1. INTRODUCTION
1.1. Epithelial cell adhesion junctions

The epithelium is a tissue that forms a lining over both the inner and outer surfaces of the body including the skin, intestine, airway passages and glands. The epithelium is made up of epithelial cells which are tightly adhered to each other and to the basement membrane in a definite orientation to form epithelial sheets. Orientation refers to formation of apical-basolateral polarity, a characteristic of epithelial cells [1]. Apical-basolateral polarity of simple epithelial cells is characterized by presence of the basal surface where cell membrane receptors contact the extracellular matrix (ECM); a lateral surface defined by cell receptors being in contact with other cells; and an apical surface facing the hollow lumen (Figure 1.1) [1]. Apical basolateral polarity is only found in the basal progenitor cells, present at the basal layer of a stratified epidermis. An essential requirement for maintaining epithelial polarity is the differentiation of protein and lipid composition of each surface (reviewed in [2]). Epithelial apical basolateral polarity is essential for maintaining tissue homeostasis (reviewed in [3, 4]), epidermal morphogenesis (reviewed in [5]) and tissue functions like maintaining homeostasis between commensal bacteria and immune cells of the gastro-intestinal tract (reviewed in [6]), regulating barrier function and maintaining symmetric and asymmetric cell division in the different compartments of the skin epidermis (reviewed in [7, 8]). Loss of epithelial cell polarity leads to increased neoplastic progression and metastasis (reviewed in [9-11]).
Figure 1.1. Epithelial cell to cell and cell to ECM junctions. Cell to cell adhesion is maintained by the tight junctions, adherens junctions and the desmosomes present at the lateral surface of the plasma membrane of the adjacent epithelial cells, while cell to ECM adhesion is maintained by the focal adhesions and hemidesmosomes present at the basal surface of the plasma membrane [12].

Establishment of the epithelial polarity requires the formation of the apical junctional complexes at the lateral surface of the cells (reviewed in [13-15]). The apical junctional complex consists of the tight junctions and the adherens junction. Tight junctions (or zona occludens) play a very important role in maintaining epithelial polarity by restricting apical/basolateral intra-membrane diffusion of lipids and by preventing paracellular diffusion of water and solutes [16]. The transmembrane proteins in the tight junctions are claudins and occludins which link the tight junctions to the actin cytoskeleton via Zonula occludens1 (ZO1) and ZO2 proteins. Decreased expression of the tight junction proteins ZO-1 [17] and occludin [18] are known to cause breast cancer.
Adherens junctions are calcium dependent adhesion junctions that anchor actin filaments and form a strong intracellular actin cytoskeleton. They are made of transmembrane classical cadherins E cadherin, P cadherin and N cadherin. These cadherins bind to actin filaments intracellularly via the catenin and vinculin family proteins. The adherens junction protein E cadherin has been demonstrated to be very important in maintaining epithelial polarity ([19] and reviewed in [20]) and homeostasis (reviewed in [21]). E cadherin expression has been shown to be decreased in colorectal [22] cancer, gastric cancer, non small cell like lung carcinoma [23] and breast cancer [24]. Loss of E cadherin has been shown to disrupt cell polarity and cause increased metastasis [25]. It was demonstrated that expression of multiple transcription factors are induced upon E cadherin loss. One of these transcription factors, Twist is required for E cadherin loss mediated metastasis [25]. Thus, loss of cell to cell adhesion in between epithelial cells can lead to loss of cell polarity and also increased neoplastic progression and metastasis.

Epithelial cell polarity is also maintained by the attachment of cells to the ECM via the focal adhesions and hemidesmosomes present at the basal side of the plasma membrane (reviewed in [26]). The focal adhesions are formed by transmembrane integrins and linked to actin filaments by adaptor proteins talin, filamin and vinculin (reviewed in [27]). Hemidesmosomes are formed by α6 β4 integrins and bullous pemphigoid antigen-2 (BPAG2) and are linked to intermediate filaments through plectin and the epithelial form of bullous pemphigoid antigen-1 (BPAG1e) (reviewed in [27]). The cell to ECM adhesion is important not just to maintain epithelial cell polarity but has been demonstrated to regulate epidermal morphogenesis (reviewed in [28, 29]) and cell motility (reviewed in [30, 31]). Thus, loss of cell to ECM adhesions also leads to increased neoplastic progression and metastasis (reviewed in [26, 32]).
1.2. The Desmosome

Desmosomes are specialized cell-cell adhesion junctions that are present abundantly in cells, which constantly encounter mechanical stress such as epithelial cells and myocardial cells ([33] reviewed in [34, 35]). They are also found in purkinje cells, meningeal cells and the follicular dendritic cells of the lymph nodes and thymus [36-38]. Desmosomes are calcium dependant junctions, thus, cell-cell adhesion is based on the presence of extra-cellular calcium [39-41]. In confluent cultures of epithelial cells and in tissues such as the epidermis, desmosomes become “hyper-adhesive” and acquire calcium independence and do not require calcium for cell-cell adhesion [41-43]. Desmosomes anchor intermediate filaments in adjacent cells of the epithelium and allow the formation of an inter-cellular intermediate filament network, which helps maintain epithelial tissue integrity and helps epithelial cells withstand mechanical stress [44-46]. Desmosomes have been demonstrated to regulate epithelial polarity (reviewed in [47, 48]), epidermal morphogenesis and cell positioning [20]. It was demonstrated that desmosomes can regulate alveolar morphogenesis by the luminal epithelial cells and cell positioning of the luminal and myo-epithelial cells of the breast [20].

1.2.1. Ultra-structure and molecular architechtue of the desmosome.

The desmosome is made up of proteins belonging to four major protein families: the desmosomal cadherins, armadillo repeat (ARM) containing proteins and the plakin family of proteins (Figure 1.1) [49, 50]. Under the electron microscope, desmosomes appear as a pair of electron dense plaques that are bilaterally symmetrical to each other and present at the cell-cell border of adjoining epithelial cells (Figure 1.2). The plasma membranes of adjacent cells are separated by an intercellular space of approximately 30 to 35 nm. In mature desmosomes, this space is bisected by an electron dense midline (DM). The DM represents the site of interaction between
the desmosomal cadherins, the desmogleins (Dsg) and desmocollins (Dsc). The Dsgs and Dscs engage in calcium dependent homophilic and heterophilic adhesive interactions.

**Figure 1.2. Desmosome ultra structure and molecular composition.** The figure depicts a cartoon representing the molecular architecture of the desmosome superimposed on an electron micrograph of the desmosome. Abbreviations: DM= dense midline, PM= plasma membrane, ODP= outer dense plaque, IDP= inner dense plaque, DSG=desmoglein, DSC=desmocollin, PG=plakoglobin, PKP=plakophilin, DP=desmoplakin (adapted from [51]).

The intracellular side of the plasma membrane consists of two large discoid or oval shaped electron dense plaques called outer dense plaque (ODP) (closer to the plasma membrane) and inner dense plaque (IDP) (further away from the plasma membrane than ODP). Both ODP and IDP are approximate 15 to 20 nm thick and the distance between these two plaques are approximately 8 nm thick. The ODP represents the site where the intracellular domains of DSGs and DSCs interact with armadillo repeat family proteins plakoglobin (Pg) and plakophilin (Pkp) and the plakin family protein desmoplakin (DP). The IDP is at about 50 to 70 nm away from the plasma membrane. At the IDP, DP interacts with intermediate filament (IF) proteins like keratin
and vimentin, thus linking the desmosome with intermediate filaments [51]. The intermediate filaments loop at the inner face of the IDP in the form of an electron dense wide arc and seem to spread out in the cytoplasm ([49, 52-55] and reviewed in [56]).

1.2.2. Desmosomal cadherins:

The cadherin protein family consists of glycosylated transmembrane proteins [57, 58]. Cadherins are broadly classified into six subfamilies namely classical cadherins (type I cadherins), atypical cadherins (type II cadherins), desmogleins (DSGs), desmocollins (DSCs), protocadherins and flamingo cadherins (reviewed in [59]). DSGs and DSCs are the cadherins present in the desmosome and are therefore called desmosomal cadherins (reviewed in [59, 60]). In humans there are four DSG genes (DSG1-4) [61-65] and three DSC genes (DSC1-3) and all DSC gene products undergo alternate splicing to form two splice variants. The longer form is designated as “a” and the shorter form “b” (Figure 1.4) [63, 66-68]. Unlike the “a” form, the “b” form lacks binding site for Pg and Pkps, but can still localize to functional desmosomes [69].

Desmosomal cadherins are structurally very similar to classical cadherins (Figure 1.3). The N terminus of DSGs and DSCs are made of five highly conserved extracellular cadherin (EC) like domains named EC 1-5. The fifth EC domain which is closest to the plasma membrane is also called the extracellular anchor (EA) domain. The EC and EA domains are connected by flexible linker peptides which serve as binding sites for calcium ions. Each binding site can hold three calcium ions. The EC domains are important for the homophilic and heterophilic interactions between cadherins. This interaction between cadherins of adjacent cells leads to formation of the dense midline in a desmosome. Multiple EC domain repeats are followed by a short transmembrane motif (TM) which spans the membrane only once. The cytoplasmic side of DSGs
and DSCs are made up of intracelllar anchor (IA) domain and an intracellular catenin binding site (ICS). The intercellular proline rich linker (IPL), a variable number of repeat unit domains (RUDs) and a desmoglein terminal domain (DTD) are present exclusively in DSGs.

The intracellular domains of the desmosomal cadherins associate with the armadillo repeat containing proteins and the plakin family proteins ([70] and reviewed in [59, 71]). The desmosomal cadherins have cell type and tissue type specific expression patterns. DSG2 and DSC2 are expressed in most human tissues, while, DSG1, DSG3, DSC1 and DSC3 are only expressed in the stratified epithelium. DSG4 is expressed only in the highly differentiated epithelial cells and in the hair shaft cortex, the lower hair cuticle, and the upper inner root sheath (IRS) cuticle [72-76].

The expression of desmosomal cadherins is stringently regulated during the formation of the epidermis. At approximately 5 to 8 weeks after embryonic gene activation, the developing epidermis exists as a two layered structure, one layer made up of peridermal cells and the other made up of basal cells. The peridermal cells stop proliferating, become

![Diagram of structural domains, isoforms and splice variants of desmosomal cadherins.](image)

*Figure 1.3. Structural domains, isoforms and splice variants of desmosomal cadherins.*

Each of the four DSG genes and three DSC genes encode separate desmosomal cadherin
isoforms. All DSC isoforms are alternatively spliced to “a” and “b” forms. The “b” form has a shorter ICS domain. Abbreviations: Pro= propeptide, EC= extracellular cadherin, EA= extracellular anchor, TM= transmembrane, IA= intracellular anchor, ICS= intracellular catenin binding site, IPL= intercellular proline rich linker, RUD= repeat unit domain [51].

flattened, develop into the first layers of the cornified epithelium and are eventually shed off. The basal cells give rise to the three layers of the epidermis namely the spinous layer, the granular layer and the cornified layer (stratum corneum). During this process, the highly proliferating stem like cells of the basal layer gradually migrates to the upper layers undergoing differentiation at each layer of the epidermis. These cells undergo terminal differentiation just before entering into the stratum corneum [77-79]. During epidermal morphogenesis, desmosomal cadherins undergo stratification dependent alterations in gene expression (Figure1.4). DSG1 expression increases while DSC2 level decreases as cells move from the basal layer to the granular layer. DSG2 is only expressed in the basal layer. DSG3 and DSC3 levels decrease as cells move from basal layer to spinous layer. Both these proteins are not expressed in the granular layer. DSG4 is expressed only in the granular layer. DSC1 is not expressed in the basal layer but its expression increases as cells move from the spinous layer to the granular layer [80]. The differentiation dependant expression of desmosomal cadherins raised the possibility that DSGs and DSCs may have direct or indirect roles in epidermal differentiation. To understand the function of different DSG and DSC isoforms, individual gene knockout mice models were generated and the phenotypes were analyzed (Table 1.1). While, DSG3 knockout mice have hair loss, acantholysis of the suprabasal layers of the oral and acantholysis of skin epidermis resulting in skin crusting [81]; the DSC1 knockout mice develop acantholysis of the granular layer of the skin epidermis leading to flaky skin and skin barrier
defects [82]. DSG2 knockout mice die as embryos just after implantation into the uterus [83] while DSC3 mice die at embryonic day (E2.5) stage even before formation of mature desmosomes [84]. The phenotypes observed in DSG2 and DSG3 mice, show that these desmosomal cadherins may play desmosome independent roles and may also regulate stem cell functions.

Desmosomal cadherins are very important regulators of epidermal differentiation (reviewed in [60]). DSG1, which is present abundantly in the granular layer of the epidermis, promotes terminal differentiation. DSG1 binds to the protein, Erbin at the cell surface of differentiating cells. The membrane localized Erbin binds to SHOC2 (a scaffolding protein which allows the formation of RAS/RAF complexes, thus activate the ERK signaling pathway). The DSG1-Erbin-SHOC2 interactions deplete the availability of SHOC2 to bind to RAS/RAF, thus inhibiting ERK activation. ERK inhibition in turn leads to terminal differentiation of keratinocytes. DSG1 loss due to haplo-insufficiency in patients suffering from Striate palmoplantar keratoderma (SPPK) (Table 1.2), lead to less Erbin-SHOC2 interactions and strong ERK activation [85]. DSG3, which is expressed maximally in the basal layers of the skin epidermis and absent in the upper layers, inhibit keratinocyte terminal differentiation. To analyze the role of DSG3 in epidermal differentiation, transgenic mice having keratin 1 promoter driven over-expression of DSG3 was generated. Since keratin 1 is expressed in the spinous and granular layers of the epidermis, DSG3 expression was forced to be expressed in these layers. The skin and hair follicles of these transgenic mice showed phenotypes of hyper-proliferation and abnormal differentiation [86]. DSG3 expression driven by involucrin promoter (expression in stratum corneum) exhibited lack of terminal differentiation in the skin epidermis [87]. Thus desmosomal cadherins regulate epidermal differentiation.
Figure 1.4. Differential expression of desmosomal cadherins in skin epidermis. Desmoglein and desmocollin isoforms show stratification specific expression in the different layers of the epidermis.

<table>
<thead>
<tr>
<th>Genes encoding desmosomal cadherin</th>
<th>Type of knockout</th>
<th>Phenotypes</th>
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<tbody>
<tr>
<td>DSG2</td>
<td>Global knockout</td>
<td>Embryos die shortly after implantation. Desmosomal independent changes are believed to occur during embryonic stem cell proliferation in the DSG2 knockout mice because at desmosome formation takes place at least 4 weeks after embryonic gene activation in mice [83].</td>
</tr>
<tr>
<td>DSG3</td>
<td>Global knockout</td>
<td>Normal at birth, but develop acantholysis of the subrabasal layer of the oral mucosa leading to oral lesions. Crusting and acantholysis of traumatized skin. Hair loss in telogen from postnatal day 20 [81].</td>
</tr>
<tr>
<td>DSC1</td>
<td>Global knockout</td>
<td>Embryos born with eyes open and develop flaky skin with punctuate barrier defects. Acantholysis in the granular layer, resulting in epidermal fragility. Develop ulcerating lesions resembling chronic dermatitis. Exhibit local hair loss from 6 weeks of age [82].</td>
</tr>
<tr>
<td>DSC3</td>
<td>Global knockout</td>
<td>Embryos die before E2.5, even before formation of the mature desmosome [84].</td>
</tr>
</tbody>
</table>

Table 1.1. Phenotypes of mice having knockout of individual desmosomal cadherin genes.

The table represents the phenotypes associated with global knockout or tissue specific knockout of different desmosomal cadherins in mice models.
Desmosomal cadherins are associated with multiple human diseases (Table 1.2). DSG2 and DSC2 are the only desmosomal cadherins expressed in the myocardium, thus mutations in DSG2 and DSC2 genes lead to severe heart defects like Arrhythmogenic right ventricular dystrophy/ cardiomyopathy (ARVD/C) [88, 89]. ARVC/D is a heritable heart disease that affects the myocardial cells of the right ventricle leading to right ventricular enlargement, irregular heartbeats and life threatening cardiac arrests. ARVD/C starts with loss of function mutants in cardiac desmosomes of the right ventricle which leads to detachment of cardiac myocytes and subsequent death of the myocytes. The damaged muscle is replaced by fibro-fatty tissue (reviewed in [90]). DSC2 mutations also cause the wooly hair syndrome in which the hair of the scalp appears wiry and frizzled like wool [91]. In the skin, haplo-insufficiency of DSG1 results in SPPK characterized by thickening of the stratum corneum of the palm, knees, soles, ankles and finger knuckles [92]. Mutations in DSC2 are also known to cause SPPK [91]. Mutations in DSC3 and DSG4 (a major component of the inner root sheath of the hair follicles) leads to hair loss or hypotrichosis [93, 94].

Circulating autoantibodies against DSG1 and DSG3 are known to cause severe skin blistering diseases like pemphigus vulgaris (PV) and pemphigus foliaceus (PF) [62, 95, 96]. Both PV and PF cause acantholysis (disruption of cell to cell adhesion) and internalization of desmosomal components from the cell surface leading to loss of desmosomes [97-99], but differ in two ways: PV antibodies target both DSG1 and DSG3 proteins while PF antibodies only target the DSG1 protein; PV antibodies lead to supra-basal skin blistering and blistering of the oral mucosa, while PF antibodies lead to only superficial skin blistering (Figure 1.5). The reason behind this is; In PF, antibodies target only DSG1, the other desmosomal isoform DSG3 (which is the major desmosomal cadherin of the basal layers), compensates for the loss of DSG1. In PV, antibodies
target both DSG1 and DSG3, other DSG isoforms cannot compensate for the loss and this causes severe suprabasal blistering of the skin (reviewed in [100, 101]).

Skin blistering and acantholysis can also be caused by proteolytic action of exfoliative toxins (ETs) produced by bacteria like Staphylococcus aureus. If the skin blisters appear to localize only to the arms, legs or trunk, then the disease is called Bullous impetigo, but if the skin blisters occur extensively throughout the body and cause superficial blistering and exfoliation, then the disease is called Staphylococcal scalded skin syndrome (SSSS) (reviewed in [102-104]). Three homologous ETs have been successfully cloned till date and characterization of these ETs have increased our knowledge about these diseases [105, 106]. These staphylococcal ETs are serine proteases [107, 108] and have been demonstrated to cleave the extracellular region of the DSG1 protein [109, 110] at glutamate 381 residue which lies between EC3 and EC4 domains of DSG1 [111]. The cleavage mechanism is not only dependent on the amino acid sequence but also on the conformation of DSG1. The ETs can cleave DSG1only when it exists in a calcium bound conformation [112, 113]. Thus alterations of desmosomal cadherins can lead to multiple heart and skin related diseases. A thorough understanding of the mechanisms regulating PV, PF, Bullous disease and SSSS can help develop ways to combat these diseases.

One excellent example when understanding the mechanism of a disease has helped in developing strategies for treatment of the disease is in PV. The PV antibodies targeting DSG3 (the most common target in PV) has been shown to activate the p38MAPK pathway. Active p38MAPK in turn phosphorylates Hsp27 (heat shock protein 27) and causes keratin filament retraction and actin re-organization. To inhibit the p38MAPK signaling pathway, a specific inhibitor SB202190 [114] was used. These were able to prevent PV disease in mice [115] and block keratin filament retraction and actin cytoskeleton reorganization [116]. The p38MAPK signaling
pathway has been considered to be a good target in developing treatment modalities for PV patients (reviewed in [117]).

**Figure 1.5. The desmoglein compensation hypothesis.** In the disease PF, auto-antibodies against DSG1 can only cause superficial skin blistering (the cleavage plane of the epidermis is at the granular layer). In PV, auto-antibodies targeted against both DSG1 and 3 lead to supra-basal skin blistering (the cleavage plane is just above the basal layer) [101].

Expression levels of desmosomal cadherins also get altered in multiple cancers (Table 1.3). Decrease in mRNA/protein expression of DSG1 is associated with pancreatic cancer and anal cancer. In colon and skin cancers, DSG2 levels increase while in pancreatic and gastric cancer, DSG2 level decreases. Similarly, increase in DSG3 levels lead to head and neck squamous cell carcinoma (HNSCC) and esophageal carcinoma while its decrease is associated with breast cancer. DSC isoforms are known to be tumor suppressors (reviewed in [118]).

Decrease in DSC1 expression is associated with lung cancer and anal cancer, while decrease in DSC2 is associated with colon cancer. In a manner similar to DSC1; a decrease in DSC3
expression is associated with lung cancer and prostate cancer. Loss of DSC2 in colon cancer cells lead to activation of the AKT/β catenin signaling pathway which in turn leads to increased neoplastic progression [119]. DSC2 loss in sporadic colorectal adenocarcinoma is also associated with a phenomenon called desmocollin switching [120].

<table>
<thead>
<tr>
<th>Desmosomal cadherin</th>
<th>Disease name (cause of disease)</th>
</tr>
</thead>
</table>
| DSG1                | Pemphigus foliaceus (Auto-antibodies) [96]  
Pemphigus vulgaris (Auto-antibodies) [121]  
Bullous impetigo (Exfoliative toxin) [109, 110]  
SSSS (Exfoliative toxin) [109, 110]  
SPPK (haplo-insufficiency) [92] |
| DSG2                | ARVC/D (Frame shift/ splicing/nonsense/missense mutations/ compound heterozygosity) [89] |
| DSG3                | Pemphigus vulgaris (Auto-antibodies) [122-124] |
| DSG4                | Hypotrichosis (intragenic deletion/ missense mutations) [93] |
| DSC2                | ARVC/D (Missense mutations) [88]  
Wooly hair syndrome with cardiomyopathy and SPPK (Frame shift mutations) [91] |
| DSC3                | Hypotrichosis with skin vesicles (nonsense mutations) [94] |

**Table 1.2. Diseases associated with desmosomal cadherins.** The table represents the diseases associated with desmosomal cadherin alterations and the experimentally validated causes of the disease.

DSC2 is the only isoform of DSCs found in normal colon epithelium. But upon DSC2 loss in colon cancers, de novo expression of DSC1 and DSC3 is observed. This desmocollin switching is postulated to promote neoplastic progression. Loss of DSG2 in colon cancers decreases neoplastic progression [125]. Investigation of the mechanism regulating this process revealed that DSG2 knockdown leads to increased DSC2 expression. Increased DSC2 in turn activated the EGFR pathway and inhibited cell proliferation [125]. These experiments showed that DSG2 has
a pro-tumorigenic role while DSC2 acts as a tumor suppressor protein in colon cancer via activating the EGFR pathway. Thus, alterations in mRNA/protein expression of desmosomal cadherins are associated with various signaling pathways which may either lead to increase or decrease in neoplastic progression.

<table>
<thead>
<tr>
<th>Desmosomal cadherin</th>
<th>Types of cancers associated with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Increased expression</td>
</tr>
<tr>
<td>DSG1</td>
<td></td>
</tr>
<tr>
<td>DSG2</td>
<td>Colon cancer [125], skin cancer [128]</td>
</tr>
<tr>
<td>DSG3</td>
<td>Head and neck cancers [130], esophageal squamous cell carcinoma [131]</td>
</tr>
<tr>
<td>DSC1</td>
<td></td>
</tr>
<tr>
<td>DSC2</td>
<td></td>
</tr>
<tr>
<td>DSC3</td>
<td></td>
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</table>

Table 1.3. Cancers associated with alterations in desmosomal cadherins. The table represents the type of cancers associated with alterations in expression of desmosomal cadherins.

1.2.3. Plakin family of proteins:

The plakin family consists of proteins having long polypeptide sequences. They join the intermediate filaments to membrane associated desmosomal plaque proteins thus playing the role of anchor proteins for both the desmosomes (cell to cell adhesion) and hemi-desmosomes (cell to ECM adhesion). The plakin family proteins present in most desmosomes are desmoplakin and plectin. The proteins periplakin, envoplakin and epiplakin are present only in cornified layers of the stratified epithelia [135].
1.2.3.1. Desmoplakin (DP):

DP is an essential component of the desmosomal plaque. It is important for desmosomal functioning and adhesion [136]. Two alternative splice variants of DP exist namely DPI and DPII. DPII as a shorter rod domain than DPI and has been proposed to exist even as monomers [137, 138] (Figure 1.6).

![Alternative splice variants of DP and their domain structure.](image)

Figure 1.6. Alternative splice variants of DP and their domain structure. The figure represents the two splice variants of DP: DPI and DPII. It also depicts the domain structure of DP. DP is made up of the N terminus head domain, the rod domain, the plakin repeat domains (A, B, C) followed by the glycine-serine-arginine (GSR) domain. DPII has a shorter rod domain [139].

The structure of DP is considered to be the best model for plakin family proteins. DP is made up of a head domain, a plakin domain followed by three plakin repeat domains (PRDs) and a glycine-serine-arginine domain (GSR) [140] (Figure 1.6). The N terminus head domain, also known as the plakin domain, is a long α helical coiled coil domain and is a characteristic feature of the plakin family of proteins. The plakin domain is made of spectrin repeats and a putative SH3 domain (Figure 1.7). DP binds to other desmosomal proteins like DSC1a, PG and PKPs via the plakin domain and is needed for recruitment of DP to the membrane associated desmosomal
plagues [141, 142]. In the DP protein structure, the plakin domain is followed by the central coiled coil rod domain which is important for protein dimerization. At the C terminal domain of DP, multiple plakin repeat domains exist followed by the GSR domain. These act as binding sites for intermediate filaments [143, 144].

**Figure 1.7. The spectrin repeats in plakin domain of DP.** The plakin domain at the N terminus of DP has 6 spectrin repeats (SRs) and consists of an Src homology region (SH3) within spectrin repeat 5 (SR5) (adapted from [140]).

Both DPI and DPII are present in all stratified and simple epithelial tissues [145]. In stratified epithelium, DP expression is highest in the granular layer and least in the basal layer (Figure 1.8) [146]. In non-epithelial cells like the human umbilical vein endothelial cells, both DPI and DPII are expressed. In myocardial and Purkinje fiber cells of the heart, only DPI is expressed where it associates with the intermediate filament component desmin [147]. DPI is also exclusively found in the meningeal cells and follicular dendritic cells where it associates with the intermediate filament component vimentin [148]. DPI mediates endothelial cell to cell adhesion by interacting with VE-cadherin, PG, p0071 and vimentin. These types of junctions are called complexus adhaerentes [33, 149-151].
The importance of DP in vivo was demonstrated by performing genetic knockout of the DP gene in mice (Table 1.4). The DP-null embryos do not survive for more than embryonic day 6.5 (E6.5). In these mice there is a significant loss of desmosomes and the structure of the desmosomes were also impaired. It was also demonstrated that in the DP-null embryos, keratin 8 (K8) / keratin 18 (K18) were not able to form the characteristic filamentous meshwork [152]. A similar phenotype was observed in mouse having epidermal specific knockout of the DP gene. In this case, the desmosomes lacked attachment to IFs and were susceptible to mechanical stress induced disruption [153]. Thus DP is an important desmosomal component that is required to maintain tissue integrity.

DP is important for maintaining tissue integrity because it is a cytolinker protein which joins the desmosome to the intermediate filaments (IFs) and this allows desmosomes to withstand mechanical stress (reviewed in [154]). It had been demonstrated that the C terminal domain of DP is required for association of DP with IF proteins like keratin and vimentin. Upon expression of the C terminal domain of DP (DP CT) in cultured cells like COS-7 and NIH3T3 cells, it was observed that the DP CT co-localizes with IFs. Desmosomes in cells having DP CT expression did not show filamentous meshwork of IFs, instead they were disrupted. It was postulated that when the expression of DP CT was high, it led to disruption of IFs [155]. Unlike DP CT, an N terminus deleted mutant of DP (DPΔN) was able to form the filamentous meshwork of IFs [155]. The interaction of IFs with the C terminal domain of DP was also found to be important for determining the spatial distribution of DP in to desmosomes, because expression of an N terminal fragment of DP (DP NTP) in cells led to DP NTP forming complexes with both desmosomal proteins and adherens junctions proteins [156]. Thus the C terminal domain of DP
is essential for both IF binding and to maintain separation of the spatial distribution of desmosomal and adherens junction proteins.

Figure 1.8. Expression of DP in the different layers of the stratified epithelium. DP expression is highest in the granular layer of the epidermis and decreases gradually in the lower layers.

DP binds to both keratin and vimentin IFs but their binding sites in DP protein were found to be different. The last 68 amino acids (which does not include the A, B and C domains) at the C terminus of DP was essential and sufficient for interaction with keratins, but for the interaction with vimentin, the C terminal plakin repeat domains A, B, C and the rod domain of DP was needed [144].
INTRODUCTION

<table>
<thead>
<tr>
<th>Gene encoding</th>
<th>Type of knockout</th>
<th>Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP</td>
<td>Global knockout</td>
<td>Embryos die at E6.5. Display abnormalities in desmosomal assembly and stability. Cells show a reduction in cell proliferation [152].</td>
</tr>
<tr>
<td>DP</td>
<td>Conditional knockout under the control of K14 promoter</td>
<td>Embryos show epidermal separation upon mechanical stresses and is most prominent in the basal layer. Desmosomes lack attachment to intermediate filaments [153].</td>
</tr>
</tbody>
</table>

Table 1.4. Phenotypes of mice having knockout of DP. The table represents the phenotypes associated with global knockout or tissue specific knockout of DP.

<table>
<thead>
<tr>
<th>Component</th>
<th>Disease name (cause of disease)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP</td>
<td>ARVC/D (Missense mutation) [157]</td>
</tr>
<tr>
<td></td>
<td>Wooly hair syndrome with or without cardiomyopathy (C terminal truncation) [158]</td>
</tr>
<tr>
<td></td>
<td>SPPK (Haplo insufficiency, Nonsense mutations) [159, 160]</td>
</tr>
<tr>
<td></td>
<td>Lethal acantholytic epidermolysis bullosa (C terminal truncation/ nonsense mutation) [161]</td>
</tr>
<tr>
<td></td>
<td>Carvajal syndrome (C terminal truncation) [162]</td>
</tr>
<tr>
<td></td>
<td>Paraneoplastic pemphigus (Auto-antibodies) [163]</td>
</tr>
</tbody>
</table>

Table 1.5. Diseases associated with DP. The table represents the diseases associated with DP and the experimentally validated causes of the disease.

The N terminal domain of DP interacts with the head domains of both plakophilin 1 (PKP1) [141, 164] and plakoglobin (PG) [142] and these interactions are important for localization of DP to desmosomes at the cell border [144]. High DP NTP expression was found to disrupt endogenous DP localization at the cell to cell border [156].

DP is an essential component of both epidermal and myocardial desmosomes (reviewed in [154]). Thus, loss of DP or mutations that cause loss of function of DP leads to many diseases.
INTRODUCTION

(Table 1.5). Genetic haploinsufficiency of DP leads to SPKK [159]. Since C terminal of DP is essential for binding of desmosomes to IFs, truncation of the C terminal domain of DP lead to many diseases like the wooly hair syndrome with or without cardiomyopathy [158], lethal acantholytic epidermolysis bullosa [161] and Carvajal syndrome (a syndrome in which patients suffer from dilated cardiomyopathy, SPKK and wooly hair syndrome) ) [162]. A missense mutation at the N terminal of DP causes the heart disease ARVC/D [157] while a heterozygous nonsense mutation in DP caused SPKK and was found to be associated with abnormal epidermal differentiation and alteration in keratin filament organization [160]. Auto-antibodies targeting DP has been known to cause diseases like the paraneoplastic pemphigus vulgaris [163].

<table>
<thead>
<tr>
<th>Desmosomal component</th>
<th>Types of cancers associated with:</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP</td>
<td>Increased expression</td>
</tr>
<tr>
<td></td>
<td>Decreased expression</td>
</tr>
<tr>
<td></td>
<td>LOH</td>
</tr>
<tr>
<td>DP</td>
<td>Uterine adenocarcinoma [165], OSCC [166], breast cancer [167], lung cancer [168]</td>
</tr>
</tbody>
</table>

Table 1.6. Cancers caused by alterations in DP. The table represents the type of cancers associated with alterations in expression of DP.

Since DP expression is obligate components of the desmosomes. Decreased expression of DP leads to loss of desmosome number and impairment of desmosomal structure (a characteristic feature of many cancers). Thus DP loss is associated with neoplastic progression in multiple cancers (Table 1.6) like uterine adenocarcinoma [165], oral squamous cell carcinoma OSCC [166], breast cancer [167] and lung cancer [168]. One of the mechanisms by which loss of DP is known to cause neoplastic progression is by activation of the Wnt/β catenin signaling [168]. In the non-small cell lung carcinoma cell line, NSCLC, it was demonstrated that DP loss leads to activation of the Wnt/β catenin signaling which resulted in increased TCF/LEF transcriptional
activity and increased expression of Wnt target genes MMP14 and plakoglobin. As a consequence of this signaling the DP knockdown clones were demonstrated to have increased cell proliferation, migration and decreased sensitivity to drug induced apoptosis [168]. In the skin keratinocyte cell line, HaCaT, loss of DP was also shown to increase cell proliferation but by increasing activation of ERK and AKT signaling pathway. [169]. Thus different signaling pathways are activated in different cell types upon loss of DP leading to increased cell proliferation, migration and increased neoplastic progression.

1.2.4. The Armadillo repeat proteins

The founding member of the Armadillo protein family is named because embryos hemizygous for the armadillo gene resembled the placental mammal, armadillo [170, 171]. β catenin (a component of the adherens junction) is known to be the human homologue of the Armadillo protein. Its amino acid sequence is 71% identical to the Drosophila Armadillo protein [172]. The armadillo repeat proteins contain multiple repeats of armadillo domains. These armadillo domains are made up of similar but non-identical sequences of 42 amino acids. Each repeat forms three alpha helices and these alpha helices are coiled together to form a superhelix (Figure 1.9). Non-helical insert sequences can bend these super helices to different degrees to form negatively or positively charged grooves which form binding sites for other proteins [173]. Two members of the armadillo repeat family namely the plakoglobin (PG) and the plakophilins (PKPs) are important constituents of the desmosome (reviewed in [51]).
1.2.4.1. Plakoglobin (PG):

PG, also known as γ catenin, is an important component of the desmosomal junctions and is also found in adherens junctions [174]. In the adherens junction, PG interacts with α catenin, E-cadherin, N cadherin and P cadherin [175-178] while in desmosomes, PG binds to desmogleins and desmocollins, plakophilins and desmoplakin. [142, 179-181]. The PG protein has 65% similarity to β catenin at the amino acid level [182]. It has a central arm repeat domain (13 repeats) and are flanked by long N terminal and C terminal domains (Figure 1.10) [183]. The arm domain forms a positively charged groove which serves as binding sites for desmosomal cadherins, adherens junction cadherins, members of the Wnt/β catenin signaling pathway like Adenomatous polyposis coli (APC) [184, 185], transcription factors like T cell factor (TCF) and Lymphoid enhancer binding factor (LEF) [186].

PG is found in all cell and tissue types including the skin epidermis and the myocardium. In the stratified epithelium, expression of PG increases as cells move from the basal layer to the granular layer (Figure 1.11) [187]. The importance of PG in the skin epidermis and myocardium can be understood upon examining the phenotypes of the PG knockout mice made in C57B/L6 strain of mice (Table 1.7). PG-null embryos die sometime in between E10.5 till before birth [188]. Most embryos die due to heart defects at about E10.5, but some survive and after E17.5 (the time when the adult pattern of epidermal differentiation starts), these embryos have very fragile skin and show severe skin blistering phenotypes.
**Figure 1.9. Secondary structure of a typical armadillo protein, β catenin.** The secondary structure of β catenin consists of 12 repeats of three alpha helices which coils together to form a superhelix [173].

**Figure 1.10. Structural domains of PG.** PG is made up of armadillo repeats flanked by a head and a tail domain at N and C terminus respectively [51].

Like acantholysis and apoptosis of cells of the granular layer and loss of the stratum corneum [189]. In vivo cytochemical and immuno-histochemical studies of the skin of PG-/- mice [189] and in vitro studies by culturing the PG-/- skin keratinocytes [190] have demonstrated that although PG-/- keratinocytes form cadherin clusters due to a compensatory effect of β catenin binding to the desmosomal cadherins, they lack effective binding to intermediate filaments and are more susceptible to cell rupture than those of PG+/+ cells. The reason behind this effect was found to be decreased localization of plakophilin1 (PKP1) and DP to the desmosomes. Thus,
The presence of PG in the desmosome is essential to withstand normal mechanical stresses generated during stratification of epidermis because PG regulates intermediate filament binding to the desmosome via DP and PKP1 [189].

PG is also important for initiating desmosome assembly and function in skin keratinocytes [188-190]. It has been demonstrated that PG binding to E-cadherin or P cadherin in the adherens junction is essential for initiation of desmosome formation [191]. In the myocardium, PG has been shown to regulate cell differentiation by regulating the Wnt signaling pathway. It has been demonstrated that nuclear plakoglobin can interact with the transcription factor, c-kit, in the cardiac progenitor cells (CPCs) to inhibit the canonical Wnt signaling pathway and activate a pro-adipogenic gene expression. This causes CPCs to differentiate into adipocytes leading to ARVC/D [192]. Since PG is an important component of the desmosome in both the epidermis and myocardium, mutations that cause loss of function of PG lead to many skin and heart diseases (Table 1.8). A C terminal truncation mutation in PG gene has been documented to cause Naxos disease (a cardiocutaneous disease consisting of the heart disease ARVC/D and cutaneous abnormalities like the palmoplantar keratoderma (PPK) and wooly hair) [193]. A novel dominant mutation involving addition of one Serine at amino acid position 39 was found to cause only ARVC/D without cutaneous abnormalities [194]. Nonsense mutations in the PG gene has been known to cause diseases like cardiomyopathy with PPK and Alopecia totalis (total baldness) [195] and also lethal congenital epidermolysis bullosa [196]. Auto-antibodies against PG are known to cause the skin blistering pemphigus vulgaris [197, 198].
Figure 1.11. Expression of PG in the different layers of the stratified epithelium. An expression gradient of PG exists in the epidermis where PG expression increases in the upper layers of the epidermis.

PG is a tumor suppressor (reviewed in [199]). PG expression at the mRNA/protein level decreases in thyroid cancer [200], bladder cancer [201, 202], neuroblastoma [203], lung cancer [204], oral cancers [205] and renal cancer [206]. Decrease in PG expression can also occur due to LOH like in the cases of prostate cancer [207], ovarian cancer and breast cancer [208] (Table 1.9). Some of the tumor suppressor activities of PG have been correlated to its role in competing with β-catenin for binding to the TCF/LEF transcription factors ([186] and reviewed in [199]). It has been demonstrated that in NSCLC cell line (where PG levels are very low or absent), exogenous expression of PG decreased the β-catenin/TCF signaling and consequently decreased the anchorage independent growth of the NSCLC clones [209]. Thus, in addition to regulating tissue integrity, PG also regulates the Wnt/β-catenin signaling pathway and acts as a tumor suppressor in multiple cancers.
Gene encoding armadillo repeat protein | Type of knockout | Phenotypes
--- | --- | ---
PG | Global knockout | Embryos die between E10.5 and birth. Develop severe heart defects, skin blistering and sub-corneal acantholysis. The embryos also show defects in desmosome number and morphology [188].
PKP2 | Global knockout | Embryos die at E11 due to altered heart morphogenesis [210].
PKP3 | Global knockout | Normal at birth. Hair coat pelage occurrence was delayed and the resulting hair follicles were morphologically abnormal. In the stages P8 to P10, PKP3 null mice showed increased cell proliferation of basal keratinocytes, thus leading to increased thickness of the suprabasal layers of the epidermis. But, in epidermis of adult PKP3 null mice, no such changes were observed. The number of desmosomes in the basal layers of the epidermis was decreased to half, while in the suprabasal cells, desmosome numbers were unaltered. PKP3 null mice also had increased susceptibility to cutaneous inflammation and showed phenotype similar to atopic dermatitis [211].

Table 1.7. Phenotypes of mice having knockout of individual armadillo repeat protein encoding genes. The table represents the phenotypes associated with global knockout or tissue specific knockout of different armadillo repeat protein encoding genes in mice models.

<table>
<thead>
<tr>
<th>Desmosomal component</th>
<th>Disease name (cause of disease)</th>
</tr>
</thead>
</table>
| PG | Naxos disease (C terminal truncation mutation) [193]  
ARVC/D without cutaneous abnormalities (Addition of one Serine at amino acid position 39) [194]  
Cardiomyopathy with PPK and Alopecia totalis (Nonsense mutation) [195]  
Lethal congenital epidermolysis bullosa (Nonsense mutation) [196]  
Pemphigus vulgaris (Auto-antibodies) [197, 198] |
Table 1.8. Diseases associated with desmosomal proteins. The table represents the diseases associated with desmosomal alterations and the experimentally validated causes of the disease

1.2.4.2. Plakophilins (PKPs):

PKPs belong to the p120 catenin (ctn) family of armadillo proteins. The p120ctn is further classified into two classes: the plakophilins and the p120ctn related proteins [216, 217]. Three isoforms of plakophilins exist, namely PKP1 (earlier known as the “band 6” protein), PKP2 and PKP3 [218-220]. A protein called p0071 is sometimes classified into the PKP sub-family and is called PKP4 although p0071 is more related to the p120ctn related proteins (Figure 1.9) [221]. PKPs have a central nine armadillo repeats flanked by a long N terminal and a very short C terminal domains. Between the 5th and the 6th arm repeat PKPs have a polypeptide sequence which causes a kink in the protein structure [222]. The N terminal domain of the PKP isoforms is majorly non-conserved except for a highly conserved small region near the amino terminus called the homologous region 2 (HR2) (Figure 1.9).
Desmosomal component | Types of cancers associated with: | Increased expression | Decreased expression | LOH
--- | --- | --- | --- | ---
PG | Thyroid cancer [200], bladder cancer [201, 202], neuroblastoma [203], lung cancer [204], Oral cancers [205], renal cancer [206] | | Prostate cancer [207], ovarian and breast cancer [208] |
PKP2 | Breast cancer [228], lung cancer [229], prostate cancer [224] | | Oropharyngeal SCC [230], colon cancer [231-233], gastric cancer [227], bladder cancer [225] |
PKP3

Table 1.9. Cancers caused by alterations in armadillo repeat proteins. The table represents the type of cancers associated with alterations in expression of armadillo repeat proteins.

1.2.4.2.1. PKP1

Two splice variants of PKP1 are formed due to alternative splicing of the respective gene transcripts (Figure 1.12), the shorter form called “a” variant and the longer form called variant “b”. The PKP1“b” variant has an addition of 22 amino acids. PKP1b localizes exclusively to the nucleus while PKP1a has been found to localize to both the desmosomes and nucleus [234].PKP1 is expressed only in the stratified and complex epithelia [219, 235]. In the epidermis, PKP1 expression increases from the basal to the granular layer [235] (Figure 1.13).

PKP1 exhibits dual localization in cell cultures, it can either be desmosomal or nuclear [234]. The in-vivo role of PKP1 has not been deciphered because no knockout mice models of PKP1 are available. But in vitro, PKP1 plays an important role in desmosomal assembly. While the C
terminus (686-726 amino acids) of PKP1 is required for its own localization to the cell-cell border, the N terminal domain of PKP1(1-235 amino acids) binds to multiple desmosomal proteins like DSG1, DSC1, DP and keratins and increases recruitment of these proteins to the desmosome in cultured keratinocytes [164, 236, 237].

PKP1, at the cell border, also regulates actin cytoskeleton re-organization. The armadillo domain of PKP1 (235-686 amino acids) has been demonstrated to indirectly bind to actin filaments and induce the formation of lamellipodia and filopodia of skin keratinocyte cells [237].

PKP1 also localizes to the nucleus (except the nucleolus) in both epithelial and non-epithelial cell lines [234]. Localization of PKP1 protein to the nucleus is regulated by two nuclear localization signals present in its N terminal and armadillo domains. Using the human squamous carcinoma derived A431 cell line, it was demonstrated that PKP1 binds to chromatin and single stranded DNA. It was also observed that upon treatment with DNA damaging agents like etoposide, the nuclear PKP1 partially redistributed to the nucleolus. In the same cell line, PKP1 knockdown resulted in increased cell survival in response to DNA damage [238]. Thus although the nuclear function of PKP1 was not established directly, it was postulated that it may be important in regulating cell survival after DNA damage.
INTRODUCTION

Figure 1.12. Structural domains of PKPs and alternative splice variants of PKP1 and PKP2. All PKPs have armadillo repeat domains flanked by N and C terminal domains but the PKP subfamily proteins have a distinctive kink due to the presence of a polypeptide sequence in between their armadillo repeat domain 5 and 6. The homology region 2 (HR2) is a highly conserved region in the N terminal domain of all the PKPs. Spliced variants PKP1b and PKP2b are formed by alternative splicing of the gene transcripts involving insertion of 21 amino acids between exons 3 and 4 in the PKP1 protein and 44 amino acids between exons 2 and 3 in the PKP2 protein (adapted from [51]).
Figure 1.13. Expression of PKP isoforms in the different layers of the stratified epithelium.

Different expression gradients of PKP isoforms exist in the epidermis.

PKP1 and PKP3, but not PKP2, has also been found to localize to stress granules (sites for stalled mRNA-protein complexes). It was postulated that PKP1 may regulate mRNA translation [239]. Later, PKP1 was also shown to increase eIF4A dependent translation by binding to the eukaryotic initiation factor (eIF4A1) and promote the adenosine triphosphate (ATP) activity of eIF4A1. PKP1 was also shown to increase eIF4A dependent translation [240]. Thus, PKP1 is not just an important component of the desmosome, it also regulates desmosomal assembly, actin cytoskeleton organization, cell survival and eIF4A dependent translation.

Since PKP1 regulates multiple processes in the epidermis, loss of function mutations like frameshift mutations, nonsense mutations or compound heterozygosity of the PKP1 gene, cause the disease called skin fragility ectodermal dysplasia syndrome characterized by thickening of skin on the palms and soles, abnormal nails and fragile, blistering skin [212, 213] (Table 1.8). Loss of PKP1 has also been reported in multiple cancers (Table 1.9) like the oropharyngeal SCC,
colon and pancreatic adenocarcinoma [223] and prostate cancer [224]. Thus PKP1 acts as a tumor suppressor just like PG.

1.2.4.2.2. **PKP2**

The PKP2 gene transcript undergoes alternative splicing to produce two splice variants (Figure 1.12), the shorter form called “a” variant and the longer form called variant “b”. The “b” variant for PKP2 has addition of 44 amino acids in between arm repeats 3 and 4. Both PKP2a and 2b variants show dual localization, they localize to the nucleus and desmosomes [218].

PKP2 is expressed in all simple, complex and stratified epithelium and is also expressed in some non-epithelial tissues like cardiomyocytes (of the heart) and follicle cells of the lymph node [218, 241]. In the epidermis, PKP2 expression decreases as keratinocytes differentiate and move from the basal layer to the spinous layer [241] (Figure 1.13). PKP2, like PKP1 localizes to the both the desmosomes and nucleus (except the nucleolus) of all cell lines derived from stratified or simple epithelium, while in non-epithelial derived cell lines that lack desmosomes like SV80 fibroblasts, PKP2 localizes only to the nucleus [218].

PKP2 is the only plakophilin isoform expressed in the heart, so upon loss of PKP2, no other plakophilin can compensate its function. The importance of PKP2 in the heart was understood while trying to generate the PKP2 knockout mice. No transgenic mice were generated. It was observed that the PKP2 null embryos died at E11 due to altered heart morphogenesis [210] (Table 1.7). Analysis of the developing heart of the PKP2 null mice showed altered cytoskeletal organization, ruptures of the cardiac walls, and blood leakage into the pericardiac cavity. It was also found that DP of the embryonic myocardium did not localize to the cell border but formed granular aggregates in the cytoplasm. DP was also found to co-localize with adherens junction
proteins, thus making it difficult to distinguish the adherens junction and the desmosomal junction. Surprisingly, the ultra structure of desmosomes of the epidermis and the stomach mucosa were found to be unaltered. Therefore, it was demonstrated that PKP2 is essential for cardiac junction formation and also for recruitment of DP to the desmosomes [210]. Consequently frameshift, missense or nonsense mutations in the PKP2 gene lead to the heart disease ARVC/D [214] (Table 1.8).

PKP2 has been shown to play a very important role in the formation of the desmosome by recruiting DP to the cell border. PKP2 performs this function by interacting with protein kinase Cα (PKC-α). PKP2 binds to PKC-α and recruits it to the cytoplasmic pool of DP. PKC-α phosphorylates DP at Ser 2849 and this phosphorylation causes DP to interact with intermediate filaments and initiate nascent desmosome formation. Since PKP2 recruits PKC-α to the cytoplasmic complexes consisting of DP and other desmosomal proteins, it prevents PKCα to interact with other substrates. In absence of PKP2, PKC-α was shown to phosphorylate its downstream targets more effectively. [242]. Thus, although PKP2 positively regulates desmosome assembly by regulating PKCα activity, it negatively regulates the global PKCα activity.

PKP2 localizes to the nucleus and it was demonstrated that Cdc25C associated kinase 1 (C-TAK1) and the 14-3-3 family proteins regulate the nuclear localization of PKP2. C-TAK1 phosphorylates PKP2 at serine 82 residue, thus forming a 14-3-3 binding site. Binding of 14-3-3 proteins sequester PKP2 to the cytoplasm and prevent its nuclear localization. The mechanism regulating the upstream C-TAK1 activation is not known [243].
INTRODUCTION

The functions of nuclear PKP2 are unclear but it has been demonstrated that PKP2 associates with the DNA directed RNA polymerase III subunit C155 (RPC155), which is the largest subunit of the RNA polymerase III holoenzyme. PKP2 was also found to be associated with other subunits of the RNA polymerase III, RPC39, RPC82 and transcription factor IIIB (TFIIIB) but not TFIIC. Due to the absence of TFIIC, the PKP2-RPC155 complexes were postulated to be inactive RNA polymerase complexes [244]. Another important function of nuclear PKP2 is its regulation of the WNT signaling pathway. PKP2 can bind to β catenin (a central member of the canonical WNT signaling pathway) and inhibit the association of β catenin with E-cadherin (of the adherens junction). This allows more β catenin to move into the nucleus and activate the transcription factors TCF and LEF.

PKP2 plays a dual role in cancer (Table 1.9). In the case of bladder cancer [225] and breast cancer [226], increase in PKP2 is associated with increased neoplastic progression, while in case of colon cancer and gastric cancer [227], PKP2 functions as a tumor suppressor. One of the mechanisms by which PKP2 regulates breast cancer progression is by associating with EGFR and activating EGFR in a ligand dependent and ligand independent manner which in turn promotes tumor formation in breast cancer [226]. Thus, PKP2 functions not just as a desmosomal protein but regulates multiple signaling pathways like WNT/β catenin, PKCα and the EGFR signaling pathways.

1.2.4.2.3. PKP3

Alternative splice variants of PKP3 have recently been reported. In the case of PKP3, the known variant is called PKP3a while the new variant is called PKP3b (Figure 1.14). The PKP3b variant utilizes a new 1st exon upstream of the known gene. This new exon contains a functional
translation start site which is used during translation of PKP3b protein. PKP3a is expressed in most epithelial cells while PKP3b variant is expressed predominantly in stratified epithelial cells and absent or heterogeneous in simple epithelial cells [245].

PKP3 is ubiquitously expressed in most simple and stratified epithelial tissues with the exception of hepatocytes [220]. PKP3 is also expressed uniformly in all the layers of the epidermis [220] (Figure 1.13). PKP3 binds to multiple proteins like all isoforms of DSGs and DSCs. It is the first protein found to bind to the “b” splice variant of all DSC isoforms. It also binds to PG, DP [246] and cytokeratin 18 (K18) [232, 246].

Figure 1.14. Alternative splice variants of PKP3. The alternate spliced variant of PKP3 mRNA is generated by introduction of a new exon at the N terminal (exon 1b). This leads to the splice variant protein having an alternate N terminal amino acid sequence [245]

PKP3, along with PG and E-cadherin play a crucial role in the initiation of desmosome formation. To analyze the process of initiation of desmosomes in cell cultures, calcium switch assays were performed. This assay is used to analyze cell junction disassembly and reassembly. It is based on the concept that desmosomes and adherens junctions are calcium dependent junctions and decreasing calcium concentrations (low calcium) or EDTA treatment to cells (for chelating calcium) from the culture media may lead to disruption or internalization of desmosomes. Thereafter, adding media having normal calcium concentrations lead to formation
of nascent desmosomes [247, 248]. Calcium switch assays were performed in PKP3 knockdown clones derived from the skin keratinocyte cell line, HaCaT and the colon carcinoma cell line HCT116, along with their respective vector control clones. It was observed that PKP3, PG and E-cadherin stayed at the cell border even after 16 to 18 hours incubation in low calcium conditions, while other desmosomal proteins like DSC2/3, DP and PKP2 disassembled from the desmosome both in the vector control and PKP3 knockdown clones. Upon addition of normal calcium, DSC2/3, DP and PKP2 showed a time dependent increase in localization to the cell border in the vector control clones but in the PKP3 knockdown clones, these proteins did not localize to the cell border. The cell border localization of PKP3, PG and E-cadherin were found to increase with time after addition of normal calcium medium in the vector control and PKP3 knockdown clones. Thus PKP3, PG and E-cadherin were proposed to be involved in initiation of desmosome formation and presence of PKP3 was demonstrated to be essential for desmosome formation. It was observed that in the PKP3 knockdown clones, PG and E-cadherin localization at the cell border was not hampered but localization of DSC2/3, DP and PKP2 were altered. Thereafter PG knockdown clones derived from HCT116 were generated. In the PG knockdown cells it was observed that cell border localization of PKP3 was decreased. Localization of DSC2/3, DP and PKP2 were also decreased but E-cadherin localization was unaltered. To analyze the role of E-cadherin in desmosome formation, three HCT116 derived E-cadherin knockdown clones were generated. In these E-cadherin knockdown clones, cell border localization of PKP3, DP and PKP2 were decreased but localization of PG remained unaltered. It was proposed that since PG can bind to both E-cadherin and P cadherin [249], E-cadherin loss may allow PG to bind to P cadherin and thus stay at the cell border. It was also shown that PKP3 co-localizes with both PG and E-cadherin at the cell border. All these experiments proved that
both PG and E-cadherin recruits PKP3 to the cell border and PKP3 is essential for initiation of desmosome formation because only in the presence of PKP3, the other desmosomal proteins like DP, DSC2/3 and PKP2 are recruited to the cell border [250].

Recently, PKP3 has been shown to collaborate with PKP2 for desmosome formation in the squamous cell carcinoma 9 (SCC9) and HaCaT cell lines. It has been demonstrated that while PKP3 assembles the cytoplasmic population of DP bound desmosomal proteins (precursors for desmosome formation); PKP2 is required to transfer these precursors to the membrane. One of the mechanisms by which PKP3 promotes desmosome assembly is by physically binding to and activating the Rap1 GTPase protein. A calcium switch assay was performed to analyze the activation of Rap1GTPase at different time intervals after addition of normal calcium to the medium. Rap1 GTPase activity was found to increase with time in the vector control clones as reported earlier [251], but it did not increase in the SCC9 and HaCaT derived PKP3 knockdown clones. Thus, it was demonstrated that PKP3 is required for activation of Rap1GTPase and expression of wild type Rap1GTPase in the PKP3 knockdown clones were able to efficiently localize DP to the cell border. PKP3 was also shown to govern E-cadherin maturation. It was demonstrated that the Rap1/PKP3 complex is required for formation of the Rap1/E-cadherin complex which in turn is required for adherens junction formation because upon PKP3 loss, Rap1GTPase was not able to bind to E-cadherin. To analyze if inability to bind Rap1GTPase would alter E-cadherin mediated adherens junction formation, it was observed that in the PKP3 knockdown clones, the adherens junctions formed were highly disorganized and even the cell border localization of E-cadherin was decreased as compared to the vector control clones [252]. Some of these observations are contradictory to [250]. Upon PKP3 loss, E-cadherin cell border localization was not altered in [250] but in [252], it is decreased. Thus, even though there are
some contradictions between [250] and [252], both these articles demonstrate the importance of PKP3 in desmosome formation.

Since PKP3 is very important in the formation and maintenance of desmosomal structure, loss of PKP3 was expected to decrease the ability of cells to form desmosomes. This was exactly what was observed in PKP3 knockout mice (Table 1.7). There was a decrease of approximately half of the lateral desmosomes in between the basal cells of the epidermis of PKP3-null mice [211], but desmosome numbers in the suprabasal cells were unaltered. It was demonstrated that the phenotypes of the PKP3 null mice were not as severe as those of PKP2 or PG null mice because in the PKP3 null mice, expression of PKP2 was found to increase and it was hypothesized that PKP2 may partly compensate for the loss of PKP3. The PKP3 null mice were normal at birth, but started showing abnormalities in the later stages. In these mice, hair coat pelage occurrence was delayed and the resulting hair follicles were morphologically abnormal. In the stages between postnatal day 8 (P8) to P10, PKP3 null mice showed increased cell proliferation of basal keratinocytes, thus leading to increased thickness of the suprabasal layers of the epidermis. This was ascertained to increased cell proliferation due to increased β-catenin signaling in the basal keratinocytes of PKP3 null mice. Unexpectedly, in the epidermis of adult PKP3 null mice, no such changes were observed. PKP3 null mice also showed increased susceptibility to cutaneous inflammation. PKP3 null mice kept in specific pathogen free environment showed less skin alterations but when kept in the conventional facilities (consisting ectoparasites), the PKP3 knockout mice developed inflammatory responses much stronger than the wild type mice [211]. Thus it was demonstrated that PKP3 is important for desmosome formation, maintaining normal hair coat structure and for preventing skin inflammation.
Supporting the role of PKP3 in prevention of inflammation, auto-antibodies that disrupt PKP3 at the cell border, causes a precancerous skin blistering disease called Paraneoplastic pemphigus [215] (Table 1.8) which is similar to the skin blistering disease PV. Interestingly, it has been recently demonstrated that PV antibodies induce Src dependent tyrosine phosphorylation of PKP3. This phosphorylation causes PKP3 detachment from the desmosome because of loss of binding to DSG3 [253]. A similar tyrosine phosphorylation of PKP3 by Src which can cause detachment of PKP3 from the desmosome has also been reported in cells subjected to oxidative stress and the tyrosine residue was found to be at position 195 [254]. A detailed understanding of the mechanisms and effects of Src mediated PKP3 phosphorylation during PV could probably help in devising ways to treat paraneoplastic pemphigus. One of the mechanisms by which Src regulates PV is by activating the downstream p38MAPK pathway. The Src/p38MAPK pathway can be an efficient target against PV because inhibition of Src has been demonstrated to block PV disease in vivo via inhibition of p38MAPK pathway [255, 256].

PKP3 loss is associated with multiple forms of cancer like oropharyngeal SCC [230], colon cancer [231-233], gastric cancer [227], bladder cancer [225]. Surprisingly, increase in PKP3 has also known to be associated with cancers like breast cancer [228], lung cancer [229], prostate cancer [224] (Table 1.9). To analyze the effect of PKP3 in colon cancer tumorigenesis, PKP3 knockdown clones were generated in the simple epithelial cell line (HCT116) and two stratified epithelial cell lines (HaCaT and FBM). It was demonstrated that PKP3 loss leads to decreased cell-cell adhesion, increased anchorage independent growth and increased in-vitro cell migration. The HCT116 and HaCaT derived PKP3 knockdown clones formed larger tumors in vivo and was shown to have a higher ability to metastasize to the lungs as compared to the vector control clones [233, 257, 258]. There were some cell type specific effects of PKP3 loss also reported. In
HaCaT, PKP3 loss increased cell growth and proliferation while in HCT116, cell growth and proliferation remained unaltered [233]. To understand the mechanisms regulating tumor formation and metastasis upon PKP3 loss, it was hypothesized that PKP3 loss may cause alterations in the normal functioning of its binding partners. Since one of the binding partners of PKP3 was cytokeratin 18 (K18) [232, 246], it was hypothesized that loss of PKP3 may regulate K18 function or stability. Since, keratins are always expressed in pairs of type I and type II keratins [259-261], and in simple epithelia K18 (type I keratin) and K8 (type II keratin) are known to form pairs [262], protein expression of K8 and K18 were analyzed in the HCT116 derived PKP3 knockdown clones and the vector control clone [232]. It was observed that both K8 and K18 protein levels were higher in the PKP3 knockdown clones than the vector control clones. Since, K8/K18 over-expression had been associated with squamous cell carcinoma progression [263-266], metastasis [267] and poor prognosis [268], it was hypothesized that increased K8 levels can regulate neoplastic progression upon PKP3 loss. To test this hypothesis, two K8 double knockdown clones (K8 knockdown clones derived from a PKP3 knockdown clone) were generated. In-vitro and in-vivo experiments were performed to compare the neoplastic potential of these double knockdown clones with the corresponding vector control clones. It was demonstrated that K8 loss causes decreased in vitro cell migration, decreased lamellopodia formation in cells. Nude mice were subcutaneously injected with the K8 double knockdown clones and its respective vector control clones and after 5 weeks, the percentage of metastasis to the lungs were analyzed. It was observed that less number of mice in K8 double knockdown group as compared to the vector controls showed greater than 30% metastasis in the lung sections of mice. Thus, it was demonstrated that elevated K8 levels were required for neoplastic transformation upon PKP3 loss. To understand the mechanism regulating increase in
K8 protein levels upon PKP3 loss, K8 mRNA levels were analyzed and it was found that K8 mRNA levels were not altered. Thus, it was hypothesized that PKP3 loss may be causing increased K8 protein stability by regulating post-translational modifications like phosphorylation or dephosphorylation. Analysis of the known phosphorylation sites of K8 [269, 270] demonstrated that K8 was dephosphorylated at Serine 473 residue in PKP3 knockdown clones. A phosphatase associated with colon cancer metastasis called Phosphatase of Regenerating Liver3 (PRL-3) had been earlier reported to increase K8 protein stability [271]. PRL-3 expression has been known to increase in colorectal cancer tissues and positively correlate with metastasis of colon cancer cells to the lung and to the liver [272-275]. PRL-3 has also been demonstrated to regulate tumor formation because in chemically induced colitis associated colon cancer model, targeted deletion of the PRL-3 encoding gene (PTP4A3) was able to decrease incidence of tumor formation [276]. Thus, since PRL-3 was known to dephosphorylate K8, it was hypothesized that PRL-3 was the phosphatase that regulates K8 protein stability upon PKP3 loss. Inhibition of PRL-3 in the PKP3 knockdown clones using a specific PRL-3 inhibitor (PRL-3i) was able to decrease K8 protein levels, K8 S473 phosphorylation and also decrease in-vitro cell migration. These experiments confirmed the above hypothesis. On analyzing PRL-3 protein and mRNA expression, it was found that PRL-3 protein levels were higher in the PKP3 knockdown clones than the vector control clones, but PRL-3 mRNA levels remain unaltered. The mechanism regulating increased PRL-3 protein levels in the PKP3 knockdown clones is not known, but it has been proposed that PKP3 may regulate translation of PRL-3 [232].

In support of the role of PKP3 regulating translation, it has been demonstrated that both PKP3 and PKP1 localize to stress granules (sites for stalled mRNA-protein complexes) and PKP3 forms complexes with RNA binding proteins like the Fragile X mental retardation autosomal...
homologue 1 (FXR1), GTPase activating protein binding protein 1 (G3BP1) and the poly A binding protein Cytoplasmic 1 (PABPC1) [239]. It has also been demonstrated that binding of PKP3 to FXR1 is mRNA independent, while binding to G3BP1, PABPC1 and another RNA binding protein NMD factor up-frameshift 1 (UPF1) is mRNA dependent. A functional role of PKP3 in regulating translation was deciphered when it was shown that by virtue of binding to the RNA binding proteins, PKP3 can regulate PKP2 mRNA stability in prostate cancer cell lines [277].

1.3. Radio-resistance of cancer cells

Radiotherapy is one of the most widely used treatment modality for cancer. It can be used as a single modality treatment or in combination with surgery and chemotherapy (Table 1.10). Although, rapid progress has been made in radiotherapy due to advanced imaging technologies, computerized treatment planning and improved radiation treatment machines, yet the outcome of radiotherapy for metastatic cancers remain very poor. (reviewed in [278]). One of the major reasons for this poor outcome is the phenomenon of local recurrence and distant metastasis. Local recurrence of cancers after irradiation occurs due to a phenomenon called long term radio-resistance. This is a property of tumor cells to withstand radiotherapy and retain the ability to proliferate (i.e. clonogenic survival) ([279] and reviewed in [280]). It has been demonstrated that there are two types of radio-resistance, intrinsic resistance and radiation induced resistance [281, 282].

1.3.1. Intrinsic radio-resistance

Intrinsic radio-resistance is a property of the inbuilt genetic makeup of cells to withstand DNA damage and repair the damaged DNA after radiation exposure [283-286]. Since irradiation (both
\(\gamma\) and X-ray irradiation) induces double stranded breaks, the double stranded DNA damage sensing proteins like DNA-PKcs, ATM and ATR (reviewed in [287]) can bind to the damaged DNA and activate cell cycle checkpoint pathways like the G1/S and G2/M checkpoints (Figure 1.15) (reviewed in [288, 289]).

Cell cycle checkpoints are non-essential regulatory pathways that prevent cell cycle progression in response to stress such as DNA damage or incomplete S-phase [290]. Ataxia telangiectasia mutated (ATM) and Ataxia telangiectasia and Rad3 related (ATR) proteins also recruit proteins belonging to the double stranded DNA damage repair (dsDDR) mechanisms namely homologous recombination (HR) and the non-homologous end joining (NHEJ) (Figure 1.16) ([291, 292] and reviewed in [293]). HR uses an identical template for repairing DNA and thus the DNA repair is error free. HR repair is active only in late S and G2 phases of the cell cycle of mammalian cells because of the availability of sister chromatids at these stages (reviewed in [294, 295]). The NHEJ repair does not use any homologous template and is therefore error prone. NHEJ repair remains active throughout the cell cycle but is majorly important in the G1 phase of the cell cycle when no sister chromatids are available for HR to work (reviewed in [295, 296]). Thus, a coordination of the cell cycle checkpoint pathways along with the HR and NHEJ pathways ensure dsDDR (reviewed in [297]). For cancer cells to be intrinsically radio-resistant, DNA damage needs to be either repaired or tolerated before entering the M phase of the cell cycle; because in the M phase, cells have compact chromatin and in this stage, cells are more prone to DNA double stranded breaks [298].

It has been demonstrated that intrinsically radio-resistant cells show higher efficiency in DNA double stranded break repair [299]. Glioblastoma Multiforme (GBM) tumors are known to be highly radio-resistant tumors (reviewed in [300]). One of the reasons which cause radio-
resistance in GBM tumors is the presence of Glioma initiating cells (GICs). GICs have been demonstrated to have a highly efficient HR mechanism of DNA repair and an abnormal cell cycle progression due to presence of low levels of p53 protein when

<table>
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<th>Early cancers curable with radiation therapy alone</th>
<th>Cancers curable with radiation therapy in combination with other modalities</th>
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<tr>
<td>Skin cancers (squamous and basal cell)</td>
<td>Breast carcinomas</td>
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<tr>
<td>Prostate carcinomas</td>
<td>Anal and rectal carcinoma</td>
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<tr>
<td>Lung carcinomas</td>
<td>Advanced cervical carcinoma</td>
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<td>Cervix carcinomas</td>
<td>Bladder carcinoma</td>
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<tr>
<td>Lymphomas (Hodgkin’s and low grade Non-Hodgkin’s)</td>
<td>Endometrial carcinoma</td>
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<td>Head and neck carcinomas</td>
<td>Tumors of the central nervous system</td>
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<td>Colorectal Cancer</td>
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<td>Pediatric tumors</td>
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Table 1.10. Some cancers can be cured with only radiation if detected early while others require combination of other modalities. The table represents cancers curable with radiation therapy alone and those requiring other modalities like surgery or chemo-radiotherapy [278, 301] compared with neural progenitor cells but efficiency of the NHEJ pathway remains unaltered [302] Another glioma stem cell type characterized by Cluster of differentiation 133 (CD133) positivity known to be highly tumorigenic and radio-resistant as compared to the CD133- cells, was demonstrated to have a high basal activity of DNA repair signaling including presence of activated ATM, Checkpoint kinase 1 (Chk1) and Checkpoint kinase 2 (Chk2) proteins. Treatment with Chk1/Chk2 inhibitors was able to decrease the radio-resistance of the CD133
positive glioma stem cells [303]. Similar to GBMs, it has been demonstrated that efficiency of DNA repair by HR is significantly elevated in breast cancer cells as compared to the normal mammary cells [304]. Thus, abnormal cell cycle checkpoints and increased efficiency of the HR pathway regulate intrinsic radio-resistance of tumor cells.

**Figure 1.15. The cell cycle, the cell cycle checkpoints and the contribution of the HR and NHEJ pathways in the cell cycle.** There are four phases in the cell cycle namely G1, S, G2 and M phase [305]. There are two checkpoints: the G1/S checkpoint checks DNA integrity before the DNA enters replication while the G2/M checkpoint checks DNA integrity before the DNA enters the M phase. The NHEJ and the HR pathways are the main double stranded DNA repair mechanisms present in mammalian cells. The NHEJ (dark grey color) predominates in the G1 to early S phase while both NHEJ and HR (light grey color) contribute to repair DNA damage during the late G2 phase (adapted from [305]).
The NHEJ repair pathway utilizes DNA damage sensors complexes like the Ku70-Ku80 protein complex. This complex is loaded onto the broken ends and this signals recruitment of the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) and p53-binding protein 1 (53BP1). Ataxia telangiectasia mutated (ATM)-mediated phosphorylation of histone H2A.X (γH2A.X) and the recruitment of 53BP1 protects the broken DNA ends from being cleaved. The DNA-PKcs regulates the stability of DSB ends through phosphorylation of Artemis and other substrates. Artemis in turn facilitates the end processing and, subsequently, DNA ligase 4 (LIG4), X-ray repair cross-complementing protein 4 (XRCC4) and XRCC4-like factor (XLF) ligate the broken ends to complete repair. repair by the NHEJ pathway is error-prone (reviewed in [306]).
HR utilizes the sister chromatids (being identical to the DNA to be repaired) and repairs the double stranded break without any error. Double stranded breaks are recognized by MRN complex composed of proteins Mre11, Rad50 and Nbs1. Recognition of DNA double stranded break signals ATM and ATR proteins which rapidly phosphorylate multiple DNA repair factors including H2A.X, CtBP-interacting protein (CtIP), breast cancer type 1 susceptibility (BRCA1) and exonuclease 1 (EXO1). MRE11 induces endo-nucleolytic cleavage at double stranded breaks allowing the resection of a part of the 5’ end of the damaged DNA region; this is mediated by CtIP and EXO1 in the presence of BRCA1 and Bloom's syndrome helicase (BLM). In addition, $\gamma$H2A.X spreads around the damaged site, thereby stabilizing the DNA repair complex. The single-stranded DNA generated by resection is rapidly coated by replication protein A (RPA) protein and is subsequently replaced by RAD51 in the presence of BRCA2. RAD51 coated DNA invades the sister chromatid to search for a homologous DNA strand. The fidelity of this process is maintained by anti-recombinases such as the PCNA-associated recombination inhibitor (PARI). The invading strand is extended by DNA polymerase and ligates to form D-loop structures called Holiday junctions. The final product of the homologous recombination-mediated repair is then determined by the resolution of the D-loops leading to either DNA undergoing crossover or no crossover (reviewed in [306]).

Intrinsic radio-resistance can be also be caused by constitutive activation of cell signaling pathways that in turn activates the cell cycle checkpoints, HR or NHEJ signaling pathway. Some of the key cell signaling pathways that are associated with intrinsic radio-resistance are the Epidermal growth factor receptor (EGFR) [307], Insulin growth factor receptor (IGFR) [308] and the Phosphoinositide 3-phosphate (PI3K)/AKT signaling pathways which work via
activation of the three different Mitogen activated protein kinase pathways namely the extra-
cellular signal regulated kinase (ERK), p38 Mitogen activated protein kinase (p38 MAPK) and
the Janus kinase (JNK) pathways. The N terminal deleted constitutively active mutant of EGFR
has also been demonstrated to cause increase in intrinsic radio-resistance by activation of both
the MAPK pathways and the PI3K/AKT signaling pathway [309]. The hepatocyte growth factor
receptor (c-Met) signaling has been shown to increase intrinsic radio-resistance by activating the
downstream nuclear factor light chain enhancer of activated B cells (NFκB) signaling pathway
[310].

Mutations in pro-apoptotic proteins can also lead to intrinsic radioresistance. The best example is
the transcription factor p53 protein; an essential pro-apoptotic protein in mammalian cells which
mediates cell cycle checkpoint activation upon DNA damage [311]. Mutations in the p53
encoding gene, TP53, which results in loss of function of the ability of p53 to induce the
expression of the pro-apoptotic protein p21 (BAX) expression has been associated with increased
intrinsic radio-resistance in glioblastoma [312]. Signaling pathways that lead to epithelial
mesenchymal transitions (EMT) are also known to cause intrinsic radio-resistance (reviewed in
[313]). In breast cancers, HOXB9 (a transcription factor) is over-expressed and is known to
increase the transcription of TGFβ, thus inducing autocrine TGFβ induced EMT [314]. It was
demonstrated that HOXB9 induces spontaneous DNA damage, thus leading to basal activation of
DNA damage response like activation of ATM, phosphorylation of histone 2AX (H2AX) and
foci formation of p53 binding protein 1 (53BP1) in the nucleus of cells. Probably this selection
pressure kept the cells ready for dsDDR and it was demonstrated that upon γ irradiation, the
HOXB9 overexpressing cells showed hyperactivation of ATM and faster accumulation of
phospho-H2AX and 53BP1 at the DNA double stranded break. This ensured faster repair and better cell survival.

The radio-resistance of these HOXB9 overexpressing cells was shown to depend on TGFβ mediated EMT because inhibition of the TGFβ signaling pathway inhibited both EMT and dsDDR responses [315]. Thus, activation of the EMT pathway can increase intrinsic radio-resistance. Supporting this hypothesis, loss of E-cadherin mediated EMT has also been demonstrated to increase intrinsic radio-resistance [316]. In this work, it was shown that treatment of MCF7 (non transformed mammary epithelial cell line) and A549 (lung carcinoma derived cell line) cells with hypoxia, TGFβ or EGFRvIII can induce EMT and radio-resistance. In all three cases E-cadherin expression was lost and vimentin expression was increased. It was hypothesized that loss of cell to cell adhesion during EMT due to E-cadherin loss may be regulating radio-resistance. To assess this hypothesis, cells were seeded at high and low confluences, irradiated at different doses of γ irradiation and cell survival analysis was performed. It was demonstrated that at high confluences, E-cadherin showed extensive border staining and when these cells were subjected to irradiation, they were more radiosensitive while sparsely seeded cells were more radio-resistant. As controls, the E-cadherin levels were analyzed and it was shown that in the densely and sparsely seeded cells, E-cadherin levels did not change. To test whether E-cadherin restoration can decrease radio-resistance, E-cadherin was stably expressed in the highly metastatic cell line MDA-MB-231 (breast carcinoma derived cell line with very less endogenous E-cadherin expression). E-cadherin restoration caused reversion of cells from mesenchymal (shown by parental MDA-MB-231 cells) to epithelial phenotype and the E-cadherin restored clones were more radio-sensitive as compared to the mock transfected MDA-MB-231 clones [316]. Thus, cell to cell adhesion was demonstrated to be important for
regulation of regulate cell death and survival upon radiation treatment because loss of cell to cell adhesion can lead to in intrinsic radio-resistance in cancer cells.

1.3.2. Radiation induced radio-resistance

Radiation induced radio-resistance develops only after radiation exposure and involves activation of signaling pathways that induce cell survival and alterations in gene expression of anti-apoptotic and radio-protective genes in cells that has been exposed to radiation [317] (Figure 1.17). Some of the mechanisms that lead to radiation induced radio-resistance are enrichment of cancer stem cells, oxidative stress and activation of cell signaling pathways activated as a consequence of the DNA damage by irradiation (reviewed in [318-320]).

Cancer stem cells (CSCs) are defined as cells within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor (reviewed in [321]). Intrinsically cancer stem cells possess the ability of accelerated DNA repair and less production of ROS which makes them intrinsically radio-resistant ([322] and reviewed in [323]).
Figure 1.17. **Radiation induced radio-resistance.** Radiation therapy kills most cells but CSCs and some residual non-CSCs survive. The CSCs undergo enrichment while non CSCs undergo mutations, phenotypic plasticity mediated changes or radiation induced activation of signaling pathways leading to repopulation of these cells thus causing cause cancer relapse [324].

Radiation therapy kills cancer cells and causes an apparent decrease of tumor cell mass. This causes a survival pressure on the cancer cells to select radiation resistant CSCs, thus leading to CSC enrichment (Figure 1.18). These enriched CSCs differentiate into cancer cells which are more radio-resistant and aggressive leading to tumor recurrence (reviewed in [325]). The hypothesis of CSC selection has been validated by treatment of non small cell like lung carcinoma (NSCLC) derived cells to 5 Grays of irradiation and grown in ultra low adherent plates to form spheres. As controls, the un-irradiated cells were also grown in the same type of plates. It was demonstrated that the cells that formed spheres in the irradiated group had higher expression of stem cell markers, Oct4, Sox2, CD44, Snail and PDGF-beta and were more radio-resistant than the control cells [326].
**Figure 1.18. Radiation induced resistance is caused by CSC enrichment.** Upon radiation treatment, most tumor cells get destroyed causing reduction in tumor volume, but CSCs are not killed due to their intrinsic property of faster DNA damage repair. These CSCs undergo enrichment under the selection pressure of hypoxia. Enrichment causes proliferation of CSCs, increased hypoxia, quiescence, high DNA damage repair capacity, low ROS production, slow progression through the cell cycle and high expression of antioxidant or scavenger proteins. Moreover, during proliferation, CSCs undergo asymmetric division and differentiate into all cell types that make up the tumor [325].

Thus selection of CSC upon irradiation seemed to be an important mechanism of radiation induced radio-resistance. Radiation is also known to induce formation of CSCs or induce phenotypic plasticity in tumor cells which have survived radiation treatment. This theory was built on the consideration that the cells in any tumor are heterogeneous populations of clones having different phenotypes and can exhibit phenotype plasticity upon alterations in tumor
microenvironment. Thus, even non-CSCs tumor cells can dedifferentiation into CSC cells or into cell types that are more radio-resistant (reviewed in [327-329]). Phenotypic plasticity allows tumor cells to acquire a resilient, stress coping state and since this process does not require selection process, it occurs very early after irradiation and can cause tumor recurrence (reviewed in [330]). Ionizing radiation has been demonstrated to induce CSC induction in a hepatocellular carcinoma cell lines HepG2 and Huh7 by increasing stem cell markers like Sox2 and Oct3/4 expression. This caused increase in spheroid formation (a cell biology technique to analyze stem like phenotypes) and radio-resistance [331]. Even in breast cancer cells, ionizing radiation has been demonstrated to cause radiation induced radio-resistance by inducing expression of stem cell associated markers like Oct4, Nanog and Sox2 [332]. Thus, one of the mechanisms involved in radiation induced radio-resistance is the dedifferentiation of non-CSCs into CSCs.

Irradiation also activates many cell signaling pathways which are involved in conferring intrinsic radio-resistance to cells and are implicated to be involved in radiation induced radio-resistance [318]. Radiation induced activation of the PI3K/AKT pathway has been demonstrated to cause radioresistance in glioblastomas [333] and prostate cancer [334]. In medulloblastomas, radiation induced activation of the Wnt/β catenin signaling pathway has been shown to cause radioresistance. This signaling was demonstrated to be mediated by the increase in expression of the urokinase plasminogen activator receptor (uPAR) [335]. Low dose clinically relevant radiation exposure of 1 to 2 grays can cause activation of the EGFR (also called ErBb1) and human epidermal growth factor receptor 3 (HER3, also known as ErBb3) pathway leading to activation of the downstream kinases Raf, MEK, ERK [336].

Radiation induced radio-resistance can also be developed in neighboring cells which have not been directly exposed to radiation treatment by the bystander effect. In this phenomenon, the
cells exposed to radiation secrete cytokines that act in a paracrine manner to activate cell signaling pathways that increase radio-resistance in the neighboring cells (Figure 1.19). Thus, when these neighboring cells are exposed to radiation, they would be more radio-resistant than their counterparts (reviewed in [337, 338]). This mechanism of radio-resistance utilizes cell communicating junctions like gap junctions for cells attached to each other or secretion of cytokines, hormones and other soluble factors in a paracrine or endocrine manner to activate radio-protective signals in distant cells [337]. Bystander effects can cause either radio-resistance or radio-sensitivity based on the cytokine profile of the secretome and also probably on the relative concentration of the cytokines. Most bystander effects are carried out in cell cultures by transferring conditioned medium (CM) from irradiated cells to non-irradiated cells, followed by incubation and analysis of radio-sensitivity [337]. To identify proteins involved in the CM, mass spectrometric analysis is performed. Using the above techniques, cytokines Interleukin 6 (IL6) and Interleukin 8 (IL8) have been found to be associated in bystander effect signaling in glioblastoma cells after treatment with γ irradiation. A similar study in multiple human tumor cell lines HT1080, U373MG, HT29, A549 and MCF-7 showed that these cell lines endogenously secreted cytokines IL-1β, IL-6, IL-8, GMCSF and VEGF in the CM and correlated with the relative radio-resistivity of these tumor cell lines. On the basis of the order of radio-resistance, the HT1080 was the most radio-resistant followed by U373MG, HT29, A549 and MCF-7 cell lines. In all other cell lines used in this study except in the MCF7 cells, irradiation using acute doses of 2 and 6 grays (Gy) caused increased secretion of these cytokines in the CM but irradiation using fractionated doses of radiation (2 Gy given three times) did not change secretion of the cytokines. When bystander A549 cells were treated with the CM obtained from irradiated A549 cells, the cells showed decrease in clonogenic survival. Thus, the bystander effect on the
A549 cells was demonstrated to result in radio-sensitivity and may be caused due to the presence of less concentration of the radio-protective cytokines [339].

**Figure 1.19. Schematic representation of the bystander effect.** Chemical signals produced in an irradiated cell (with red borderline) are passed on to the neighboring cells through gap junctions. The irradiated cells also secrete hormones, proteins (cytokines) and other soluble factors that can act in a paracrine or endocrine manner to transfer radio-protective signals to distant cells or organs (adapted from [337]).

Thus, although radiation therapy has been used in treatment of cancers, there are multiple mechanisms by which radio-resistance can be induced by the same radiation treatment leading to local recurrence and poor prognosis of cancer.

**1.4. PKP3 loss and radio-resistance**

PKP3 loss causes decrease in cell to cell adhesion and loss of cell adhesion in EMT and metastatic cancers has been known to cause both intrinsic and radiation induced radio-resistance ([316, 340-342] and reviewed in [325]). To analyze if PKP3 loss can cause radio-resistance, HCT116 derived vector control and PKP3 knockdown clones were subjected to 4 Gy of \( \gamma \) irradiation or left un-irradiated. Twenty four hours post-irradiation, cells were trypsinized and 10,000 cells of each clone were plated onto 10cm plates and allowed to grow for 14 days. During...
this time cells formed visible colonies which were stained with crystal violet and images of the plates were taken. It was demonstrated semi-quantitatively that irradiated PKP3 knockdown clones formed more colonies as compared to the irradiated vector control clones [257].

Thus, multiple mechanisms may exist by which PKP3 loss can regulate tumor formation and metastasis. PRL-3 mediated K8 de-phosphorylation leading to K8 protein stability and increase in K8 protein levels is just one of the mechanisms by which PKP3 loss in HCT116 cells can regulate neoplastic progression. But it still does not explain all the phenotypes found in HCT116 derived PKP3 knockdown clones like even though cell proliferation was not altered, what causes increased anchorage independent growth in vitro and increased size of tumors in vivo. More work is also required to understand the cell type specific effects of PKP3 loss in HaCaT, HCT116 and FBM cell lines. Further, as the mechanisms regulating radio-resistance observed upon PKP3 loss were not clear, experiments needed to be performed to identify the mechanisms underlying the acquisition of radio-resistance in cells lacking PKP.