Oral-facial-digital syndrome type I cells exhibit impaired DNA repair; unanticipated consequences of defective OFD1 outside of the cilia network

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Abstract
Defects in OFD1 underlie the clinically complex ciliopathy, Oral-Facial-Digital syndrome Type I (OFD Type I). Our understanding of the molecular, cellular and clinical consequences of impaired OFD1 originates from its characterised roles at the centrosome/basal body/cilia network. Nonetheless, the first described OFD1 interactors were components of the TIP60 histone acetyltransferase complex. We find that OFD1 can also localise to chromatin and its reduced expression is associated with mis-localization of TIP60 in patient-derived cell lines. TIP60 plays important roles in controlling DNA repair. OFD Type I cells exhibit reduced histone acetylation and altered chromatin dynamics in response to DNA double strand breaks (DSBs). Furthermore, reduced OFD1 impaired DSB repair via homologous recombination repair (HRR). OFD1 loss also adversely impacted upon the DSB-induced G2-M checkpoint, inducing a hypersensitive and prolonged arrest. Our findings show that OFD Type I patient cells have pronounced defects in the DSB-induced histone modification, chromatin remodelling and DSB-repair via HRR; effectively phenocopying loss of TIP60. These data extend our knowledge of the molecular and cellular consequences of impaired OFD1, demonstrating that loss of OFD1 can negatively impact upon important nuclear events; chromatin plasticity and DNA repair.

Introduction
Oral-Facial-Digital syndrome, Type I (OFD1; OMIM 311200), belongs to a heterogeneous group of developmental conditions; the oral-facial-digital syndromes (OFDS) (1–3). OFD Type I is caused by mutations in OFD1 (formerly Cxorf5) which tend to occur sporadically, segregating in an X-linked dominant fashion and causing male lethality, usually in the 1st/2nd trimester (4,5). Nevertheless, rare cases of male OFD Type I have been reported (2,6,7). Heterozygous deletion and genomic rearrangements involving OFD1 have also been reported as disease mechanisms (8–10). OFD Type I is clinically complex and variable, yet features affecting the craniofacial region...
Additionally, TIP60 acetyltransferase complexes have established a direct interaction with the RUVBL1 subunit (19). HAT chromatin acetylation, including DMAP1, TIP60, TRRAF and RUVBL2; interacting distally with the centrosome/basal body/cilia (19). Furthermore, OFD1 patient fibroblasts also exhibit profound neurological impacts including cognitive and behavioural abnormalities and complex structural brain abnormalities (9,11).

OFD1 encodes a 1011 amino acid protein composed of five α helical coiled-coil domains (α-CC) and N-terminally localised Lis1 (Lis1 homology) domain, but lacks homology to proteins of known function (5). Lis1 motifs have been postulated to mediate an array of activities including cell migration, nucleokinesis, protein dimerization and transcription (12). The α-CC is a highly versatile motif mediating protein-protein interaction and subunit oligomerization (13). Nevertheless, OFD1 amino acid sequence lacks any obvious and characteristic indicators of cellular functionality. Importantly, generation of an Ofd1 knockout mouse model established a role for Ofd1 in cilia formation and function (14). These mice recapitulated the human disorder, although with increased severity as a likely consequence of the differing nature of X-inactivation between mouse and humans (14). Ofd1 knockout mice exhibited markedly reduced tissue expression on several interconnected aspects of the DDR, including DSB-induced histone modification, chromatin remodelling and DSB repair (Fig. 1D). We next performed sub-cellular fractionation of WT and OFD Type I LCLs following transient overexpression of FLAG-tagged OFD1 in HEK293, and then immunoprecipitating it with the FLAG-tag before blotting for captured endogenous TIP60 (Fig. 1A). In a complementary reciprocal approach, we showed that OFD1 was detectable following immunoprecipitation of endogenous TIP60 from HeLa cell extract (Fig. 1B).

It is likely that only a fraction of total cellular OFD1 interacts with TIP60, yet importantly, the functional significance of the association between OFD1 and the RUVBL-TIP60 HAT complex has not been investigated. Using indirect immunofluorescence detection of TIP60 in OFD Type I patient fibroblasts from two separate individuals (FB.1 and FB.2), we unexpectedly observed a marked accumulation of cytoplasmic staining, in contrast to wild-type (WT) fibroblasts (Fig. 1C). OFD: FB.1 fibroblasts, bearing a frameshift mutation in OFD1 (1360delCTA), are derived from an OFD Type I individual described previously (Family 4) by Christel Thauvin-Robinet and colleagues (36). Ofd1 knockout mice exhibited marked failure in left-right axis specification, cystic kidneys and a failure to form cilia in the embryonic node (14). This was associated with defective sonic hedgehog (Shh) and canonical WNT signalling (14–16). Many of these findings have been recapitulated in other model organisms such as zebrafish (Danio rerio) (17). Consequently, cell biological research has primarily focused on dissecting the precise role(s) of OFD1 at the centrosome/basal body/cilium.

OFD1 can localise to the centrosome, co-localizing with γ-tubulin, but specifically to the centriolar satellite region surrounding the centrosome and basal body (18,19). OFD1 has been shown to interact with PCM-1 (20). OFD1 regulates the length and structure of distal centrioles (21). Recently, autophagic degradation of the centriolar satellite pool of OFD1 specifically was found to be required for cilia formation, indicating a context-specific regulation of OFD1 in controlling ciliogenesis (22,23).

There now exists a compelling body of evidence demonstrating that impaired OFD1 function negatively impacts upon cilia mediated paracrine signalling from multiple pathways including Shh, WNT and NOTCH, firmly establishing OFD Type I as a ciliopathy (24).

Interestingly, OFD1 has also been shown to localise to the nucleus, suggesting it may also have additional functions outside of the centrosome/basal body/cilium (19). Furthermore, OFD1 was found to co-immunoprecipitate several components of the TIP60 histone acetyltransferase (HAT) multi-subunit complex, including DMAP1, TIP60, TRRAF and RUVBL2; interacting directly with the RUVBL1 subunit (19). HAT chromatin acetylation, including that mediated by RUVBL1/2 and TIP60 containing complexes, controls transcriptional regulation (25). Additionally, TIP60 acetyltransferase complexes have established roles in activating ATM, an apical controlling kinase of the DNA damage response (DDR), in chromatin remodelling at DNA double strand breaks (DSBs) and in controlling DSB repair (26–35). The functional implications of OFD1’s association with TIP60 complexes have not been explored hitherto. Here, using OFD Type I patient cells, we characterise the impact of reduced OFD1 expression on several interconnected aspects of the DDR including DSB-induced histone modification, chromatin remodelling and DSB repair. We provide evidence that reduced OFD1 expression causes mis-localization of TIP60, and thereby effectively phenocopies impaired TIP60 function in the DDR. We describe several novel cellular defects in OFD Type I patient cells demonstrating that this condition is characterised by compromised DSB-induced chromatin dynamics and impaired HRR-mediated DSB repair.

Results

TIP60 is mis-localized in OFD type I patient fibroblasts

Much of our understanding of OFD1 function and the consequences of its dysfunction have focused on its roles at the centrosome/basal body/cilia. Yet, one of the first described OFD1 interactors was in fact the RUVBL-TIP60 HAT complex, where it was found to directly interact with at least one constituent subunit, RUVBL1 (19). We confirmed the association between OFD1 and TIP60 following transient overexpression of FLAG-tagged OFD1 in HEK293, and then immunoprecipitating it with the FLAG-tag before blotting for captured endogenous TIP60 (Fig. 1A). In a complementary reciprocal approach, we showed that OFD1 was detectable following immunoprecipitation of endogenous TIP60 from HeLa cell extract (Fig. 1B).
Type I LCLs compared to WT (Fig. 2E). Finally, we found that stable lentiviral shRNA knockdown of OFD1 in U2OS resulted in reduced TIP60 in chromatin preparations (Fig. 2F). Collectively, these findings (Fig. 1C and D and Fig. 2C–F) indicate that reduced OFD1 is associated with a subcellular redistribution of TIP60 and suggest that TIP60 functionality could be compromised through mis-localization in this context. Importantly, altered TIP60 distribution in the context of reduced OFD1 levels was observed in proliferating non-ciliated cells.

**OFD type I patient cells exhibit impaired TIP60 HAT activity and DSB-induced histone modification**

ATM activation at DSBs induces a complex series of events involving a range of histone modifications including phosphorylation, acetylation and ubiquitination. This enables the ordered recruitment of various signal transduction, chromatin remodelling and repair factors to enact effective repair (37). TIP60 plays key roles at several levels in these processes; its HAT function is activated by DSBs, TIP60 activates ATM via direct acetylation, and TIP60 acetylates specific histones facilitating chromatin remodelling and transcriptional regulation (28,31,38,39,39).

We observed reduced IR-induced ATM-dependent phosphorylation of several DDR factors in OFD Type I patient LCLs, although the impacts upon ATM autophosphorylation were modest (Supplementary Material, Fig. 1). Interestingly, we observed a marked reduction in IR-induced acetylation of histone H2A on lysine-5 (H2A-AcK5) in OFD Type I fibroblasts compared to WT (Fig. 3A). H2A-AcK5 is a TIP60-dependent acetylation (40). TIP60 also regulates DSB-induced ubiquitination of histone H2AX, an event requiring prior acetylation (39). We observed only a modest decrease in IR-induced $\gamma$H2AX in OFD Type I patient fibroblasts (Fig. 3A). This is not unexpected considering other kinases can, redundantly with ATM, conduct this phosphorylation (41). Nonetheless, we did observe reduced levels of slower migrating $\gamma$H2AX species (modified; Mod-$\gamma$H2AX), consistent with impaired IR-induced post-translational modification (e.g. ubiquitinated, acetylated and phosphorylated) of $\gamma$H2AX (Mod-$\gamma$H2AX) in OFD Type I fibroblasts, compared to WT (Fig. 3A). Furthermore, siRNA mediated knockdown of either OFD1 or TIP60 in control fibroblasts recapitulated the reduced IR-induced H2A-AcK5 and Mod-$\gamma$H2AX seen in the OFD1 Type I patient fibroblasts (Fig. 3B).

Figure 1. OFD1 associates with TIP60 and OFD Type I patient cells exhibit mis-localization of TIP60. (A) Interaction between OFD1 and TIP60 was demonstrated by co-immunoprecipitation (IP). HEK293 cells were transfected with p3XFlag-CMV10 containing OFD1 (FLAG-OFD1) or empty vector (FLAG-Ev) and IP’d using M2 anti-FLAG agarose-conjugated antibody beads. Total lysate (TL) inputs and IPs were subsequently blotted for TIP60. (B) Co-IP analysis of endogenous OFD1-TIP60 was performed on total lysates (TL) from Hela cells. IgG was used as loading control. (C) Unperturbed exponentially growing 1BR.3 control wild-type (WT) fibroblasts exhibited pronounced nuclear staining for TIP60. This was in contrast to TIP60 staining pattern in two distinct OFD Type I patient fibroblast lines (OFD-FB-1 and OFD-FB-2). Both patient fibroblasts exhibited a more dispersed staining pattern with a preponderance of TIP60 appearing to localise to the cytoplasm, in contrast to WT fibroblasts. Scale bar: 20μm. (D) The left-hand panel represents OFD1 expression analysis following siRNA mediated OFD1 knockdown in 1BR.3 control wild-type (WT) fibroblasts (OFD1 siRNA) compared to treatment with the control (Ctrl) scrambled siRNA. The right-hand immunofluorescence panels for these respective treatments show that following siRNA of OFD1, TIP60 localisation is dispersed from the nucleus; a similar staining pattern to that observed in the patient fibroblasts (C). Scale bar: 20μm.
Acetylation of histone H4 on K16 plays roles in transcriptional activation, maintenance of euchromatin and in controlling higher order chromatin structure (42,43). Several HAT complexes can acetylate histone H4 on K16 (44). Notable amongst these are MOF (MYST1/KAT8) containing HATs, which have also been implicated in the ATM-dependent DNA damage response (DDR) (45–47). DSB-induced acetylation of histone H4 on lysine-16 (H4-AcK16) has recently been shown to be an important TIP60-dependent event directing DSB repair (30). Using WT fibroblasts, we observed a transient time-dependent increase in H4-AcK16 levels up to 15 min following irradiation (Fig. 3C). Importantly, this was in contrast to the rather unresponsive H4-AcK16 levels observed in OFD Type I fibroblasts (Fig. 3C). Further, siRNA mediated knockdown of OFD1 in U2OS cells similarly affected DSB-induced H4-AcK16 as observed in the patient fibroblasts (Fig. 3D). Interestingly, siRNA of TIP60 completely inhibited H4-AcK16 acetylation under these conditions, likely indicating that the negative impact of reduced OFD1 upon TIP60 function is not absolute, in this case. Nonetheless, these findings show that reduced OFD1 expression results in a detectable impairment of DSB-induced histone modification, including two TIP60-dependent histone acetylation events; H2A-AcK5 and H4-AcK16 formation. Importantly, these defects are also observed in OFD Type I patient-derived cells.

**OFD type I fibroblasts exhibit impaired chromatin remodelling**

Localised chromatin remodelling at DSBs, including histone eviction and variant histone exchange are required for optimal repair (37,48–52). Histone H4-AcK16 specifically, has been shown to control higher order chromatin structure, even impairing the action of the ACF nucleosome re-modeller in mobilizing mononucleosomes (53). DSB-induced chromatin relaxation as a consequence of enhanced histone dynamics can be assessed by monitoring the kinetics of micrococal nuclease
MNase digestion of chromatin (54). We undertook a modified version of this approach using a bioanalyser rather than agarose gel electrophoresis to monitor the appearance of fully digested chromatin down to mononucleosomes (Fig. 4A). The rate of appearance of mononucleosomes was not obviously different between unperturbed untreated (Unt) WT and OFD Type I fibroblasts (Fig. 4A: Unt). Strikingly, MNase digestion to mononucleosomes following treatment with the radiomimetic DSB-inducing agent neocarzinostatin was impaired in OFD Type I patient compared to WT (Fig. 4A: Neo and Fig. 4B). These results are consistent with reduced chromatin plasticity/relaxation potential following DSB formation in OFD Type I fibroblasts. These data are also consistent with the altered DSB-induced histone modifications described in Fig. 3. Furthermore, we obtained a similar attenuation of MNase accessibility after neocarzinostatin treatment following stable lentiviral mediated shRNA knockdown of OFD1 in U2OS cells, compared to control cells (Fig. 4C and D).

Reduced OFD1 expression impairs DSB-induced homologous recombination repair

Considering the abnormalities in DSB-induced histone modification and chromatin plasticity we observed, we next examined DSB repair capability in the context of reduced OFD1 expression. We monitored the repair of IR-induced DSBs using 53BP1 foci formation and disappearance overtime. In G0 cells we found DSB repair to be unaffected in fibroblasts from two distinct OFD Type I patients compared to those of WT, consistent with functional non-homologous end-joining (NHEJ)-mediated DSB repair (Fig. 5A).

TIP60 HAT complexes are directly implicated in the repair of DSBs via the Homologous Recombination Repair (HRR) pathway (29,30,34,35,55). There has been a lot of interest in dissecting the signalling networks that control the DSB repair pathway choice between NHEJ and HRR. Active competition between BRCA1 and 53BP1 influence DSB end resection, determining whether repair...
Figure 4. OFD Type I patient cells exhibit reduced micrococcal nuclease chromatin digestion following DSBs. (A) Nuclei isolated from wild-type (WT) and OFD Type I patient fibroblasts (OFD: FB-2) were subjected to micrococcal nuclease (Mnase) digestion to evaluate chromatin dynamics. The appearance of fully digested chromatin down to mononucleosome-sized DNA fragments was monitored using the Agilent 2100 Bioanalyzer and DNA chips, with the digestion profiles generated using the 2100 Expert DNA dsDNA 1200 ladder. A typical digestion profile from untreated fibroblasts (Unt) is shown in the left-hand side with mononucleosome-sized DNA fragments and various polynucleosome species indicated (Mono, Di, Tri). No significant change in profiles was observed between WT fibroblasts and OFD Type I patient fibroblasts following digestion up to 10 mins under these conditions. In contrast, digestion profiles generated from cells treated with the radiomimetic neocarzinostatin (Neo) did show differences in digestion profiles. The right-hand panel shows a profile generated following 6 mins digestion with Mnase, clearly demonstrating the reduced level of digestion products (mononucleosomes and polynucleosomes) detected from the OFD Type I patient cells. (B) The rate of generation of mononucleosome species was determined over time as indicated, normalised to mononucleosome levels following 2 mins Mnase digestion. A statistically significant difference (*P < 0.05 Student’s t-test) in Mnase accessibility is observed from 6 mins (NS, not significant) wherein the OFD Type I patient fibroblasts demonstrate a reduction in Mnase accessible chromatin following treatment with neocarzinostatin. Data represents the mean ± S.D. of five separate digestion profiles. (C) OFD1 expression is markedly reduced in U2OS cells stably transduced with lentiviral shRNA directed against OFD1 (OFD1 shRNA) compared to cells transduced with control (Ctrl) shRNA vector. (D) Similar to the OFD Type I patient fibroblasts (B), Mnase accessibility following neocarzinostatin treatment is reduced in OFD1 shRNA cells compared to control (Ctrl) from 6 mins digestion with Mnase (*P < 0.05 Student’s t-test. NS, not significant). Data represents the mean ± S.D. of four independent determinations.

The impaired HRR capacity observed following reduced OFD1 expression was associated with modestly elevated sensitivity of OFD Type I LCLs (Fig. 6A) and shRNA mediated knockdown of OFD1 (Fig. 6B) to killing by IR. Impaired HRR is synthetically lethal to inhibition of PARP using small molecule inhibitors such as olaparib (61–63). We found that OFD Type I LCLs also exhibited elevated levels of apoptosis following treatment with increasing concentrations of olaparib, as measured by the appearance of the 85 kilodalton form (p85) of caspase-cleaved PARP (Fig. 6C). Furthermore, we observed elevated sensitivity to killing by olaparib via clonogenic assay following stable lentiviral knockdown of OFD1 (Fig. 6D). Collectively, these data show that impaired OFD1 function (siRNA, shRNA), and in the context of OFD Type I patient cells, reduces DSB-induced HRR, a phenotype observed following TIP60 acetyltransferase deficiency and consistent with sub-optimal TIP60 function when OFD1 levels are reduced. These results show for the first time that reduced OFD1 expression results in impaired DNA repair in cycling non-ciliated cells.

**OFD Type I LCLs exhibit a hypersensitive DSB-induced G2-M checkpoint activation and impaired recovery from arrest**

An important consequence of activation of the DDR signal transduction network is cell cycle checkpoint activation,
particularly at the G2-M boundary. Unexpectedly, we found that OFD Type I LCLs exhibited a hypersensitive G2-M checkpoint activation phenotype compared to WT LCLs, showing marked G2 arrest at IR doses as low as 0.1Gy (Fig. 7A). Furthermore, we found that G2-M arrest was prolonged in OFD Type I LCLs compared to WT LCLs, with only approximately 30% of G2 cells progressing into mitosis 16hrs after 1Gy IR (Fig. 7B). We observed a similar marked delay in recovery from IR-induced G2-M arrest following siRNA of Ofd1 in normal human fibroblasts, and importantly, also following siRNA of Tip60 (Fig. 7C). The latter observation suggests that hypersensitive and prolonged G2-M checkpoint response can occur as a consequence of impaired Tip60 acetyltransferase function and again we uncover evidence that reduced Ofd1 expression mimics reduced Tip60 expression.

In summary, our data show that reduced Ofd1 levels are associated with mis-localization of Tip60, and that reduced Ofd1 expression results in a series of DSB-mediated histone modification, chromatin remodelling and DSB-repair deficits overlapping with those observed upon Tip60 deficiency. Furthermore, we can detect these defects in OFD Type I patient cells and under conditions independent of cilia formation (e.g., using LCLs and exponentially growing fibroblasts). Our data strongly suggest that reduced Ofd1 expression can phenocopy loss of Tip60, thereby expanding our understanding of the complex consequences of impaired Ofd1 function.

**Discussion**

OFD Type I patients present with several core clinical features common to ciliopathies (e.g., cystic disease, patterning and limb abnormalities) (2,64). Our understanding of the functional and clinical consequences of reduced Ofd1 derive entirely from its documented roles at the centrosome/basal body/cilia network. Cilia formation and cilia-dependent paracrine signalling are markedly impaired in Ofd1 knockout mice (14,24,65). Here, using patient-derived cell lines, we extend our knowledge of the cellular consequences of reduced Ofd1; identifying and characterising several novel phenotypes relating to pathways controlling genomic stability, chromatin dynamics and cell cycle checkpoint control.

Whilst it is difficult to untangle the influence of the specific cellular defects described here from cilia-dependent defects in a paracrine signalling, it is possible that the former may also play a contributing role to the clinical spectrum of OFD Type I syndrome. Emerging evidence suggests a growing link between congenital defects in genes encoding proteins with canonical roles in DNA repair, DDR-signalling or DNA replication and/or mitotic cell division, with impaired cilia function (66). For example, defects in components of the DNA replication licencing Origin Recognition Complex (ORC) that underlie Meier-Gorlin syndrome (MGS; a microcephalic primordial dwarfism (MPD)), were subsequently found to cause profound impairments in...
OFD Type I patient cells exhibit elevated sensitivity to DNA breakage. (A) Wild-type (WT) and OFD Type I patient LCLs irradiated with 6Gy IR and % cell death determined 72 h post-IR using flow cytometry-based Annexin V/Sytox Green analysis. OFD Type I LCLs exhibited elevated cell death compared to control wild-type (WT) LCLs under these conditions. Data represents the mean ± S.D. of three independent experiments (* P < 0.05 Student’s t-test). (B) Clonogenic survival analysis of U2OS transduced with control vector (Ctrl) or lentiviral shRNA towards OFD1 (OFD1 shRNA) following 6Gy IR. Surviving colonies were scored 14 days following plating. OFD1 shRNA in U2OS was associated with reduced clonal survival compared to control (Ctrl) shRNA under these conditions. Data represents the mean ± S.D. of three independent experiments (* P < 0.05 Student’s t-test). (C) Wild-type (WT) and OFD Type I patient LCLs were treated with increasing concentrations of olaparib (0, 50, 100, 150µM) for 48 h. Apoptosis was detected by western blotting for the presence of the p85KD caspase-cleaved form of PARP. Elevated levels of p85-PARP were detectable at all concentrations of olaparib following treatment of the OFD Type I LCLs compared to WT LCLs. (D) Clonogenic survival analysis of U2OS transduced with control vector (Ctrl) or lentiviral shRNA towards OFD1 (OFD1 shRNA) following treatment with different concentrations of olaparib. Consistent with the compromised HRR observed following siRNA of OFD1 and the elevated olaparib-induced apoptosis seen in OFD Type I patient LCLs, OFD1 shRNA specifically exhibited elevated sensitivity to killing by olaparib.

OFD Type I patient cells exhibit a hypersensitive DSB-induced G2-M checkpoint arrest and delayed recovery. (A) LCLs from normal wild-type (WT) and OFD Type I individuals were irradiated with increasing doses of IR and the mitotic index determined in the presence of colcemid at 4 h post-IR. OFD Type I LCLs demonstrated a robust G2 arrest at doses as low as 0.1Gy, in contrast to WT LCLs (P < 0.05 Student’s t-test). Data represents the mean ± S.D. of four independent determinations. These results indicate that OFD Type I LCLs exhibit hypersensitive activation of the DSB-induced G2-M cell cycle checkpoint. (B) Recovery from G2-M checkpoint activation and arrest following 1Gy IR was monitored in wild-type (WT) and OFD Type I LCLs. At 8h post-IR in the presence of a nocodazole trap, only approximately 30% recovery (i.e. progression from G2 into M phase) was observed in OFD Type I LCLs compared to circa 75% recovery of WT LCLs. These results indicate that OFD Type I LCLs exhibit impaired recovery from activation of the DSB-induced G2-M cell cycle checkpoint compared to WT LCLs. Error bars represent the mean ± S.D. of four independent experiments (* P < 0.05 Student’s t-test). (C) MRC5VA fibroblasts were subjected to siRNA using control (Ctrl) scrambled oligos and siRNA SmartPools against OFD1 and TIP60. 48 h post-transfection cells were irradiated with 2Gy IR and mitotic recovery determined 8h post-IR in the presence of a nocodazole trap. At this time Ctrl cells showed around a 70% recovery in contrast to approximate 30% recovery seen following siRNA of either OFD1 or TIP60. Similar to OFD Type I LCLs, siRNA knockdown of OFD1 results in impaired recovery from DSB-induced G2-M cell cycle checkpoint activation. This impaired recovery is also seen following siRNA of TIP60. Error bars represent the mean ± S.D. of four independent experiments (* P < 0.05 Student’s t-test).
cilia formation and signalling; impairments which plausibly contribute to the clinical presentation of MGS (67–70). Defects in the DDR apical kinase ATR underlie another MPD; Seckel syndrome (71,72). Atr deficiency was recently reported to underlie photoreceptor degeneration linked to cilia dysfunction in a mouse model of ATR-Seckel syndrome, and to negatively influence G1-S entry and Shh-signalling in ATR-Seckel fibroblasts along with altered left-right asymmetry in an Atr zebrafish model (73,74). Furthermore, recent exome sequencing based searches for novel nephronophthisis-related ciliopathy (NPHP-RC) genes identified pathogenic variants in several DNA repair and DDR-signalling proteins including FAN1, MRE11, CEP164 and ZNF432 (75,76). Conversely, reduced expression of multiple NPHP-RC genes has been shown to induce replication stress, S phase accumulation and elevated levels of DNA damage in several model systems (77–81). Therefore, accumulating evidence indicates complex and reciprocal functional interactions between DDR, DNA repair and cell cycle machinery with components of the cilia network. Our findings concerning OFD1 provide another layer of complexity to these relationships, where reduced OFD1 expression results in attenuated DDR signalling and DSB-induced histone modification, altered chromatin plasticity, impaired HRR and hyperactive G2 checkpoint arrest.

Disorders caused by defects in proteins with canonical roles in DDR-signalling and in DNA repair, including HRR, present a variable clinical landscape (reviewed in (82,83)). Several interesting parallels exist between some of these conditions and OFD Type I syndrome. For example, mutations in ATM underlie Ataxia Telangiectasia, a progressive neurodegenerative condition characterised by progressive ataxia, ocular apraxia and dysarthria (84,85). These motor abnormalities are characteristically observed in Joubert syndrome patients carrying OFD1 mutations (JBT10; OMIM 300904) (86). Structural brain abnormalities are a frequent feature of DDR-defective conditions (83). Similar abnormalities (e.g. agenesis of the corpus callosum, cerebellar hypoplasia/atrophy, cortical malformation) are also reflected in the clinical spectrum of OFD Type I patients (9,11). Polycystic kidney disease is a characteristic feature of OFD Type I patients, often requiring dialysis and ultimately transplantation in late childhood-early adulthood (1). Interestingly, Chaki and colleagues reported the co-localisation of specific NPHP-RC proteins with TIP60 nuclear foci; and one of these NPHP-RC proteins (NPHP10/SYCCAG8) has previously been shown to directly interact with OFD1 (75,87). Interestingly, we note that TIP60 has also recently been identified as a constituent of the wider CPLANE (ciliogenesis and planar polarity effector) protein network (88). Collectively, these findings underscore the complex relationship between impaired DDR and normal development.

Our novel findings that reduced OFD1 expression is associated with altered DSB-induced histone modification and chromatin dynamics may have implications not just in DNA repair, but also in transcriptional regulation. These could have complex adverse impacts upon the normal development via alterations of the epigenome that may also influence the clinical presentation of OFD Type I syndrome. Histone H4-AcK16, a histone post-translational modification we found to be altered in OFD Type I cells, is particularly important for the establishment and maintenance of euchromatin and in controlling higher order chromatin structure (42,43,53). Integrating the genome-wide distribution of this modification with the transcriptome profile of OFD Type I patient tissues may help in identifying novel abnormally regulated genes and/or networks in this context. Furthermore, altering the epigenome using various chemical inhibitors has shown therapeutic efficacy in models for other developmental disorders, such as histone deacetylase inhibitors in Kabuki syndrome (89). Whether this approach has any merit in OFD Type I models is worthy of investigation based on the histone modification abnormalities documented here.

Defects in DDR and DNA repair cause genomic instability that is often reflected by elevated cancer predisposition. Elevated cancer incidence is nonetheless not a core clinical feature of OFD Type I syndrome, although hamartomas have been described here (2). Reduced histone H4-AcK16 is a common hallmark of cancers and TIP60 is frequently under-expressed in cancer (90,91). Importantly, these associations are complex and context specific. Loss of histone H4-AcK16 in cancer is strongly associated with coincident loss of histone H4-trimethylation at K20. Furthermore, other HATs can form H4-AcK16 and it is notable that this histone mark is not completely absent in OFD Type I patient cells (Fig. 3). In fact, it is perhaps even elevated above background in untreated cells compared to WT, but then unresponsive to DSBs (Fig. 3C and D). Whilst TIP60 has been described as a tumour suppressor, TIP60 holoenzyme can counteract Myc-driven lymphomagenesis in Eμ-myc transgenic mice (91). Our findings suggest that OFD Type I patient cells are compromised in TIP60 acetyltransferase activity likely due to subcellular mis-localization; though importantly, they are not completely defective in TIP60 function.

Finally, the novel G2-M checkpoint phenotypes we identified when OFD1 is under-expressed may be particularly relevant with regards to cancer development or lack thereof, in OFD Type I syndrome. We found a hypersensitive and prolong G2 arrest following DSB formation in OFD Type I patient cells. Compromised ATM-dependent DDR signalling is typically associated with a defective DSB arrest at G2-M. It is this defect in G2-M arrest that plays a major influence in enabling the propagation of genomic instability into malignant transformation (92). A hypersensitive G2-M checkpoint would counteract this effect, removing these cells from the cell cycle until either effective DSB repair is completed, or the cells are permanently exited from the cycling population (senescence or apoptosis) (93). Furthermore, spatio-temporal timing of mitosis is fundamental to normal cortical development (94). Embryonic brain development is associated with elevated levels of endogenous DSBs that require effective cell cycle checkpoint surveillance and coordinated repair (95). A hyper-sensitive and prolonged G2-M arrest, as observed here following reduced OFD1, could conceivably disrupt this delicate balance, impacting normal cortical development and thereby potentially contributing to the cortical abnormalities observed in OFD Type I patients (neuronal migration/organisation disorders, pachygyria, grey matter heterotopias, cortical dysplasia, polymicrogyria and schizencephaly) (11). Our data show that the G2-M checkpoint abnormality we identified in OFD Type I patient cells is identical to that observed following knockdown of TIP60. Again, this illustrates that impaired OFD1 can phenocopy loss of TIP60 in certain circumstances.

In conclusion, we identify a series of novel and unanticipated cellular phenotypes associated with reduced OFD1 expression in OFD Type I patient cells. Our findings provide strong evidence that reduced OFD1 expression results in compromised DSB-induced histone modification, altered chromatin plasticity, defective HRR-mediated DSB repair and hypersensitive and prolonged cell cycle checkpoint arrest. Our results suggest that TIP60 mis-localization in OFD Type I likely underlie these phenotypes. Collectively, these observations provide functional contextualisation to the previously described interaction.
between OFD1 and the multisubunit TIP60 acetyltransferase complex. Furthermore, these findings extend our knowledge of the consequences of OFD1 dysfunction from its established roles at the centrosome/basal body/cilia network.

Materials and Methods

Cell culture

All cell lines were cultured at 37°C in humidified incubators under 5% CO2. LCLs were grown in RPMI1640 with 15% fetal calf serum, antibiotics (Pen-Strep) and L-Gln. Patient derived primary and hTERT-immortalised fibroblasts and control fibroblasts (1BR.3, MRC5VA) were grown in MEM with 15% fetal calf serum (FCS) supplemented with antibodies (Pen-Strep) and L-Gln, whilst HeLa and HEK293 cells were grown in MEM with 10% FCS, Pen-Strep and L-Gln. U2OS were cultured in McCoy’s 5A supplemented with 10% FCS, Pen-Strep and L-Gln.

Antibodies

The following antibodies were obtained from Cell Signalling Technology; H2A (#2578) and H2A-AcK5 (#2576). Antibodies obtained from Santa Cruz Technologies included: Lamin B (C-20. #sc-6216), RAD51 (H-92. #sc-8349), TIP60 (N-17. #sc-5725) and CENP-F (H-260. #sc-22791). For IP of endogenous protein anti-TIP60 (#07-038) from EDM-Millipore was used and anti-TIP60 (H00010524-D01) from Abnova was also used for expression analysis. Anti-KAP1 (#A300-274A) and anti-53BP1 (#A300-272) were from Bethyl. The other antibodies used included those against α-tubulin (Sigma-Aldrich, #T5168), βH2AX (S139. Millipore, #05-636), PARP (AbDSerotec, #MCA1522), p85 PARP (Promega, #G734A), OFD1 (NovusBio, #NBP1-89356), H4 (Abcam, #ab10158) and H4-AcK16 (EMD-Millipore, #07-329).

Immunoprecipitation

HEK293 cells were transfected with p3XFLAG-CMV10 expressing the human OFD1 or with the empty vector. 48 hrs post-transfection, cells were lysed (10mM Tris HCl pH7.5, NaCl 150mM, Triton 1%, supplemented with a cocktail of protease inhibitor (Sigma-Aldrich)) and total protein extracts pre-cleared twice with mouse IgG agarose beads (Sigma-Aldrich). The unbound proteins were washed out twice with the lysis buffer and the retained protein complexes eluted with 3XFLAG peptide, and loaded on polyacrylamide SDS-PAGE.

For IP of endogenous proteins from HeLa, cells were lysed in Buffer A (50mM Tris-HCl, 1mM EDTA, 10mM MgCl2, 5mM EGTA, 0.5% Triton X-100 pH 7.3) and 500μg of total lysates were incubated for 1h with 5μg of specific antibodies or rabbit IgG as control, followed by for 1h of incubation with protein A-Sepharose beads. The unbound proteins were washed out twice with the Buffer A and once with the Buffer ST (50mM Tris-HCl, 150mM NaCl pH 7.3), while the bound fraction was analysed by western blot. Proteases Inhibitors from SIGMA (P8340) and PhosSTOP phosphatase inhibitors (both from Roche) were included throughout.

Cellular fractionation

5×10^6 LCLs were extracted in 900 μl of 0.1% NP40/PBS supplemented with complete protease inhibitors (Sigma-Aldrich). The supernatant (cytoplasmic fraction) was retained and nuclear pellet washed (PBS) and extracted with 100 μl of 9M Urea/50mM Tris pH7.5 with β-mercaptoethanol and sonicated (20s at 30% amplitude using a microprobe) to solubilise the chromatin.

For chromatin extract preparation 1×10^6 cells were washed in cold PBS, resuspended in 200 μl. Hypotonic buffer (10 mM HEPES pH 7.5, 5mM KCl, 1.5 mM MgCl2, 1mM DTT, 10mM NaF, 1 mM Na2VO3, 10 mM β-glycerophosphate, 0.5% IGEPAL and Roche protease inhibitor cocktail) and incubated on ice for 15 min. The pelleted sample was washed twice in 200 μl of hypotonic buffer and resuspended in 100 μl Hypotonic buffer containing 0.5 M NaCl and incubated for 15 min on ice. Chromatin was pelleted (17,000xg for 10 min), and solubilised in urea buffer (9M Urea, 50 mM Tris-HCl pH 7.5 with β-mercaptoethanol). Samples were sonicated for 10sec at 30% Amplitude and resuspended in SDS buffer for loading on SDS-PAGE gels.

siRNA

Cells were transfected using Metafectene Pro (Biontex; #T040-5.0) using ON-TARGETplus siRNA SmartPools (4+ validated oligo mix per target). OFD1 (#L-009300-00-0005) and TIP60 (#L-006301-00-0005).

shOFD1 lentiviral transduction

Lentiviral shRNA against OFD1 were used as pre-made viral particles from Santa Cruz Technologies (#sc-91245-V) according to manufacturer’s instructions on U2OS cells. Transduced clones were selected using 5μg/ml puromycin.

Immunofluorescence

Fibroblasts grown on glass coverslips were washed in PBS, permeabilized for 30sec in 0.05% Triton/PBS, washed (PBS) and then fixed with 3% paraformaldehyde (PFA) with 2% sucrose for 10 min. Coverslips were then washed (PBS), blocked in 5% BSA/ PBS for 30 min, prior to incubation with anti-TIP60 (Abcam; ab62644) for 1h. Following washing in PBS, coverslips were incubated with secondary anti goat-Cy3 (Sigma-Aldrich; #C2821) for 1h, counterstained with DAPI, and mounted in Vectashield (Vector Laboratories; #H-1000).

DSB repair analysis was assessed using IR-induced 53BP1 foci. Primary fibroblasts were grown to confluence for repair analysis in G0. For repair analysis in G2, cells were treated with 10μM aphidicolin following irradiation to prevent S-G2 transit, whilst 53BP1 foci were enumerated in G2 cells identified by strong staining with CENP-F.

Micrococcal nuclease chromatin digestion

5×10^6 cells were used per digestion (1BR.3 and OFD:FB-2 or U2OS and U2OS shOFD1). Cells were either untreated (Unt) or treated with 2μg neocarzinostatin (Sigma; #N9162-100UG) for 30 min (Neo). To isolate nuclei cells were washed in PBS and resuspended in 300 μl NIB buffer (15mM Tris HCl, pH 7.5; 25mM KCl; 10mM NaCl, 1mM MgCl2; 1mM CaCl2; 250mM Sucrose; 0.5mM DTT). Cells were subjected to two rounds of freeze/thaw using liquid nitrogen and pelleted by centrifugation at 4°C for 10 min. Supernatant was removed and the nuclear pellet resuspended in 350 μl NIB buffer and treated with 3 μl of MNase (Roche; #107921; 10U/μl) and digested at room temperature. 50μl samples were taken from the master digestion for each time point.
(2, 3, 4, 6, 8 and 10 min) and 50μl of stop buffer (2.4% SDS, 40mM EDTA, 20μg Proteinase K) added immediately and incubated at 37°C for 10 min. Samples were then left on ice for DNA isolation. To each sample 50 μl 1×TE buffer was added followed by 150 μl of phenol:chloroform:isoamylalcohol (25:24:1). Following phase separation, 100 μl of soluble fraction was transferred to a new tube and then mixed with 10 μl 3M sodium acetate pH5.2, 750 μl 100% ethanol and 1 μl 10mg/ml glyceron (Sigma-Aldrich; #G8751). The resultant pellet was washed with 300 μl 70% ethanol, dried and resuspended in 20 μl/100 of phenol:chloroform:isoamylalcohol (25:24:1). Following phase separation was then run on Agilent 2100 Bioanalyser using Agilent DNA dsDNA 12000 Kit (#5067-1508) and evaluated using the 2100 Expert DNA Foundation (TMBFCB311TT) to B.F.

G2-M checkpoint analysis

For G2-M checkpoint activation analysis, LCLs were either untreated (Unt) or irradiated with increasing doses of IR (0.1, 0.2, 0.5Gy) before seeding into complete medium supplemented with 0.2mg/ml colcemid and incubated for 4 h. LCLs were pelleted, swollen in 75mM KCl (10 min) and fixed in 3% PFA (10 min) prior to cytopsining onto poly-L-lysine coated slides. Cells were counter-stained with DAPI and mounted in Vectashield prior to analysis on the Zeiss Axioplan platform. For the checkpoint recovery analysis, cells were allowed to recover for 8 h in the presence of a nocodazole trap (300nM).

HRR analysis using I-SceI GFP-assay

DR-U2OS GFP cells were seeded at 2×10⁵ and subjected to transfection with the appropriate siRNA SmartPool system. 6 h post-transfection cells were transfected with the appropriate vector systems (I-SceI expression vector, empty pCMV or GFP vector). Cells were incubated for 48 h, harvested and run as live cell suspensions on the BD-Accuri FACS platform with data analysis via BD-CSampler Software.

Cell death and clonogenic survival

Cell death analysis on LCLs was analysed 72 h post-IR (6 Gy) using the Dead Cell Apoptosis (Annexin V/Sytox Green) flow cytometry kit from ThermoFisher Scientific (V35113) according to the manufacturers’ instructions. For clonogenic survival studies different numbers of cells (Ctrl: control vector. OFD1 shRNA: lentiviral transduced with OFD1 silencing shRNA) were seeded into 10-cm dishes with increasing concentrations of olaparib as indicated or following 4 Gy IR. After 14 days colonies were fixed in 10-cm dishes with increasing concentrations of IR (0.1, 0.2, 0.5Gy) before seeding into complete medium supplemented with 0.5Gy) before seeding into complete medium supplemented with 0.2mg/ml colcemid and incubated for 4 h. LCLs were pelleted, swollen in 75mM KCl (10 min) and fixed in 3% PFA (10 min) prior to cytopsining onto poly-L-lysine coated slides. Cells were counter-stained with DAPI and mounted in Vectashield prior to analysis on the Zeiss Axioplan platform. For the checkpoint recovery analysis, cells were allowed to recover for 8 h in the presence of a nocodazole trap (300nM).

**Supplementary Material**

Supplementary Material is available at HMG online.

**Conflict of Interest statement.** None declared.

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