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# The metabolic sensor PASK is a histone 3 kinase that also regulates H3K4 methylation by associating with H3K4 MLL2 methyltransferase complex

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## ABSTRACT

**The metabolic sensor Per-Arnt-Sim (Pas) domain-containing serine/threonine kinase (PASK) is expressed predominantly in the cytoplasm of different cell types, although a small percentage is also expressed in the nucleus. Herein, we show that the nuclear PASK associates with the mammalian H3K4 MLL2 methyltransferase complex and enhances H3K4 di- and tri-methylation. We also show that PASK is a histone kinase that phosphorylates H3 at T3, T6, S10 and T11. Taken together, these results suggest that PASK regulates two different H3 tail modifications involving H3K4 methylation and H3 phosphorylation. Using muscle satellite cell differentiation and functional analysis after loss or gain of Pask expression using the CRISPR/Cas9 system, we provide evidence that some of the regulatory functions of PASK during development and differentiation may occur through the regulation of these histone modifications.**

## INTRODUCTION

The histone tail undergoes different types of post-translational modifications (PTMs), namely, acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and ADP ribosylation. These PTMs directly modify the compaction of chromatin and indirectly recruit downstream effector proteins, ultimately impacting transcription, replication and other essential biological processes (1,2). Histone 3 Lysine 4 (H3K4) can undergo mono-, di- and tri-methylation (3,4) and is generally associated with euchromatin. In yeast, the Set1 histone methyltransferase is responsible for H3K4 methylation, while in mammals these PTMs are catalyzed by the SET domain-containing (hSET1a and hSET1b) and mixed-lineage leukemia (MLL1, MLL2, MLL3 and MLL4) family of histone methyltransferases (5,6). H3K4 methyltransferases induce mono-, di- and tri-methylation based on the methyltransferase complex. These complexes form transcription activating complexes at the promoters and enhancers of genes, whose core members include, Absent Small or Homeotic like (ASH2), the WD40-repeat protein 5 (WDR5), Retinoblastoma-binding protein 5 (RBBP5) and Dpy-30-like protein (DPY-30) (4,7–11).

Phosphorylation of histones plays critical roles in transcriptional regulation, DNA damage repair, chromosome condensation, chromosome segregation and cell cycle regulation (12–15). Different serines (H3S10 and H3S28) and threonines (H3T3, H3T6 and H3T11) of the H3 tail are phosphorylated during cell division, transcriptional regulation and dosage compensation (16–19). Several histone-associated kinases have been associated with specific biological functions. These include, histone H3 associated protein kinase (HASPIN)- (H3T3), protein kinase C beta I (PKCβ1)- H3T6, Death-associated protein kinase (DLK/ZIP kinase)- (H3T11), Aurora kinase B (AURKB)- (H3S10 and H3S28) and Pim-1 proto-oncogene, serine/threonine kinase (PIM1)- (H3S10) (1,12,19–24). It has also been suggested that rather than a single post-translational modification, multiple histone PTMs, involving non-histone proteins by direct or indirect interactions are responsible for distinct biological functions (25). To this end, there are multiple cross-talks (i.e. phosphorylation affecting methylation or acetylation) among different PTMs (24–28).

Pas domain-containing proteins, so named because of the first identified proteins, Period (Per) (29), Aryl hydrocar-

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bon receptor nuclear translocator (Arnt) (30) and Single-minded protein (Sim) (31), are evolutionarily conserved group of proteins present in all species from bacteria, archaea to eukaryotes (32). They form complexes with other proteins and act as sensor domains in many signalling proteins (33), including transmembrane channel proteins and activators of transcription (32).

We identified a Pas domain-containing protein, Per-Arnt-Sim (Pas) domain-containing serine/threonine kinase (PASK) from a microarray analysis using Cynomolgus macaques aimed at identifying factors that respond to subtle changes in the developmental environment of the fetus (34). In lower organisms, the PASK acts as a sensor for light intensity, gas pressure, redox potentials and certain organic ligands (32,35,36). Genetic alteration studies in mice also identified PASK as a key metabolic regulatory gene and was proposed to be a metabolic sensor (33,37). Consistent with these findings, PASK was found to be expressed in developing pancreatic epithelium, the islets of Langerhans and other tissues of metabolic relevance, where it exhibits a tissue-specific metabolic phenotype (34,37,38). PASK is expressed predominantly in the cytoplasm, although a small fraction is detectable in the nucleus (39). Although the role of PASK as a signaling molecule in metabolic pathways such as insulin secretion and lipid metabolism has been elucidated (37,40–44), the molecular mechanism(s) and the roles played by it in the nucleus remain to be established. Herein using *in vitro* kinase and methyltransferase assays, we show that PASK associates with the mammalian H3K4 MLL2 methyltransferase complex and this association enhances H3K4 di- and tri-methylation. We also show that PASK is a histone kinase that phosphorylates H3 at T3, T6, S10 and T11. Further, by using C2C12 muscle satellite cell differentiation as a model, we provide evidence that PASK may have a regulatory role in normal development and differentiation by regulating the histone modifications involving H3K4 methylation and H3 phosphorylation.

## MATERIALS AND METHODS

### Cell lines and cell culture

Mouse satellite cells (C2C12) and HEK293T cells were obtained from American Type Culture Collection (ATCC) and maintained in growth media, GM (DMEM supplemented with 10% FBS (fetal bovine serum) and 1% PS (penicillin and streptomycin) at 37°C in a humidified, 5% $CO_2$ atmosphere. To induce differentiation of C2C12 myoblasts to myotubes, 90–95% confluent cultures were washed with PBS and incubated in differentiation media (DM: DMEM with 2% horse serum and 1% PS), with media change every 24 h.

### Plasmids, transfection and protein purification

C2C12 and HEK293T cells expressing mouse Pask and human PASK-V5, respectively and their corresponding vector controls were generated by transfecting the plasmids using Viafect transfection reagent (Promega, Cat. No. E4981) according to manufacturer's instructions.

For generating the mouse Pask expression plasmid, a sequence verified, full-length Pask cDNA clone (4432411M05: imaGenes) was obtained and the Pask insert was released with Not1/Kpn1 enzymes. The purified insert was cloned into Not1/Kpn1 sites of pcDNA3.1- vector and the insert verified by sequencing. Stable C2C12 cells expressing PASK and Vector control cells were generated by transfecting the mPASK expression plasmid pcDNA3.1(-)-Pask or the pcDNA3.1(-)-Vect. Mouse Pask cDNA in pcDNA3.1$^+$ C-Flag and pcDNA3.1$^+$ C-eGFP were purchased from GeneScript. The human full-length PASK pcDNA 3.1$^+$ hPASK-V5 and kinase dead (KD) mutated version (pcDNA3.1$^+$ K1028R hPASK-V5) were gifted by Dr Jared Rutter. The deletion mutations of human PASK protein, 949–1315, 405–1315 and 200–1315 in pcDNA3.1 hPASK V5 were made using Q5 site directed mutagenesis kit from Qiagen.

Endogenous Pask from C2C12 cells was forced to overexpress by employing an inducible CRISPR Tet-on system for controllable gene activation. For this, a plasmid PB-TRE-dCas9-VPR, a gift from George Church (Chavez *et al.*, 2015) (Addgene #63800) containing a nuclease null Cas9 (dCas9) and inducible Tet-on system conjugated with tripartite activator, VP64-p65-Rta (VPR) for induction was co-transfected along with the sgRNA plasmid (Addgene # 68463) (45).

The sgRNA sequences for mPask activation are:

| | | |
|---|---|---|
| 1 | F | 5′-GGA CGC GCG AAG AAA TCG GGT CTT T-3′ |
| | R | 5′-TTT CAA AGA CCC GAT TTC TTC GCG C-3′ |
| 2 | F | 5′-GGA CGA GGT AGA ACG GTA GCC AAC A-3′ |
| | R | 5′-TTT CTG TTG GCT ACC GTT CTA CCT C-3′ |

The successfully transfected cells were induced with doxycycline at a concentration of 1 μg/ml and used for further analysis. Transfected but non-induced cells were used as controls.

CRISPR silencing of endogenous Pask gene was accomplished by cloning the gRNA sequence targeting the coding region of endogenous mouse Pask into the vector pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene #62988), deposited by Feng Zhang (46). After transfection, silenced C2C12 cells were subjected to quick selection with 4–5 μg/ml of puromycin followed by serial dilution, plating and selection of single clones with inactivation of Pask gene.

The sgRNA sequences for mPask silencing are:

| | | |
|---|---|---|
| 1. | F | 5′-CAC CGG TGG ACA TCG TCA GCC GAA T-3′ |
| | R | 5′-AAA CAT TCG GCT GAC GAT GTC CAC C-3′ |
| 2. | F | 5′-CAC CGC TTG TGC TGT GTA GTG GTT C-3′ |
| | R | 5′-AAA CGA ACC ACT ACA CAG CAC AAG C-3′ |

V5-tagged Pask proteins were purified using anti-V5 agarose affinity gel clone V5-10 (#A7345; Sigma) according manufacturer's instructions.

### Real-time PCR analysis

Extraction of total RNA and real-time RT-PCRs were performed as previously described (34). Briefly, total RNA was converted to cDNA using Applied Biosystem's high-capacity cDNA reverse transcription kit using 1 μg of total RNA in a reaction volume of 20 μl as per manufacturer's instructions (Applied Biosystems, USA). PCR reactions were carried out in a volume of 25 μl of power SYBR Green

Master Mix with 200 ng of cDNA and 200 nM of each primer, using QuantStudio 3 real time PCR system (Applied Biosystems, CA, USA). The comparative $C_t$ method was used to calculate the relative gene expression (47). 18S RNA was used as the internal control.

The primers used were as follows:

- Pask F 5′ GGACTGGAAATGTTTGTCTGAGA 3′
- Pask R 5′ TCAGGTGTTTGTGGGCTATGG 3′
- Uni18SrRNA F5′ AGTCCCTGCCCTTTGTACACA 3′
- Uni18SrRNA R5′ GATCCGAGGGCCTCACTAAAC 3′

### Immunofluorescence

Mouse satellite (C2C12 cells) and human embryonic kidney (HEK293T cells) were grown in cavity slides, fixed in 4% paraformaldehyde/PBS, pH 7.4, washed, and blocked with BBX (PBS 0.1% Triton, 0.1% bovine serum albumin, 250 mM NaCl). The cells were incubated with the primary antibody for 2 h at room temperature or overnight at 4°C. They were washed again and blocked with BBX and incubated with the anti-rabbit-Alexa Fluor 488 secondary antibody in BBX for 1 h at dark, washed and mounted. Labeled cells were visualized with a Nikon C1 laser scanning microscopy system. All images were converted to the tagged information file format and processed with the Adobe Photoshop program.

### Western blot analysis

Total protein was extracted using the RIPA lysis buffer (1× PBS, 50 mM NaF, 0.5% Na deoxycholate (w/v), 0.1% SDS, 1% IGEPAL, 1.5 mM $Na_3VO_4$, 1 mM PMSF and 1× Halt protease and phosphatase inhibitor cocktail, (Thermofisher Scientific) at 4°C. The supernatant was collected and quantitated with Bio-Rad protein microassay using BSA as standard (Cat no. 500–0006). Nuclear and cytoplasmic extracts were prepared using NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology, IL, USA) as per the manufacturer's instructions. Western blot analysis was performed with 20 μg of nuclear, cytoplasmic or total cellular extracts. The following antibodies were used: GAPDH (2118; Cell Signaling Technology), H3 (Abcam; ab1791), PASK (3086; Cell Signaling Technology), PASK (MA-1-700; Thermo Scientific) Myf5 (SC-302; Santa Cruz Biotechnology), MyoD (SC-304; Santa Cruz Biotechnology), ASH2 (5019; Cell signaling Technology), MLL1 (A300-086; Bethyl lab), MLL2 (ABE 206; Merck-Millipore), hSET1a (A300-289A; Bethyl lab), hSET1b (A302-281A; Bethyl lab), RBBP5 (61405; Active Motif), WDR5 (D9E11; Cell Signaling Technology), phospho H3T3 (9714S; Cell signaling Technology), phospho H3T6 (ab14102; Abcam), phospho H3S10 (9701S; Cell Signaling Technology), phospho H3T11 (ab5168; Abcam), H3K4 monomethyl (39635; Active Motif) H3K4 monomethyl (MABE636 (Merk), H3K4 dimethyl (39141; Active Motif), H3K4 trimethyl (39915; Active Motif), Cyclin D1 (134175; Abcam), Cyclin E (4132T; Cell Signaling Technology) and Cyclin B (12231T; Cell Signaling Technology). Secondary antibodies used were obtained from Bio-Rad, USA.

### Co-immunoprecipitation analysis

For co-immunoprecipitation analysis, the nuclear extracts were prepared from C2C12 cells using the universal magnetic Co-IP kit (Active motif, 54002) as per manufacturer's instructions. Proteins bound to the respective antibody were separated with protein A/G magnetic beads (#88803, Pierce-Thermofisher Scientific) followed by washing five times with IP wash buffer. The bound protein complexes were separated from the beads by denaturation using SDS loading dye and analyzed by western blot analysis.

### Histone isolation

Histones were isolated using the acid extraction protocol as described by Shechter *et al.* (48).

### *In vitro* methyltransferase assay

*In vitro* methyltransferase assay was performed as described by Wu *et al.* (49). MLL2 complex was prepared by mixing equimolar (5 nM) concentrations of the core complex components, MLL2 (M342-381G-10; SignalChem), ASH2 (A372-30BG-50; SignalChem), WDR5 (W325-30H-50; SignalChem) and RBBP5 (R315-30H-50; SignalChem). Assays were performed in a volume of 20 μl comprising 4 μl of 5× HMTase buffer (250 mM Tris-HCl, pH 8.8, 2.5 mM DTT, 100 mM KCl, 50 mM $MgCl_2$), 1 μl of protease inhibitor cocktail (Roche #11836170001), 4 μl of the MLL2 complex, 1 μl of s-adenosyl-L-methionine (SAM; 2 mM in water, MP Biomedicals, Inc.), 0.5 μl of 1 mg/ml recombinant Histone H3 (Millipore) and 100 ng of PASK full-length (TP-309096; Origene) or PASK kinase only active protein (PV3972; Thermofisher Scientific). For the assays with V5-purified PASK and mutants, PASK proteins were replaced with the respective mutant. For PASK inhibition by Bio E-115, the specified concentration of the inhibitor was pre-incubated at 37°C for 15 min with the assay components except for the substrate, SAM. After incubation at 37°C for 3 h, sodium dodecyl sulphate loading buffer was added to stop each reaction mixture. Methylation of H3 was determined by western blotting using specific antibodies.

### *In vitro* kinase assay

Kinase assays were performed in a total volume of 20 μl containing the buffer components (50 mM HEPES, pH 7.5, 10 mM MgCl2 and 1 mM DTT). MLL2 complex was prepared as detailed above and added to a final concentration of 1 nM. About 0.5 μl of 1 mg/ml recombinant H3 (Millipore) and 100 ng of PASK full-length, PASK kinase only active or PASK mutants were added. For PASK inhibition by Bio E-115, the specified concentration of the inhibitor was pre-incubated at 30°C for 15 min with the assay components except for the substrate, 50 μM ATP. After the addition of ATP, assay mixture was incubated at 30°C for 2

h. The reaction was stopped by the addition of SDS loading dye and the resultant phosphorylation was analysed by western blotting.

### Direct interaction assays of PASK with the subunits of the human H3K4 MLL2 complex

To assess the direct interaction of PASK with the subunits of human H3K4 MLL2 complex, 0.3 picomoles of hPASK-V5 was incubated with 0.3 picomoles of recombinant MLL2, ASH2, WDR5 or RBBP5 in high salt IP buffer (1×) in the presence of 100 mM NaCl, detergent, DTT and Protease inhibitors (Nuclear Complex Co-IP Kit, Active motif Cat #, 54001) for 4 h at room temperature. The reaction products were then incubated with 1 μg of V5 peptide antibody (SC- 271944, Santa Cruz) overnight at 4°C. The complex was captured with V5 agarose beads (A7345, Sigma), washed five times with IP wash buffer (Nuclear Complex Co-IP Kit, Active motif Cat #, 54001) and eluted with SDS loading dye and analyzed by western blotting. Ten percent of the reaction was saved as input before the bead capture.

### Haematoxylin–eosin staining

Cells grown on plastic coverslips, at different stages of differentiation, were fixed with formalin–acetic acid–ethanol for 30–45 min. The fixed cells were rinsed and stained with Harris hematoxylin for 7 min, rinsed and counterstained with 1% eosin for 2 min followed by differentiation in 70–95% ethanol and dehydration with 100% ethanol. The dehydrated and air dried cells were mounted with DPX and imaged.

For treatment with the kinase inhibitor of PASK, BioE 1115 (532306, Calbiochem), the C1C12 cells were grown and differentiated at a concentration of 50 μM (41). To verify the effect of WDR5, the C2C12 cells were grown and differentiated in the presence of WDR5 inhibitor, WDR5-0103 (SML0859, Sigma) at a con of 100 μM and assessed the differentiation as above.

### Synchronization of C2C12, C2C12-Pask-KO, C2C12-BioE-1115 cells and FACS analysis

C2C12, C2C12-Pask-KO, C2C12-BioE-1115 and C2C12-CRISPR-vect control cells were synchronized at different cell cycle stages. The cells were trypsinized and the cell suspension was incubated in a methyl cellulose (4000cP, #M0512-250G; Sigma) DMEM suspension media for 48 h followed by plating with media containing hydroxy urea (#400046; Merck-Millipore, 1 mM) and incubated for 16 h (G1/S). After 16 h, HU was removed to release the cells from G1/S and were collected at 10 and 24 h, respectively after HU removal. The cells were also arrested at G2 by treating them with colcemid (10295892001; Roche Diagnostics GmbH) for 16 h at a concentration of 50 ng/ml, following which they were harvested for analyses by FACS and western blotting of proteins and histones for control cells. Propidium iodide-stained C2C12, C2C12-Pask-KO, C2C12-BioE-1115 and C2C12-CRISPR-vect control cells were also treated as above analyzed using FACS calibre for the proportion of cells in different stages of cell cycle.

### Chromatin immunoprecipitation

C2C12 cells were cross-linked for 15 min at RT using PBS containing 1% formaldehyde followed by the halt of cross-linking with glycine (125 mM) for 5 min at room temperature. Cells were then washed with PBS, scraped, pelleted and re-suspended in lysis buffer [50 mM HEPES, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1× Halt protease and phosphatase inhibitor cocktail, (Thermofisher Scientific) and 0.15% SDS] and incubated in ice for 10 min followed by sonication (Bioruptor Plus, Diagenode, Liege, Belgium) of 8 cycles of 45 s on and off at high setting that is repeated 3 times with a rest of 1 min on ice between cycles. The sonicated samples were clarified by centrifugation, diluted with lysis buffer and the resultant cross-linked chromatin was incubated with ChIP specific primary antibodies overnight at 4°C along with IgG and H3 controls. Antibody–Protein–DNA complexes thus formed were immunoprecipitated using protein A/G beads and washed extensively before eluting with 1% SDS at 70°C, for 10 min with continuous shaking at 1200 rpm. Cross-linking was reversed by the addition of NaCl (to a final concentration 0.2 M) to the sample and incubating at 65°C for 16 h with continuous shaking at 1200 rpm. The immunoprecipitated and reverse cross-linked DNA thus obtained was purified using Qiagen PCR purification columns and subjected to real-time PCR assays.

The list of primers used is:

- Myo D PRR -200bp F 5′-GAG TAG ACA CTG GAG AGG CTT GG-3′
- Myo D PRR -200bp R 5′-GAA AGC AGT CGT GTC CTG GG-3′
- Myo D CER -20Kb F 5′-GGG CAT TTA TGG GTC TTC CT-3′
- Myo D CER -20Kb R 5′-CTC ATG CCT GGT GTT TAG GG-3′
- Myo D CD1 +1Kb F 5′-CAT CTG ACA CTG GAG TCG CTT TG-3′
- Myo D CD1 +1 Kb R 5′ -CAA GCA ACA CTC CTT GTC ATC AC-3′
- Myo D CD2 +2 Kb F 5′-GTG AGC CTT GCA CAC CTA AGC C-3′
- Myo D CD2 +2 Kb R 5′-GTT GCA CTA CAC AGC ATG CCT G-3′
- Myf 5 PRR -315 bp F 5′-AAT GTC TTG CTA CCG TGC TG-3′
- Myf 5 PRR -315 bp R 5′-CTG GTC CCT TTG ACG CTA AT-3′
- Myf 5 CD1 +1.4 Kb F 5′-GAG TTA GGG GGC TGG TAT GA-3′
- Myf 5 CD1 +1.4 Kb R 5′-TCA AAG CTG CTG TTC TTT CG-3′
- Myf 5 CD2 +1.5 Kb F 5′-ACA GCA GCT TTG ACA GCA TC-3′
- Myf 5 CD2 +1.5 Kb R 5′-GGA CAG ACT GCC ATG ACT GA-3′
- Myf 5 CD3 +1.6 Kb F 5′-ATG GCA GTC TGT CCT TCA AA-3′
- Myf 5 CD3 +1.6 Kb R 5′-GCG TGT ATG CTG GTC TGT CT-3′

### Statistics

All data are expressed as means ± SEM of triplicate experiments unless state otherwise. Data were analysed using the two-tailed *t*-test or ANOVA.

## RESULTS

### PASK associates with the H3K4 MLL2 methyltransferase complex

PASK has been shown by a large scale protein interaction mapping to be a potential binding partner of WDR5 (50). WDR5 is a major H3K4-methyl-associated protein, and a well-established essential component of the human MLL and SET1 H3K4 methyltransferase complexes (3,4). This observation raised the possibility that PASK might be associated with the H3K4 methyltransferase complex in the nucleus.

To verify this possibility, we examined by immunohistochemistry, the cytoplasmic and nuclear expression of PASK in 293T cells and observed that PASK was distinctly discernible in the cytoplasm and the nucleus of 293T cells (Figure 1A). To verify this further, we prepared nuclear and cytoplasmic extracts from 293T cells and assessed the expression of PASK by western blotting. As it was demonstrated by immunohistochemistry, western blotting also revealed that while PASK was found predominantly in the cytoplasm, a substantial proportion was also detectable in the nuclear fraction (Figure 1B).

To verify the association of PASK with H3K4 methyltransferase complex, we transfected 293T cells with a full-length human PASK expression plasmid with a V5 tag (33) and generated nuclear extracts. We then performed co-immunoprecipitation (Co-IP) experiments using the V5 tag antibody followed by western blot analysis using a PASK antibody to verify the presence of the PASK protein in the immunoprecipitate. Our results showed the presence of PASK in the immunoprecipitate (Figure 1C). We then used a WDR5 antibody and did western blot analysis to verify the reciprocal interaction between the two proteins: PASK and WDR5. Our results showed that WDR5 co-immunoprecipitated with PASK (Figure 1C). We further verified that PASK associates with other members of the mammalian H3K4 methyltransferase complexes, by successfully immunoprecipitating ASH2 and RBBP5 (Figure 1C). These results suggested that PASK indeed associates with members of the H3K4 methyltransferase complexes.

We then probed the PASK-V5 immunoprecipitate for members of the mixed-lineage leukemia (MLL1 and MLL2) and SET domain-containing (hSET1a and hSET1b) family of histone methyltransferases based on their known association with the WDR5–ASH2–RBBP5 core complex (51,52) to identify the histone methyltransferase responsible for the methyltransferase activity of PASK H3K4 methyltransferase complex. PASK interacted with MLL2 (Figure 1C) but not with MLL1, hSet1A or hSet1B (Supplementary Figure S1), indicating that MLL2 most probably associates with PASK in the nucleus.

Having seen that PASK associates with the H3K4–MLL2 complex, we then verified the direct interaction of PASK with the subunits of the human H3K4–MLL2 complex by incubating hPASK-V5 with recombinant MLL2, ASH2, WDR5 or RBBP5 individually and captured with V5 agarose beads after V5 peptide antibody incubation. These were assessed by western blotting for the presence of core members of the H3K4 MLL2 complex, MLL2, ASH2, WDR5 and RBBP5. Our results suggested that PASK associates directly with WDR5 of the H3K4 MLL2 methyltransferase complex (Figure 1D).

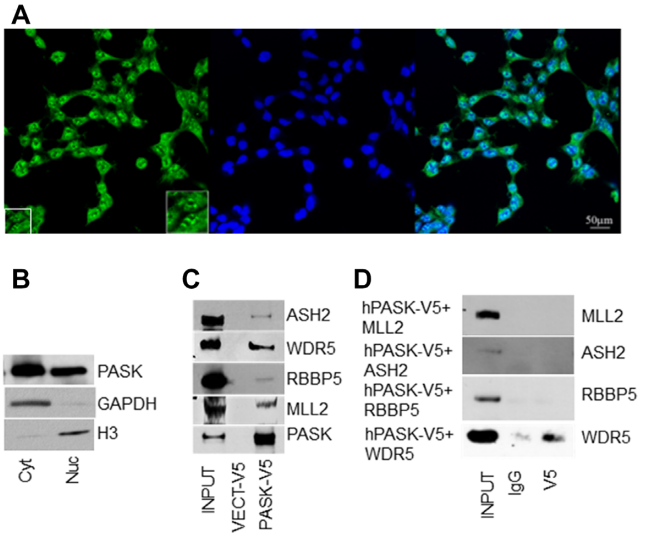



**Figure 1.** PASK is expressed in the cytoplasm and the nucleus of muscle satellite cells and associates with H3K4 MLL2 methyltransferase complex. (**A**) Confocal micrograph of HEK 293T cells immunostained with anti-rabbit PASK antibody (green) and TOPRO3 (blue) to stain the nucleus. Note that PASK is present in both the cytoplasm and nucleus. Note also the speckled appearance in the nucleus (insert). (**B**) Western blot analysis of the cytoplasmic and nuclear fractions of HEK293T cells with anti-rabbit PASK antibody reveals expression of PASK in both the cytoplasmic and nuclear fractions. GAPDH and H3 were used as markers for cytoplasmic and nuclear fractions. (**C**) PASK immunoprecipitated with V5 antibody from the nuclear extract of HEK 293T cells transfected with a hPASK expression plasmid with a V5 tag. PASK along with the core members of the H3K4 MLL2 methyltransferase complex, WDR5, ASH2 RBBP5 and the methyltransferase enzyme MLL2 are co-immunoprecipitated in the presence of V5 antibody. Nuclear extract from V5 vector transfected HEK 293T cells was used as control. About 10% of the nuclear extract was used as input. The results shown are a representative of three independent experiments. (**D**) Direct interaction analysis of PASK with the core members of the H3K4 MLL2 methyltransferase complex was analyzed using recombinant WDR5, ASH2 RBBP5, MLL2 and PASK-V5 proteins. Western blot analysis of V5 agarose beads capture shows PASK directly interacts with WDR5 protein. About 10% of the reaction was used as input. IgG was used as control.

### PASK enhances H3K4 methylation in assays in the presence of the recombinant proteins of the core members of the H3K4 MLL2 methyltransferase complex *in vitro*

To determine whether PASK modulates the activity of WDR5–ASH2–RBBP5-MLL2 complex and contributes to H3K4 methylation, we performed *in vitro* methyltransferase assays with recombinant histones in the presence of the methyl-donor S-adenosyl-methionine (SAM) and human recombinant proteins of core members of the H3K4 MLL2 methyltransferase complex, WDR5–ASH2–RBBP5-MLL2. Experiments were conducted in the pres-

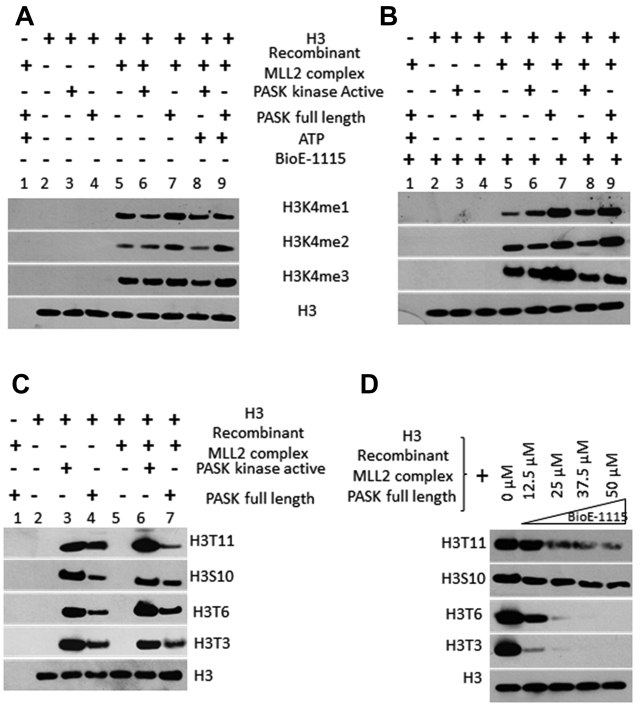



**Figure 2.** PASK promotes transcriptionally active chromatin through H3K4 mono-, di- and tri- methylation and also acts as a Histone 3 kinase. (**A**) *In vitro* methyltransferase assays with PASK full-length (lanes 1,4,7 and 9) and PASK kinase active proteins (lanes 3, 6 and 8) in the presence (lanes 1, 8 and 9) and absence (lanes 2–7) of ATP. Compared to the basal methylation (lane 5), H3K4 mono, di- and tri-methylation increased in the presence of PASK full-length protein only (lanes 7 and 9). The presence of ATP did not alter this increase (lanes 7 and 9). (**B**) *In vitro* methyltransferase assays with PASK full-length and kinase active proteins in the presence of PASK inhibitor, BioE-1115 which has no effect on the increase in H3K4 mono, di and tri-methylation levels by full-length PASK (lanes 7 and 9). *In vitro* methyl transferase assays without H3, MLL2 complex and PASK full-length and kinase active proteins (lanes 1–4) were used as controls in panels (A) and (B). (**C**) *In vitro* kinase assays with PASK full-length and kinase active PASK proteins. Both full-length and kinase active PASK proteins induced significant phosphorylations of H3T3, H3T6, H3S10 and H3T11 irrespective of the presence of recombinant proteins of the H3K4 MLL2 complex (lanes 3,4,6 and 7). *In vitro* kinase assays without H3, MLL2 complex and PASK full-length and kinase active proteins (lanes 1, 2, 5) were used as controls. (**D**) *In vitro* kinase assays in the presence of increasing concentrations of PASK inhibitor, BioE-1115 (0–50 μM), which shows significant decrease in the phosphorylations of H3T3 and H3T6. H3T11 phosphorylation was inhibited to a much lesser extent, and H3S10 was very minimally affected. The results shown are a representative of three independent experiments.

ence of both full-length and kinase active (aa 879–1323) PASK protein and without H3, MLL2 complex and PASK full-length or kinase active proteins (Figure 2A, lanes 1–4) as controls. PASK full-length and kinase active proteins in the presence of ATP were used in the assays to exclude the possibility of methylation resulting from the phosphorylation by PASK, since it is a kinase (Figure 2A, lanes 8–9). *In vitro* methyltransferase assays in the presence of the full-length PASK alone showed elevated levels of H3K4 mono-, di- and tri-methylation compared to that observed for WDR5–ASH2–RBBP5-MLL2, demonstrating that PASK might be promoting transcriptionally active chromatin (Figure 2A). The presence of kinase-only active PASK and the inclusion of ATP with PASK full-length and kinase active proteins in the methyltransferase assays had no effect on H3K4 methylation. This is suggestive of the fact that the observed increase in methylation might not be through phosphorylation of any of the members of the H3K4–MLL2 complex.

To confirm these observations, we added quinoxaline-6-carboxylic acid, BioE-1115 (532306 - Calbiochem), a cell-permeable, selective and reversible kinase inhibitor of PASK at a concentration of 50 μM (41) and repeated the experiments. The presence of the PASK kinase inhibitor did not affect the increase in the levels of H3K4 mono-, di- and tri-methylation with the full-length PASK suggesting that the role of PASK in methylation is independent of its kinase activity (Figure 2B).

## PASK is a H3 Kinase

As PASK is a serine/threonine kinase, we examined whether PASK might be a H3 kinase by performing *in vitro* kinase assays with full-length and kinase active PASK proteins in the presence of ATP (Figure 2C; lanes 3, 4 and 6, 7), the human recombinant proteins of the core members of the H3K4 MLL2 methyltransferase complex and determined the phosphorylation levels of H3T3, H3T6, H3S10 and H3T11. In the presence of both the full-length and kinase active PASK proteins, the *in vitro* kinase assays revealed significant levels of H3T3, H3T6, H3S10 and H3T11 phosphorylations suggesting that PASK might be a H3 kinase (Figure 2C). The addition of the MLL2 complex did not affect the levels of phosphorylation of any of these histone markers (Figure 2C). The presence of increasing concentrations of the PASK inhibitor BioE-1115 attenuated significantly the phosphorylation levels of H3T3 and H3T6. H3T11 phosphorylation was inhibited to a lesser extent and H3S10 was minimally affected (Figure 2D).

## H3K4 methylation occurs in the presence of full-length PASK

We expressed and purified human full-length PASK (PASK-full-V5), kinase dead KD (K1028R) (PASK-KD-V5) plasmids with a V5 tag (33) and protein from deletion constructs of PASK (PASK-949-V5, PASK-400-V5 and PASK-200-V5) (Supplementary Figure S2A–C). We then performed *in vitro* methyltransferase assays with recombinant histones with the methyl-donor S-adenosylmethionine (SAM) and human recombinant proteins of the core members of the H3K4 methyltransferase complex, WDR5–ASH2–RBBP5-MLL2 in the presence of both full-length and kinase dead PASK protein (Figure 3A, lanes 1–4). We also included PASK-full-V5 and PASK-KD-V5 proteins in the methyltransferase assays in the presence of ATP since PASK is a kinase to exclude the possibility of methylation resulting from the phosphorylation by PASK, (Figure 3A, lanes 8–10). In the presence of both the PASK-full-V5 and PASK-KD-V5 proteins, the assay revealed an increase in the levels of H3K4 mono-, di- and tri-methylation compared to that produced by the WDR5–ASH2–RBBP5-MLL2 complex (Figure 3A, compare lanes 5 to 6–7, and 8 to 9–10). This seems to indicate that the effect of PASK in H3K4 methylation is independent of its kinase activity.

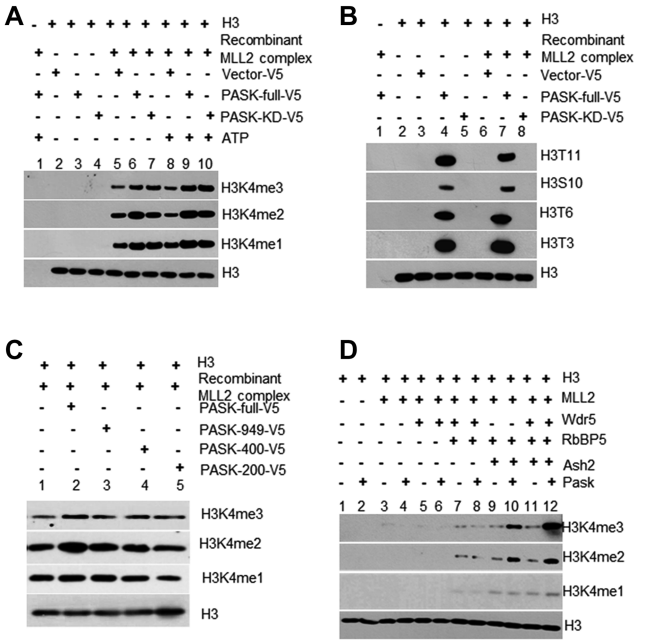



**Figure 3.** The presence of full-length PASK is needed to promote H3K4 mono, di- and tri-methylation. (**A**) *In vitro* methyl transferase assays with PASK-full-V5 (full length) and PASK-KD-V5 (kinase dead) proteins in the presence (lanes 1, 8, 9 and 10) and absence of ATP (lanes 2–7). Both PASK-full-V5 and PASK-KD-V5 proteins increased H3K4 mono, di and tri- methylation of H3K4 (lane 6, 7, 9 and 10). Vector V5 was used as control. (**B**) *In vitro* kinase assay with PASK-full-V5 and PASK-KD-V5 proteins. Only PASK-full-V5 protein was able to induce phosphorylations of H3T3, H3T6, H3S10 and H3T11 (lanes 4 and 7) as opposed to PASK-KD-V5 protein (lanes 5 and 8). The addition of recombinant proteins of H3K4 MLL2 complex did not affect the levels of phosphorylation (compare lane 4 to 7). H3 was used as loading control. (**C**) *In vitro* methyltransferase assays with the V5 purified PASK deletion proteins (PASK-949-V5, PASK-400-V5 and PASK-200-V5) and PASK-full-V5 protein. Only the PASK-full-V5 protein showed an increase in the levels of H3K4 mono, di- and tri-methylation (lane 2). (**D**) *In vitro* methyltransferase assays in the presence of PASK and in the presence and absence of the core components of H3K4 MLL2 complex. The methyltransferase assays in the presence of all the core components showed an increase in the levels of H3K4 mono, di- and tri-methylation with the addition of PASK (compare lane 11 to 12). Note the increase in the levels of H3K4 di- and tri-methylation seen with the H3K4 complex in the absence of WDR5 as well (compare lane 9 to 10). The results shown are a representative of three independent experiments.

To test the specificity of PASK as a histone kinase, we then performed *in vitro* kinase assays with PASK-full-V5 and PASK-KD-V5 proteins in the presence of ATP (Figure 3B; lanes 4, 5, 7 and 8) and the human recombinant proteins of the core members of the MLL2 complex (Figure 3B; lanes 1 and 6–8). We then looked for phosphorylation levels of H3T3, H3T6, H3S10 and H3T11. *In vitro* kinase assays in the presence of the full-length PASK protein alone showed significant levels of phosphorylation of H3T3, H3T6, H3S10 and H3T11 suggesting that the methyltransferase activity of PASK-WDR5-ASH2-RBBP5-MLL2 is independent of its H3 kinase activity (Figure 3B; lanes 4 and 7). The addition or omission of the MLL2 complex did not affect the levels of phosphorylation of any of these histone markers (Figure 3B, compare lane 4 with 7).

To further verify the contribution of PASK to H3K4 methylation, we performed *in vitro* methyltransferase assays with recombinant histones with the methyl-donor S-adenosyl-methionine (SAM) and the core members of the H3K4 MLL2 methyltransferase complex, in the presence of both full-length and PASK deletion proteins (PASK-949-V5 PASK-400-V5 and PASK-200-V5). *In vitro* methyltransferase assays in the presence of only the PASK-full-V5 protein produced an increase in the levels of H3K4 mono-, di- and tri-methylation compared to that produced by the WDR5–ASH2–RBBP5-MLL2 complex (Figure 3C, lane 2), demonstrating that PASK full-length form may be needed to promote H3K4 methylation.

## PASK facilitates H3K4 di- and tri-methylation in the presence of ASH2 and RBBP5 with MLL2

Mammalian H3K4 methyltransferase complexes are involved in transcriptional activation and their core complexes consisting of WDR5, ASH2 and RBBP5 alone are sufficient to induce transcriptional activation (3,4,7). It has also been suggested that WDR5 does not read the level of methylation but presents the histone to the catalytic subunit of the MLL1 complex (52,53). Therefore, to independently confirm the function of PASK in H3K4 MLL2 complex, we have used recombinant H3 as substrate and performed *in vitro* methyltransferase assays with MLL2 methyltransferase, in the presence or absence of PASK and of the core components, WDR5, ASH2 and RBBP5. *In vitro* methyltransferase assays in the presence of PASK and all of the core components showed a comparable increase in the levels of H3K4 mono-, di- and tri-methylation (Figure 3D, compare lane 11 with 12). Interestingly, a very clear increase in the levels of H3K4 di- and tri-methylation was observed without WDR5 (Figure 3D, compare lane 9 with 10) suggesting that PASK might be able to promote H3K4 di- and tri-methylation even in the absence of WDR5. However, there was no change in the levels of H3K4 mono methylation without WDR5 (Figure 3D, compare lane 9 with 10). It is noteworthy that recent studies on the structure of H3K4 methyltransferase complex have shown that WDR5 might stabilize the complex, which in turn promotes H3K4 trimethylation (10). Conversely, it was also demonstrated that in differentiating myoblasts WDR5 could contribute to H3K4 tri-methylation at promoters of specific genes independently of the H3K4 complex through its phosphorylation by PASK (42).

## Pask is a member of the endogenous H3K4 Mll2 methyltransferase complex

It has been shown that PASK promotes the differentiation of embryonic stem cells, adipogenic and myogenic progenitor cells (42). Therefore, to determine the functional relevance of Pask in H3K4 methylation and H3 phosphorylation endogenously, we used the muscle satellite cells, (C2C12), and determined the expression of Pask by immunohistochemistry. Immunostaining showed clear localization of a fraction of Pask in the nucleus (Figure 4A). We then generated cytoplasmic and nuclear fractions and determined its presence in both the fractions (Figure 4B).

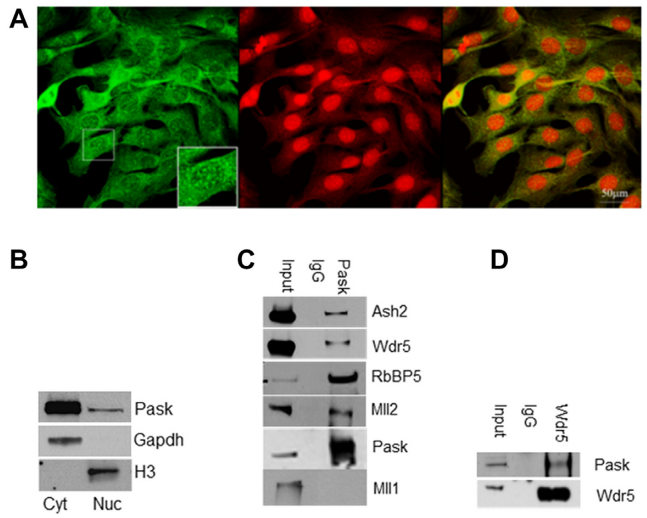



**Figure 4.** Pask is expressed in the cytoplasm and the nucleus of C2C12 muscle satellite cells and associates with the endogenous mammalian H3K4 Mll2 methyltransferase complex. (**A**) Confocal micrograph of C2C12 muscle satellite cells immunostained with anti-rabbit Pask antibody (green) and propidium iodide (red) depicting the presence of Pask in both the cytoplasm and nucleus. Insert shows its speckled appearance in the nucleus. (**B**) Western blot analysis of the cytoplasmic and nuclear fractions of C2C12 cells with anti-rabbit Pask antibody reveals expression of Pask in both the cytoplasmic and nuclear fractions. Gapdh and H3 were used as markers for the cytoplasmic and nuclear fractions. (**C**) Pask co-immunoprecipitated the core members of the H3K4 Mll2 methyltransferase members Wdr5, Ash2, RbBP5 and Mll2 methyltransferase enzyme from the C2C12 muscle satellite cells when brought down with Pask antibody. Western blots of Pask and Mll1 were also shown for the specificity of the immunoprecipitation. (**D**) Wdr5 co-immunoprecipitated Pask. Normal rabbit IgG was used as control for immunoprecipitation (C and D). About 10% of the nuclear extract was used as input in both experiments, (C and D). The results shown are a representative of three independent experiments.

We next verified whether Pask associates with the endogenous H3K4 Mll2 methyltransferase complex by performing Co-IP experiments using anti-PASK antibody followed by western blotting analysis for Pask itself and for the core members Ash2, Wdr5, RbBP5 and for the methyltransferase Mll2. As observed *in vitro,* Pask co-immunoprecipitated all of the core members and Mll2 methyltransferase (Figure 4C). Taken together, these results suggest that PASK associates with the members of the H3K4 Mll2 methyltransferase complex endogenously. We investigated this interaction further by Co-IP using anti-WDR5 antibody followed by western blotting for Wdr5 and Pask. As expected, both Wdr5 and Pask were detected in the immunoprecipitates of Wdr5 (Figure 4D) further confirming that Pask and Wdr5 interacted physically.

### Pask expression changes during the cell cycle in C2C12 muscle satellite cells

Skeletal muscle satellite cells that are normally reversibly arrested at G0 stage would re-enter into the cell cycle in response to activation by the MyoD family of transcription factors and proliferate. We examined the expression of Pask during different stages of the cell cycle, using the double synchronization protocol (54). C2C12 cells were cultured in suspension for 48 h in 1.5% methocel in GM followed by culture in GM containing 1 mM hydroxyurea (HU) for 16 h to arrest at G1/S border. Cells were subsequently released from G1/S arrest and collected at the different time points (0, 10, 24 h). We also synchronized C2C12 cells at G2 phase, by treating them with Colcemid at a concentration of 50 ng/ml for a period of 16 h. The cells harvested at different time points of the arrest and released as indicated were analyzed by FACS to assess the percentage of cells in each stage of the cell cycle (Figure 5 A) followed by the estimation of Pask expression at different cell cycle stages by western blotting (Figure 5B). Our results suggest that the expression of Pask increased as the cells entered the G2 stage of the cell cycle.

We next verified the effect of absence of Pask expression and the inhibition of phosphorylation by Pask to cell cycle progression by using C2C12-Pask-KO, C2C12-BioE-1115 and C2C12-CRISPR-vect control cells. The different combinations of C2C12 cells were cultured and were analyzed as above by FACS to assess the percentage of cells in each stage of the cell cycle (Figure 5C and D). Absence of Pask expression or the phosphorylation by Pask by the addition of Pask kinase inhibitor affected the progression of cell cycle. This resulted in less number of cells in G2 stage, the stage of the cell cycle where the Pask expression was high (Figure 5B) while there were more cells in the S phase. This effect was more pronounced in the complete absence of Pask expression compared to the inhibition of Pask phosphorylation (Figure 5A, C and D).

### Expression changes in Pask affects H3K4 methylation and H3 phosphorylation

To comprehend the functional role of Pask in H3K4 methylation and H3 phosphorylation, we generated a C2C12 muscle satellite cell line with increased expression of Pask by stable transfection of mouse Pask cDNA (C2C12- Pask) along with vector transfected (C2C12-Vect) control cells. The expression of Pask in C2C12-Pask cells was compared with C2C12-Vect cells by western blotting that showed higher levels of Pask expression in the C2C12-Pask cells (Supplementary Figure S3A). We also generated C2C12 cells overexpressing endogenous Pask (C2C12-CRISPR-Act-Pask) by using the modified Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/dCas9 activation system containing a nuclease null Cas9 (dCas9) under the control of tet-on system (55) and confirmed the expression of dCas9 and Pask upon induction with doxycycline (Supplementary Figure S3B). We first examined the expression levels of the core members of H3K4 Mll2 complex namely, Ash2, Wdr5, RbBP5 along with Mll2 methyltransferase and found no difference in their expression when Pask was overexpressed (Supplementary Figure S3C).

To test specifically the type of H3 tail modifications regulated by Pask, we isolated histones (Supplementary Figure S3D) from C2C12-Pask and C2C12-Vect cells and C2C12-CRISPR-Act-Pask cells in the presence or absence of doxycycline induction and analyzsed them for H3T3, H3T6, H3S10 and H3T11 phosphorylation and H3K4 mono-, di- and tri-methylation levels using specific antibodies. The levels of H3T3, H3T6, H3S10, H3T11 phosphorylation

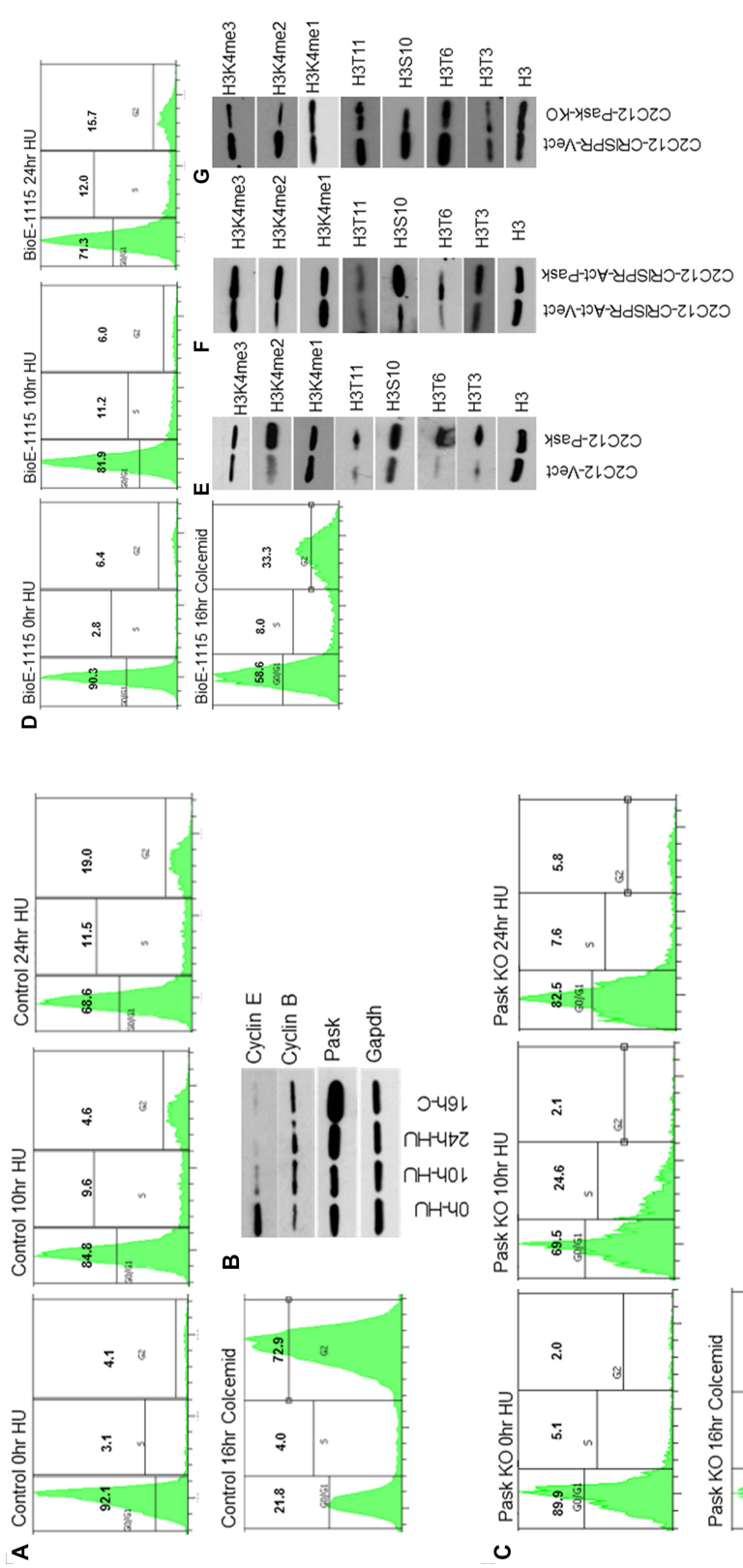



**Figure 5.** Pask expression changes during cell cycle in muscle satellite C2C12 cells. (**A**) FACS analysis of synchronized C2C12 cells at 48 h after suspension culture, after 16 h of HU treatment (G1/S), at 0, 10, 24 h after HU removal and after 16 h of colcemid treatment. The percentage of cells at G0/G1, S and G2 stages of cell cycle are shown. (**B**) Western blot analysis shows an increase in the expression of Pask as cells advance from G0 to G2. Gapdh was used as control and Cyclin E and B as markers for cell cycle. (**C** and **D**) FACS analysis of C2C12-Pask-KO and C2C12 BioE-1115 cells synchronised and assessed as above. The percentage of cells at G0/G1, S and G2 stages of cell cycle are shown. Western blot analysis of H3K4 mono-, di- and tri methylations and H3T3, H3T6, H3S10, H3T11 phosphorylation of C2C12 cells overexpressing Pask from an exogenous expression plasmid, (C2C12- Pask) or vector control (C2C12- Vect) (**E**) or endogenous promoter, CRISPR-Act system (C2C12-CRISPR-Act-Vect and C2C12-CRISPR-Act-Pask (**F**). H3K4, di- and tri methylations and H3T3, H3T6, H3S10, H3T11 phosphorylation are elevated when Pask is overexpressed. H3 was used as loading control. (**G**) Western blot analysis of H3K4 mono-, di- and tri methylations and H3T3, H3T6, H3S10, H3T11 phosphorylation of C2C12 cells edited for the expression of Pask by CRISPR/CAs9 system (C2C12-CRISPR-Vect and C2C12-Pasl-KO). H3K4, di- and tri methylations and H3T3, H3T6, H3S10, H3T11 phosphorylation are downregulated in the absence of Pask expression. The results shown are a representative of three independent experiments.

were increased in histones from C2C12-Pask cells compared to histones from C2C12-Vect cells confirming that Pask is a H3 kinase. Furthermore, the fact that H3K4 di- and tri-methylation were also increased supported the fact that Pask might be promoting transcriptionally active chromatin by its association with Wdr5-Ash2-RbBP5-Mll2 (Figure 5E and F).

To substantiate further the functional effect of overexpression of Pask, we generated C2C12 cell lines that were edited for the Pask gene, C2C12- Pask-KO cells and C2C12-CRISPR-Vect cells using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system (56). We have generated single cell clones and assessed the expression of Pask using western blotting. Based on expression, we picked five clones of C2C12-Pask-KO cell lines that did not show any Pask expression (Supplementary Figure S4A) and assessed the change by sequencing (Supplementary Figure S4B). Three of them (clones 2A6, 1C2, 2C3) had 94 bp deletions at the 5th exon while clone 2B6 had a 91 bp deletion with an insertion of 17 bp. The clone 1A3 had a loner deletion extending beyond exon 5 (Supplementary Figure S4B). To reduce the clonal effects that may be caused by picking single cells and generating the KO cells we pooled clones 2A6 and 2B6 together and assessed the expression levels of the core members of H3K4 Mll2 complex, Ash2, Wdr5, RbBP5 along with Mll2 methyltransferase in those C2C12 cells that were edited for Pask and the results showed that they were unaltered (Supplementary Figure S4C). We used these for all the analyses involving Pask-KO cells.

We isolated histones from C2C12-Pask-KO and C2C12-CRISPR-Vect cells as described above and analyzed them for H3K4 mono-, di- and tri-methylation and levels of H3T3, H3T6, H3S10, H3T11 phosphorylation using specific antibodies. H3K4 di- and tri-methylation decreased in histones from C2C12- Pask-KO cells compared to histones from C2C12-CRISPR-Vect cells supporting the fact that Pask associates with H3K4 Mll2 methyltranferase complex and contributes to H3K4 di- and tri-methylation. (Figure 5G). In contrast to our previous observations, in our endogenous assays, in the absence of Pask, H3K4 monomethylation did not change (Figure 5G). This could be due to the possibility that other regulators, which may modulate the methylation in the endogenous assay might be absent in the *in vitro* assays. In addition, the phosphorylation levels of H3T3, H3T6, H3S10 and H3T11 were decreased in the histones from C2C12- Pask-KO cells compared to histones from C2C12-CRISPR-Vect cells confirming further that Pask is H3 kinase (Figure 5G). Again, the effect is variable and could be due to the possibility that other kinases may be contributing to the final effect and/or the activity might be more specific for some modification/genomic location.

### Pask expression is downregulated upon differentiation of muscle satellite C2C12 cells

Muscle fibers are formed by the fusion of myoblasts under the control of muscle specific transcription factors, myogenic differentiation (MyoD), myogenic factor 5 (Myf5), myogenin (MyoG) and myosin heavy chain (MHC) (57,58). To understand the functional relevance of Pask during muscle differentiation, we seeded C2C12 cells in proliferation media and shifted them to differentiation media at 90–95% confluence as described in the 'Materials and Methods' section. Cells were harvested at 0, 12, 24, 48, 72, 96 and 120 h in differentiation media and the expression of Pask mRNA and protein were compared by RT-qPCR and western blotting analysis at the different time points. As shown in Figure 6 A and B, there was a decrease in the mRNA and protein expression of Pask upon differentiation of C2C12 cells. We also tested the expression level of nuclear Pask upon differentiation and found it to be downregulated especially after 24 h of differentiation (Figure 6B). The fact that Pask expression is downregulated during C2C12 differentiation suggests a role for Pask in maintaining the undifferentiated status and proliferation during the early stages of muscle satellite cells.

### Overexpression or loss of Pask expression in muscle satellite (C2C12) cells interferes with myogenic differentiation

To gain insight into the role of Pask during myogenic differentiation, we seeded C2C12 cells that stably expressed or were edited for Pask with their corresponding vector transfected cells (C2C12-Pask or C2C12-Vect), (C2C12-Pask-KO or C2C12-CRISPR-Vect) on plastic coverslips in proliferation media. At 90–95% confluence, they were shifted to differentiation media and their capability to differentiate into myotubes was assessed by haematoxylin and eosin staining at 24, 48 and 96 h.

The capability of C2C12-Pask cells to differentiate into myotubes was severely compromised (Figure 6C) while the C2C12-Vect cells formed multinucleated myotubes within 96 h of incubation in the differentiation media (Figure 6C). The number of myotubes ($P < 0.001$***) (Figure 6D) and fusion index ($P < 0.01$**) (Figure 6E) was also reduced significantly in C2C12-Pask cells.

As observed in cells with forced expression of Pask, the ability of C2C12 cells to differentiate into myotubes was severely compromised in the absence of Pask as well, as observed in the C2C12-Pask-KO cells. At 96 h the C2C12-Pask-KO cells did not differentiate and form multinucleated myotubes (with at least four or more nuclei) while the C2C12-CRISR-Vect cells formed multinucleated myotubes (Figure 6F). However, we noticed few very thin elongated cells with one or two nuclei (Figure 6F). These results suggest that normal expression of Pask might be a requirement for proper myogenic differentiation and any change in its expression level might adversely alter the process.

To differentiate between the role of Pask during myogenic differentiation which is mediated through phosphorylation or through phosphorylation of its downstream target Wdr5 (42), we seeded C2C12 cells on plastic coverslips in proliferation media with the vehicle, DMSO (C2C12 control), the kinase inhibitor of PASK, BioE 1115 or the inhibitor of WDR5. At 90–95% confluence, they were shifted to differentiation media with the vehicle or the inhibitors and their capability to differentiate into myotubes was assessed by haematoxylin and eosin staining at 24, 48 and 96 h as above. The capability of C2C12 cells to differentiate into myotubes was compromised in the presence of both the PASK kinase and WDR5 inhibitors (Figure 6F). The num-

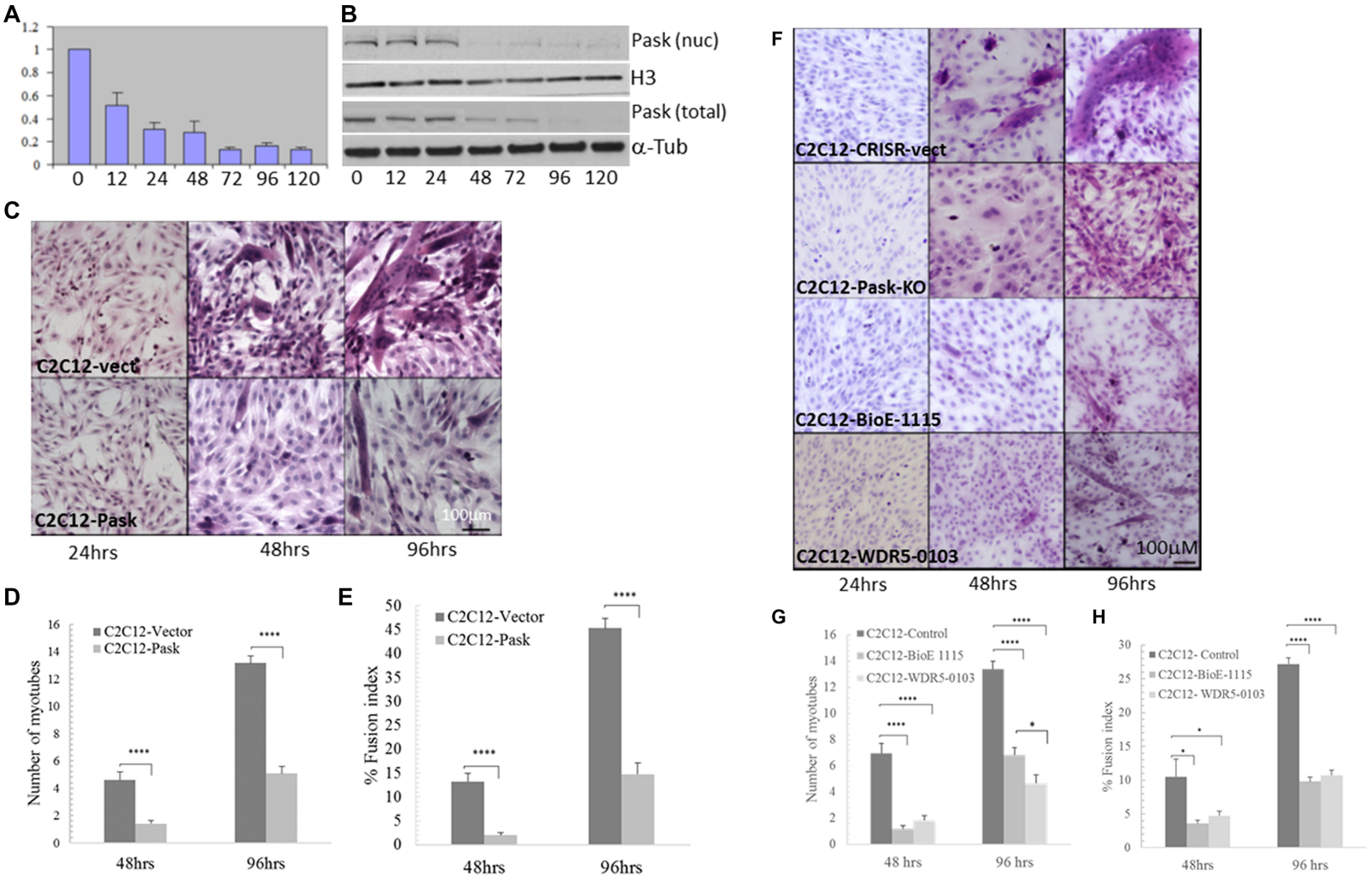



**Figure 6.** Pask expression is downregulated upon differentiation of muscle satellite C2C12 cells and overexpression or loss of Pask expression interferes with myogenic differentiation. C2C12 were seeded in proliferation media and then shifted to differentiation media after 24hrs. Cells were harvested at the different time points (0–120 hrs) and were analyzed for Pask expression using qRT-PCR (A) and western blotting (B). The expression of Pask mRNA and protein were decreased in C2C12 cells which had been shifted to the differentiation media. The expression of nuclear Pask was also analysed after shifting to differentiation media at different time points and after generation of nuclear extract (B). The expression of nuclear Pask protein was decreased in the C2C12 cells which had been shifted to the differentiation media from 24 hrs. 18s rRNA was used as internal control for qRT-RCR and α-Tubulin and Histone 3 were used as loading control for Western blotting. (C, F) Stable C2C12 muscle satellite cells over expressing Pask (C2C12- Pask) or vector control (C2C12- Vect) or those which were edited for Pask expression (C2C12-Pask-KO), or treated with Pask kinase inhibitor, BioE1115, or with WDR5 inhibitor, 0103 or vector control (C2C12- CRISPR-Vect) were seeded in proliferation media and then shifted to differentiation media after 24 hrs. Cells were analyzed at 24, 48 and 96 hrs for myotube formation (C, F). Quantitative analysis of the number of myotubes (D, G) and the percentage fusion index (E, H) of C2C12- Pask over expression cells or C2C12 cells in the presence of Pask kinase and Wdr5 inhibitors are shown. Forced, loss of expression or the treatments with Pask kinase and Wdr5 inhibitors inhibits myogenic differentiation. Columns represent mean of triplicate determinations ± SEM (D). P value = ($P$<0.05*, $P$< 0.01** and $P$<0.001***).

ber of myotubes ($P < 0.001$***, both 48 and 96 h) (Figure 6G) and fusion index ($P < 0.01$*, 48 h and $P < 0.001$*** 96 h) (Figure 6H) was also reduced significantly in presence of both the PASK kinase and WDR5 inhibitors. The number of myotubes was significantly more in the presence of the PASK kinase inhibitor compared to that of WDR5 inhibitor at 96 h. On the other hand, the percentage fusion index was higher with the WDR5 inhibitor at 96 h although this difference did not reach the significance level (Figure 6G and H). While the complete absence of Pask severely compromised C2C12 myogenic differentiation, the effect of Pask mediated through phosphorylation or through phosphorylation of its downstream target Wdr5 is not very distinct as the phenotypic difference is very shubtle.

### Altered myogenic differentiation following overexpression or loss of Pask expression might be due to altered regulation of the muscle specific transcription factors Myf5 and MyoD

To understand the mechanism by which Pask regulates myogenic differentiation, we analyzed the expression of the early muscle specific transcription factors, MyoD and Myf5 by western blotting in C2C12 cells that overexpressed or were edited for Pask. The expression of both MyoD and Myf5 was increased in cells that overexpressed Pask (Figure 7A and B), while their expression was downregulated in the absence of Pask (Figure 7C). Since MyoD and Myf5 play important roles in proliferation versus differentiation of myoblasts (59), it could be inferred that Pask might be regulating myogenic differentiation through the regulation of the expression of these myogenic transcription factors. Further, these transcription factors are needed during specific time points of myogenic differentiation, mainly during myoblast specification. Alteration in them, either an increase or a decrease will result in defective differentiation that may be a reason why both over and loss of Pask expression affect C2C12 differentiation.

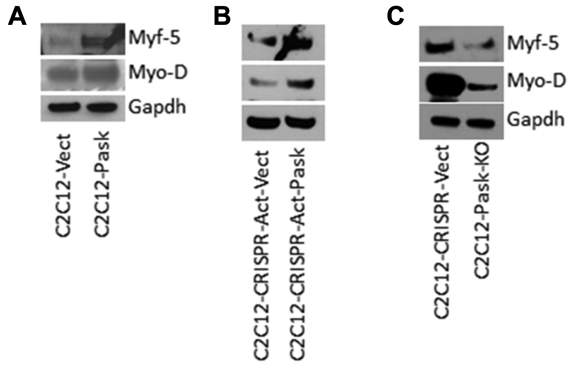
### Expression changes in Pask affects H3K4 methylation and H3 phosphorylation levels at the promoters of MyoD and Myf5

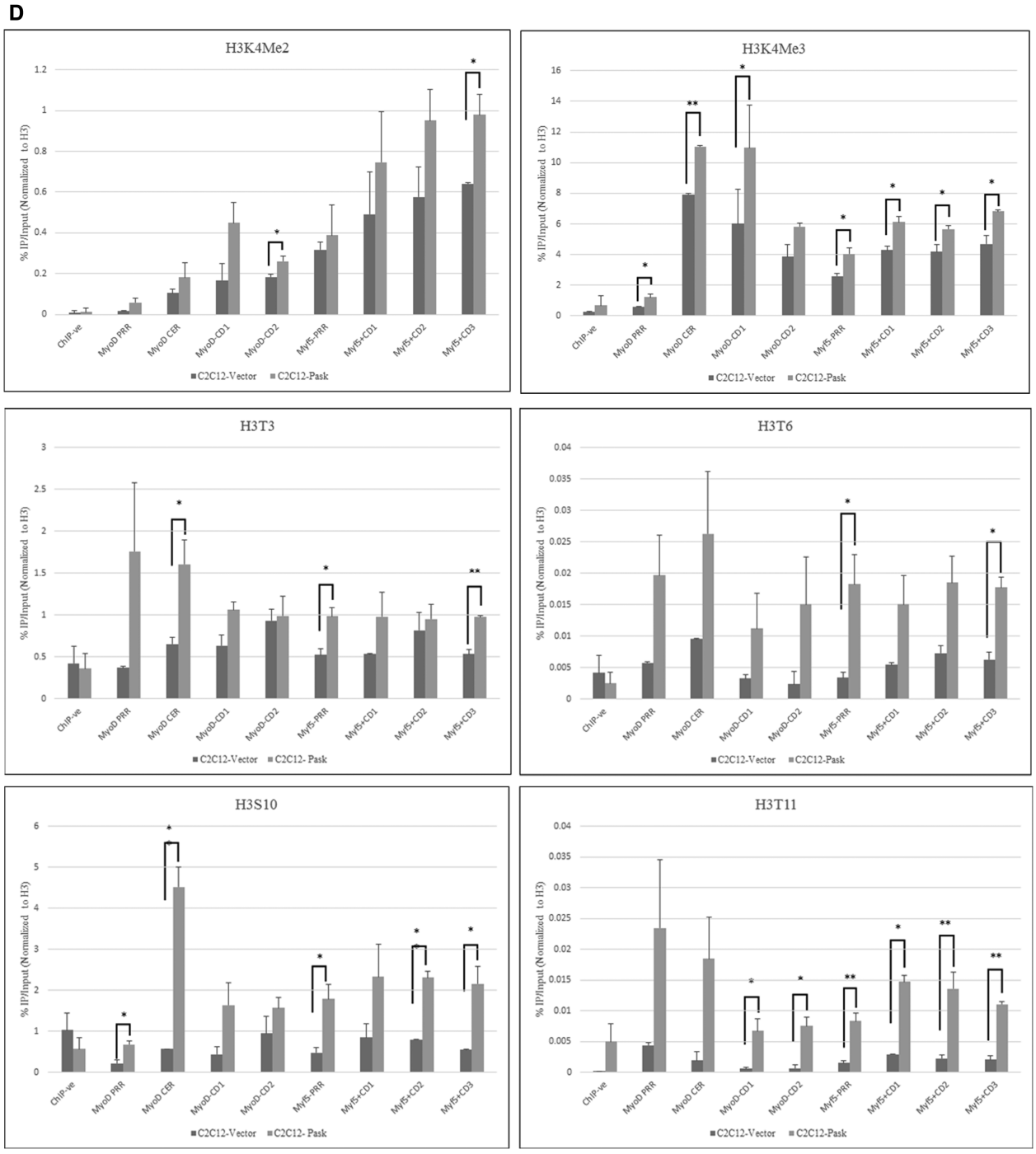
Since Pask affected MyoD and Myf5 expression and also influenced myogenic differentiation, we examined whether H3K4 di- and tri-methylation and H3T3, H3T6, H3S10 and H3T11 phosphorylation changes occurred at the promoter region of MyoD and Myf5 by Chromatin immunoprecipitation (ChIP) analysis using antibodies specific for these modifications. We then compared the levels of these modifications between C2C12-Pask cells and C2C12-Vect cells (Figure 7D) and between C2C12-Pask-KO and C2C12-CRISR-Vect cells combined clones 2A6 and 2B6) (Figure 7E). For MyoD, we examined the core enhancer region (CER) -20 kb, proximal regulatory region (PRR) -200 bp, coding region 1 (CD1) +1 kb and coding region 2 (CD2) +2 kb regions (60). We examined, the proximal regulatory region (PRR) -315 bp, coding region 1 (CD1) +1.4 kb, coding region 2 (CD2) +1.5 kb and coding region 3 (CD3) +1.6 kb of the of the Myf5 transcription factor (61) (Figure 7C and D). In both genes, H3K4 methylation and H3 phosphorylation were altered in a manner that correlated to the presence or absence of Pask expression (Figure 7C and D). Pask overexpression was associated with an increase in the levels of both H3K4 di- and tri-methylation in most of the regions tested. Similarly, H3T3, H3T6, H3S10 and H3T11 phosphorylation levels were also increased with many of these regions tested showing significant changes (Figure 7D). Complimenting to what was seen with overexpression, the absence of Pask expression resulted in the decrease in the levels of H3K4 di- and tri-methylation and H3T3, H3T6, H3S10 and H3T11 phosphorylation levels (Figure 7E). Although variations were seen in the level of changes observed for the different modifications and the regions tested in these promoters and in the coding region, they changed according to the increase or decrease in the Pask expression. Taken together these results suggest that indeed the global changes seen in cells that either overexpressed or lacked Pask lead to specific changes in the promoters of these genes which regulate myogenic differentiation and might also be responsible for the altered differentiation observed in muscle satellite cells.

## DISCUSSION

PASK was reported to be a metabolic sensor based on knock-out studies in mice which were resistant to high-fat diet-induced obesity and T2DM (37). Earlier studies also suggested that PASK might play a pivotal role in glucose metabolism and energy homeostasis through the regulation of insulin secretion, lipid metabolism and mitochondrial respiration (37,40,43,44). In an earlier study using Cynomolgus macaques, the expression of the PASK gene changed in response to alterations in the developmental environment (34). Thus if PASK could sense changes in the developmental environment and induce responses to them, it should be able to mediate these adjustments genome-wide by regulating multiple pathways at the same time. The best fit mechanism by which this could be achieved, we opined, was probably the epigenetic modifications of histones and DNA methylation both of which occur in the nucleus. PASK must be present in the nucleus to be involved in these epigenetic regulatory processes. The appearance of PASK in the nucleus as speckle-like structures has been reported in the testes (39) and in cultured cells (33), although the studies did not elucidate the physiological relevance of PASK in the nucleus. The results of the present study also demonstrate the presence of a fraction of endogenous PASK in the nucleus of HEK293T and C2C12 muscle satellite cells, lending credence to the belief that PASK could be functioning genome-wide.

In addition to being expressed in the nucleus, PASK appears to be a binding partner of WDR5, a major H3K4-methyl-associated protein. It co-immunoprecipitated with WDR5 and other members of the H3K4 methyltransferase complex (ASH2, RBBP5 and MLL2) from HEK293T and C2C12 muscle satellite cells suggesting that PASK might be associated with the mammalian H3K4 MLL2 methyltransferase complex. Our results also show PASK specifically associates with WDR5 of the H3K4 MLL2 methyltransferase complex. Earlier studies have suggested that the subunit composition of the different mammalian methyltransferases, hSet1 (hSet1A and B) and MLLs (MLL1–


A
Myf-5
Myo-D
Gapdh
C2C12-Vect
C2C12-Pask
B
C2C12-CRISPR-Act-Vect
C2C12-CRISPR-Act-Pask
C
Myf-5
Myo-D
Gapdh
C2C12-CRISPR-Vect
C2C12-Pask-KO


D
H3K4Me2
H3K4Me3
H3T3
H3T6
H3S10
H3T11
% IP/Input (Normalized to H3)
ChIP-ve
MyoD PRR
MyoD CER
MyoD-CD1
MyoD-CD2
Myf5-PRR
Myf5+CD1
Myf5+CD2
Myf5+CD3
C2C12-Vector
C2C12-Pask
C2C12- Pask

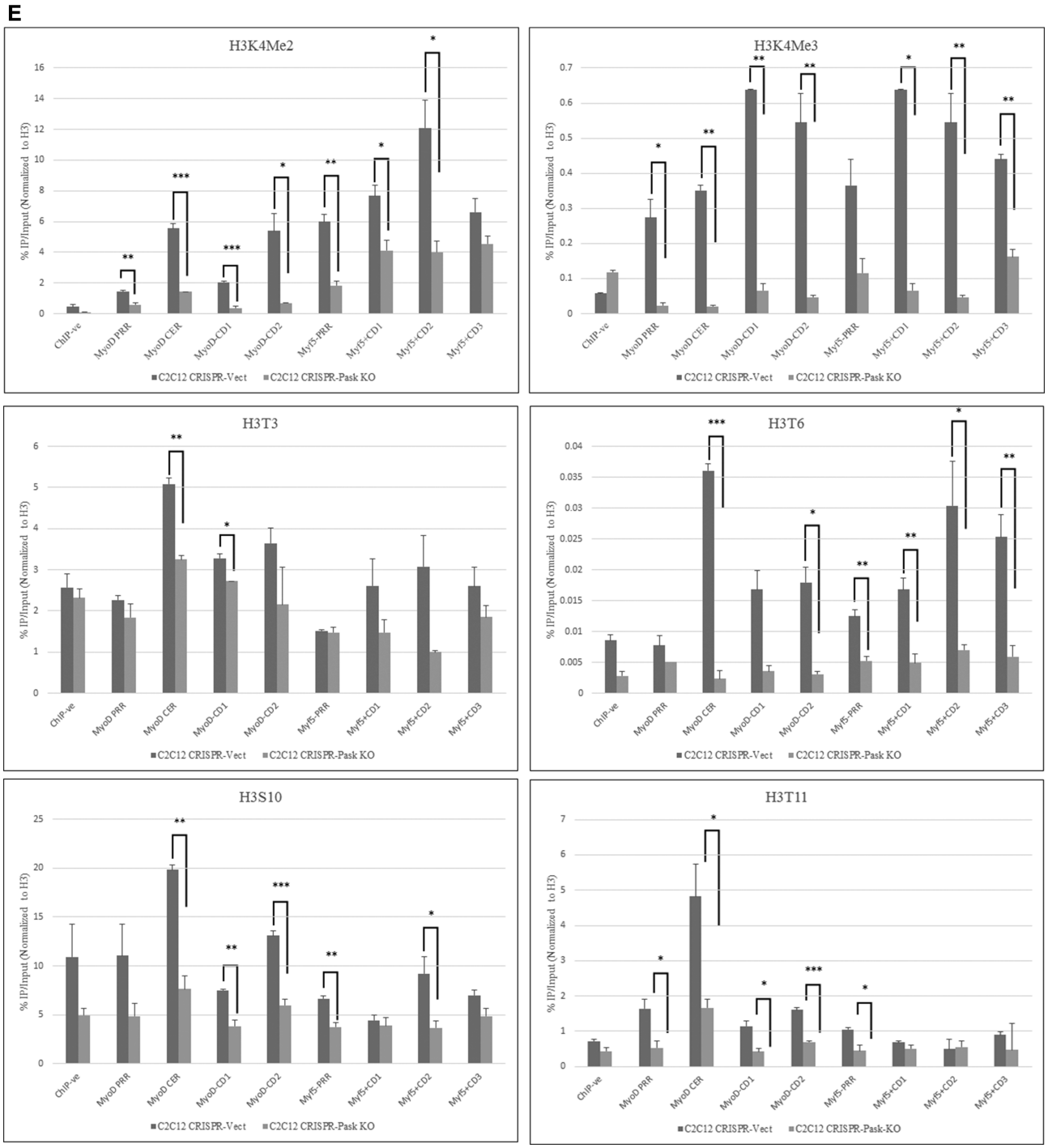



**Figure 7.** Over or loss of Pask expression alters the expression of muscle specific transcription factors, MyoD and Myf5 and affects the H3K4 methylation and H3 phosphorylation at their promoters. Expression of early myogenic factors MyoD and Myf5 was analyzed in cells over expressing Pask from an exogenous expression plasmid, (C2C12- Pask) or vector control (C2C12- Vect) (**A**) or endogenous promoter, CRISPR-Act system (C2C12-CRISPR-Act-Vect and C2C12-CRISPR-Act-Pask (**B**) or Pask deficient C2C12 cells (C2C12-Pask-KO) or vector control (C2C12- CRISPR-Vect) (**C**) by western blotting. The expression of Myf5 and MyoD were increased concurrent with the expression of Pask (A and B) and their expression decreased when Pask expression was lost (C). Chromatin immunoprecipitation (ChIP) analysis at the promoter region of MyoD and Myf5 genes for H3K4 di- and tri-methylation and H3T3, H3T6, H3S10, H3T11 phosphorylation from these cells. H3K4 methylation and H3 phosphorylation were altered which could be correlated to the increase or loss of Pask expression (**D** and **E**). The data shown are normalized to total H3. Columns represent mean of three independent ChIP experiments with three replicate determinations ± SD. *P* value = ($P < 0.05$*, $P < 0.01$** and $P < 0.001$***).

4) are different. A similar example was shown for the tumour suppressor protein Menin, which was suggested to be present only in MLL1/2 methyltransferase complex (8). H3K4 methylation functions in gene transcriptional activation and undergoes three different types of modifications namely mono-, di- or tri-methylation (4,62). Our results suggest that the association of PASK with the H3K4 MLL2 methyltransferase complex enhances transcriptionally active chromatin. Its presence elevated the levels of mono-, di- and tri-methylation, in *in vitro* assays and di- and tri-methylation on the analysis involving endogenous complex. One possibility for this variation may be in endogenous assays, additional modifications from other regulators and effectors which are not present in the *in vitro* assays. As PASK enhances di- and tri-methylation in endogenous assays, we suggest it preferentially enhances di- and tri-methylation by its association with H3K4 MLL2 methyltransferase complex. These results also imply that PASK might be mediating some of its functions through the regulation of histones as was demonstrated by our analysis of C2C12 differentiation. The role Pask in C2C12 differentiation mediated through H3K4 methylation is clearer. However, the role mediated through H3 phosphorylations, which is supported by the changes in the H3 phosphorylations of the muscle specific genes, Myf5 and MyoD needs further characterization to demarcate its role mediated through WDR5 phosphorylation.

A similar mechanism was recently shown to underlie the activity of the paired-box transcription factor (Pax7) which recruits histone methyltransferase complex and is indispensable for the maintenance of muscle stem cells and activation of myogenic genes during differentiation (61).

It is becoming increasingly evident that 'the histone code' or the combinations of histone tail modifications such as methylation, acetylation or phosphorylation recognized by effector molecules together is responsible for the regulation, activation or inhibition of different biological processes (2,25). It is also known that these different histone modifications could be stable as in the case of methylation of lysines and arginines or highly dynamic, as in phosphorylation of serines and threonines and acetylation of lysines (63). H3 phosphorylations, H3S10, H3S28 (64–66), H3T11 (21) and H3T3 (22,67) are well conserved and play critical roles during cell division (18,19,68).

Recent studies have shown that the interactions that occur between such covalent modifications as methylation, acetylation and phosphorylation at the same time at the histone tail elicit positive or negative biological responses (25). For example, mass spectrometry and immunoassays have shown a positive phospho/methyl interaction of H3K9 methylation and H3S10 phosphorylation (17). Phosphorylation of H3T3 as part of a combinatorial pattern with H3K4 tri-methylation and H3R8 dimethylation that configure mitotic chromatin has also been reported (69). The presence of H3T3 phosphorylation with other modifications such as H3S10 phosphorylation and H3K3 di-methylation, H3K9 tri-methylation, and H3K4 tri-methylation and H3K27 tri-methylation have also been reported (69). A binary switch involving the modification of H3S10 phosphorylation and H3K9 tri-methylation has

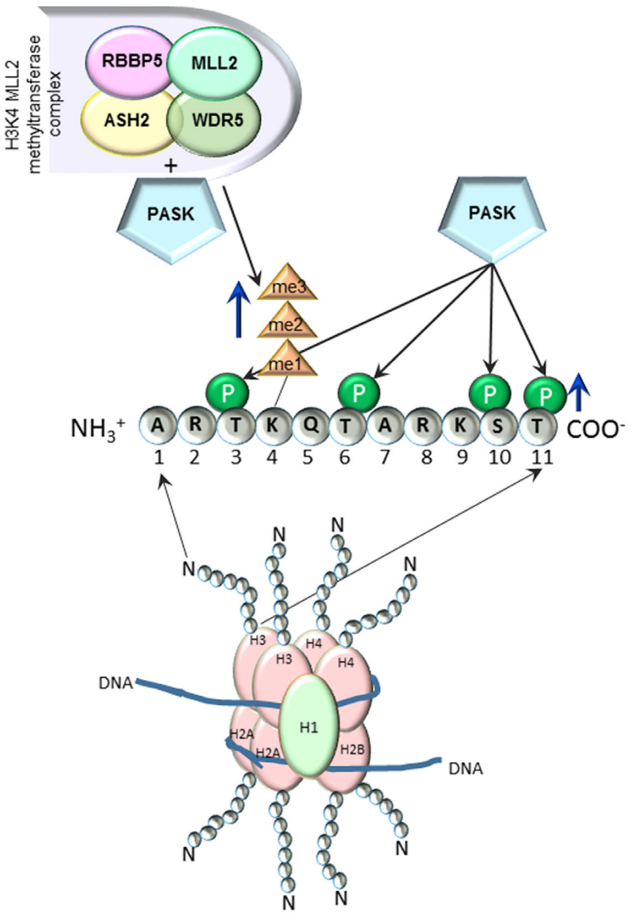



**Figure 8.** The model depicting the association of PASK with the mammalian H3K4 MLL2 methyltransferase complex. PASK associates with the H3K4 MLL2 methyltransferase complex and this association promotes H3K4 methylation. PASK is also a histone kinase and phosphorylates H3 at T3, T6, S10 and T11.

been shown to play a critical role in the interaction of the effector protein HP1 with the pericentromeric heterochromatin (25).

There are also studies, which show an inverse correlation between methylation and phosphorylation. Based on *in vitro* kinase analysis, it has been shown that the activities of H3T3 specific kinase HASPIN could be impaired by the presence of H3K4 methylation (70). Other effector molecules such as PHD finger-containing proteins, which recognize and bind to the histone tail modification of H3K4 methylation, are also compromised by the presence of H3T3 phosphorylation during mitosis (71). However, our results suggest that PASK phosphorylation of H3 is independent of its role in methylation of H3K4 MLL2 complex. Although our studies have shown that PASK phosphorylates H3 at T3, T6, S10 and T11, it is also possible it will phosphorylate other serine's and threonine's in the other regions of H3 and histones as well.

While these studies together establish the co-existence of more than one histone tail modification and act as part of a complex read out involving different readers, writers and

erasers (72–75), the molecules which affect these regulatory histone codes together, if any, are not known. Herein, we show that PASK could affect both phosphorylation and methylation. It contributes to H3K4 di- and tri-methylation through its association with the H3K3 MLL2 methyltransferase complex and to phosphorylation of H3T3, H3T6, H3S10 and H3T11 as a histone kinase (Figure 8).

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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