Subnuclear localization and mobility are key indicators of PAX3 dysfunction in Waardenburg syndrome

Gareth N. Corry¹, Michael J. Hendzel² and D. Alan Underhill¹,²,*

¹Department of Medical Genetics, University of Alberta, Edmonton, Alberta, Canada T6G 2H7 and ²Department of Oncology, Cross Cancer Institute, Edmonton, Alberta, Canada T6G 1Z2

Mutations in the transcription factor PAX3 cause Waardenburg syndrome (WS) in humans and the mouse Splotch mutant, which display similar neural crest-derived defects. Previous characterization of disease-causing mutations revealed pleiotropic effects on PAX3 DNA binding and transcriptional activity. In this study, we evaluated the impact of disease alleles on PAX3 localization and mobility. Immunofluorescence analyses indicated that the majority of PAX3 occupies the interchromatin space, with only sporadic colocalization with sites of transcription. Interestingly, PAX3 disease alleles fell into two distinct categories when localization and dynamics in fluorescence recovery after photobleaching (FRAP) were assessed. The first group (class I), comprising N47H, G81A and V265F exhibit a diffuse distribution and markedly increased mobility when compared with wild-type PAX3. In contrast, the G42R, F45L, S84F, Y90H and R271G mutants (class II) display evidence of subnuclear compartmentalization and mobility intermediate between wild-type PAX3 and class I proteins. However, unlike class I mutants, which retain DNA binding, class II proteins are deficient for this activity, indicating that DNA binding is not a primary determinant of PAX3 distribution and movement. Importantly, class I properties prevail when combined with a class II mutation, which taken with the proximity of the two mutant classes within the PAX3 protein, suggests class I mutants act by perturbing PAX3 conformation. Together, these results establish that altered localization and dynamics play a key role in PAX3 dysfunction and that loss of the underlying determinants represents the principal defect for a subset of Waardenburg mutations.

INTRODUCTION

The PAX3 transcription factor is a key regulator of developmentally important processes in metazoan organisms and also carries out distinct postnatal functions (1–3). The mammalian PAX gene family comprises nine members, all of which feature a DNA-binding paired domain (PD) related to that of the Drosophila paired (Prd) protein (4–6). Like Prd, PAX3 contains a homeodomain (HD), which is known to functionally interact with the PD to modulate DNA binding activity (7–12). During murine embryogenesis, Pax3 is expressed in the dorsal neural tube, neural crest cells, and dermomyotome (3,4), all of which are affected by mutation of Pax3 in the mouse Splotch (Sp) mutants (13–15). Specifically, homozygous Sp embryos exhibit neural tube and cardiac defects, as well as an absence of limb and diaphragm muscle, while heterozygotes are characterized by pigmented defects (16–18). In humans, PAX3 haploinsufficiency causes Waardenburg Syndrome (WS) types 1 and 3 (19–22), where individuals present with sensorineural deafness, pigmentary defects and dystopia canthorum. In addition, WS3 patients are distinguished by limb musculature defects. Lastly, a translocation involving PAX3 in the childhood cancer alveolar rhabdomyosarcoma results in an oncogenic fusion protein (PAX3-FKHR) comprising both DNA-binding domains of PAX3 and the carboxy-terminal region of the FOXO1A transcription factor (23,24).

Characterization of the PAX3 locus from WS patients has revealed a broad spectrum of lesions, including roughly 30 missense mutations, most of which occur in either the PD or
Biochemical analyses of these mutants have revealed pleiotropic effects on DNA binding that range from relatively inert to greatly reduced to elevated (9,25–28). In addition, reporter gene assays using two melanocyte-specific targets of PAX3, microphthalmia-associated transcription factor (MITF) (28) and tyrosinase-related protein-1 (Tyrp-1) (29), indicate disease-causing mutations have disparate effects on promoter activity (26). As a result, it has not been straightforward to understand the molecular basis of PAX3 loss of function or establish relationships between phenotype and genotype, particularly in accounting for the differences in WS types 1 and 3. This is further complicated by the fact that the same PAX3 mutant allele can produce distinct phenotypes, even within the same family. As a result, it appears other aspects of PAX3 function may be compromised by disease-causing mutations and could potentially account for the variable phenotypic expressivity seen in WS.

The eukaryotic nucleus is a highly organized structure (30–33). Notably, the positioning of nuclear constituents relative to each other plays a vital role in the regulation of nuclear processes, and disruptions to this organization can be pathogenic (reviewed in 34). Within this scheme, transcription factors can exhibit distinct distributions and also display varying degrees of mobility that likely facilitates movement between different compartments in response to signaling events or interactions with other nuclear elements (33,35,36). Live cell studies show that chromosomes undergo constrained diffusion within a given territory (37,38) and long-range, actin-dependent chromosomal movement has also been proposed (39,40). On the other hand, individual chromatin loci have been shown to exhibit fast, short-range motion (41,42). Together, these observations suggest that the coordinated and dynamic interaction between the transcriptional machinery and target chromosomal loci is critical for proper regulation of eukaryotic gene expression (43). With this in mind, we have assessed the subnuclear localization and mobility of PAX3 and disease-causing variants. Collectively, our analyses indicate that PAX3 contains multiple determinants for subnuclear localization and dynamics, although DNA binding does not appear to be a significant determinant of either. Importantly, we find that adjacent mutations can have markedly distinct effects on PAX3 dysfunction and establish altered dynamics as a key aspect of PAX3 disease alleles, regardless of their effect on DNA binding or reporter gene activity.

RESULTS

Subnuclear localization of wild-type PAX3

To investigate the subnuclear distribution of PAX3, we performed immunofluorescence on endogenous PAX3 in B16F10 mouse melanoma cells and primary limb bud cultures, as well as in mouse 10T1/2 cells after transient transfection with PAX3 expression plasmids. An important objective of these analyses was to establish a reference point for the characterization of PAX3 disease alleles. In B16F10 cells, PAX3 adopted a reticular pattern, which was largely excluded from Hoechst-stained pericentromeric heterochromatin, perinuclear and perinucleolar heterochromatin, and nucleoli (Fig. 1A). Characterization of PAX3 localization in primary limb bud cultures also revealed a reticular distribution that did not overlap with condensed chromatin (Fig. 1B). As in the B16F10 cells, several foci of elevated PAX3 staining were present and typically occupied the nuclear periphery (arrows, Fig. 1A and B). Moreover, PAX3 immunofluorescence in whole embryo sections (10.5 dpc) was excluded...
from heterochromatin over the entire PAX3 expression domain (cells of the dorsal neural tube, dorsal root ganglia, dermomyotome and migrating myoblasts; data not shown) and is therefore consistent with data from B16F10 and primary cells. In both B16F10 cells and primary cultures, an identical pattern was observed with a second PAX3-specific antibody that recognizes a different epitope (data not shown; described in Materials and Methods). In 10T1/2 fibroblast cells transiently transfected with plasmids expressing untagged PAX3 (Fig. 1C, top row) or PAX3 with a carboxy-terminal green fluorescent protein (GFP) tag (PAX3-GFP; Fig. 1C, bottom row), we observed similar intranuclear distributions to that of endogenous PAX3. In the latter case, this pattern was observed in ~80% of transfected cells, while in the remaining cells, PAX3-GFP co-localized to a large extent with heterochromatic areas (data not shown).

We also monitored PAX3 localization throughout mitosis in unsynchronized B16F10 cells stained with Hoechst (Fig. 1D). In prophase, PAX3 was initially confined to the nucleus at early stages and eventually redistributed to the cytoplasm. This pattern was maintained until cells entered telophase and the chromosomes began to decondense, at which point PAX3 once again appeared in proximity to loosely packed chromatin, with the majority of PAX3 found in nuclei by late telophase. As observed during interphase, PAX3 was clearly excluded from regions of Hoechst staining, which was most apparent with highly condensed chromosomes at metaphase. Despite the redistribution of PAX3 during mitosis, the protein retained evidence of the reticular pattern observed during interphase where PAX3 immunofluorescence often appears as 'strands' (arrows in Fig. 1D). Together, these analyses reveal that the steady state distribution of PAX3, both in vivo and in situ, involves a mesh-like pattern during interphase (and to a lesser extent in mitosis) and does not show obvious localization to heterochromatic landmarks demarcated by Hoechst staining at any point during the cell cycle.

Localization of wild-type PAX3 and post-translational histone modifications

The DNA-binding dyes Hoechst 33258 and 4',6-diamidino-2-phenylindole (DAPI) exhibit a preference for AT-rich DNA, which is more abundant in heterochromatin and, as a result, do not efficiently demarcate the euchromatic compartment. To further assess PAX3 subnuclear localization, immunofluorescence was used to delimit either active or inactive chromatin using antibodies that recognize histone modifications enriched in these chromatin domains. These included acetylated histone H3 and H4, which mark transcriptionally competent euchromatin (44), trimethyl Lys4 of histone H3 (H3K4me3), which marks promoter regions of transcriptionally active genes (45,46), and trimethyl Lys36 of histone H3 (H3K36me3), which is associated with transcriptional elongation by RNA Polymersase II (47–49). Modifications associated with heterochromatin and silenced chromatin were also investigated; these included trimethyl Lys9 of histone H3, found primarily in condensed silenced heterochromatin (50,51) and trimethyl Lys20 of histone H4, a marker of pericentromeric heterochromatin (52). Not surprisingly, PAX3 failed to localize with these heterochromatin marks, given that they largely recapitulate the Hoechst pattern (data not shown).

As the euchromatin-associated histone modifications revealed qualitatively similar localization with respect to each other, H3K4me3 was used to demonstrate their relationship with PAX3. Although more diffuse than PAX3, H3K4me3 also produced a reticular pattern that was excluded from Hoechst (Fig. 2A). PAX3 immunofluorescence was in close proximity to H3K4me3 throughout the nucleus, where the signals appeared interdigitated or intertwined and also intersected at some points (arrows in Fig. 2B). This is more obvious upon introducing an intensity threshold (Fig. 2C) and reveals that PAX3 is closely apposed to euchromatin with a small subset displaying co-localization. Lastly, rendering images in three dimensions (3D) illustrate the clear difference in localization of PAX3 with respect to euchromatin and heterochromatin (Fig. 2D). Based on this spatial relationship, we propose that the vast majority of PAX3 occupies the interchromatin space and that only a subset appears to be engaged with euchromatin.

The spatial relationship of PAX3 with euchromatin was further evaluated in cells incubated with 5-fluorouridine (5-FUrd), a nucleotide analog that is incorporated into newly synthesized RNA and can be recognized by a bromodeoxyuridine (BrdU)-specific antibody. We incubated B16F10 cells plasmid with 5-FUrd for 30 min and then performed co-immunofluorescence with PAX3 and BrdU-specific antibodies. The BrdU antibody recognized numerous small punctate nuclear foci corresponding to sites of 5-FUrd incorporation (Fig. 2E) and the PAX3 signal showed partial overlap with several 5-FUrd foci per nucleus. A similar pattern of co-localization was observed in 10T1/2 nuclei transiently transfected with PAX3 (data not shown). Consistent with the juxtaposition of PAX3 and H3K4me3, these results provide evidence that PAX3 is found in regions of transcriptional activity, but suggest that only a small number of PAX3 sites contain transcriptionally active loci at any given time.

Effects of disease-associated mutations on PAX3 subnuclear localization

The above findings establish that PAX3-GFP recapitulates the distribution of untagged and endogenous PAX3, and provides a means to characterize PAX3 mutants independent of effects the mutations may have on antibody recognition. For this purpose, seven missense mutations causing WS were selected (F45L, N47H, G81A, S84F and Y90H in the PD, and V265F and R271G in the HD), as well as the G42R PD mutation found in the Splotch-delayed (Spd) mouse (15). We have previously shown that this cohort of mutations has variable effects on DNA binding and reporter gene transactivation (26). We also assessed the effects of two WS-associated mutations outside the DNA-binding domains, A196T in the linker and Q391H in the carboxy-terminus (see Fig. 3A for positions of mutations). To compare the localization of wild-type and mutant PAX3 proteins, each was expressed as a GFP fusion protein in 10T1/2 cells. As noted for wild-type PAX3-GFP (Fig. 3B), all mutants were excluded from Hoechst-stained chromatin (Fig. 3C–E). The Spd mutant displayed a more uniform distribution than wild-type
PAX3-GFP (Fig. 3C, top row), was less abundant in perinucleolar areas, and tended to display one or two large bright foci in some cells rather than the multiple bright foci characteristic of PAX3-GFP. The F45L mutant displayed a similar pattern to the wild-type protein, with a number of bright foci and concentrations around nucleoli (Fig. 3C, second row). Both the N47H (Fig. 3C, third row) and G81A (Fig. 3C, fourth row) proteins showed consistently lower expression.
levels than the other PAX3 variants, tended to be excluded from perinucleolar areas, and generally showed a more diffuse staining pattern than the wild-type protein. These mutants also showed a moderate level of cytoplasmic localization, suggesting the N47H and G81A mutations may interfere with nuclear localization or retention of PAX3. In addition, the N47H mutant consistently showed exclusion from the nuclear periphery. The S84F mutant was similar in...
appearance to wild-type and F45L (Fig. 3C, fifth row), although it tended to exhibit a noticeably more diffuse background localization pattern than either. Likewise, the Y90H mutant displayed numerous bright foci against a less intense, diffuse background (Fig. 3C, bottom row). The HD mutant V265F was distributed throughout the nucleus and cytoplasm (Fig. 3D, top row), closely resembling the N47H and G81A mutants. Finally, the R271G HD variant showed a pattern similar to $Sp^d$, having a uniform distribution in Hoechst-free areas with less enrichment around the nucleoli and one or more large bright foci (Fig. 3D, bottom row). The A196T and Q391H mutants showed similar characteristics to wild-type PAX3-GFP, including a punctate pattern and enrichment in perinucleolar areas (Fig. 3E), although we did not observe the same bright foci distinctive of PAX3-GFP. In this regard, the A196T and Q391H mutants more closely resemble the $Sp^d$ and R271G patterns. Together, these observations demonstrate that PAX3 disease mutations influence subnuclear compartmentalization to differing degrees, which was most severe for N47H, G81A and V265F, and represents another aspect of PAX3 dysfunction.

Nuclear dynamics of wild-type and mutant PAX3

In addition to transcription factor compartmentalization within the cell, protein mobility has emerged as essential component of gene regulation. We therefore used fluorescence recovery after photobleaching (FRAP) experiments to analyze the mobility of wild-type PAX3-GFP and disease-causing mutants. At the same time, this provided an opportunity to compare the localization of wild-type PAX3-GFP fusions to those containing disease-causing mutants in live and fixed cells. The post-bleach recovery profiles of wild-type and mutant PAX3-GFP proteins show that their localization in live cells can be generally correlated with that observed in fixed cells and also allow categorization of the mutants into two groups. The first includes $Sp^d$, F45L, S84F, Y90H and R271G, which retain a compartmentalized appearance similar to that of wild-type PAX3 (Fig. 4A; cf. Fig. 3). The N47H, G81A and V265F mutants form a second group characterized by diffuse distribution and, as noted in fixed cells (Fig. 3), display varying degrees of cytoplasmic localization, although fluorescence intensity in the nucleus is greater in each case (Fig. 4B). As will be elaborated below, the presence or absence of a compartmentalized distribution correlates with mobility.

The analysis of PAX3 disease allele dynamics provides two major findings: with the exception of PAX3-GFP A196T and Q391H, all mutants tested displayed an obvious increase in mobility when compared with the wild-type protein and had essentially recovered to 100% by 90s (Fig. 4C; see also Supplementary Material, Fig. S1), indicating they lack the immobile fraction seen with wild-type PAX3. Nevertheless, the mutants fell into two categories that could be distinguished in terms of overall mobility and free pool sizes, the free pool being estimated by the value at the first time point after photobleaching (1.5 s). The first group, designated as class I, which includes the N47H and G81A mutations in the PD and V265F in the HD, showed the highest recovery rates and possessed the largest freely diffusing pool of all the PAX3-GFP proteins (Fig. 4C). The remaining mutants ($Sp^d$, F45L, S84F, Y90H and R271G), which we have called class II, displayed recovery rates and ‘free pools’ intermediate between wild-type PAX3 and class I mutants (Fig. 4C), with $Sp^d$ being the fastest and Y90H the slowest amongst this cohort. As noted above, these differences in mobility also correlate with localization, where the class I mutants displayed a diffuse distribution that included a cytoplasmic fraction, while the class II mutants retained clear evidence of subnuclear compartmentalization. The specificity of these findings is underscored by the properties of the only two WS missense mutations outside of either DNA-binding domain, A196T and Q391H. Consistent with the idea they occur at intron–exon junctions and are thought to contribute to PAX3 dysfunction by aberrant splicing, neither mutant had an appreciable affect on PAX3 mobility (Fig. 4C). Importantly, the differences in mobility among the two mutant classes and wild-type PAX3-GFP are independent of protein expression levels. Specifically, none of the PAX3 variants were expressed at abnormally high levels, indicating that the increased mobility and unbound fraction of the fastest-recovering PAX3 variants is not due to the saturation of binding sites or other nuclear constituents that constrain PAX3 movement. Together, these observations indicate that disease-causing alleles of PAX3 exert significant effects on mobility and provide the first measure of PAX3 activity that is consistently altered in this collection of mutations.

The segregation of PAX3 mutants into two discrete categories based on localization and dynamics was unanticipated. To address whether these reflect completely separate effects and to further refine their mechanistic basis, representative mutants from each class were combined. This involved the creation of F45L/G81A (class II/class I) and F45L/S84F (class II/class II) PAX3-GFP fusions. In each case, DNA binding was assessed and compared with wild-type PAX3 and the singly mutated counterparts (F45L, G81A and S84F). As previously shown (26), the presence of a class II mutation was associated with a loss or reduction in PDHD DNA binding compared with wild-type (Fig. 5A). Significantly, this impairment was increased in severity in the class II/class II double mutant, suggesting that the effects of these mutations on DNA binding are additive. Nevertheless, the class II/class II combination did not display defects in localization or mobility over and above each individual mutation (Figs 5B and C). The class II/class I double mutant, however, fully recapitulated the mobility and localization of the class I mutant (Figs 5B and C), but with reduced (MIIF) or absent (Tyrp-1) DNA binding (Fig. 5A). From a mechanistic standpoint, this indicates that robust DNA binding by class I proteins is not required for their increase in mobility or diffuse distribution and that these attributes supersede those of class II mutants when combined in cis. Moreover, it clearly establishes that class I and II mutations lead to PAX3 dysfunction via distinct means, such that altered nuclear dynamics appears to be the primary defect of the N47H, G81A and V265F proteins.

DISCUSSION

In this study, we have examined the subnuclear distribution and dynamics of the developmentally important transcription factor PAX3 and numerous disease-causing mutants. Immunofluorescence of endogenous and exogenous PAX3
revealed a reticular pattern that was excluded from regions of heterochromatin (Fig. 1), but closely apposed to euchromatin (Fig. 2). This was most apparent with its global proximity to epigenetic marks of transcriptionally active chromatin, notably H3K4me3 and H3K36me3, which are associated with promoter (45,46) and transcribed regions (47–49), respectively. In this regard, the distribution of PAX3 resembles that described for proteins that localize to the interchromatin space, exemplified by the RNAPII-associated transcription factor TFIIH (53,54), the ubiquitous SP1 and SP3 proteins (55), the POU domain transcription factor OCT1 (53), the HOX family member TLX1 (56), and the forkhead protein...
FOXC1 (57), among others (33). This subnuclear compartment appears as a complex, channel-like network that traverses between and within chromosome territories in the interphase nucleus (58). Moreover, PAX3 mobility \( (t_{50} \text{ of } \sim 7.9 \text{ s}) \) is similar to many other DNA-binding and chromatin-associated proteins with half maximal recovery times on the order of several seconds (59). Lastly, the fact that only a small subset of PAX3 co-localizes with H3K4me3 or sites of transcription delineated by S'-FUrd incorporation also correlates with studies that found only a fraction of the total nuclear transcription factor population coincided with foci formed by RNAPII (53,60–62). As a result, our findings with PAX3 are consistent with models where transcription factors accumulate predominantly in subnuclear domains within the interchromatin compartment, which may modulate their local concentration or serve as assembly sites for regulatory complexes and allow dynamic exchange with target sites (63–65).

Disease mutations have been shown to disrupt or alter the \textit{in vivo} action and \textit{in vitro} biochemical properties of numerous transcription factors (66–71). In the case of \textit{PAX3}, disease-causing alterations range from complete deletions of the locus to single nucleotide substitutions. These lesions can lead to an absence of protein product, internal deletions or truncations, or missense mutations, which together establish that the WS phenotype arises from haploinsufficiency. With only two exceptions, A196T and Q391H, all known missense mutations occur in one of the two PAX3 DNA-binding domains (72), and loss-of-function analyses have therefore focused on DNA binding and reporter gene assays. These studies have revealed a range of effects on PAX3 DNA binding and reporter gene activity and demonstrate that these parameters do not always correlate—reductions in DNA binding did not necessarily lead to decreased values in reporter gene assays (7–9,25–28). Given this disparity, it is significant that disease alleles were found to have more consistent effects on PAX3 mobility and that these correlate with localization and DNA binding (discussed below). Specifically, all mutations present within the PD or HD were characterized by increased mobility, which is further underscored by the fact that A196T and Q391H resembled wild-type PAX3 in terms of localization and mobility. This latter finding was not unexpected, as both mutations occur at intron–exon junctions and have been suggested to disrupt PAX3 function through aberrant splicing. From a mechanistic standpoint, the increase in mobility of PAX3 mutants represents a thermodynamic defect that may impede their ability to form functional complexes during transcription, an idea that is supported by the dynamic interdependence of the glucocorticoid receptor and high mobility group-B1 proteins (73). Lastly, based on the fact that increased mobility was a shared feature amongst a randomly selected cohort of seven WS mutations (and one \textit{Sp} mutation), we suggest this may be a general characteristic of disease-causing missense alleles.

The increase in the mobility of PAX3 mutants reflects a loss of molecular interactions that constrain movement of the wild-type protein, the severity of which led to stratification of mutants into two groups. Accordingly, the mutants exhibiting the greatest mobility (class I: N47H, G81A and V265F) can be considered to manifest the most severe defect in these interactions and is consistent with their diffuse localization and lack of compartmentalization. Likewise, the intermediate effect of class II mutants (\textit{Sp} \textsuperscript{70}, F45L, S84F, Y90H and R271G) on PAX3 movement suggests they retain sufficient interactions to confer a compartmentalized appearance. In this regard, PAX3 is similar to other transcription factors, where altered nuclear distribution (68,70,71,74,75) and increased mobility (76–79) have been seen upon mutation of the DNA-binding domain, and are thought to derive from defects in protein-DNA...
interactions. For instance, analyses of wild-type and mutant forms of the NF-kB subunit p65 found that high mobility correlated with low DNA binding affinity and a more random distribution in the nucleus (78), and an analogous relationship was also demonstrated for the glucocorticoid receptor (80). Similarly, disease-causing mutations in the POU-class HD factor PIT1/POU1F1 lead to reductions in DNA binding activity and increase nuclear mobility (67). A key difference in the
behavior of PAX3 mutants, however, is that their DNA binding properties are inversely correlated with their intranuclear localization and mobility, since these parameters are most severely affected by mutations (class I) that permit interaction with target sites at levels similar to wild-type PAX3. Although this suggests that DNA binding might be required for the rapid mobility and diffuse distribution of class I mutants, these attributes are retained even with the loss or reduction of DNA binding that occurs by combining with a class II mutation (Fig. 5). Together, these data indicate altered localization and mobility are important aspects of PAX3 dysfunction in WS, (Fig. 5). Together, these data indicate altered localization and mobility, since these parameters are most severely affected by mutations (class I) that permit interaction with target sites at levels similar to wild-type PAX3. Although this suggests that DNA binding might be required for the rapid mobility and diffuse distribution of class I mutants, these attributes are retained even with the loss or reduction of DNA binding that occurs by combining with a class II mutation (Fig. 5). Together, these data indicate altered localization and mobility are important aspects of PAX3 dysfunction in WS, and represent the principal defect in class I mutants where spatially close were just as likely to segregate to different classes. This can be illustrated by the proximity of F45L (class II) and N47H (class I), which both project side chains toward the minor groove in the PD crystal structure, while G81A (class I) and S84F (class II) lie on the same surface of helix 3 of the amino-terminal PD subdomain, facing the major groove (Fig. 6A). As a result of this spatial relationship, it is unlikely that each mutational class affects a distinct interaction surface. Rather, these data suggest that class II proteins retain determinants that constrain their localization, but attenuation of the underlying interaction(s) that support compartmentalization leads to increased mobility (Fig. 6B). In contrast, class I mutations would cause a loss of key interaction surfaces altogether, which is underscored by finding that rapid mobility and diffuse localization prevail when class I and II mutations are present in the same polypeptide (Fig. 6B).

When combined with our observation that all class I mutants exhibit a cytoplasmic fraction, reflecting a defect in nuclear import or retention, the most likely explanation for the behavior of class I mutants would involve altered conformational changes (indicated by the change in structure, middle) appear to underlie the further increase in mobility of class I mutants. Schematic diagrams on the right summarize the compartmentalized pattern of class II mutants (bottom) and diffuse, uncompartmentalized phenotype of class I mutants (top). Green, PAX3; red, Hoechst; black, pericentromeric heterochromatin foci (PCH).

**Figure 6.** Disease mutations exert differential effects on determinants that constrain PAX3 activity in the nucleus. (A) Ribbon structures of the PAX3 homeodomain (left) and amino-terminal paired subdomain (right) showing the spatial proximity of selected class I (dark gray) and class II (light gray) mutants. The model depicts the DNA binding surface of the PD and HD oriented as they would appear on a composite PH0 motif. (B) Summary of the effects of class I and II mutations on PAX3 behavior in vivo. Wild-type PAX3 and proteins from the two mutant classes each display unique recovery profiles in FRAP experiments; the increase in mobility among class II mutants reflects impairments in molecular interactions that constrain local movement of PAX3, while conformational alterations (indicated by the change in structure, middle) appear to underlie the further increase in mobility of class I mutants. Schematic diagrams on the right summarize the compartmentalized pattern of class II mutants (bottom) and diffuse, uncompartmentalized phenotype of class I mutants (top). Green, PAX3; red, Hoechst; black, pericentromeric heterochromatin foci (PCH).

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Class I and II mutants can be separated by a single amino acid in the PAX3 primary structure, as seen for F45L and N47H. Moreover, when these mutants are examined in the context of the DNA-bound PD or HD crystal structures, or models where the domains are bound to the composite PH0 motif (81), there is no obvious correlation between the position of mutations and their effects on localization and mobility (summarized in Fig. 6A). For instance, mutations in separate DNA-binding domains could belong to the same mutant class, while those that were spatially close were just as likely to segregate to different classes. This can be illustrated by the proximity of F45L (class II) and N47H (class I), which both project side chains toward the minor groove in the PD crystal structure, while G81A (class I) and S84F (class II) lie on the same surface of helix 3 of the amino-terminal PD subdomain, facing the major groove (Fig. 6A). As a result of this spatial relationship, it is unlikely that each mutational class affects a distinct interaction surface. Rather, these data suggest that class II proteins retain determinants that constrain their localization, but attenuation of the underlying interaction(s) that support compartmentalization leads to increased mobility (Fig. 6B). In contrast, class I mutations would cause a loss of key interaction surfaces altogether, which is underscored by finding that rapid mobility and diffuse localization prevail when class I and II mutations are present in the same polypeptide (Fig. 6B).

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**Materials and Methods**

**Cell culture**

Mouse embryonic fibroblast C3H 10T1/2 cells and B16F10 murine melanoma cells were obtained from American Type Culture Collection. Cells were maintained in DMEM supplemented with 2 mM L-glutamine and 10% FBS at 37°C and 5% CO2.

**Antibodies**

Two commercially available PAX3-specific antibodies were used, one, a mouse monoclonal antibody developed by C. Ordahl (University of California, San Francisco) and
obtained from the Developmental Studies Hybridoma Bank, NICHD/University of Iowa (1:200) and the other, a goat polyclonal antibody from R&D Biosystems (1:100). Other antibodies were specific for BrdU (1:1000; Sigma) and H3K4me3 (1:500; Abcam).

Expression constructs
The untagged PAX3 expression plasmid has been described previously (26). An EcoRI-BamHI PCR fragment containing the entire PAX3 coding sequence was inserted into pEGFP-N1 (Clontech) to allow expression of PAX3 with a carboxy-terminal GFP tag (PAX3-GFP); primers used were: GFP.PAX3.F 5’-CAGAATTCTGATGACCAGCGCTGGGC and GFP.PAX3.R 5’-CAGGATCTCTGGAACGTCCAAGGCTT. Mutant PAX3-GFP constructs were created by exchanging an XmaI fragment from wild-type PAX3-GFP for one containing the mutation from a construct previously mutated by site directed mutagenesis (26).

The wild-type PAX3 PDHD expression construct has been described previously (26). Mutant versions of the pET21a-PDHD plasmid were created by PCR-amplifying an insert containing amino acids 34–279 of PAX3 from the corresponding pEGFP-N1-PAX3 construct (discussed above). Primers used were: pET.PD.F 5’- CAGGATCCGGCCAGGGCCGAGTCAA and pET.HD.R 5’- CAGGATCCGGCCAGGGCCGAGTCAA and pET.HD.R 5’-CCTGCGGCCGGAAGCTTTTGCTTGTTTCTCC.

Immunocytochemistry
Cells were seeded on glass cover slips in 6-well plates at a density of ~1.0 x 10^6 cells/ml. After 24 h growth, cells were transfected with 1 μg DNA in DMEM containing polyethyleneimine (PEI). At 24 h post-transfection, cells were washed in PBS and fixed for 5 min at room temperature with 4% paraformaldehyde in PBS. Cells were then permeabilized with 10% Triton X-100 in PBS. Cells transfected with GFP constructs were mounted onto glass slides with 20 μl Mowiol containing 1 μg/ml Hoechst 33258 (Sigma). For immunofluorescence, cells were blocked using 5% PBS–BSA for 1 h and washed 3 x with 1% PBS–BSA prior to application of primary antibodies diluted in 1% PBS–BSA. Cells were washed 3 x with 1% PBS–BSA then incubated with a secondary antibody (Sigma; 1:400 in 1% PBS–BSA) conjugated to a fluorescent moiety for 1 h. Coverslips were washed with PBS and mounted onto glass slides with 20 μl Mowiol containing 1 μg/ml Hoechst 33258 (Sigma). Fluorescine isothiocyanate (FITC) staining of B16F10 cells or PAX3-transfected 10T1/2 cells was performed by diluting 5-FUrd in DMEM to a final concentration of 1 mM, then adding this mixture to cells and incubating for 30 min at 37°C.

Fluorescence imaging was performed using a Zeiss Axioskop 2 optical microscope equipped with a Photometrics CoolSnap HQ CCD camera (Roper Scientific Inc.). Images were captured using Metamorph 2.6r6 (Molecular Devices) and processed with Huygens deconvolution software (Scientific Volume Imaging). Three-dimensional rendering and image construction was performed using Imaris (Bitplane AG).

Fluorescence recovery after photobleaching
PAX3-GFP constructs were transfected into 10T1/2 cells and images were collected with a Zeiss Laser Scanning Confocal Microscope (LSM 510, software version LSM 3.2) mounted on a Zeiss Axiovert M100 inverted microscope with a 40 x apochromatic lens (numerical aperture 1.3). The 488 nm laser line (from a 25 mW argon laser) was used to image the PAX3-GFP constructs. A long pass filter (505 nm) was used to collect emission from the PAX3-GFP constructs. A 2 μM rectangle was bleached across the center of the nucleus and images were taken at 1 s intervals for the first 4 s, 2 s intervals from seconds 4 to 14, and 5 s intervals from seconds 14 to 90 following the bleach.

Recombinant protein expression and electrophoretic mobility shift assay
Expression and purification of the PDHD proteins and mobility shift assays were performed as previously described (26).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

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