SaOS-2 cells. Inspection of the region 5′ of the *EphA4* gene reveals at least one potential Pax3 PD binding site (Chalepakis *et al*., 1994b; Chalepakis and Gruss, 1995; Underhill *et al*., 1995; Underhill and Gros, 1997) at −83 (5′CACGTCACCGGC). Likewise, *EFNB2*, another factor involved in somite development (Durbin *et al*., 1998; Johnson *et al*., 2001; Barrios *et al*., 2003) and polarized trafficking of axons and neural crest cells through the somite (Wang and Anderson, 1997; Koblar *et al*., 2000; De Bellard *et al*., 2002), was induced by Pax3 and Pax3/FKHR in SaOS-2 cells. *EFNB2* harbours two Pax3 DNA-binding consensus sequences upstream of the transcript start (−3 and −355). Furthermore, we observed further that *MSX2*, a known target for repression by Pax3 (Kwang *et al*., 2002), was repressed by Pax3/FKHR in SaOS-2 cells (data not shown). More direct evidence of transcriptional regulation of factors by Pax3 and Pax3/FKHR was determined through chromatin immunoprecipitation. Pax3 and Pax3/FKHR coimmunoprecipitated with a segment of *CNR1* promoter region 25 bp upstream of a Pax3 PD consensus sequence (−357) and 900 bp downstream of three additional Pax3 DNA-binding consensus sequences. PD sites are also found in the analogous region of the putative murine *Cnr1* gene, although not strictly conserved positionally. Expression of *CNR1* during embryogenesis in the developing brain and neural tube (Buckley *et al*., 1998) in a pattern overlapping that of Pax3 (Goulding *et al*., 1991; Dietrich *et al*., 1993; Gerard *et al*., 1995; Terzic and Saraga-Babic, 1999) as well as the role of CNR1 (Song and Zhong, 2000) and Pax3 (Epstein *et al*., 1996; Conway *et al*., 1997a; Tremblay *et al*., 1998) in cell migration are consistent with Pax3 potentially regulating *CNR1* expression directly.

Other studies have attempted to identify transcriptional targets of Pax3 or Pax3/FKHR through microarray analysis (Khan *et al*., 1999) or using the 'CASTing' method (Khan *et al*., 1998). Comparison between our data with those from these other studies shows a striking lack of overlap in the potential targets. Notably, the gene expression profiles obtained in these studies were determined following expression of Pax3 or Pax3/FKHR in distinct cell lines. However, as we illustrated in Figure 10, Pax3- or Pax3/FKHR-regulated transcription of at least some factors was cell-type specific. *CNR1* was induced, for example, by Pax3/FKHR in both SaOS-2 and RD cells and is expressed in the Pax3/FKHR-expressing RH30 cells. In the case of *BMP4*, however, we observed repression of expression in SaOS-2 cells but Pax3/FKHR-dependent induction in RD cells. Additionally, *SLIT2* appears to be a target of Pax3/FKHR in SaOS-2 cells and is expressed in RH30 rhabdomyosarcomas, but is refractory to Pax3/FKHR activity in RD cells. We also determined that while *CXCR4* is strongly induced by Pax3/FKHR in RD cells (data not shown), as was demonstrated previously (Libura *et al*., 2002), no change in expression was seen in Pax3/FKHR-expressing SaOS-2 cells. Thus, comparisons between NIH3T3 cells (Khan *et al*., 1999) or medulloblastomas (Mayanil *et al*., 2000, 2001) with SaOS-2, RD or RH30 cells is likely to reveal divergent sets of gene targets. These data further imply significant limitations in extrapolating microarray data derived from individual cell lines to other rhabdomyosarcomas or to identification of target genes during, for example, Pax3-dependent neural crest cell migration, somite development or in rhabdomyosarcomas.

The data presented in this study, therefore, demonstrated both quantitative and qualitative differences in target gene regulation between Pax3 and its oncogenic variant, Pax3/FKHR. Our genome-wide survey of genes regulated by Pax3/FKHR revealed a number of important potential targets, which are expected to significantly influence the phenotype of rhabdomyosarcomas. Large-scale comparison between Pax3 (RD cells) and Pax3/FKHR-expressing cells (RH30 cells) as well as identification of direct transcriptional targets of these factors in primary cells using large-scale chromatin immunoprecipitations (Weinmann *et al*., 2001; Wells *et al*., 2003) is currently in progress.

Materials and methods

Cell culture

The human osteosarcoma, SaOS-2, and human rhabdomyosarcoma, RD, were maintained in Dulbecco's minimal essential medium supplemented with 10% foetal bovine serum (FBS; Sigma, Oakville, Canada). Human RH30 rhabdomyosarcoma and human U2-OS osteosarcoma cell lines were grown in α-MEM supplemented with 15% FBS and L-glutamine.

Antibodies

Rabbit polyclonal α-Pax-3 was obtained from Geneka Biotechnology (Montreal, Canada), α-pol II monoclonal antibody (clone 8WG16) from Covance (New Jersey, USA), goat polyclonal α-β-catenin from Santa Cruz Biotechnology (Santa Cruz, USA), mouse monoclonal α-β-tubulin and TRITC-labelled phalloidin from Sigma chemicals (Oakville, Canada) and α-acetylated histone H4 from Upstate Biotechnology (New York, USA).

Adenoviruses and infection

Adenoviruses expressing GFP and βgal or flagged-tagged Pax3 have been described previously (Wiggan and Hamel, 2002;

Wiggan *et al*., 2002). Pax3/FKHR^{HA}-expressing adenovirus was produced by ligating and HA-tagged Pax3/FKHR (gift from J Epstein; Sublett *et al*., 1995) into pAd-Track-CMV. Bacterial-mediated recombination and adenovirus amplification in 293 cells were performed as described previously (He *et al*., 1998). Infection of cell lines were performed as we described previously (Wiggan and Hamel, 2002; Wiggan *et al*., 2002).

Microscopy and immunofluorescence staining

Morphology of living cells was documented using a Nikon Eclipse TE200 microscope equipped with a Nikon DMX1200 digital camera. Immunofluorescent detection of actin, tubulin and β-catenin in paraformaldehyde-fixed SaOS-2 cells was performed as described previously (Wiggan and Hamel, 2002; Wiggan *et al*., 2002).

RNA isolation, microarray analysis and RT–PCR

Total RNA was isolated from SaOS-2, RD and RH30 cells using Tri-reagent (Sigma) and the RNeasy Mini Kit. Microarray analysis was performed using the Affymetrix Human U133A microarray according to the protocol of the manufacturer. For verification of expression, RT–PCR was performed using Superscript One step RT–PCR system with Platinum Taq (Invitrogen). PCR products were analysed on 2% agarose gels and quantified using NIH Image software. Primers pairs for specific human factors are as follows:

CNR1	ATC GTG TAT GCG TAC ATG TAT AT
	ACA GAG CCT CGG CAG ACG T
BMP4	TGG ACT TGG CCG ACT ACA CC
	CCA GTG AAG TTT CCC CCA ATG
BMP	ATG ATT CCT GGT AAC CGA ATG
	GCT CAG GAT ACT CAA GAC CA
CITED1	ACT TCT GCC AAG GCT CTG AAA
	GCA GCT AGA TGG AAA GTC CG
DLX3	ATG AGT GGC TCC TTC GAT CG
	GTA CAC AGC CCC AGG GTT G
SLIT2	GGT TCC CTC GGA GCA GCA A
	CCA CGG AAA GCT TTC CTT GC
TWSG	GCT GTA AGG AGT GCA TGC TG
	CCA GAT TCT CAT GAT GTG AA
PLN	CAG ACT TCC TGT CCT GCT GGT ATC
	AGA TCT AGA GGT TGT AGC AGA ACT
PTHLH	ATG CAG CGG AGA CTG GTT CA
	GTC TTG AGC GGC TGC TCT TT
EPHB2	AAA ATT GAG CAG GTG ATG GG
	TCA CAG GTG TGC TCT TGG TC
EFNB2	GCA AGT TCT GCT GGA TCA AC
	AGG ATG TGT TTG TTC CCC GAA TG
EPHA4	AGT TCC AGA CCG AAC ACA GCC TTG
	GCC ATG CAT CTG CTG CAT CTG
GAPDH	GTC AGT GGT GGA CCT GAC CT
	AGG GGT CTA CAT GGC AAC TG

Chromatin immunoprecipitation

At 48 h postinfection, cells were crosslinked for 10 min in 1% formaldehyde. A total of 3×10^6 cells were washed in ice-cold PBS. Chromatin immunoprecipitations were then performed as described previously (Popkin *et al*., 2003). The predicted promoter regions of the human *CNR1*, *EPHA2* and *EPHA4* genes were obtained from Genbank. Primers were as follows: *CNR1* −357 to −457 upstream of the annotated gene – forward primer: GCACACTTGTGTCACCAACCTGCTCAT, reverse primer: CCTCAGGTGATCCACCTGCCTCGGCCT; *EPHA2* +102 to –104 upstream of the annotated gene – forward primer: AGACATTCCTGAGGGCGGGC, reverse primer: TCTCGCTCTCGGTCCGATCC; and *EPHA4* −25 to −126 upstream of the annotated gene – forward primer: TGTCTGCGCCGCCATTGGCC, reverse primer: AGTTAG GAGAGCAGCGGGCTG.

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References

Anderson J, Gordon A, Pritchard-Jones K and Shipley J. (1999). *Genes Chromosomes Cancer*, **26,** 275–285.
Anderson J, Ramsay A, Gould S and Pritchard-Jones K. (2001). *Am. J. Pathol.*, **159,** 1089–1096.
Baldwin CT, Lipsky NR, Hoth CF, Cohen T, Mamuya W and Milunsky A. (1994). *Hum. Mutat.*, **3,** 205–211.
Barrios A, Poole RJ, Durbin L, Brennan C, Holder N and Wilson SW. (2003). *Curr. Biol.*, **13,** 1571–1582.
Bennicelli JL, Edwards RH and Barr FG. (1996). *Proc. Natl. Acad. Sci. USA*, **93,** 5455–5459.
Bernasconi M, Remppis A, Fredericks WJ, Rauscher III FJ and Schafer BW. (1996). *Proc. Natl. Acad. Sci. USA*, **93,** 13164–13169.
Blake J and Ziman MR. (2003). *Histol. Histopathol.*, **18,** 529–539.
Bober E, Franz T, Arnold HH, Gruss P and Tremblay P. (1994). *Development*, **120,** 603–612.
Bouizar Z, Spyratos F, Deytieux S, de Vernejoul MC and Jullienne A. (1993). *Cancer Res.*, **53,** 5076–5078.
Buckley NE, Hansson S, Harta G and Mezey E. (1998). *Neuroscience*, **82,** 1131–1149.
Bundred NJ, Walls J and Ratcliffe WA. (1996). *Ann. R. Coll. Surg. Engl.*, **78,** 354–358.
Cao Y and Wang C. (2000). *J. Biol. Chem.*, **275,** 9854–9862.
Chalepakis G and Gruss P. (1995). *Gene*, **162,** 267–270.
Chalepakis G, Goulding M, Read A, Strachan T and Gruss P. (1994a). *Proc. Natl. Acad. Sci. USA*, **91,** 3685–3689.
Chalepakis G, Wijnholds J and Gruss P. (1994b). *Nucleic Acids Res.*, **22,** 3131–3137.
Chang C, Holtzman DA, Chau S, Chickering T, Woolf EA, Holmgren LM, Bodorova J, Gearing DP, Holmes WE and Brivanlou AH. (2001). *Nature*, **410,** 483–487.
Chirgwin JM and Guise TA. (2000). *Crit. Rev. Eukaryot. Gene Expr.*, **10,** 159–178.
Conway SJ, Henderson DJ and Copp AJ. (1997a). *Development*, **124,** 505–514.
Conway SJ, Henderson DJ, Kirby ML, Anderson RH and Copp AJ. (1997b). *Cardiovasc. Res.*, **36,** 163–173.
De Bellard ME, Ching W, Gossler A and Bronner-Fraser M. (2002). *Dev. Biol.*, **249,** 121–130.
Dietrich S, Schubert FR and Gruss P. (1993). *Mech. Dev.*, **44,** 189–207.
Dunbar ME and Wysolmerski JJ. (1999). *J. Mammary Gland Biol. Neoplasia*, **4,** 21–34.

Durbin L, Brennan C, Shiomi K, Cooke J, Barrios A, Shanmugalingam S, Guthrie B, Lindberg R and Holder N. (1998). *Genes Dev.*, **12,** 3096–3109.
Elbashir SM, Lendeckel W and Tuschl T. (2001). *Genes Dev.*, **15,** 188–200.
Epstein JA, Shapiro DN, Cheng J, Lam PY and Maas RL. (1996). *Proc. Natl. Acad. Sci. USA*, **93,** 4213–4218.
Epstein JA, Song B, Lakkis M and Wang C. (1998). *Mol. Cell. Biol.*, **18,** 4118–4130.
Farquharson C, Jefferies D, Seawright E and Houston B. (2001). *Endocrinology*, **142,** 4131–4140.
Foley J, Dann P, Hong J, Cosgrove J, Dreyer B, Rimm D, Dunbar M, Philbrick W and Wysolmerski J. (2001). *Development*, **128,** 513–525.
Fortin AS, Underhill DA and Gros P. (1997). *Hum. Mol. Genet.*, **6,** 1781–1790.
Fredericks WJ, Ayyanathan K and Rauscher III FJ. (2001). *Cancer Lett.*, **162** (Suppl)**,** S23–S32.
Fredericks WJ, Galili N, Mukhopadhyay S, Rovera G, Bennicelli J, Barr FG and Rauscher III FJ. (1995). *Mol. Cell. Biol.*, **15,** 1522–1535.
Gerard M, Abitbol M, Delezoide AL, Dufier JL, Mallet J and Vekemans M. (1995). *C.R. Acad. Sci. Ser. III*, **318,** 57–66.
Goulding MD, Chalepakis G, Deutsch U, Erselius JR and Gruss P. (1991). *EMBO J.*, **10,** 1135–1147.
Guise TA, Yin JJ, Taylor SD, Kumagai Y, Dallas M, Boyce BF, Yoneda T and Mundy GR. (1996). *J. Clin. Invest.*, **98,** 1544–1549.
He TC, Zhou S, da Costa LT, Yu J, Kinzler KW and Vogelstein B. (1998). *Proc. Natl. Acad. Sci. USA*, **95,** 2509–2514.
Hollenbach AD, Sublett JE, McPherson CJ and Grosveld G. (1999). *EMBO J.*, **18,** 3702–3711.
Hoth CF, Milunsky A, Lipsky N, Sheffer R, Clarren SK and Baldwin CT. (1993). *Am. J. Hum. Genet.*, **52,** 455–462.
Hu H. (1999). *Neuron*, **23,** 703–711.
Johnson J, Rhee J, Parsons SM, Brown D, Olson EN and Rawls A. (2001). *Dev. Biol.*, **229,** 176–187.
Kaestner KH, Knochel W and Martinez DE. (2000). *Genes Dev.*, **14,** 142–146.
Kawasaki H, Takayama J, Nagasaki K, Yamaguchi K and Ohira M. (1998). *J. Pediatr. Hematol. Oncol.*, **20,** 327–329.
Khan J, Bittner ML, Saal LH, Teichmann U, Azorsa DO, Gooden GC, Pavan WJ, Trent JM and Meltzer PS. (1999). *Proc. Natl. Acad. Sci. USA*, **96,** 13264–13269.
Khan J, Simon R, Bittner M, Chen Y, Leighton SB, Pohida T, Smith PD, Jiang Y, Gooden GC, Trent JM and Meltzer PS. (1998). *Cancer Res.*, **58,** 5009–5013.
Kissin MW, Henderson MA, Danks JA, Hayman JA, Bennett RC and Martin TJ. (1993). *Eur. J. Surg. Oncol.*, **19,** 134–142.
Koblar SA, Krull CE, Pasquale EB, McLennan R, Peale FD, Cerretti DP and Bothwell M. (2000). *J. Neurobiol.*, **42,** 437–447.
Kwang SJ, Brugger SM, Lazik A, Merrill AE, Wu LY, Liu YH, Ishii M, Sangiorgi FO, Rauchman M, Sucov HM, Maas RL and Maxson Jr RE. (2002). *Development*, **129,** 527–538.
Lagutina I, Conway SJ, Sublett J and Grosveld GC. (2002). *Mol. Cell. Biol.*, **22,** 7204–7216.
Lam PY, Sublett JE, Hollenbach AD and Roussel MF. (1999). *Mol. Cell. Biol.*, **19,** 594–601.
Libura J, Drukala J, Majka M, Tomescu O, Navenot JM, Kucia M, Marquez L, Peiper SC, Barr FG, Janowska-Wieczorek A and Ratajczak MZ. (2002). *Blood*, **100,** 2597–2606.
Luo T, Matsuo-Takasaki M, Lim JH and Sargent TD. (2001). *Int. J. Dev. Biol.*, **45,** 681–684.
Mayanil CS, George D, Freilich L, Miljan EJ, Mania-Farnell B, McLone DG and Bremer EG. (2001). *J. Biol. Chem.*, **276,** 49299–49309.
Mayanil CS, George D, Mania-Farnell B, Bremer CL, McLone DG and Bremer EG. (2000). *J. Biol. Chem.*, **275,** 23259–23266.
Nguyen Ba-Charvet KT, Brose K, Marillat V, Kidd T, Goodman CS, Tessier-Lavigne M, Sotelo C and Chedotal A. (1999). *Neuron*, **22,** 463–473.
Park GT and Morasso MI. (2002). *Nucleic Acids Res.*, **30,** 515–522.
Popkin DL, Watson MA, Karaskov E, Dunn GP, Bremner R and Virgin IV HW. (2003). *Proc. Natl. Acad. Sci. USA*, **100,** 14309–14314.
Poser I and Bosserhoff AK. (2004). *Histol. Histopathol.*, **19,** 173–188.
Relaix F, Polimeni M, Rocancourt D, Ponzetto C, Schafer BW and Buckingham M. (2003). *Genes Dev.*, **17,** 2950–2965.
Ross JJ, Shimmi O, Vilmos P, Petryk A, Kim H, Gaudenz K, Hermanson S, Ekker SC, O'Connor MB and Marsh JL. (2001). *Nature*, **410,** 479–483.
Scheidler S, Fredericks WJ, Rauscher III FJ, Barr FG and Vogt PK. (1996). *Proc. Natl. Acad. Sci. USA*, **93,** 9805–9809.
Schubert FR, Tremblay P, Mansouri A, Faisst AM, Kammandel B, Lumsden A, Gruss P and Dietrich S. (2001). *Dev. Dyn.*, **222,** 506–521.
Scott IC, Blitz IL, Pappano WN, Maas SA, Cho KW and Greenspan DS. (2001). *Nature*, **410,** 475–478.
Sebag M, Henderson J, Goltzman D and Kremer R. (1994). *Am. J. Physiol.*, **267,** C723–C730.
Shapiro DN, Sublett JE, Li B, Downing JR and Naeve CW. (1993). *Cancer Res.*, **53,** 5108–5112.
Song ZH and Zhong M. (2000). *J. Pharmacol. Exp. Ther.*, **294,** 204–209.
Sublett JE, Jeon IS and Shapiro DN. (1995). *Oncogene*, **11,** 545–552.
Tassabehji M, Newton VE, Leverton K, Turnbull K, Seemanova E, Kunze J, Sperling K, Strachan T and Read AP. (1994). *Hum. Mol. Genet.*, **3,** 1069–1074.
Terzic J and Saraga-Babic M. (1999). *Int. J. Dev. Biol.*, **43,** 501–508.
Tremblay P, Dietrich S, Mericskay M, Schubert FR, Li Z and Paulin D. (1998). *Dev. Biol.*, **203,** 49–61.
Tremblay P, Kessel M and Gruss P. (1995). *Dev. Biol.*, **171,** 317–329.
Underhill DA and Gros P. (1997). *J. Biol. Chem.*, **272,** 14175–14182.
Underhill DA, Vogan KJ and Gros P. (1995). *Proc. Natl. Acad. Sci. USA*, **92,** 3692–3696.
Vachtenheim J and Novotna H. (1999). *Cell. Mol. Biol. (Noisy-le-grand)*, **45,** 1075–1082.
Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM and Tabin CJ. (1996). *Science*, **273,** 613–622.
Wang B, Xiao Y, Ding BB, Zhang N, Yuan X, Gui L, Qian KX, Duan S, Chen Z, Rao Y and Geng JG. (2003). *Cancer Cell*, **4,** 19–29.
Wang HU and Anderson DJ. (1997). *Neuron*, **18,** 383–396.
Wardle FC, Angerer LM, Angerer RC and Dale L. (1999a). *Dev. Biol.*, **206,** 63–72.
Wardle FC, Welch JV and Dale L. (1999b). *Mech. Dev.*, **86,** 75–85.

Weinmann AS, Bartley SM, Zhang T, Zhang MQ and Farnham PJ. (2001). *Mol. Cell. Biol.*, **21,** 6820–6832.
Wells J, Yan PS, Cechvala M, Huang T and Farnham PJ. (2003). *Oncogene*, **22,** 1445–1460.
Wiggan O and Hamel PA. (2002). *J. Cell Sci.*, **115,** 531–541.
Wiggan O, Fadel MP and Hamel PA. (2002). *J. Cell Sci.*, **115,** 517–529.
Wiggan O, Taniguchi-Sidle A and Hamel PA. (1998). *Oncogene*, **16,** 227–236.
Xia SJ, Pressey JG and Barr FG. (2002). *Cancer Biol. Ther.*, **1,** 97–104.
Yu K, Srinivasan S, Shimmi O, Biehs B, Rashka KE, Kimelman D, O'Connor MB and Bier E. (2000). *Development*, **127,** 2143–2154.

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